

Interactions between marine facultative epiphyte *Chlamydomonas* sp. (Chlamydomonadales, Chlorophyta) and ceramiacean algae (Rhodophyta)

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Abstract: Previously unrecorded marine *Chlamydomonas* that grew epiphytic on ceramiacean algae was collected from the western coast of Korea and isolated into a unialgal culture. The isolate was subjected to 18S rDNA phylogenetic analysis as well as ultrastructure and life cycle studies. It had an affinity with the marine *Chlamydomonas* species and was less related to freshwater/terrestrial representatives of this genus. It had flagella shorter than the cell body, two-layered cell wall with striated outer surface and abundant mucilaginous material beneath the innermost layer, and no contractile vacuoles. This alga grew faster in mixed cultures with ceramiacean algae rather than in any tested unialgal culture condition; the cells looked healthier and zoosporangia and motile flagellated vegetative cells appeared more often. These results suggested that this *Chlamydomonas* might be a facultative epiphyte benefiting from its hosts. Several ceramiacean algae were tested as host plants. Meanwhile, cell deformation or collapse of the whole thallus was caused to *Aglaothamnion byssoides*, and preliminary study suggested that a substance released from *Chlamydomonas* caused the response. This is first report on harmful epiphytic interactions between *Chlamydomonas* species and red ceramiacean algae.

Key words: *Aglaothamnion*, Ceramiacean algae, *Chlamydomonas*, Host-epiphyte interaction, Ultrastructure

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Introduction

Members of the genus *Chlamydomonas* may be found in practically all water types, on soil or in its upper layers, and on the bark of trees (Graham and Wilcox, 2000; John and Tsarenko, 2002). Little is known about the ecology of the majority of the species but many are abundant in nutrient-rich waters. A small number of *Chlamydomonas* species inhabit snow-fields and some are described as symbionts of foraminifers (Lee *et al.*, 1974; John and Tsarenko, 2002; Pocock *et al.*, 2004). Some species are well adapted to grow in unfavorable environments, *i.e.* *C. acidophila* in acidic freshwater of pH 3.3 (Rhodes, 1981), *C. pitschmannii* in thermoacidic freshwater, or *C. pulsatilla* in saltwater, brackish and fresh waters (www.bio.utulsa.edu). Some taxa are epiphytic, *i.e.* freshwater *C. dinobryonii* inhabiting empty *Dinobryon* loricas, *C. epiphytica* adhering to *Microcystis* colonies, and marine *C. olifanii* (Konovalova, 1999) and *C. epibiotica* (Ettl, 1976) living on other organisms.

In the past decades *Chlamydomonas* has become the premier model system for diverse areas of cell and molecular biology, although only a few representatives were studied in comparison to a total number of described species. Over five hundred *Chlamydomonas* taxa were recognized, mostly freshwater and terrestrial, whereas data on the marine and brackish members (~35 species) have been fragmentary (Ettl, 1976; Hirayama *et al.*, 2001). Many species were identified on the basis of morphological observations, including size and shape of the cell, shape and position of the chloroplast and pyrenoids, length of the flagella, papillae shape, number of contractile vacuoles, and other visible features (Butcher,

1959; Dangeard, 1965; Cann and Pennick, 1982; Ettl, 1976, 1983; Hirayama *et al.*, 2001). However, identification of *Chlamydomonas* in the traditional sense poses difficulties at present, since Pröschold *et al.* (2001, 2005) proposed *C. reinhardtii* as conserved type of *Chlamydomonas* so that taxa not related to 'Reinhardtii' clade would have to be excluded from the genus (Pröschold *et al.*, 2001, 2005; Pröschold and Silva, 2007).

In the autumn of 2003 we isolated a unique *Chlamydomonas*-like microalga, which was epiphytic on ceramiacean algae from the western coast of Korea. Although our phylogenetic data reveal that this strain belongs within 'Moewusii' clade according to classification of Pröschold *et al.* (2001), we use the traditional name *Chlamydomonas* sp. until further information is available, since its morphology is typical to the genus. Contamination with this microalga caused cell deformation or collapse in some red algae and these seemed to be caused by a substance released from this *Chlamydomonas* strain. The present paper aims to describe epiphyte's morphology, reproduction, and 18S rDNA molecular phylogeny since this microalga was previously unrecorded, and also results of its epiphytism on the ceramiacean algae.

Materials and Methods

Culture of *Chlamydomonas* strain: The cells of *Chlamydomonas* sp. (hereafter: *Chlamydomonas* sp. KNU-M-2003-C2) were found as a contaminating epiphyte in the culture of *Aglaothamnion* spp. collected in Daechon, Korea in September 2003 (Table 1). Three different culture media were used to maintain the unialgal culture of



Table - 1: List of algae used in this study, their collection site or source and conditions for laboratory culture

Species	Conditions for laboratory culture			Collection site and date
	Temperature (°C)	Irradiance, $\mu\text{mol photons m}^{-2} \text{s}^{-1}$	Light: Dark photoperiod (hr)	
<i>Aglaothamnion byssoides</i> (Amott ex Harvey) L'Hardy-Halos et Ruen Le Jolis (male and female plants, tetrasporophytes, carposporophytes)	15, 23	15	12 : 12	UTEX culture collection
<i>Aglaothamnion oosumiense</i> Itono (male and female plants)	15, 23	15	12 : 12	Eochungdo (Korea) June 1994, Daecheon (Korea) September 2003
<i>Antithamnion callocladus</i> Itono (male plants)	15, 23	15	12 : 12	Shimoda (Japan) May 1995
<i>Antithamnion nipponicum</i> Yamada et Inagaki (male plants)	15, 23	15	12 : 12	Kachen (Korea) May 1995, Daecheon (Korea) September 2003
<i>Antithamnion sparsum</i> Tokida (male plants)	15, 23	15	12 : 12	Kangreung (Korea) July 1986
<i>Pterothamnion yezoense</i> (Inagaki) Athanasiadis and Kraft (tetrasporophytes)	15, 23	15	12 : 12	Songsanpo (Korea) 1998
<i>Chlamydomonas</i> sp. KNU-M-2003-C2	12, 15, 20, 23	<2-4, <15-30	12 : 12	Daecheon (Korea) September 2003

Table - 2: List of organisms used in phylogenetic analysis and their GenBank accession numbers for the 18S rDNA sequences

Species name	Habitat	GenBank accession number
<i>Chlamydomonas parkeae</i> Ettl	Saltwater	DQ009747
<i>Chlamydomonas hedleyi</i> Lee, Crockett, Hagen et Stone	Brackish or saltwater, symbiotic	DQ009746
<i>Chlamydomonas</i> sp. MBIC 10471	Saltwater	AB058350
<i>Chlamydomonas</i> sp. KNU-M-2003-C2 (this study)	Brackish or saltwater	DQ459878
<i>Chlamydomonas noctigama</i> Korschikov	Freshwater	AF008242
<i>Chlamydomonas bilatus</i> Ettl	Freshwater	AF517098
<i>Chlamydomonas moewusii</i> Gerloff	Freshwater	U70786, U41174
<i>Chlamydomonas pitschmannii</i> Ettl	Aero-terrestrial, thermoacidic freshwater	U70789, AJ628982
<i>Chlamydomonas acidophila</i> Negoro	Acid freshwater	AJ628977
<i>Chlamydomonas pseudogloeogama</i> Gerloff	Aeroterrestrial, freshwater	AF517097
<i>Chlamydomonas pulsatilla</i> Wollenweber	Saltwater, brackish or freshwater	AF514404
<i>Chlamydomonas chlamydogama</i> Bold	Freshwater	AY220560
<i>Chlamydomonas incerta</i> Pascher	Freshwater	AY781664
<i>Chlamydomonas reinhardtii</i> Dangeard	Freshwater	AY665726
<i>Chlamydomonas debaryana</i> Goroschankin	Aeroterrestrial, freshwater	AF008240
<i>Chlamydomonas rubrifilum</i> Korschikov	Aeroterrestrial, freshwater	AJ410455
<i>Chlamydomonas tetragama</i> (Bohlin) Ettl	Freshwater	AB007370
<i>Bracteacoccus aerius</i> Bischoff et Bold	Aeroterrestrial, freshwater	U63101
<i>Bracteacoccus minor</i> (Chodat) Petrova	Aeroterrestrial, freshwater	U63097

Chlamydomonas: modified Provasoli's medium (MPM/2, West and McBride, 1999), IMR medium (Klochkova et al., 2006), and modified TAP (Tris-Acetate Phosphate) medium of pH 7.2, including 2.42 g of Tris base, 12.5 ml of Filner's Beijerincks Solution (FBS), 5 ml of KPO_4 mix, 5 ml of Trace Mineral Solution (TMC), 1.0 ml of glacial acetic acid, and 980 ml of filtered sterilized seawater. Composition of

FBS, KPO_4 mix, and TMC solutions is referred to www.lclark.edu/~bkbaxter/200lab/chlamy_recipes.htm.

Host-epiphyte interactions: The red algae used in this experiment and conditions for their laboratory culture are given in Table 1. All red algal isolates were maintained in IMR medium. Studies of

Table - 3: Comparison between some marine and brackish *Chlamydomonas* and strain used in this study

Species	Cell size (µm)		Eyespot	Contractile vacuole
	Motile / Non-motile			
<i>Chlamydomonas</i> sp. KNU-M-2003-C2 (this study)	3.2-6.4 x 2.6-4.6 / 3.2-6.4 x 2.6-4.6		Present	Absent
<i>C. concordia</i> Green, Neuville et Daste ^{7,9}	10-15 (cell diameter)		?	2
<i>C. parkeae</i> Ettl ¹⁰	5-9 (cell diameter)		Present	Absent
<i>C. perigranulata</i> Hirayama, Ueda, Nakayama et Inouye ⁶	7-12 x 5.5-9 / 10-16		Present	Absent
<i>C. semiample</i> Butcher ¹	6-8 x 4.5-6 / 6.5-7 x 7-7.5		Present	Absent?
<i>C. bullosa</i> Butcher ^{1,2}	9-12 x 7-8 / 15-20		Present	Absent
<i>C. kuwadae</i> Gerloff ^{1,3,4}	9-15 x 7-11 / ND		Present	2
<i>C. uva-maris</i> Butcher ¹	8-15 x 6-8 / 9 x 8		Present	Absent
<i>C. salinophila</i> Ettl ^{3,5}	8-10 x 7-8 / ND		Present	Absent
<i>C. subehrenbergii</i> Butcher ¹	6-9 x 5-7 / 10-12		Present	Absent
<i>Chlamydomonas</i> sp. (CCMP 220) ⁸	ND / ND		Present	Absent
<i>Chlamydomonas</i> sp. (CCMP 222) ⁸	5-10 x 7-14		Present	Absent
<i>Chlamydomonas</i> sp. (CCMP 230) ⁸	ND / ND		Absent	Absent
<i>Chlamydomonas</i> sp. (CCMP 235) ⁸	5-9 x 6-10		Present	Absent
<i>Chlamydomonas</i> sp. (CCMP 236) ⁸	2-4 x 5-7		?	Absent
<i>Chlamydomonas</i> sp. (CCMP 1236) ⁸	4-6 x 6-8		Present	Absent

ND - no data available. ¹Butcher (1959). ²Cann and Pennick (1982). ³Ettl (1976). ⁴Ettl (1983). ⁵Dangeard (1965). ⁶Hirayama *et al.* (2001). ⁷Biegala *et al.* (2003). ⁸www.bio.utulsa.edu/ccmp.bigelow.org, ⁹planktonnet.sb-roscoff.fr, ¹⁰seasquirt.mbio.co.jp

interactions of *Chlamydomonas* sp. with ceramiacean algae were performed as follows; to maintain a combination of host and epiphyte, *Chlamydomonas* cells were added to cultures containing other potential host algae (Table 1) in different amounts (diluted/ dense suspensions of cells) in 90 X 15-mm Petri dishes. Samples were examined daily with Olympus BX50 microscope.

To test the effect of water-soluble metabolites of *Chlamydomonas* on various ceramiacean algae, IMR medium containing *Chlamydomonas* cells from the unialgal culture was filtered after 3-4 weeks. The filtered solution was centrifuged two times at 2,000 g for 2 min to remove remaining *Chlamydomonas* cells. The red algae from clean unialgal cultures (Table 1) were transferred into the *Chlamydomonas*-culture filtrate and observed daily under the Olympus BX50 microscope.

DNA extraction and phylogenetic analysis of 18S rDNA: The DNA of *Chlamydomonas* sp. KNU-M-2003-C2 was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The 18S region was amplified and sequenced with the primers SR1-SR7 and SR4-SR12 (Nakayama *et al.*, 1996). 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, USA). The electrophoregram outputs for each sample were checked using Sequence Navigator v. 1.0.1 software (Applied Biosystems, Korea). The 18S rDNA sequence was aligned using the multisequence editing program, SeqPup (Gilbert, 1999), to compare with those deposited to the GenBank (Table 2). *Bracteacoccus aerius* and *B. minor* were used as outgroup species.

Bayesian analysis were conducted with MrBayes v.3.1 (Ronquist and Huelsenbeck, 2003) using the general time reversible (GTR) + the shape parameter of the gamma distribution (Γ) + proportion of invariable sites (I) model. This model of sequences evolution was chosen based on results from Modeltest v.3.6 (Posada and Crandall, 1998). The Akaike information criterion (AIC) selected GTR + Γ + I model as the best-fitting model for the SSU data. The GTR rates, the shape parameter of the gamma distribution and proportion of invariable site value were not fixed. For the data matrix, two independent 1.5 million generations were performed with four chains and trees sampled every 100 generations. The burn-in period can be identified graphically by tracking the likelihoods at each generation to determine whether the likelihood values reach a plateau. After preliminary analyses, a burn-in period of 500,000 generations was determined to be appropriate for the data. The 20,000 trees sampled at stationarity were used to infer the Bayesian posterior probability. Majority-rule consensus trees were calculated using PAUP*.

Application of fluorescent probe: For cell wall staining, Calcofluor White M2R (Fluorescent Brightener 28, Sigma) was diluted in PBS (phosphate-buffered saline; Kim *et al.*, 2006) buffer to a concentration of 0.1 mg ml⁻¹, and *Chlamydomonas* cells were incubated in this solution for 5 min. After washing with seawater the cells were resuspended in IMR medium and examined with Olympus BX50 microscope under the UV filter.

Electron microscopic observations: Cells were fixed in PBS buffer containing 2% glutaraldehyde at 4°C for 2 hr. The glutaraldehyde was then rinsed out with PBS buffer and the cells were postfixated with 2% osmium tetroxide containing 1% KFe(CN)₂ at 4°C for 1.5 hr. Thereafter, the cells were rinsed out with PBS buffer and were dehydrated in a graded acetone series, embedded in



Spurr's epoxy resin and polymerized overnight in a 70°C oven (Polysciences Inc., USA). Sections stained with uranyl acetate and Reynolds's lead citrate (Reynolds, 1963) were viewed and photographed on a Phillips Bio Twin Transmission Electron Microscope.

Results and Discussion

Isolation of the strain, observations of morphology and reproduction, phylogenetic position: A unique marine *Chlamydomonas* strain KNU-M-2003-C2 was found during the culture of *Aglaothamnion* and *Antithamnion* species collected from Daechon (western coast of Korea), and contaminated newly added red algae quickly (Table 1). This contamination made all the red algal isolates weak. *Chlamydomonas* cells were detached from the thalli of *Aglaothamnion* sp. with a brush and isolated into a unialgal culture (Fig. 1).

This *Chlamydomonas* strain could grow in salinity ranges from 150 to 450 mM NaCl. In salinity lower than 150 mM the cells swelled and subsequently died. In order to establish the most suitable condition for the unialgal culture of this strain different culture media were used to grow cells either on shaking incubator or in stationary culture. The cells showed no difference in the growth rate in MP/2 and IMR media and mostly stayed non-motile (99.2±0.3%). When cultured in the modified TAP medium that included glacial acetic acid many cells developed flagella and became motile; however the growth rate did not increase as comparing to MP/2 or IMR media, even though TAP is a rich medium that allows *Chlamydomonas* to grow either heterotrophically or photoheterotrophically.

The growth of *Chlamydomonas* cells was much faster when they were maintained in mixed cultures with ceramiacean algae (Table 1, Fig. 1A, E), and the zoosporangia and motile flagellated vegetative cells (Fig. 1D) appeared more often. The mixed culture was obviously better growth condition for this *Chlamydomonas* strain than unialgal culture in all conditions tested in different media, temperatures, light intensities, and in stationary culture and shaking incubators.

The motile cells were morphologically similar to non-motile cells, except for the presence of flagella (Fig. 1C-D). Cell body was ellipsoidal in lateral view, from above circular, small anterior papilla was present. In all culture conditions most cells did not develop flagella and remained at the bottom of the culture vessels or attached to the host algae (Fig. 1A). The cell size was same in motile and non-motile cells; 3.2-6.4 µm in length and 2.6-4.6 µm in width, which is significantly smaller than other saltwater *Chlamydomonas* (Table 3). The flagella were shorter than the cell body, inserted apically (Fig. 1D). The cross-sections of flagella revealed the typical 9 + 2 tubule construction. The proportion of non-motile cells was higher in this isolate, but most cells had developed basal bodies beneath the cell wall. This implies that the alga is basically motile in its vegetative phase, but this particular species may have developed the tendency toward being non-motile in the vegetative stage. The contractile vacuoles were absent and did not appear when the cells were

incubated for 2 d in IMR medium diluted with double-distilled water to 2/3 and 1/3 (<150 mM NaCl). The vacuoles seem to be often absent in the saltwater and brackish *Chlamydomonas* (Table 3) and are present in a majority of freshwater taxa. Several large lipid droplets were present in the cytosol (Fig. 2). The cells freshly inoculated in a new medium had less lipid droplets, but they began to appear after several days of culture. The cells sometimes contained bright yellow-orange globules in the cytosol.

Subdivision of the genus *Chlamydomonas* into nine major morphological groups according to chloroplast shape and pyrenoid number (Ettl, 1976), which was traditionally used as a taxonomic character, was not readily applicable to this isolate. As ultrastructural studies showed, it had a single grass-green parietal chloroplast belonging to the *Chlorogoniella* group, occupying at least half of cell (Fig. 2A), with one large pyrenoid surrounded by 3 to 4 starch grains (Fig. 3A-B). But sometimes cells with a chloroplast of cup- and saucer-like shapes could be seen too. Therefore, variable taxonomic characters appeared within different individuals belonging to the same species. This could have occurred because of the lipid droplets scattered in the cytosol (Fig. 2, 3C); they became larger and more abundant in the older cells and occupied more space, therefore other organelles were dislocated. The pyrenoid matrix was intersected by thylakoids. In the chloroplast matrix thylakoids were appressed together very closely. In addition to the thick starch grains surrounding the pyrenoid, the chloroplast contained independent starch bodies and plastoglobules embedded in the matrix. Hoham et al. (2002) also suggested that variation of chloroplast morphology could occur between individual species within the same population complex, as do the presence or absence and the size of eyespots. In this isolate, the eyespot (Fig. 2A) appeared bright orange at the light microscope level; it was around 1-1.1 µm in length, closely pressed up against cell membrane, with globules of varying sizes (55.5-222 nm in diameter) packed close together. Normally, the nucleus was situated next to the basal bodies (Fig. 3D), but sometimes was found at the posterior half of the cell. The Golgi body was often located nearby the nucleus (data not shown). The plasma membrane was irregular and had invaginations (Fig. 2). Similar irregular membranes were seen in the cross-sections of *C. noctigama* and *C. raudensis* (Pocock et al., 2004). The cell wall of *Chlamydomonas* was reported to be thin and smooth (John and Tsarenko, 2002; Pocock et al., 2004) except *C. reinhardtii* with seven-layered wall consisting primarily of hydroxyproline-rich glycoproteins that resembled plant extensins (Harris, 2001). The cell wall of marine *C. perigranulata* was shown to consist of three layers, with outermost layer having lamellate pattern (Hirayama et al., 2001). The cells of closely related *C. noctigama* were surrounded by a thick extracellular wall-bound matrix (Pocock et al., 2004). In *Chlamydomonas* sp. KNU-M-2003-C2, the cell wall was 66.5-91 nm thick, composed of two layers including the innermost layer and the outermost layer with very small spiny projections arranged in a striated pattern (Fig. 3E-G). Calcofluor White did not stain the outermost layer (data not shown). Calcofluor White stains a range of polysaccharides, including cellulose, β-D-glucans, xyloglucan, substituted celluloses (Wood,

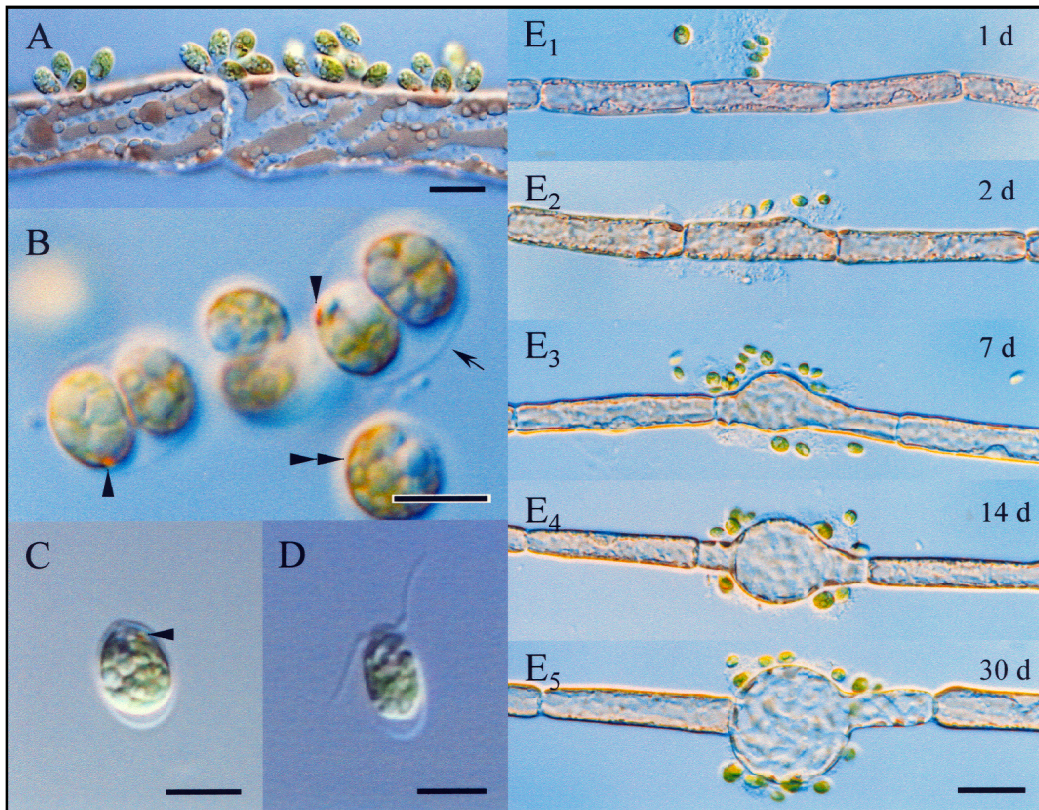


Fig. 1: Light micrographs of *A. byssoides* filaments contaminated by *Chlamydomonas* sp. KNU-M-2003-C2. A. *Chlamydomonas* cells attached to the red algal filament by anterior ends. B-D. *Chlamydomonas* cells. B. Non-motile cell (double arrowhead) and aplanosporangia (arrow). Arrowheads point to the eyespot (same in Fig. C). C. Non-motile cell lacking flagella but possessing anterior papilla as in motile cells. D. Motile cell. E. Cell swelling in *A. byssoides* contaminated with *Chlamydomonas*. The times indicated on each figure represent the period, which had past since inoculation with *Chlamydomonas*. Scale bars: A, E = 20 μm , B-D = 5 μm

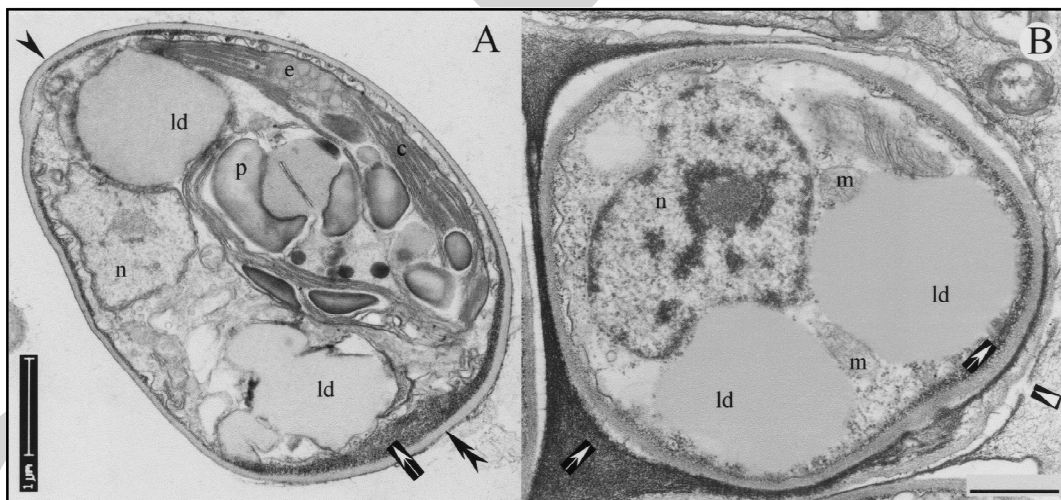


Fig. 2: Electron microscopy of *Chlamydomonas* cells, A. Longitudinal section of non-motile cell, showing major cellular organelles. Single and double arrowheads point to anterior and posterior ends of the cell, respectively. Arrow points to abundant intracellular material located beneath the cell wall. B. Cross section of aplanospore, which is still within the wall of the parent cell (arrowhead). Arrow shows abundant intracellular material filling whole space between the aplanospores. Same material is present inside the cell (arrow). c - chloroplast, e - eyespot, ld - lipid droplet, m - mitochondrion, n - nucleus, p - pyrenoid. Scale bars: A = 1 μm , B = 500 nm

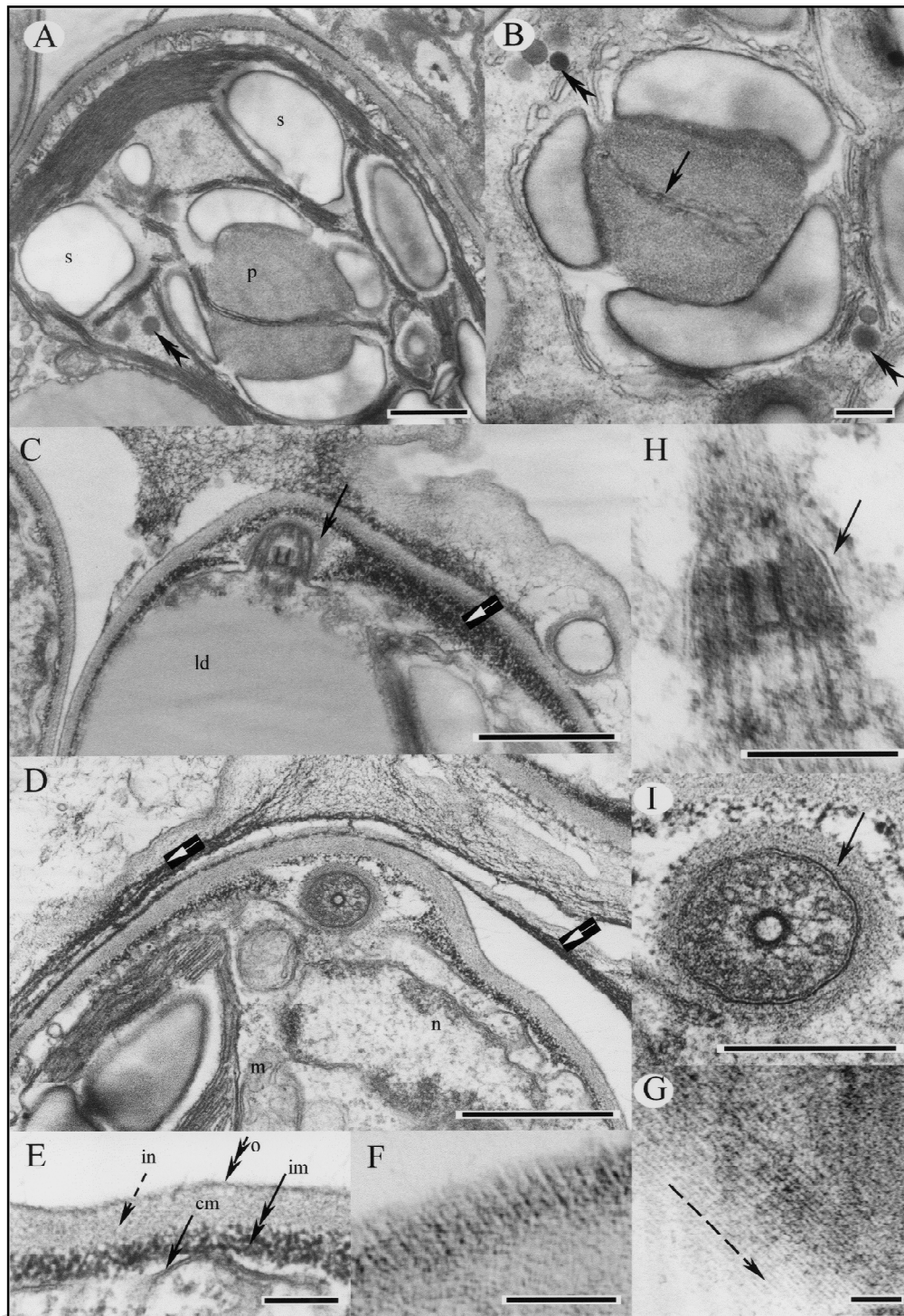


Fig. 3: Electron microscopy of *Chlamydomonas* cells. A. Chloroplast containing pyrenoid surrounded with starch grains, numerous independent starch bodies, and plastoglobules (B, double arrowheads). Thylakoids were appressed together very closely. B. Pyrenoid matrix intersected by thylakoids (arrow). C-D. Longitudinal (C) and transverse (D) sections through the cells' anterior end. Note dense material inside and outside the cells and surrounding the basal bodies (arrows). E. Cell wall composed of two layers. F-G. Tangential longitudinal section showing striated pattern of cell wall. H. Enlarged micrograph of longitudinal section through flagellum showing that its surface is not striated (arrow). I. Enlarged micrograph of transverse section of the transition region showing the stellate structure connecting with the doublets. Note dense material surrounding flagellum (arrow). c - chloroplast, cm - cell membrane, im - intracellular matrix, in - innermost layer of cell wall, ld - lipid droplet, m - mitochondrion, n - nucleus, o - outermost layer of cell wall, p - pyrenoid, s - starch. Scale bars: A - 400 nm, B, H-I = 200 nm, C-D = 500 nm, E-G = 100 nm

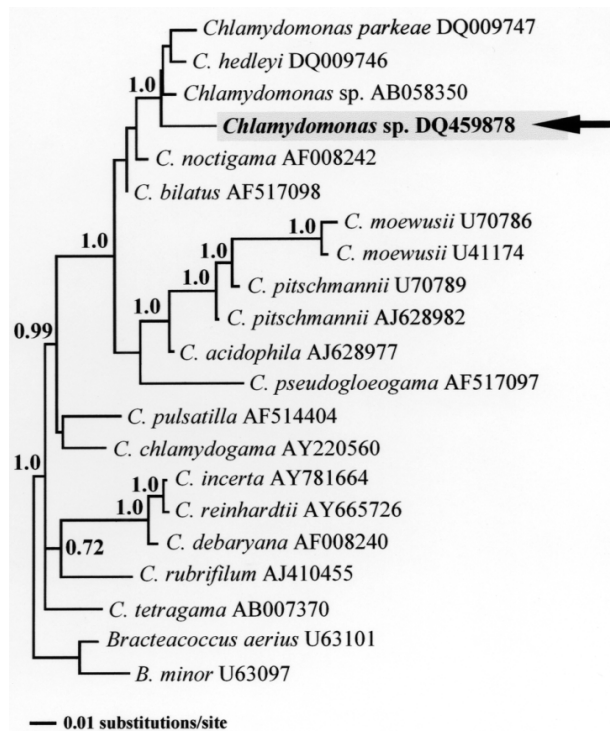


Fig. 4: The Bayesian consensus tree. The branch lengths represent expected number of substitution per site. Estimated posterior probabilities from 1 million post burn-in generations are indicated above branches. -lnL = 6209.63; RAC=0.7589, RAG=2.3722, RAT=1.1522, RCG=0.7483, RCT=4.7153, RGT=1; π A=0.2372, π C=0.2196, π G=0.2847, π T=0.2585; I=0.5966; $\Gamma(\alpha)$ =0.7081

1980), and even chitin (Ruzin, 1999). Unlike the cell surface, the flagella surface was not striated (Fig. 3H-I). We recently reported on a *Chlorococcum* sp., which had striated pattern on the cell surface similar to this *Chlamydomonas*, as well as on the zoospore flagella surface (Klochkova *et al.*, 2006). This difference in flagella fine structure is of interest because these phylogenetically related genera, *Chlamydomonas* and *Chlorococcum* [Nakayama *et al.*, 1996; Pocock *et al.*, 2004 (Fig. 3)], possess the same flagellar apparatus, with basal bodies displayed in a clockwise (CW) orientation (Lewis *et al.*, 1992).

An intracellular matrix, seemingly mucilaginous material, was present in cells in large amounts between the plasma membrane and cell wall (Fig. 2, 3 C-D). Same material covered flagella basal bodies (Fig. 3 C-D, I). When the protoplast was contracted, mucilage filled up the space 4-5.5 times thicker than the cell wall. In dividing cells, the whole space between daughter cells was filled with this mucilage (Fig. 2B), which was later released into seawater when parent wall ruptured. Thus non-motile cells often formed colonies embedded in mucilage. The mucilage is not typical for *Chlamydomonas* species except *C. bipapillata*.

In unialgal culture, cells propagated by division of protoplast into 2 to 4 daughter protoplasts released from parent as aplanospores after parent wall rupture (Fig. 1B). Most aplanospores

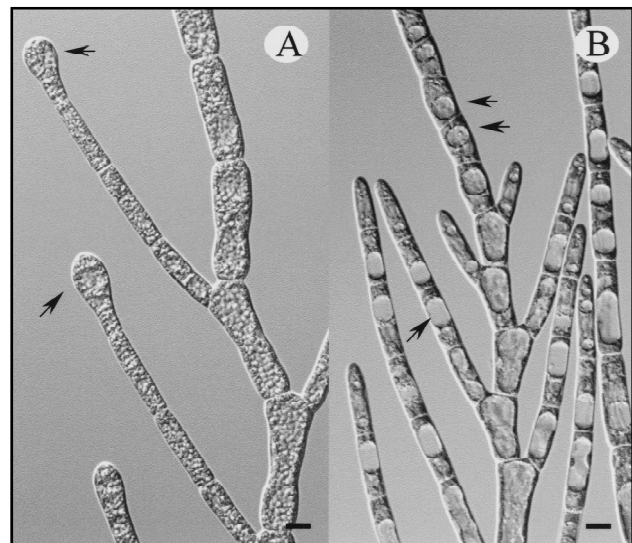


Fig. 5: Plants of *A. byssoides* grown in IMR medium collected from the culture vessels where diluted (A) and dense (B) suspensions of *Chlamydomonas* cells were kept for several weeks. A. Swelled terminal cells on the lateral branches (arrows) that developed within 2 d of incubation in the *Chlamydomonas*-culture filtrate from diluted suspension of cells. B. Vacuole collapse (arrows) and subsequent cell death occurred within 1-2 d in the *Chlamydomonas*-culture filtrate from dense suspension of cells. Scale bars: 20 μ m

had basal bodies beneath the cell wall (Fig. 3C). They could be induced to assemble flagella and become motile during several hr-long exposures to high light condition under light microscope or by adding a droplet of glacial acetic acid to the culture medium.

Reproduction by zoospores was often observed in mixed cultures with ceramiacean algae. The zoosporangial cells contained 2-32 zoospores of 2.8-5.5 μ m in length and 2.2-3.2 μ m in width. Sexual reproduction was not observed regardless of the growth conditions.

For the phylogenetic analysis, a total of 1555 nucleotides were determined and 1575 sites were aligned together with 20 known sequences (Table 2) including gaps. The 18S rDNA sequence of the isolate showed no match to those reported in the GenBank. This new isolate belonged within the sub-group of marine *Chlamydomonas* species, including *C. parkeae*, *C. hedleyi*, and *Chlamydomonas* sp. (MBIC 10471) and was less related to freshwater/terrestrial *Chlamydomonas* (Fig. 4). It belonged within so-called 'Moewusii' clade following the designation according to Pröschold *et al.* (2001) and was closely related to a saltwater *Chlamydomonas* strain MBIC 10471 and freshwater *C. noctigama*. It is interesting that *Chlamydomonas* sp. KNU-M-2003-C2 was phylogenetically placed between the marine foraminifer symbiont *C. hedleyi* and freshwater *C. noctigama*. Pawlowski *et al.* (2001) suggested *C. noctigama* to be the ancestor to foraminifer symbionts, including *C. hedleyi*. However, the morphology is distinct from *C. hedleyi* and *C. noctigama* (Pocock *et al.*, 2004). The data on morphology of *Chlamydomonas* sp. MBIC 10471 is absent and the strain is no longer available in MBIC algae collection, thus the comparison is not possible.

Host-epiphyte interactions: One of the interesting physiological characteristics of *Chlamydomonas* sp. KNU-M-2003-C2 was that it apparently colonized surfaces of other algae and also benefited from growing together with the hosts. We therefore suggested that it behaved in the same way in its natural environment as it did in artificial culture and was a facultative epiphyte.

The *Chlamydomonas* cells caused specific cell deformation or thallus collapse in some ceramiacean species. One response to contamination with *Chlamydomonas* was swelling and subsequent disruption of host cells observed in *Aglaothamnion byssoides*. When several cells of *Chlamydomonas* settled on *A. byssoides* (Fig. 1A) the cell surrounded by contaminants swelled dramatically over a period of several days (Fig. 1E) and then burst. The number of *Chlamydomonas* cells surrounding swelling *A. byssoides* cell increased noticeably according to time, which could be because damaged host leaked cytoplasm that attracted epiphytes. Another response was prompt necrosis of the whole plant by dense suspensions of *Chlamydomonas* cells ($\sim 48\text{-}65 \times 10^4$ cells per ml) within 1-2 days.

Some substances from *Chlamydomonas* released in seawater could affect *A. byssoides* directly. When healthy *A. byssoides* plants were transferred to the *Chlamydomonas*-culture filtrate from diluted suspension of cells ($\sim 20\text{-}40 \times 10^4$ cells per ml), the terminal cells on lateral branches swelled within 2 days ($40.9 \pm 9.5\%$, Fig. 5A). The growth type of *A. byssoides* is apical, and thus newly developing walls of the growing terminal cells are less rigid than in other cells of the thalli. This may explain why they were easily damaged.

The response was different when the *Chlamydomonas*-culture filtrate from dense suspension of cells ($\sim 48\text{-}65 \times 10^4$ cells per ml) was tested. First, the vacuole disrupted in almost all *A. byssoides* cells (Fig. 5B) and then every cell in the thallus collapsed within 1-2 days. On the contrary, the control plants incubated in normal IMR medium remained healthy and growing well. Therefore, a substance released from *Chlamydomonas* seemed to cause cell deformation and necrosis of *A. byssoides* even without direct host-to-epiphyte contact. Although we do not know the nature of this substance yet, the fact that these *Chlamydomonas* cells excrete lot of materials is evident from the ultrastructural studies. In aplano- and zoosporangia, thick intracellular mucilage occupied whole space between the daughter cells and was released to seawater upon the rupture of the parent wall.

Species *Aglaothamnion oosumiense*, *Antithamnion callocladus*, *A. nipponicum*, *A. sparsum* and *Pterothamnion yezoense* were not severely damaged by *Chlamydomonas* although there were collapsed cells in the thalli because of contamination compared to the control plants from unialgal cultures.

In destructive association of parenchymatous red alga *Chondrus crispus* and the pathogenic filamentous green alga

Acrochaete operculata the interaction is governed by recognition by the pathogen of the carrageenan oligomeric degradation products of the host cell walls (Potin et al., 2002). More studies need to be conducted to understand recognition process in *A. byssoides*-*Chlamydomonas* association and verify the nature of presumed allelochemicals released by *Chlamydomonas*. To our knowledge, this is the first report on harmful epiphytic interactions between *Chlamydomonas* species and red ceramiacean algae, and it contributes to the understanding of interactions of plants in the marine environment.

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References

- Biegala, I.C., F. Not, D. Vaultot and N. Simon: Quantitative assessment of picoeukaryotes in the natural environment by using taxon-specific oligonucleotide probes in association with tyramide signal amplification-fluorescence *in situ* hybridization and flow cytometry. *Appl. Environ. Microbiol.*, **69**, 5519-5529 (2003).
- Butcher, R.W.: An Introductory Account of Smaller Algae of British Coastal Waters. I. Chlorophyceae. Fish. Investigations, London (1959).
- Cann, J.P. and N.C. Pennick: The fine structure of *Chlamydomonas bullosa* Butcher. *Arch. Protistenk.*, **125**, 241-248 (1982).
- Dangeard, P.: Sur quelques algues vertes marines nouvelles observees en culture. *Botaniste*, **49**, 1-45 (1965).
- Ettl, H.: Die Gattung *Chlamydomonas* Ehrenberg. *Beih. Nova Hedwigia*, **49**, 1-1122 (1976).
- Ettl, H.: Chlorophyta I: Phytomonadina. In: Süßwasserflora von Mitteleuropa, Bd. 9 (Eds.: H. Ettl, G. Gartner, H. Heynig and D. Mollenhauer). Gustav Fischer, Stuttgart. pp. 1-807 (1983).
- Gilbert, D.G.: SeqPup, biosequence editor and analysis software for molecular biology. Version 0.9. Genome Informatics Lab, Biology Dep., Indiana Univ., Bloomington (1999).
- Graham, L.E. and W. Wilcox: Algae. Prentice-Hall, Inc., Upper Saddle River, NJ 07458, USA (2000).
- Harris, E.H.: *Chlamydomonas* as a model organism. *Annu. Rev. Plant Physiol. Mol. Biol.*, **52**, 363-406 (2001).
- Hirayama, S., R. Ueda, T. Nakayama and I. Inouye: *Chlamydomonas perigranulata* sp. nov. (Chlamydomonadales, Chlorophyceae) from the Red Sea. *Bot. Mar.*, **44**, 41-46 (2001).
- Hoham, R.W., T.A. Bonome, C.W. Martin and J.H. Leebens-Mack: A combined 18S rDNA and *rbcl* phylogenetic analysis of *Chloromonas* and *Chlamydomonas* (Chlorophyceae, Volvocales) emphasizing snow and other cold-temperature habitats. *J. Phycol.*, **38**, 1051-1064 (2002).
- John, D.M. and P.M. Tsarenko: Order Chlorococcales. In: The freshwater algal flora of the British Isles. An identification guide to freshwater and terrestrial algae (Eds: D.M. John, B.A. Whitton and A.J. Brook). Cambridge University Press. pp. 327-409 (2002).
- Kim, G.H., T.A. Klochkova, K.S. Yoon and K.P. Lee: Purification and characterization of a lectin, Bryohealin, involved in the protoplast formation of a marine green alga *Bryopsis plumosa* (Chlorophyta). *J. Phycol.*, **42**, 86-95 (2006).
- Klochkova, T.A., S.H. Kang, G.Y. Cho, C.M. Pueschel, J.A. West and G.H. Kim: Biology of a terrestrial green alga *Chlorococcum* sp. (Chlorococcales, Chlorophyta) collected from the Miruksazi stupa in Korea. *Phycologia*, **45**, 349-358 (2006).

- Konovalova, G.V.: Addition to the flora of planktonic flagellated algae of the Kronotzky Inlet (Kamchatka). *Botanical Journal (Botanicheskij Zhurnal)*, **84**, 136-140 (1999).
- Lee, J.J., L.J. Crockett, J. Hagen and R.J. Stone: The taxonomic identity and physiological ecology of *Chlamydomonas hedleyi* sp. nov., algal flagellate symbiont from the foraminifer *Archaias angulatus*. *Br. Phycol. J.*, **9**, 407-422 (1974).
- Lewis, L.A., L.W. Wilcox, P.A. Fuerst and G.L. Floyd: Concordance of molecular and ultrastructural data in the study of zoosporic chlorococcalean green algae. *J. Phycol.*, **28**, 375-380 (1992).
- Nakayama, T., S. Watanabe, K. Mitsui, H. Uchida and I. Inouye: The phylogenetic relationships between the Chlamydomonadales and Chlorococcales inferred from 18S rDNA sequence data. *Phycol. Res.*, **44**, 47-56 (1996).
- Pawlowski, J., M. Holzmann, J.F. Fahrni and P. Hallock: Molecular identification of algal endosymbionts in large miliolid Foraminifera: I. Chlorophytes. *J. Eukaryot. Microbiol.*, **48**, 362-367 (2001).
- Pocock, T., M. A. Lachance, T. Pröschold, J.C. Prisco, S.S. Kim and N.P.A. Huner: Identification of a psychrophilic green alga from lake Bonney Antarctica: *Chlamydomonas raudensis* Ettl. (UWO 241) *Chlorophyceae. J. Phycol.*, **40**, 1138-1148 (2004).
- Posada, D. and K.A. Crandall: MODELTEST: Testing the model of DNA substitution. *Bioinformatics*, **14**, 817-818 (1998).
- Potin, P., K. Bouarab, J.P. Salaun, G. Pohnert and B. Kloareg: Biotic interactions of marine algae. *Cur. Opinion in Plant Biol.*, **5**, 308-317 (2002).
- Pröschold, T., B. Marin, U.G. Schlosser and M. Melkonian: Molecular phylogeny and taxonomic revision of *Chlamydomonas* (Chlorophyta). I. Emendation of *Chlamydomonas* Ehrenberg and *Chloromonas* Gobi, and description of *Oogamochlamys* gen. nov. and *Lobochlamys* gen. nov. *Protist*, **152**, 265-300 (2001).
- Pröschold, T., E.H. Harris and A.W. Coleman: Portrait of a species: *Chlamydomonas reinhardtii*. *Genetics*, **170**, 1601-1610 (2005).
- Pröschold, T. and P.C. Silva: Proposal to change the listed type of *Chlamydomonas* Ehrenb., nom. cons. (Chlorophyta). *Taxon*, **56**, 595-596 (2007).
- Reynolds, E.S.: The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell. Biol.*, **17**, 208-212 (1963).
- Rhodes, R.G.: Heterothallism in *Chlamydomonas acidophila* Negoro isolated from acidic strip-mine ponds. *Phycologia*, **20**, 81-82 (1981).
- Ronquist, F. and J.P. Huelsenbeck: MRBAYES 3: Bayesian phylogenetics inference under mixed models. *Bioinformatics*, **19**, 1572-1574 (2003).
- Ruzin, S.E.: Plant Microtechnique and Microscopy. Oxford University Press Inc., New York (1999).
- West, J.A. and D.L. McBride: Long-term and diurnal carpospore discharge patterns in the Ceramiaceae, Rhodomelaceae and Delesseriaceae (Rhodophyta). *Hydrobiologia*, **398-399**, 101-113 (1999).
- Wood, P.J.: Specificity in the interaction of direct dyes with polysaccharides. *Carbohyd. Res.*, **85**, 271-287 (1980).