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Effect of high salinity on cell growth and protein production of Antarctic ice microalgae *Chlamydomonas* sp. ICE-L

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Abstract Antarctic ice microalgae *Chlamydomonas* sp. ICE-L can survive and thrive in Antarctic sea ice. In this study, *Chlamydomonas* sp. ICE-L could survive at the salinity of 132‰ NaCl. SDS-PAGE showed that the density of 2 bands (26 and 36 kD) decreased obviously at the salinity of 99‰ NaCl compared to at the salinity of 33‰ NaCl. The soluble proteins in *Chlamydomonas* sp. ICE-L grown under salinity of 33‰ and 99‰ NaCl were compared by 2-D gel electrophoresis. After shocking with high salinity, 8 protein spots were found to disappear, and the density of 28 protein spots decreased. In addition, 19 protein spots were enhanced or induced, including one new peptide (51 kD). The changes of proteins might be correlated with the resistance for *Chlamydomonas* sp. ICE-L to high salinity.

Key words Antarctic ice alga, *Chlamydomonas* sp. ICE-L, soluble proteins, two-dimensional polyacrylamide gel electrophoresis gel electrophoresis (2-D PAGE) analysis, high-salinity press.

1 Introduction

Sea ice is an ephemeral feature of polar regions, and its average covering area is about 4×10^6 km^2 in summer and 2×10^7 km^2, even 6.6×10^7 km^2[1] in winter. Unlike freshwater ice, frozen seawater forms a semisolid matrix, permeated by a network of channels and pores. These vary in size from a few micrometers to millimeters, and are filled with brine formed from expelled salts as the ice crystals freeze together[2]. Scientists were first able to see these channels clearly in 1992, which highly branched frazil crystal interstices 200 to 300 μm wide look like an untidy bird's nest. The elongated interstices of congelation crystals resemble close-ups of human hairs. It is within this labyrinth that the sea-ice microalgae live with the only liquid

being pockets of concentrated brines. Sea-ice microalgae can thrive in the ice, and their prolific growth ensures that they play a fundamental role in polar ecosystems[3]. of a A diverse group of pteropods, copepods, amphipods, and fish feed on the ice algae. Apart from their ecological importance, the algae species found in sea ice have become the focus for novel biotechnology, as well as being considered as proxies for possible life forms on ice-covered extraterrestrial bodies.

Dehydration, caused by the high brine salinities, is a major stressor for ice-trapped organisms, which may experience salinities three times (even five times)[4] that of seawater. Surviving osmotic stress requires the adjustment of cellular concentrations of osmolytes, so as to restore the osmotic balance between the external medium and the inside of the cells. In response to the changing external osmotic pressure osmolytes, including inorganic ions and organic solutes (such as proline, mannitol, and glycine betaine), are accumulated or synthesized in hypersaline conditions and broken down or released during hyposaline shock.

The plant ability of plants to salinity-resistance is determined by multiple biochemical pathways that facilitate retention and/or acquisition of water, protect chloroplast functions, and maintain ion homeostasis[5]. Essential pathways include those that lead to synthesis of osmotically active metabolites, specific proteins, and certain free radical scavenging enzymes that control ion and water flux and support scavenging of oxygen radicals or chaperones[5]. Basic research on the halotolerance mechanism in plants is important for the improvement of salinity tolerance of crops by genetic engineering. The green alga *Chlamydomonas* attracts much interest as a biological model system for photosynthetic organisms, and thus might possibly serve as a convenient model system for the biology of plant salinity tolerance.

Mechanisms of dehydration tolerance in plants may include common gene products and regulatory pathways[6], and it is likely that these occur in sea-ice organisms too. The expression of DnaK1 from a halotolerant cyanobacterium *Aphanothece halophytica* improved the salinity tolerance of the tobacco plant[7]. Many scientists found that the salinity-resistance of some microalgae was correlated with proteins. The term of proteomics was put forward by Wilking *et al*. in 1994[8]. It was to analyze the structure and function of one genome, one cell, or tissue. The key step of gel-based proteomics approach is two-dimensional gel electrophoresis (2-DE)[9,10]. In this paper, the effect of salinity on the proteins expression of *Chlamydomonas* sp. ICE-L was analyzed by 2-DE. Changes of protein spots appeared to be correlated with the resistance to high salinity.

2 Materials and methods

2.1 Alga materials and culture conditions

Microalga *Chlamydomonas* sp. ICE-L was isolated from Antarctic sea ice collected in 18th Chinese Antarctic expedition during 2001/2002. *Chlamydomonas* sp. ICE-L was grown axenically at 6—8 ℃ in autoclaved Provasoli medium, with illumination of 200—300 μE $m^{-2}s^{-1}$ on a 12 :12 h light/dark cycle. Cultures were main-

tained and shaken 3 times everyday. Mesophilic alga *Chlamydomonas monadina* was cultured in the same conditions as in *Chlamydomonas* sp. ICE-L, except that *Chlamydomonas monadina* grew at 20—25 ℃.

For salinity stress study, NaCl was added to the culture medium at a final concentration of 33‰, 66‰, 99‰, 132‰ and 165‰. The cell density was measured in triple by cell counts after incubation for 0, 2, 4, 6, 8, 10, 12, 14, 16 and 18 days, respectively.

2.2 High salinity stress culture

Previous salinity-tolerant experiments showed that *Chlamydomonas* sp. ICE-L could grow well in salinity of 99‰ NaCl. So in present study we took alga grew grown under salinity of 99‰ NaCl as salt-stressed material and under salinity of 33‰ NaCl as control. After 14 days culture under same conditions same to above, *Chlamydomonas* sp. cells were collected for farther SDS-PAGE or 2-DE analysis.

2.3 SDS-PAGE analysis

Proteins of *Chlamydomonas* sp. ICE-L were extracted for SDS-PAGE according to Wang[11]. SDS extraction buffer components were 64 mM Tris-HCl, 10% glycerol, 2% SDS and 5% β-mercaptoethanol. Supernatant samples were separated in 12.5% polyacrylamide gel (3% stacking and 12.5% separation gel) at changeable current of 15 mA in stacking gel (3%) and 25 mA in separation gel (12.5%). Separation gel was visualized by staining with Coomassie Brilliant Blue (CBB). The stained gel was analyzed using software Bandscan 5.0.

2.4 2-DE analysis

The protein samples for 2-DE were prepared by improved Trichloroacetic acid (TCA)-acetone fractional preparation method as described previously (unpublished). A total of 40 mg alga powder was lysed in 400 μl lysis buffer (9 M urea, 2% (W/V) CHAPS, 1% (W/V) DTT, 0.5% Phamarlyte pH 3～10, and 5 mM PMSF). The concentration of the total proteins was determined with 2-D Quant Kit (Amersham Biosciences) with bovine serum albumin as standard. Then protein was packed respectively in 500 μl eppendorf tubes and stored at —80 ℃ until test use.

The 2-DE was performed according to the method of 2-D protocol (Amersham Biosciences). Isoelectric focusing for the first dimension was performed in precast Immobiline DryStrip gels with non-linear gradient pH 3-10 (Amersham Biosciences). Proteins were first subjected to isoelectric focusing (Ettan IPGphor system, Amersham Biosciences) for a total of 19.66 kVh at 20 ℃. The second dimension was a vertical SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the SE600 Ruby system (Amersham Biosciences). After SDS-PAGE, gels were fixed overnight in a solution of 10% acetic acid and 40% ethanol and then visualized by silver-based staining technique.

2.5 Analysis of protein patterns

The stained 2-DE gels were scanned with Uniscan C720 system. ImageMasterTM 2D Platinum software 5.0 (Amersham Biosciences) was used for spot intensity calibration, spot detection, background abstraction, matching, and the establishment of average-gel. Intensity of each spot was quantified by calculation of spot volume after normalization of the image using the total spot volume normalization method multiplied by the total area of all the spots. The molecular masses were interpolated from marker proteins (Shanghai Institute of Biochemistry, Shanghai) co-electrophoresed with sample in SDS-PAGE.

3 Results

3.1 Effect of salinity on the growth of ice microalga Chlamydomonas sp. *ICE-L*

The effects of salinity on Antarctic ice microalga *Chlamydomonas* sp. ICE-L and mesophilic microalga *C. monadina* were shown in figure 1. It proved that both microalgae could grow well at the salinity of 33‰ NaCl. At a salinity of 33‰ NaCl, *Chlamydomonas* sp. ICE-L grew slowly at the beginning, but the growth rate increased quickly after 6 days. It entered the stationary phase on the 14th day, when the cell density was 1.8×10^7/mL. Mesophilic *C. monadina* grew quickly, and the peroid from the 2nd to 6th day was its exponential growth phase. At the salinity of 66‰ and 99‰ NaCl, the growth curves of ice microalga were similar. After adaptation for 8-10 days in high salinity, *Chlamydomonas* sp. ICE-L began to grow rapidly, and got to the maximal density on the 14th day. For mesophilic *C. monadina*, the exponential growth phase were 2-8 days and 6-12days at the salinity of 66‰ and 99‰ NaCl, respectively, and their maximal densities were 107/ml and 8.5×10^6/ml respectively on the 12th day. The density of *Chlamydomonas* sp. ICE-L changed little at salinity of 132‰, about 5×10^6/ml in 12 days, and increased slightly after the 12th day. However, the salinity of 132‰ NaCl was fatal to mesophilic microalga. Both of ice microalga and mesophilic microalga ones could not survive at the salinity of 165‰ NaCl, and all cells were bleached and dead at the 8th and 6th day, respectively.

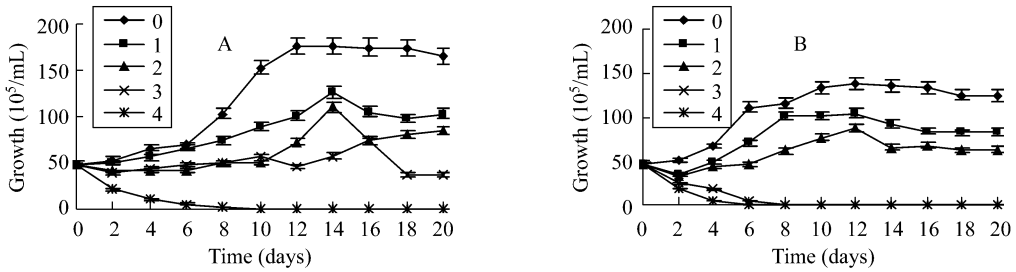

Fig. 1 The growth curve of Chlamydomonas sp. ICE-L (A) and Chlamydomonas monadina(B) at differ-

ent salinity. 0: the salinity of 33‰; 1: the salinity of 66‰; 2: the salinity of 99‰; 3: the salinity of 132‰; 4: the salinity of 165‰.

3.2 SDS-PAGE analysis of Chlamydomonas sp. *ICE-L*

Result of SDS-PAGE of soluble proteins in *Chlamydomonas* sp. ICE-L was shown in Fig. 2. There was no change of band number with or without high salinity treatment, and there were a total of 37 bands. However, the band intensity was changeable. Intensity of two bands decreased: One band was a 26 kD protein, whose intensity decreased from 7.0% to 2.4%. Another was a 36 kD protein, decreasing from 6.8% to 1.4%.

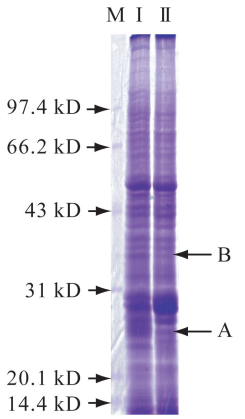

Fig. 2 SDS-PAGE map of soluble proteins from Antarctic ice alga *Chlamydomonas* sp. ICE-L stressed by high salinity. Ⅰ. Control, cultured in the salinity of 33‰ NaCl, Ⅱ. Treatment, cultured in the salinity of 99‰ NaCl, M. Protein marker.

3.3 2-DE analysis of differential proteins in Chlamydomonas sp. *ICE-L under high salinity shock*

The 2-DE patterns of untreated and treated Antarctic ice alga *Chlamydomonas* sp. ICE-L were shown in Fig. 3. The protein spots spread in pH 3～10, most distributed in the acidic terminal of 2-DE gel. Most of the proteins were no higher than 100 kD. For ice alga treated with high salinity, a total of 652 spots were detected. For untreated cells, a total of 626 spots were obtained. There were a total of 598 spots matched between the untreated and treated ice microalga cells. After treatment with the salinity of 99‰, a new peptide (MW 51 kDa, pI 6.90) was found (Fig. 4), and 18 protein spots were enhanced or induced in ice alga (Table 1). Most of the 19 protein spots were acidic or neutral. At the same time, 28 protein spots were weakended (Table 2) and 8 protein spots were disappeared (Table 3), most of which were also acidic or neutral.

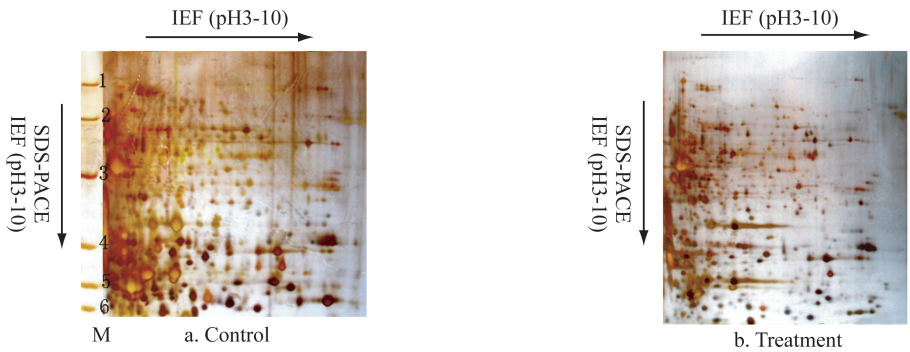

Fig. 3 a: 2-DE map (pH 3-10) of Antarctic ice alga *Chlamydomonas* sp. ICE-L stressed by high salinity for 14 days. M. marker proteins of 97.4, 66.2, 43, 31, 20.1 and 14.4 kDa (1 to 6). b: Fig. 4 3D image of increased protein in

Chlamydomonas sp. ICE-L treated with high salinity, arrow for newly-synthesized protein.

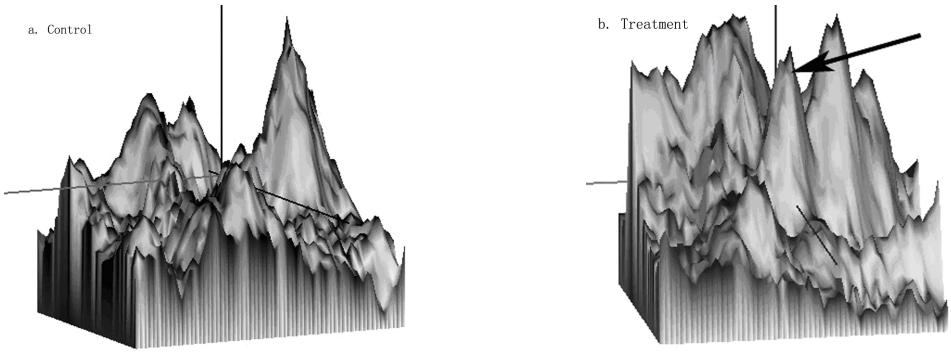

Fig. 4 3D image of increased protein in Chlamydomonas sp. ICE—L treated with high salinity, arrow for newly—synthesized protein.

Table. 1 Protein spots with increased volume in high salinity treated Antarctic ice microalga *Chlamydomonas* sp. ICE-L

Protein number[1]	Isoelectric point(pI)	Molecular mass/kD	Protein level[2] increased/%
111	6.35	41	292.7
123	7.86	40	430.2
164	6.59	35	120.6
180	5.86	33	261.8
194	5.47	33	447.3
207	6.47	31	60.7
221	6.05	30	217.4
232	7.82	30	212.2
241	9.16	29	234.0
257	6.30	27	319.5
271	6.42	26	291.9
275	3.91	26	136.2
298	7.30	24	315.3
325	8.32	21	286.1
327	7.20	21	93.8
381	5.65	16	467.1
435	9.01	12	192.3
442	5.42	12	79.3

1: The number of protein spots in the 2-DE gel for high salinity treated ice microalga.

4 Discussion

4.1 *The salinity tolerance of Chlamydomonas* sp. *ICE-L*

Antarctic ice microalga is very important resource and can contribute up to 30% of the annual primary production of seasonally ice-covered areas[3]. Many researches on the resistance to different salinity were carried out. Kottmeier and Sullivan[12] provided that some taxa are apparently euryhaline and maintain their growth rates over a salinity range of 10-50‰, while ice algae can still be physiologically active at salinities as high as 100‰. Our researches Present study found that ice microalga could

survive at the salinity of 132‰, while mesophilic microalga *C. monadina* only maintained life at salinity under 99‰. In the medium of 66‰ and 99‰ salinities, *Chlamydomonas* sp. ICE-L cell division was halted following a hyper-osmotic shock in 10 days, but proliferation was reinitiated during the late phase of the osmotic response and exponential growth was resumed soon after completion of the osmotic response. Our rResults of this paper were in accordance with former studies[13,14]. After 10 days treatment with 165‰ salinity, Antarctic ice microalga *Chlamydomonas* sp. ICE-L was bleached and dead. Active oxygen induced by high salinity may inhibit the repair of the photodamaged photosystem Ⅱ[15], resulting in that the photosynthesis ability of ice microalga was inhibited, and the microalga died at last.

4.2 2-DE analyses of proteins exposed under high salinity shock

High salinity can make plants produce much active oxygen, which increases the per-

Table. 2 Protein spots with decreased volume in high salinity treated Antarctic ice microalga *Chlamydomonas* sp. ICE-L

Proteinnumber[1]	Isoelectric point(pI)	Molecular mass/kD	Protein level[2] decreased/%
93	6.48	43	200.5
95	6.66	44	321.5
97	6.89	43	294.0
117	7.04	41	139.9
119	6.23	40	91.5
126	8.91	39	187.9
208	7.10	31	209.8
247	7.00	28	150.8
263	5.48	27	246.0
310	5.60	22	266.0
336	5.88	19	300.4
346	6.52	19	66.0
347	6.96	19	126.8
357	6.42	18	162.2
359	7.62	17	239.3
364	6.39	17	891.7
394	7.12	15	198.5
399	8.39	14	480.1
412	5.91	14	376.1
413	6.31	13	63.1
428	9.70	12	597.6
429	6.98	12	210.3
430	7.27	12	932.2
432	8.61	12	94.5
437	6.81	12	657.9
447	5.71	11	274.8
449	6.50	11	204.1
451	7.16	11	115.7

1: The number of protein spots in the 2-DE gel for high salinity treated ice microalga.

Table. 3 Protein spots disappeared in high salinity treated Antarctic ice microalga *Chlamydomonas* sp. ICE-L

Protein number[1]	Isoelectric point(pI)	Molecular mass/kD	Protein level[2] / %
95	6.68	43	0.201
97	6.88	43	0.281
134	9.88	39	0.104
319	7.88	22	0.357
345	6.39	20	0.224
376	8.81	16	0.122
409	6.88	14	0.223
458	6.16	10	0.386

1. The number of protein spots in the 2-DE gel for high salinity treated ice microalga; 2. The ratio of each spot volume to total volume of the whole spots.

meability of cell membrane, decreases the efficiency of photosynthesis and respiration, and destroys DNA and proteins in cell[16]. For Antarctic ice microalga *Chlamydomonas* sp. ICE-L, the protein content decreased obviously ($P < 0.01$) from 884.5 to 813.6 $\mu g \cdot g^{-1}$ wet weight cells after high salinity (99‰ NaCl) shock, which was accordant with Rafael and Bertha's studies[17] in green alga *Botryococcus braunii*(race a). In order to keep alive in channels or holes containing high salinity, many physiological and biochemical responses happened, including the secretion of osmoprotectant compounds, such as laminaribiose[17], osmoregulatory isoform of dihydroxyacetone (DHAP) reductase (Osm-DHAPR)[18] and glycerol[19]. In addition, many researches showed that proteins played an important role in osmoregulation. Adding high amounts of NaCl, Hagemanna *et al*.[20] found that about 64.4 and 20.5 kD proteins, which may be involved in the signal transduction chain sensing changes in the NaCl content, were induced in *Synechocystis* sp. PCC 6803. When marine microalga *Tetraselmis* (*Platymonas*) *viridis* was grown grew in high-salinity media, the synthesis of several proteins with molecular weights close to 100 kD was induced. The data obtained argue for the hypothesis, which was put forward earlier, that a novel Na^+-ATPase isoform is induced by *T. viridis* growing at high NaCl concentrations[21]. Several salinity-tolerant enzymes that function over a wide range of salinities have been characterized from psychrophilic isolates[22]. However, based on the results of osmotic adaptation of *Dunalieh sulina* and *Chlamydomonas* HS-5 utilizing protein synthesis inhibitors, Sadka *et al*.[23] and Miyasaka *et al*.[19] find that protein synthesis is not essential for the osmotic adaptation response.

In this study, a new polypeptide (MW 51 kD, pI 6.90) was found after 99‰ salinity shock in Antarctic ice green microalga *Chlamydomonas* sp. ICE-L. Except for induced protein spot, 18 protein spots with increased volume were also considered as mechanism of in protecting *Chlamydomonas* sp. ICE-L against high salinity shock.

5 Conclusions

Ice microalga can survive and thrive in channels or pores containing high salinity in Antarctic ice layer. In order to find the resistant degree to high salinity, Antarctic

ice alga *Chlamydomonas* sp. ICE-L was studied as the model material. Results showed that *Chlamydomonas* sp. ICE-L could survive at the salinity of 132‰, which was higher than that in case of mesophilic microalga *Chlamydomonas monadina*. The SDS-PAGE analysis of soluble proteins showed that the density of 2 bands (26 and 36 kD) decreased obviously after the shock of 99‰ salinity. The proteins in *Chlamydomonas* sp. ICE-L treated with and without high salinity were compared by 2-DE. A total of 8 protein spots were found to disappear and 18 decreased. At the same time, 18 protein spots were enhanced and one new peptide (51 kD) was induced. The detection of new proteins is the base of the identification of protein characters and structures with matrix-assisted laser desorption/ionization time of flying mass spectrometry (MALDI-TOF-MS), and among which the finding of new salinity-resistance proteins is expected.

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