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NOTE

Effects of different ion compositions on growth of obligately halophilic protozoan *Halocafeteria seosinensis*

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Abstract Substantial halophilic organisms have been found in 100–200‰ salinities. These ranges represent a highly specialized halophilic environment to which only a few halotolerant species have adapted. Recent studies have underlined the existence of diverse obligately halophilic protozoa in the salinity ranges of 100–200‰. The ranges of salinity under which these organisms can grow have been examined to some extent, but the balance of specific ions that will support growth has not been investigated. The heterotrophic nanoflagellate *Halocafeteria*, the type strain of which grows optimally at 150‰ salinity and 35°C, is a commonly encountered obligate halophile found in very hypersaline environments. These extreme environments can vary in their Mg:Ca ratios (i.e. weight ratios) and sulfate concentrations. To examine growth response of *Halocafeteria* to the different chemical compositions, densities of *Halocafeteria seosinensis* strain EHF34 were monitored in seven different ion composition media for 9 days at 1- to 2-day intervals (at 150‰ salinity and 35°C, with no prey limitation). *Halocafeteria* does not grow at Mg:Ca ratios of 35 and 100 and at high sulfate concentrations of 11.6 and 31.6 g l^{-1}. It grows well in 0.6 g l^{-1} sulfate media at Mg:Ca ratios of 2, 10 or 35, but not 100. The present study demonstrates that the growth of the obligate halophile *Halocafeteria* can be affected by different ion compositions in hypersaline environments. Therefore, *Halocafeteria* may not be ubiquitous in hypersaline environments due to its ionic requirements.

Keywords Bicosoecid · Growth · *Halocafeteria* · Halophile · Hypersaline · Mg:Ca ratio · Protozoa · Stramenopile · Sulfate

Introduction

Since 2006, many halophilic protozoa have been characterized in laboratory cultures using morphological and phylogenetic data (Park et al. 2006, 2007, 2009; Cho et al. 2008; Park and Simpson 2011). Most of these protozoa were identified as distinct genera. A representative of the halophilic protozoa is *Halocafeteria seosinensis* (Park et al. 2006). *Halocafeteria* is a small bicosoecid stramenopile that is aloricate and lacks flagellar hairs (Park et al. 2006). Its closest relatives include the marine taxa *Cafeteria* and *Caecitellus*, and an undescribed clade of halotolerant flagellates labeled 'Group B' by Park and Simpson (2010). *Halocafeteria*-like organisms can feed on prokaryotes in various high salinity waters (Park et al. 2003, 2006), and have frequently been observed in geographically different hypersaline environments (J. S. Park and A. G. B. Simpson, unpublished). The type strain of *Halocafeteria seosensis*, EHF34, was originally isolated from a Korean solar saltern from waters of very high salinity (300‰ salinity; Park et al. 2006). This strain of *Halocafeteria* can be grown in >300‰ salinity media, but does not grow in <75‰ salinity media, suggesting a borderline extreme halophile (Park et al. 2006). It grows best at 150‰ salinity and at a temperature 35°C (Park et al. 2006).

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Table 1 Summary of major ion concentration (g l^{-1}) in various hypersaline environments

Chemicals	Saturated brine	Saturated potash	Great Salt Lake, USA	Dead Sea, Middle East	Lake Mushiki, Canada	Don Juan Pond, Antarctic
Na^+	98.4	61.4	105	39.7	62.2	11.5
Mg^{2+}	14.5	39.3	11.1	42.4	29	1.2
Ca^{2+}	0.4	0.2	0.6	17.2	NA	114
K^+	4.9	12.8	6.7	7.6	NA	0.2
Cl^-	187	189	181	219	114	212
SO_4^{2-}	19.3	51.2	27	0.4	237	0.01
Salinity (ppt)	324	354	333	327	342	339
Mg:Ca (weight ratio)	36	197	19	9.99	NA	0.01
K:Na (weight ratio)	0.05	0.21	0.06	0.19	NA	0.02

Based on Javor (1989)

NA not available

Among the halophilic protozoa, *Halocafeteria*, and similar organisms are potentially important heterotrophs in high salinity waters, and are of considerable evolutionary interest. In salt environments, it is unclear which non-biological factors other than total salinity and temperature affect their growth. It is important to look at the ubiquity of microbes in a variety of high salt environments because these environments vary considerably in their geochemistry (Javor 1989). Natural hypersaline environments contain quite different Mg:Ca ratios (i.e. weight ratio) and ion compositions (Javor 1989). These ionic variations may affect the growth and survival of halophilic protozoa. Post et al. (1983) suggested that the Mg:Ca ratio is a dominant factor influencing the growth of protozoan communities in hypersaline environments, rather than the concentration of sodium chloride. Both magnesium and calcium are required for the growth of the halotolerant alga *Dunaliella* (Baas-Becking 1931; McLachlan 1960), which is the most ubiquitous eukaryote found in salinity ranges of 40–350‰ (Javor 1989). *Dunaliella* is commonly absent in nutrient-poor solar salterns, in particular high salinity waters with low concentrations of phosphate (Javor 1989; Oren 2002). However, very few attempts have been made at such observation in heterotrophic nanoflagellate isolated from high salinity water.

The purpose of this study is to examine growth response of *Halocafeteria* to various chemical compositions in artificial high salinity waters, and which ion compositions significantly affecting on the growth of *Halocafeteria*.

Materials and methods

Halocafeteria seosinensis strain EHF34 was isolated from high salinity water (~300‰) in a Korean solar saltern in May 2003 (Park et al. 2006). The saltern was composed of 72 salt ponds, with a total area of about 50,000 m^2 when full (Park et al. 2003). The average water depth of the ponds was about 10 cm, and the saltern was eutrophic (Park et al. 2003, 2009). The temperature of the high salinity (~300‰) water varied between 18 and 40°C from April to October in 1998–2001(Park et al. 2009). Unfortunately the ion compositions of high salinity waters in a Korean solar saltern have not been reported. However, Javor (1989) reported various chemical compositions in high salinity waters including similar environment to a Korean solar saltern (i.e. saturated brines in many salterns, see Table 1). To determine the effect of different chemical compositions on growth of *Halocafeteria* in the same salinity media and temperature (i.e. 150‰ salinity and 35°C), different chemical compositions were prepared for seven media (i.e. I to VII, Table 2). The ionic compositions studied here were similar to those of other extreme hypersaline environments (Table 1) as reported by Javor (1989). Mg:Ca (weight) ratios in the seven media ranged from 2 to 100, while sulfate concentrations were in the range of 0.6–31.6 g l^{-1} (Table 2). Inocula of *Halocafeteria seosinensis* strain EHF34 (mean ± SD of $8.2 \pm 0.5 \times 10^3$ cell ml^{-1}, $n = 2$, inoculums of 0.5 ml) were added to culture flasks containing 40 ml of medium I to VII (with the same 150‰ salinity), but different ion compositions (Table 2). These culture flasks were incubated at 35°C for 9 days with heat-killed *Idiomarina seosinensis* added (average density of 2.3×10^7 cells ml^{-1}), and observed at 1- to 2-day intervals. Heat-killed bacteria were centrifuged at 3,000×*g* for 5 min, and washed and resuspended three times with each sterile medium prior to adding to the cultures to avoid changes of salinity and chemical compositions. The incubation period is short to minimize the growth of indigenous prokaryotes from inoculums in the

Table 2 Chemical compositions (g l^{-1}) of seven different media (I to VII) used for growth of *Halocafeteria seosinensis*

Chemicals	I	II	III	IV	V	VI	VII
NaCl	144.6	137.6	121.6	68.6	137.6	121.6	76
KCl	3.8	3.8	3.8	3.8	3.8	3.8	3.8
$MgCl_2 \cdot 6H_2O$	1.51	7.8	27.18	81.5	13.45	50.53	146.9
$MgSO_4 \cdot 7H_2O$	1.65	8.5	29.7	81	1.65	1.65	1.65
$CaCl_2 \cdot 2H_2O$	0.65	0.65	0.65	0.65	0.65	0.65	0.65
Salinity (ppt)	150	150	150	150	150	150	150
Na^+	56.88	54.13	47.84	26.99	54.13	47.84	29.90
Mg^{2+}	0.34	1.77	6.18	17.73	1.77	6.20	17.72
Ca^{2+}	0.18	0.18	0.18	0.18	0.18	0.18	0.18
K^+	1.99	1.99	1.99	1.99	1.99	1.99	1.99
Cl^-	90.36	88.31	85.36	72.16	90.28	93.51	99.45
SO_4^{2-}	0.64	3.31	11.57	31.57	0.64	0.64	0.64
Mg:Ca (weight ratio)	1.94	9.99	34.85	100.03	9.99	35.00	99.99
K:Na (weight ratio)	0.04	0.04	0.04	0.07	0.04	0.04	0.07

media. All trials were performed in duplicate—two bottles for each medium.

The procedure for measuring abundance and determining growth rates was the same as reported by Park et al. (2006). In brief, a subsample was taken from each experimental bottle every 1–2 days, and *H. seosinensis* cells were immediately fixed with glutaraldehyde (1% final concentration). DAPI (4′,6-diamidino-2-phenylindole)-stained *Halocafeteria* were collected on 0.8 μm polycarbonate filters (25 mm in diameter) under a vacuum not exceeding 100 mmHg. The cells were enumerated at 1,000× magnification with UV excitation using an epifluorescence microscope. Varying volumes of samples (1 ml: <3 days after incubation, 0.5 ml: >3 days after incubation) were immediately filtered depending on cell abundance after fixing the samples.

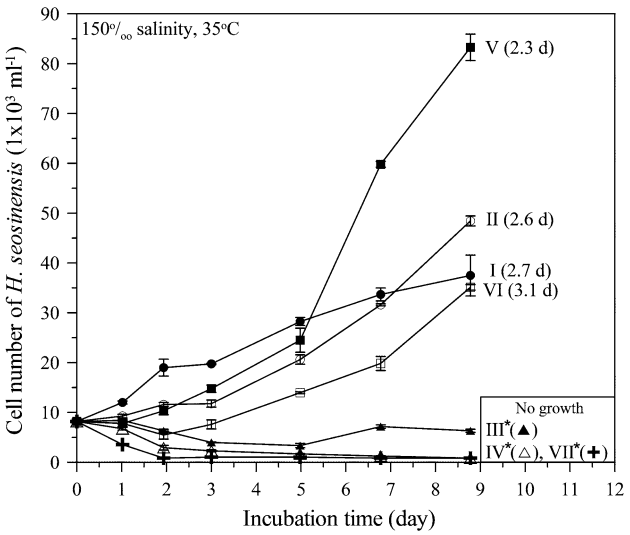

Fig. 1 Effects of seven artificial seawater media (marked I to VII, 150‰ salinity) on growth rates of *Halocafeteria seosinensis* at 35°C. Doubling times of *H. seosinensis* are shown in *parentheses*. Note that *H. seosinensis* could not grow in medium III, IV, and VII (*asterisk* no growth). For chemical compositions of each medium, see Table 2. *Error bars* show 1SD ($n = 2$)

Results and discussion

The bicosoecid *Halocafeteria seosinensis* showed different growth responses to various chemical composition media during short-term incubation, in spite of the same overall salinity (150‰ salinity), temperature (35°C) and prey density (avg. 2.3×10^7 cells ml^{-1} per inoculum). *Halocafeteria* failed to grow (i.e. significantly decreased in their abundance) in the artificial seawater media with the Mg:Ca ratio of 100 (medium IV and VII; Table 2; Fig. 1), whereas *Halocafeteria* grew with a doubling time of 2.3–3.1 days in low Mg:Ca ratios of 2, 10 or 35 (medium I, II, V, and VI; Table 2; Fig. 1). Unfortunately there has no comparable study of an extremely halophilic protist at the same trophic level as *Halocafeteria*. However, several reports have found results similar to the present study in the case of halotolerant alga *Dunaliella* though this organism is an entirely different trophic type. The non-obligately halophilic *Dunaliella* is frequently observed in hypersaline environments (Javor 1989). *Dunaliella salina* grows optimally at 120‰ salinity (Javor 1989), lower than, but comparable to *H. seosinensis* EHF34. *Dunaliella* is able to be influenced by various Mg:Ca ratios as well (McLachlan 1960). Halotolerant *Dunaliella tertiolecta* could tolerate the Mg:Ca ratios ranging from 1 to 20, and grew best at a Mg:Ca ratio of 4 (McLachlan 1960). *Halocafeteria* can

tolerate a rather higher Mg:Ca ratios than *Dunaliella*, and the optimal Mg:Ca ratio is higher as well (Table 2; Fig. 1).

It is possible that a K:Na ratio could have an effect on the growth of *Halocafeteria* since Van Auken and McNulty (1973) demonstrated that a K:Na ratio is an important factor to influence on growth of halotolerant *Dunaliella*. *Dunaliella* grew optimally at a K:Na ratio between 0.1 and 0.001, but rapidly decreased their growth at a K:Na ratio of 1.0 or higher. *Halocaferia* did not grow at a K:Na ratio ranging from 0.04 to 0.07 in medium III, IV and VII, but grew at a K:Na ratio of 0.04 in medium I, II, V, and VI (Table 2; Fig. 1). It is unlikely that the employed K:Na ratios affect the growth of *Halocafeteria*.

The growth rate of *Halocafeteria* is also sensitive to the concentrations of sulfate. *Halocafeteria* grew in relatively low concentration of sulfate (0.6 g l^{-1}, medium VI), but did not grow in relatively high concentration of sulfate (11.6 g l^{-1}, medium III) though other chemicals were almost the same concentration in the two media (Table 2; Fig. 1). In addition, *Halocafeteria* in medium V (sulfate concentration: 0.6 g l^{-1}) grew much faster than that in medium II (sulfate concentration: 3.3 g l^{-1}, Table 2; Fig. 1) under otherwise almost identical conditions. This result indicated that a sulfate concentration of 3.3 g l^{-1} appears to inhibit the growth of *Halocafeteria* as well. Therefore, the concentration of sulfate is another important determinant affecting on growth of halophilic *Halocafeteria*.

The doubling time of *Halocafeteria* is fastest in medium V (2.3 days) that contains a Mg:Ca ratio of 10 and sulfate concentration of 0.6 g l^{-1} (Table 2; Fig. 1). For liquid cultures of obligate halophilic and heterotrophic protozoa, medium V has been commonly used in previous studies (Park et al. 2009; Park and Simpson 2011). The flagellate *Pharyngomonas*, amoeba *Tulamoeba*, and amoeboflagellate *Euplaesiobystra* isolated from various hypersaline environments grew well in medium V. Thus, medium V as a base medium is recommended to isolate and subculture halophilic protozoa.

In summary, *Halocafeteria* does not grow in relatively high Mg:Ca ratio (100) and sulfate concentrations (>11 g l^{-1}) under otherwise ideal conditions (i.e. 150‰ total salinity and 35°C, with no prey limitation), indicating that *Halocafeteria* may not thrive in some hypersaline ecosystems because of its ionic requirements. It would be very interesting to test whether *Halocafeteria*-like organisms may be present in natural high salinity waters with the relatively high Mg:Ca ratios of 100 and sulfate concentrations (>11.6 g l^{-1}, Table 1).

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