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Biology of a terrestrial green alga, *Chlorococcum* sp. (Chlorococcales, Chlorophyta), collected from the Miruksazi stupa in Korea

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A terrestrial chlorophyte, *Chlorococcum* sp., was isolated from the stone walls of Miruksazi stupa, which is a national treasure of Korea. The alga was one of the dominant organisms contributing to biodeterioration of the monument and it grew extensively on the walls of the inner room of the stupa, which had been sealed for more than 5 yr before we started this experiment. *Chlorococcum* survived in darkness during that time as dormant, warty, thick-walled spores. The resting spores revived in freshwater medium and released numerous unicellular progeny, which were isolated into a unialgal culture. The isolate was subjected to 18S rDNA phylogenetic analysis as well as ultrastructure and life cycle studies. In addition, the effect of salinity stress was investigated using sterile enriched seawater as a medium. *Chlorococcum* sp. grew in seawater culture medium for more than 5 mo and reproduced by aplanospores.

KEY WORDS: resting spore, biodeterioration, *Chlorococcum*, drought resistance, salinity stress

INTRODUCTION

The order Chlorococcales is comprised of many genera that are found worldwide in various aquatic and terrestrial habitats. They are known to be effective colonizers of denuded soils, new soils, and newly formed water bodies, and are capable of growing on any surface that holds moisture, including tree bark, stone or plaster walls, glass, etc. (e.g. Graham & Wilcox 2000; John & Tsarenko 2002; Shubert 2003). Their growth may result in considerable damage to surfaces over time, including deterioration of woodwork and stone monuments.

Chlorococcum is recorded from many aquatic habitats and is frequently associated with soil and subaerial surfaces (Canter-Lund & Lund 1995; John & Tsarenko 2002; Shubert 2003). *Chlorococcum* has been the organism of choice for a number of physiological and biochemical investigations; however, not many studies have been conducted regarding its ability to tolerate environmental stress conditions (Shields & Durrell 1964; Blackwell & Gilmour 1991a, b; Dash *et al.* 1995; Masojidek *et al.* 2000). High resistance to drought (Shields & Durrell 1964) and salinity stress (Blackwell & Gilmour 1991a, b) have been documented for several species of *Chlorococcum* from different habitats. *Chlorococcum submarinum* Alvik found in inland saline lakes, estuaries, and marine coastal habitats grew in very broad salinities (pH range from 2 to 10), and in the presence of 10 ppm copper (Blackwell & Gilmour 1991b). However, a freshwater *Chlorococcum* was less tolerant to salinity stress (Masojidek *et al.* 2000). The drought resistance of *Chlorococcum* sp. from soil was reported (Shields & Durrell 1964). This was attributed to the presence of highly viscous protoplasts with small vacuoles and very low water contents (Ahmadjian 1967). The drought resistance might also be due to the development of hypnospores (aplanospores with thick walls bearing spines within a smooth-walled aplanosporangium) and hypnozygotes ('sleeping' zygotes known as cysts) in some species, with thick, spiny, or ornamented walls, for example, in *Chlorococcum hypnosporum* Starr, *Chlorococcum minutum* Starr, and *Chlorococcum echinozygotum* Starr. Development of vegetative cells with thickened ornamented cell walls in harsh environmental conditions was not previously recorded in *Chlorococcum*.

In the present paper we report on a *Chlorococcum* species that is highly resistant to environmental stress conditions. We herein consider its habitat, morphology and reproduction, 18S rDNA molecular phylogeny, and drought and salinity tolerance. A culture of this isolate was initiated from the resting spores collected from the stone walls of Miruksazi stupa in Korea during conservation of the monument and chemical treatment of Korean stone cultural properties.

MATERIAL AND METHODS

Brief description of the study area

Miruksazi stupa, located in Iksan, South Korea (national treasure No. 11), was constructed in AD 600–641. The stupa was largely destroyed during early 1900s, but its collapsed outer

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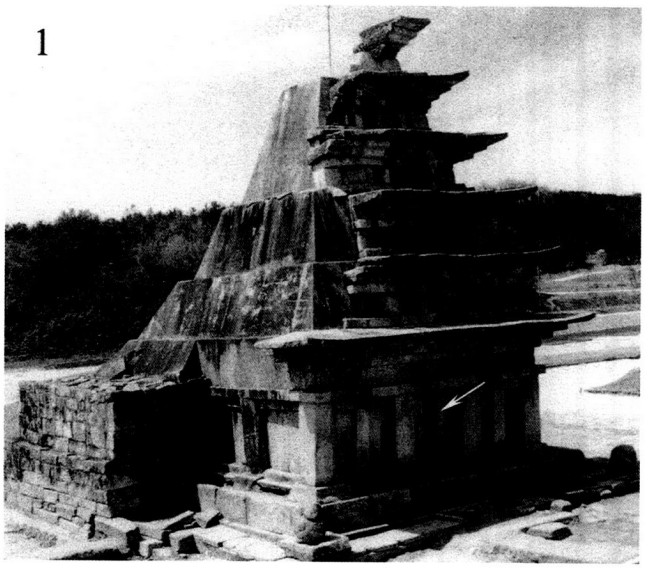


Fig. 1. Photograph of Miruksazi stupa taken from a poster at the site of the stupa. Arrow points to the entrance of the inner room.

eastern side was cemented in 1915 to prevent further structural instability (Fig. 1). At that time, the entrances leading to the inner room were sealed with cement. In 1997, a project was initiated to conserve the stupa, and the inner room was open for a month. Then the inner room was closed and sealed again, and by the time we started investigations in 2002 it had been sealed for more than 5 yr. No light penetrated the inner room and the only probable moisture might have occurred from condensation of underground water. Microorganisms dwelling on the stupa were collected to gather the basic data that could help determine the biodeterioration of the stupa and its chemical treatment.

Collection of *Chlorococcum* resting spores and their culture

In April 2002, scrapings were made from the stone wall of the inner room and were placed in a plastic container. Resting spores were observed in the scrapings under a light microscope and a culture was established by adding spores to a modified liquid ATCC Medium 625 (http://www-cyanosite.bio.purdue.edu) of pH 7.5, containing 496 mg $NaNO_3$, 39 mg K_2HPO_4, 75 mg $MgSO_4$, 36 mg $CaCl_2$, 6 mg $FeC_6H_5O_7$ (ferric citrate), 6 mg $C_6H_8O_7 \cdot H_2O$ (citric acid monohydrate), 1 mg EDTA, a 1 litre of ddH_2O in 90 by 15-mm Petri dishes. Once unicellular progeny was released, the cells were transferred to 250 ml glass flasks or to 90 by 15-mm Petri dishes and grown on a shaking incubator (Vision Scientific Co., Ltd) at 25°C with 15 μmol photons m^{-2} s^{-1} of cool-white fluorescent lighting and a 12:12 h light-dark regime.

DNA extraction and phylogenetic analysis of 18S rDNA

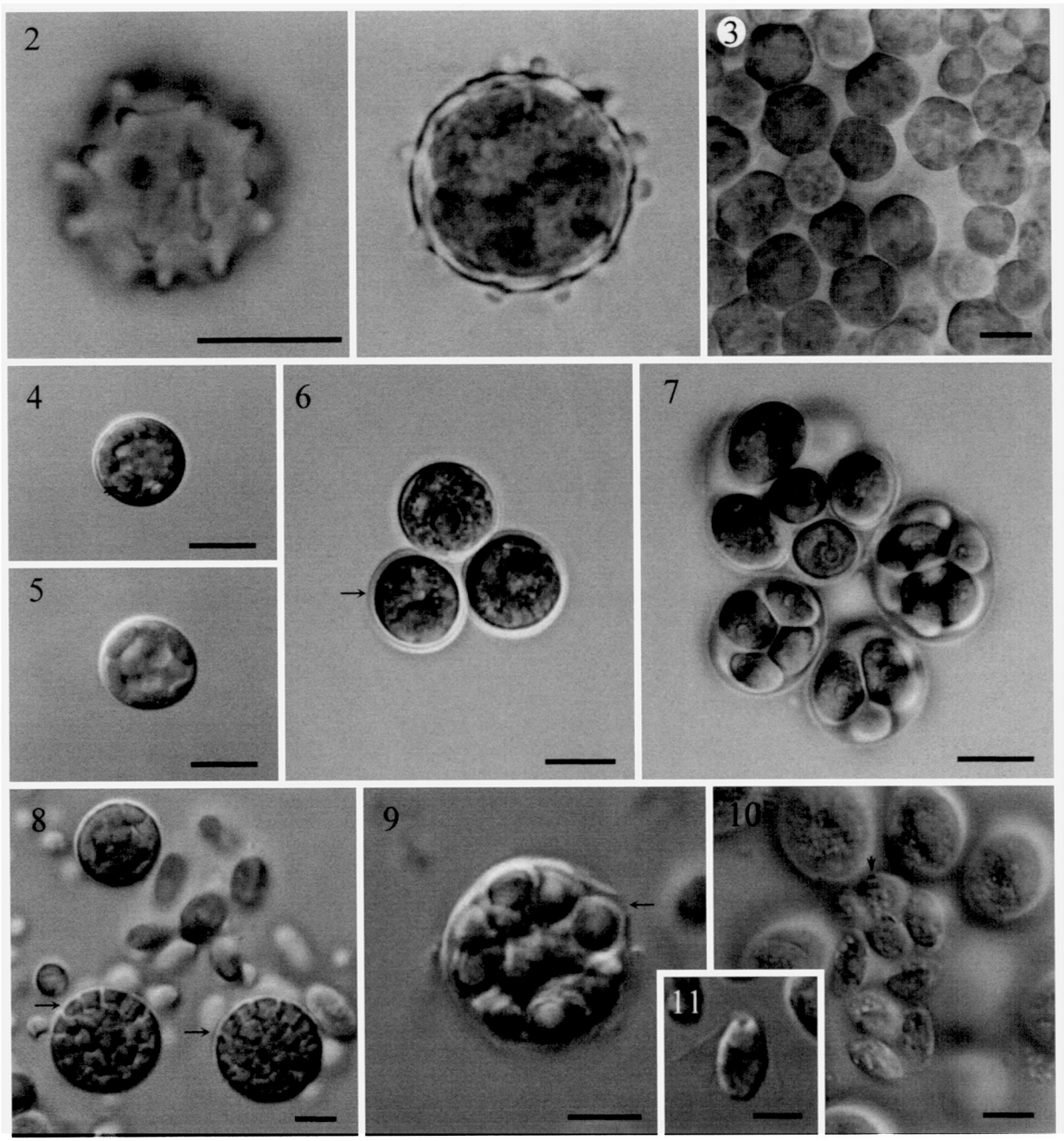
The DNA of *Chlorococcum* sp. was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Extracted DNA was stored at −20°C and used for the amplification of 18S rDNA.

The partial 18S region was amplified and sequenced with the primers SR-1 and SR-12 (Nakayama *et al.* 1996). One nested primer was newly designed for the sequencing, SSU_750F (5′-CTGTAGGACCGGAGTAATGAT-3′). The PCR products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's protocol. The sequences of the forward and reverse strands were determined using an ABI PRISM 377 DNA Sequencer (Applied Biosystems Inc., Foster City, CA) at the Center for Research, Chungnam National University (Daejon, Korea). The electrophoregram outputs for each sample were checked using Sequence Navigator v. 1.0.1 software (Applied Biosystems). The 18S rDNA sequence of *Chlorococcum* sp. was collated and aligned using the multisequence editing program SeqPup (Gilbert 1999) to compare our sequence with those published previously (Buchheim *et al.* 1996, 2001; Nakayama *et al.* 1996; Krienitz *et al.* 2003, 2004; Suda *et al.*, unpublished). A total of 1546 nucleotides were determined in this study. For the phylogenetic analysis, 1665 sites were aligned together with 10 known GenBank sequences (Table 1) including gaps. *Parachlorella kessleri* (Fott & Novakova) Krienitz, Hegewald, Hepplerle, Huss, Rohr & Wolf was used as an outgroup species based on previously published 18S rDNA phylogeny (Krienitz *et al.* 2004).

A maximum parsimony tree was constructed with PAUP* (Swofford 2001) v. 4.0b10 using a heuristic search algorithm with the following settings: heuristic search with 100 random sequence-addition replicates, TBR branch swapping, MulTrees, all characters unordered and unweighted, and branches

Table 1. Organisms used in phylogenetic analysis and their GenBank accession numbers for the 18S rDNA sequences.

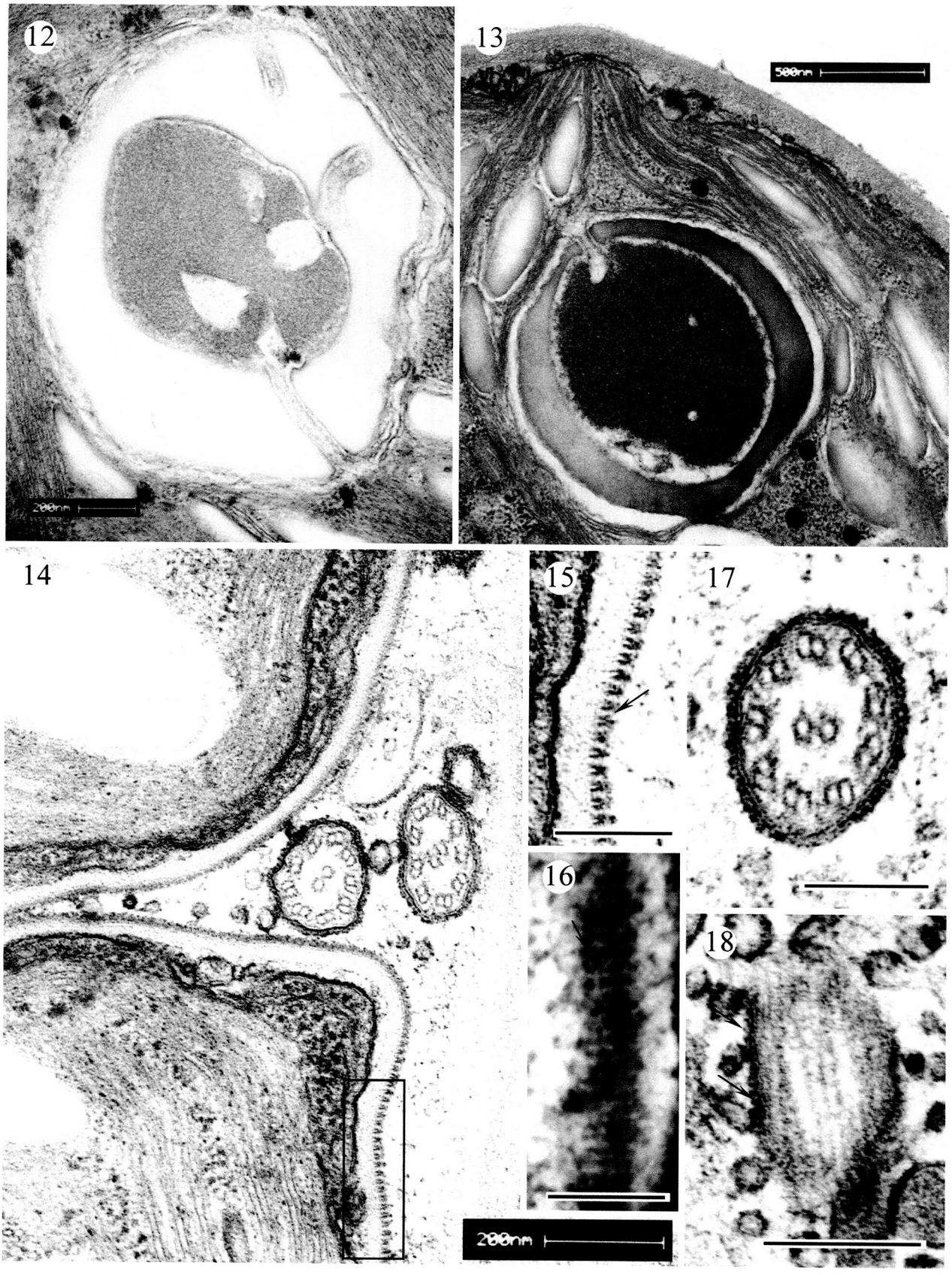
Species	Reference	GenBank accession no.
1. *Chlamydomonas* sp.	Suda *et al.* (unpublished)	AB058351
2. *Chlamydomonas* sp.	Suda *et al.* (unpublished)	AB058350
3. *Chlamydomonas chlamydogama* Bold	Buchheim *et al.* (unpublished)	AY220560
4. *Chlorococcum* sp.	Suda *et al.* (unpublished)	AB058335
5. *C. littorale* Chihara, Nakayama & Inouye	Suda *et al.* (unpublished)	AB058336
6. *C. hypnosporum* Starr	Nakayama *et al.* (1996)	U41173
7. *Chlorococcum echinozygotum* Starr	Buchheim *et al.* (1996)	U57698
8. *C. ellipsoideum* Deason & Bold	Buchheim *et al.* (2001)	U70586
9. *C. robustum* Ettl & Gärtner	Krienitz *et al.* (2003)	AY122332
10. *Chlorococcum* sp.	This study	DQ303098
11. *Parachlorella kessleri* (Fott & Novakova) Krienitz, Hegewald, Hepplerle, Huss, Rohr & Wolf	Krienitz *et al.* (2004)	AB080309



Figs 2–11. Morphology and reproduction of *Chlorococcum* cells derived from resting spores. In Figs 2–9, scale bars = 10 μm.
Fig. 2. Through-focus images of *Chlorococcum* resting spores showing surface view and middle focal plane.
Fig. 3. Progeny cells were derived from resting spores in freshwater medium.
Fig. 4. Starch sheath surrounding pyrenoid (arrowhead) observed with light microscopy.
Fig. 5. Vegetative cell with multiple chloroplast lobes.
Fig. 6. Cell wall layers thickening in old cultures (arrow).
Fig. 7. Aplanosporangia containing eight aplanospores each.
Fig. 8. Developing zoosporangia (arrows).
Figs 9–10. Zoospores were set free from an aperture at the cell surface (Fig. 9, arrow) or by cell wall rupture (Fig. 10).
Fig. 10. Notice the eyespot of the zoospores (arrowhead). Scale bar = 5 μm.
Fig. 11. Motile zoospore with flagella. Scale bar = 3 μm.

with a maximum length of zero collapsed to yield polytomies. The bootstrap values for the resulting nodes were assessed using bootstrapping with 2000 pseudoreplicates performed by 10 random sequence-additional heuristic searches.

Parameter values of the substitution model for maximum likelihood and Bayesian analyses were estimated by PAUP* from the maximum parsimony seed tree. A maximum likelihood tree was constructed under the selected model by the



heuristic search option with 100 random sequence-additions. Other settings for the heuristic search were same as those in the parsimony search. Bootstrap replications were performed using PAUP* to complete 500 pseudoreplicates by heuristic search option with 2 random sequence-additions.

Bayesian analyses were performed using MrBayes 3.0 (Huelsenbeck & Ronquist 2001). Each analysis was initiated from a random starting tree and the program was set to run four Markov chain Monte Carlo iterations simultaneously for 2,000,000 generations with trees sampled every 100th generation. The likelihood scores stabilized at approximately 300,000 generations, so the first 3000 trees were burned. The last 17,000 trees were compiled to the 50% majority rule consensus tree to obtain the Bayesian posterior probabilities.

Application of fluorescent probes

For nuclear staining, DAPI (4',6-diamidino-2-phenylindole, Sigma) was used. The cells were placed in 5 μg ml^{-1} DAPI solution in ATCC Medium for 5 min and then heated in a microwave for a few seconds. After staining, cells were mounted on slides in the DAPI solution and were examined with an Olympus BX50 microscope under a UV filter (U-MWG, 330–380 nm excitation filter, 420 nm barrier filter).

To verify cell viability, one drop of fluorescein diacetate solution (FDA; Sigma; 1–5 mg ml^{-1} in acetone) was diluted in seawater and then was applied to the cells for 5 to 10 min. These were examined with an Olympus BX50 microscope under a UV filter. FDA staining indicates activity of esterases in living cells (Oparka & Read 1994). After incubation in FDA solution living cells fluoresced yellowish-green when excited under a UV light, whereas dead cells appeared red due to the autofluorescence of chlorophyll.

Salinity experiment

To investigate the tolerance of *Chlorococcum* to high salinity, the cells from freshwater culture (ATCC Medium) were collected in a plastic tube, centrifuged, and washed two times with IMR medium (1 litre of autoclaved enriched seawater pH 8, containing 50 mg $NaNO_3$, 6.8 mg KH_2PO_4, 1 ml trace metal chelator stock solution, and 100 μl vitamin stock solution). The trace metal chelator stock solution of pH 7.5 contained 1 g $FeCl_3$, 0.62 g $MnSO_4$, 0.25 g $ZnSO_4$, 0.13 g $Na_2MoO_4 \cdot 2H_2O$, 4 mg $CoCl_2 \cdot 6H_2O$, 4 mg $CuSO_4 \cdot 5H_2O$, 6 g EDTA, and 1 litre ddH_2O. The vitamin stock solution contained 10 mg thiamin, 0.1 mg cyanocabalamin, 0.1 mg biotin, and 100 ml ddH_2O. After the wash, the cells were transferred to IMR medium and grown in 90 by 15-mm Petri dishes in the same conditions as described above.

Cell viability was verified using FDA staining. The percentage of living cells in freshwater culture before their transfer to seawater was estimated first using the equation % = 100 ÷ total number of cells × number of fluorescing cells (yellowish-green fluorescence of FDA). This was considered to be the initial percentage of living cells (100%), and the percentage of cells that remained alive after transfer to seawater was calculated at various times after transfer using the equation % = 100 ÷ % of living cells (before transfer) × % of living cells (at a given time after transfer). A fluorescing aplanosporangium was considered as one cell.

Electron microscopic observations

Cells were fixed in ATCC Medium containing 2% glutaraldehyde at 4°C for 2 h. The glutaraldehyde was then rinsed out with ATCC Medium and the cells were postfixed with 2% osmium tetroxide containing 1% $KFe(CN)_2$ at 4°C for 1.5 h. Thereafter, the cells were rinsed with ATCC Medium and were dehydrated in a graded acetone series, embedded in Spurr's epoxy resin, and polymerized overnight in a 70°C oven (Polysciences Inc.). Sections were cut with a diamond knife on a Reichert Ultracut E microtome and mounted on uncoated 200 mesh copper grids. Sections were stained with 2% uranyl acetate for 25 min and a triple lead stain for 10 min (82 ml ddH_2O, 1 g lead acetate, 1 g lead citrate, 1 g lead nitrate, 2 g sodium citrate, and 18 ml of 1 N NaOH). They were viewed and photographed on a Phillips Bio Twin Transmission Electron Microscope.

RESULTS

Collection of algae

Inside the stupa, the algae covered the stone walls of the entrances leading to the inner room (Fig. 1). Collected in different parts inside the stupa were *Phormidium*, *Microcystis*, broken frustules of a pennate diatom, and dormant, warty, thick-walled spores of *Chlorococcum* sp. The resting spores were most abundant, so that the entire stone surface was covered with a green film. Many small resting spores were inside the broken diatom frustules. Smooth-walled vegetative cells of *Chlorococcum* were not found. The spot where samples were collected was dark during daytime and dry. Collected samples were placed in Petri dishes containing ATCC medium and transferred to a culture chamber. In culture only *Chlorococcum* spores remained alive, whereas cyanobacteria and diatoms did not revive.

←

Figs 12–18. Electron micrographs of *Chlorococcum* cells.
Fig. 12. Pyrenoid surrounded with thick starch sheath with channels. No chloroplast lamellae enter the pyrenoid matrix. Scale bar = 200 nm.
Fig. 13. Pyrenoid within starch-containing chloroplast. Scale bar = 500 nm.
Fig. 14. Section through the zoosporangium. Notice thick, striated zoospore cell wall. The transverse sections of a flagella pair are also shown. Scale bar = 200 nm.
Figs 15–16. Enlarged micrographs of the transverse section (Fig. 15) and tangential longitudinal section (Fig. 16) of striated cell wall (arrows). Scale bar = 95 nm.
Fig. 17. Enlarged transverse section of flagellum where it is still within the collar of the cell. Scale bar = 100 nm.
Fig. 18. Longitudinal section through flagellum. Notice that the outer surface is not smooth (arrows). Scale bar = 100 nm.

Establishment of unialgal culture of *Chlorococcum* from resting spores

The resting spores were solitary, thick-walled and warty (Fig. 2), from 6 to 15 μm in diameter (11.2 ± 2.6 μm diameter on average), light yellowish-green in color, with uniform cell contents. Empty spores were rarely found. When cultured in ATCC medium the spores stayed unchanged for 1 mo. Then every spore swelled, and its cell wall softened and became covered with slime so that outwardly it looked as if it started to decay. The whole cell contents divided next, forming numerous small cells inside the spores. Large spores contained more than 30 cells (maximum, 36). The spherical and nonmotile cells were released from an aperture at the spore surface; however, it was not clear whether this was a specialized liberation pore or rupture of the cell wall. There was marked synchrony of transformation in all spores.

Morphology, reproduction, and phylogenetic position of *Chlorococcum* cells derived from resting spores

The released cells increased in size, attaining a width of 7.8–15.2 μm (Fig. 3). They were grass-green in color, solitary or in groups, without a mucilaginous envelope. Individual cells were spherical (Figs 3–6) or ellipsoidal (Fig. 10), uninucleate, and had a single cup-shaped parietal chloroplast with one large pyrenoid (Fig. 4). The pyrenoid was covered by a continuous starch sheath with several channels (Fig. 12) and was embedded in the center of the chloroplast. In addition to the thick starch sheath surrounding the pyrenoid, the chloroplast contained numerous independent starch grains embedded in the matrix (Fig. 13). In cells agitated on the shaker, the chloroplast had multiple lobes (Figs 4–5), but these were not observed in cells grown in stationary culture (Fig. 6). The outer cell wall layer was usually thin but sometimes thickened with age (Fig. 6). The cell wall was quite thick in mature vegetative cells (200–223.8 nm) and about 50–100 nm in zoospores and aplanospores. The outer cell wall surface was striated, which could be seen in cross-sections of zoospores, aplanospores, and young vegetative cells (Figs 14–16).

The cells released from the resting spores reproduced by aplanospores (Fig. 7) for several months, with two to four or eight aplanospores in each aplanosporangium, two and four being the usual number. Reproduction by zoospores occurred later.

Zoospore formation was induced when the cells were grown in stationary culture under a dim light (1.5–2 μmol photons m^{-2} s^{-1}). The general organization of zoospores was *Chlamydomonas*-like. The zoosporangial cells contained 2–32 zoospores (Figs 8–9) that were ellipsoidal-ovoid, 3.2–3.6 μm wide and 6.4–7 μm long, with 2 equal anterior flagella, contractile vacuoles, and an orange-red eyespot (Figs 10–11). As seen in Figs 14, 17, and 18, the flagella surface was not smooth. Zoospores were liberated from an aperture at the cell surface or by rupture of the cell wall (Figs 9–10). They became motile several hours after release, and their motile period continued for several hours (Fig. 11). At quiescence, the zoospores developed into spherical or ellipsoidal vegetative cells over a period of several days. Sexual reproduction was not observed regardless of the growth conditions.

Phylogenetic analysis revealed that the *Chlorococcum* sp. collected from the Miruksazi stupa is closely related to *Chlorococcum ellipsoideum* Deason & Bold, as the bootstrap values from maximum likelihood, maximum parsimony, and Bayesian analyses were 86%, 99%, and 100%, respectively (0.65% divergence; Fig. 19, Table 2).

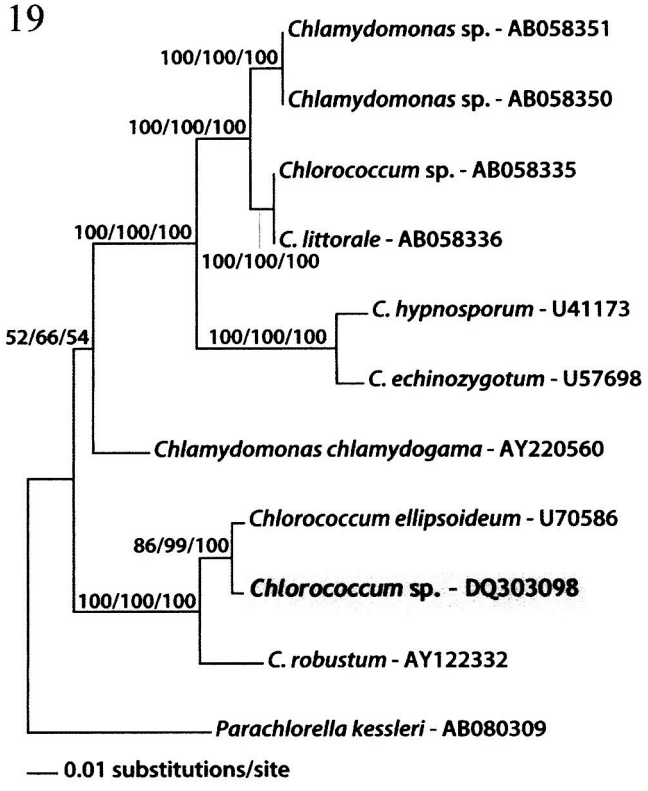


Fig. 19. The maximum likelihood tree (−ln *L* = 4516.07073) for *Chlorococcum* sp. constructed from the partial 18S rDNA sequences (1665 characters) [GRT + I + T model estimated by PAUP*; base frequencies (A = 0.2448, C = 0.2194, G = 0.2785), substitution rates (A ↔ C = 0.791303, A ↔ G = 1.532329, A ↔ T = 1.238707, C ↔ G = 0.614123, C ↔ T = 3.883597), proportion of invariable sites (0.592757), and gamma distribution shape parameter (0.573251)]. The maximum likelihood (ML) and maximum parsimony (MP) bootstrap values, and Bayesian (BA) posterior probabilities are shown above or below the branches (%, ML/MP/BA).

Salinity experiment

The tolerance of newly released *Chlorococcum* cells to high salinity was investigated (Figs 20–22). FDA staining was used to examine the viability of the cells (Fig. 23). *Chlorococcum* sp. was able to survive and reproduce in the seawater medium for more than 5 mo.

The percentage of living cells in freshwater culture (0 mmol NaCl l^{-1}) before transfer to seawater (approx. 450 mmol NaCl l^{-1}) was 23.2 ± 1.2% from the total number of cells present, and this was considered to be the initial number of living cells (100%; Fig. 23). The number of living cells dropped to 82% 1 d after transfer to seawater and to 52.6% on the second day. Thereafter, the number of living cells decreased to 37.4% until the 14th day and remained relatively constant until the 19th day. The cells increased in size up to 13–22 μm, the outer wall layer thickened slightly, the vacuole enlarged, and the cytoplasm became granular (Figs 20–21). The alpanosporan-

Table 2. Uncorrected pairwise distances of the 18S gene of *Chlorococcum* sp. from Miruksazi stupa (bold) compared with related taxa.

	1	2	3	4	5	6	7	8	9	10	11
1. *Chlamydomonas* sp. A058351	—										
2. *Chlamydomonas* sp. AB058350	0	—									
3. *C. chlamydogama* AY220560	6.28	6.28	—								
4. *Chlorococcum* sp. AB058335	1.75	1.75	6.21	—							
5. *C. littorale* AB058336	1.75	1.75	6.22	0	—						
6. *C. hypnosporum* U41173	6.02	6.02	7.30	5.96	5.96	—					
7. *C. echinozygotum* U57698	6.35	6.35	7.15	6.11	6.11	1.78	—				
8. *C. ellipsoideum* U70586	7.61	7.61	5.75	7.49	7.50	9.25	9.22	—			
9. *C. robustum* AY122332	7.33	7.33	6.54	7.57	7.57	9.75	9.71	3.09	—		
10. *Chlorococcum* sp. DQ303098	6.68	6.68	5.46	6.49	6.49	9.02	9.10	0.65	2.52	—	
11. *Parachlorella kessleri* AB080309	9.18	9.18	6.77	9.24	9.25	10.89	10.57	8.47	8.91	7.68	—

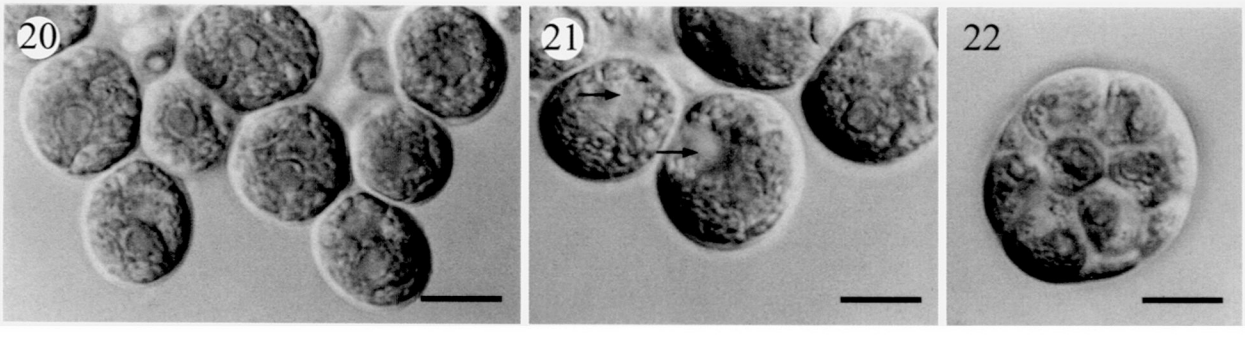
gia contained 2–16 aplanospores (Fig. 22), whereas in freshwater the maximum number of aplanospores was 8 (Fig. 7). Zoospores were never observed.

The number of living cells increased up to 70.2 ± 14% at the 20th day after transfer to seawater (Fig. 23) due to release of aplanospores from the aplanosporangial cells. After the massive release of aplanospores the number of living cells began to decrease gradually. The aplanosporangia were still present but not as abundant as before, and they contained two to four aplanospores. Living cells changed from grass green to yellowish-green and then brownish in color at 3 mo after transfer, and numerous orange globules, presumably secondary carotenoids, were observed in the cytoplasm. Such change in color was not observed in individual cells from the old freshwater cultures initiated from the resting spores, although they sometimes contained yellow-orange globules in small amounts. At all times when cells were transferred to seawater and then returned to ATCC medium, the cells revived and grew normally again.

DISCUSSION

A terrestrial green alga, *Chlorococcum* sp., was one of the dominant organisms causing biodeterioration of the Miruksazi stupa and it was abundant on the stone walls of the inner room. There are reports on the effect of algae on the biodeterioration of stone (Brook 1968; Grant 1982; Young & Urquhart 1998; Tomaselli *et al.* 2000; Kim *et al.* 2001). In particular, members of Chlorococcales, including *Chlorella, Chlorococcum, Scenedesmus,* and others are known to cause etching of the minerals (Welton *et al.* 2003).

Phylogenetic analysis revealed affinity of *Chlorococcum* from Miruksazi stupa with *C. ellipsoideum,* as the maximum likelihood, maximum parsimony, and Bayesian analyses values were 86%, 99%, and 100%, respectively. However, there were dissimilarities between our isolate and *C. ellipsoideum.* In our isolate, the outer cell wall layer sometimes thickened with age, but it was not as great as 5 μm thick as in *C. ellipsoideum.* In old cultures of *C. ellipsoideum,* the protoplast



Figs 20–22. The effect of high salinity on *Chlorococcum.* Scale bars = 10 μm.
Fig. 20. Granular appearance of cytoplasm following suspension in seawater.
Fig. 21. Cells increased in size, the outer wall layer slightly thickened and the vacuole enlarged (arrows) by the 14th day after transfer to seawater.
Fig. 22. Aplanosporangium containing 16 aplanospores grown in seawater.

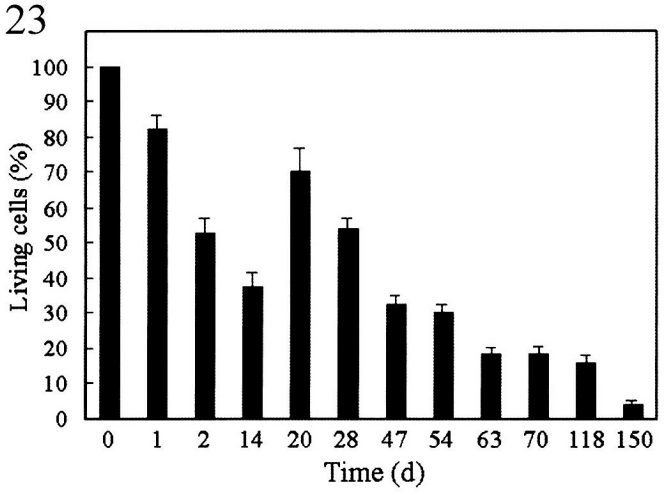


Fig. 23. Percentage of living *Chlorococcum* cells in seawater counted with FDA staining over time course.

became obscured by rather large droplets of orange-yellow oil and the cells changed from green to yellow-orange in color. Such changes were not observed in several-months-old cultures of our *Chlorococcum.* Finally, the size of zoospores was 3.2–3.6 by 6.4–7 μm in our isolate and 2.5–3.0 by 7–9 μm in *C. ellipsoideum.* The cells of our *Chlorococcum* looked different from those of *C. ellipsoideum* shown by Deason & Bold (1960). Therefore, we used the name *Chlorococcum* sp. for our isolate to distinguish it from *C. ellipsoideum* until further information is available to assign a correct name to it. The genus *Chlorococcum* still requires major phylogenetic studies because there is considerable confusion and disagreement regarding the limits of the 100 or more described species (John & Tsarenko 2002).

Drought resistance of a soil *Chlorococcum* species has been attributed to the presence of highly viscous protoplasts with small vacuoles and very low water contents (Shields & Durrell 1964; Ahmadjian 1967). Drought resistance can also be attributed to hypnospores and hypnozygotes in some taxa (Graham & Wilcox 2000; John & Tsarenko 2002). Unfortunately, we could not perform studies on the ultrastructure of the resting spores in our isolate because collection of additional samples from the stupa was prohibited. Ultrastructural studies on the cells that derived from the resting spores revealed a thick, layered, striated cell wall. The cell wall structure might have contributed to the drought resistance of this species. The cells of most representatives of the Chlorococcales are surrounded by a cellulose cell wall, but some taxa may have chitin or sporopollenin deposited on the wall (e.g. Shubert 2003), which adds strength and is believed to prevent desiccation (Graham & Wilcox 2000). The cell walls of *Chlorococcum* are known to contain cellulose and glycoprotein rich in hydroxyproline (Miller 1978).

We determined that the resting spores were not hypnozygotes (zygospores) for the following reasons. *Chlorococcum* isogametes are similar to zoospores in morphology and size (John & Tsarenko 2002) and are thus designated as facultative gametes (Bold & Wynne 1985). Because the size of zoospores was 3.2–3.6 by 6.4–7 μm in our isolate, the smallest potential hypnozygote, resulting from the fusion of two isogametes, would therefore be greater than 10 μm in diameter. This exceeds the smallest diameter of the resting spores collected (6 μm, 7.6% of spores). Furthermore, sexual reproduction was not observed in the cultured cells that were derived from the spores. Trainor (1978) suggested that sufficient nutrients and water 'trigger' the formation of planospores in *Chlorococcum,* whereas in a dry soil, aplanospores would develop. Therefore, it is likely that vegetative cells derived from the aplanospores developed thickened, warty cell walls to survive desiccation.

The morphology of *Chlorococcum* resting spores resembled that of the hypnozygotes of *Chlamydomonas nivalis* (Bauer) and *Volvox* sp. because they had multiple warts all around the cell surface, unlike Zygnematalean hypnozygotes (Graham & Wilcox 2000) and akinetes of a soil alga *Chlorosarcina longispinosa* Chantanachat & Bold (Chantanachat & Bold 1962), which had multiple long, sharp spines. However, a spiny or warty surface is not characteristic of all green algal resting cells because drawing of the hypnozygote of *Chlorococcum echinozygotum* (Chung 1993) showed that it had regular pentagonal ornamentation on the cell surface. This supports the statement by Shubert (2003) that wall ornamentation may not be a stable character with some taxa, particularly at the species level. To our knowledge, formation of vegetative cells with thickened ornamented cell walls in harsh environmental conditions was not previously recorded in *Chlorococcum* (e.g. Graham & Wilcox 2000; John & Tsarenko 2002; Shubert 2003) and supplements the taxonomic characteristics of this genus.

The formation of resting stages has been considered extensively in studies of the marine and freshwater microalgae, including dinoflagellates, diatoms, chrysophytes, and euglenoids, whereas information on the resting stages of nonmotile coccoid and colonial greens, particularly the chlorococcalean algal group, is sparse and in most cases not supported by microphotographs. Information on resting stage formation and later recruitment in response to fluctuating environments is important due to the ability of chlorococcalean taxa to produce blooms in the field (Johnson *et al.* 2003; Shubert 2003).

The synchronized pathway of spore transformation for *Chlorococcum* sp. in freshwater medium is interesting. Although the resting spores were alive, they were not stained with DAPI and FDA, even with the use of microwave fixation and detergent treatment. Our conclusion is that the cell wall was impermeable to these chemicals. It is noteworthy that formation of the resting spores could not be induced by drought in the laboratory, perhaps because conditions were different from those in the stupa.

The terrestrial *Chlorococcum* sp. possessed a high capacity to survive in darkness and drought condition for more than 5 yr and to revive in freshwater medium. Our assumption was that this alga would be tolerant to unfavorable environments in general. We investigated its tolerance to high salinity by growing cells in seawater and examining their viability with FDA staining. When *Chlorococcum* sp. grown in freshwater medium was transferred directly into seawater medium, it grew and reproduced by aplanospores for more than 5 mo, although its growth rate was significantly reduced. At all times after transfer from freshwater to seawater the cells could start normal growth when transferred to freshwater medium again. Studies by Blackwell & Gilmour (1991a, b) also showed that marine species *C. submarinum*

successfully grew in a wide range of salinities. The ability to tolerate salinity stress is, therefore, found in both marine and freshwater/terrestrial species of *Chlorococcum*, although there are some differences in the degree of tolerance among them (Blackwell & Gilmour 1991a, b; Masojidek *et al.* 2000). Some chlorococcalean algae, including *Chlorella*, *Ankistrodesmus*, and *Scenedesmus* from saline water bodies were also favored by increased salinity (Dash *et al.* 1995). Freshwater alga *Chlorella emersonii* Shihira & Kraus could grow in 335 mmol NaCl l^{-1} although the growth rate was reduced by 30–40% (Setter & Greenway 1979). Related algae, *Chlamydomonas pulsatilla* Wollenweber and *Dunaliella* spp., were also capable of growing in a wide range of salinities (Hellebust & Lin 1989; Fisher *et al.* 1997). Thus, our finding is consistent in this regard.

In conclusion, this study has demonstrated that *Chlorococcum* sp. can tolerate various unfavorable environments such as prolonged desiccation, darkness, and salinity stress. The present results and other studies (Shields & Durrell 1964; Blackwell & Gilmour 1991a, b) may explain the ubiquity of this genus in almost every biome.

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