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Lectin binding patterns of *Scrippsiella lachrymosa* (Dinophyceae) in relation to cyst formation and nutrient conditions

Anke Kremp*,[1], Donald M. Anderson

Biology Department, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, USA

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Abstract

In many dinoflagellates, it has been a challenging task to study the qualitative and quantitative processes leading to encystment because gametes are often not morphologically distinguishable from other vegetative cells. We examined whether sexual differentiation is accompanied by changes in cell surface glycoprotein properties that are reflected in the binding patterns of complementary lectins. Labeling percentages of nine different fluorescein isothiocyanate (FITC)-conjugated lectins were analyzed together with cell and cyst abundance in N-deplete and *f*/2 control cultures of *Scrippsiella lachrymosa* Lewis throughout an encystment experiment. Although labeling varied between lectins and treatments and considerable changes occurred through time, no direct correlation was observed between glycoconjugate properties and sexual life cycle processes. A conspicuous decrease in labeling of lectins that are complementary to amino sugars (in particular, with WGA, a lectin that is complementary to *N*-acetylglucosamine) was observed in the low nitrogen treatment, suggesting a link between the nutrient status of a cell and expression of surface carbohydrates. Presumably, the reduction of *N*-acetylglucosamine residues was an early indication of N stress in cell populations. Labeling experiments with phosphate-limited cells showed that the decrease in WGA-complementary amino-sugar residues was not specific for N stress, but appeared to be a general response to nutrient limitation. Our findings confirm that glycoconjugate composition of dinoflagellate cells can change depending

* Corresponding author.
E-mail address: anke.kremp@ymparisto.fi (A. Kremp).
[1] Present address: University of Helsinki, Tvärminne Zoological Station, 10900 Hanko, Finland.

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on their physiological state, which has to be considered when applying lectins for taxonomic differentiation of dinoflagellate species.

Keywords: Dinoflagellates; Gametes; Glycoconjugates; Lectins; Nutrient status; *Scrippsiella lachrymosa*; Sexual reproduction

1. Introduction

Dinoflagellate life histories involve sexual reproduction which, in many species, is coupled to the formation of thick-walled, non-motile resting cysts. In particular, for gonyaulacoid and peridinoid dinoflagellates, it has been described that zygotes, resulting from sexual fusion of gametes, encyst and become thick walled resting stages (Pfiester and Anderson, 1987; Xiaoping et al., 1989). Although sexuality and cyst formation are important for genetic diversity, survival and population dynamics of dinoflagellates (reviewed in Dale, 1983; Pfiester and Anderson, 1987), these processes have remained poorly understood. This is mainly due to the fact that cysts are often the only evidence of sexual events in a dinoflagellate population (Anderson et al., 1983; Probert, 2002), as the sexual stages preceding encystment cannot yet be unequivocally identified for the many species that are hologamous, i.e. whose gametes are not morphologically distinguishable from vegetative cells (Xiaoping et al., 1989; Parrow et al., 2002). Planozygotes (swimming zygotes that become resting cysts) are characterized in part by their two longitudinal flagella, but planomeiocytes (post-dormancy motile zygotes) also possess this feature. Size has been used to identify sexual stages (Anderson et al., 1983; Anderson and Lindquist, 1985; Probert et al., 1998; Kremp and Heiskanen, 1999; Olli and Anderson, 2002). In some species, gametes have been found to be smaller and less pigmented than vegetative cells (von Stosch, 1973; Coats et al., 1984). Planozygotes in turn, are often larger than the rest of the population (Pfiester and Anderson, 1987), and may be deeply pigmented (Anderson et al., 1983). Identification of a life cycle stage on the basis of size alone may be complicated though, as cell size may vary considerably in vegetative cells depending on their physiological state.

Microscopic observations together with photographic documentation of sexually induced cells have revealed important details of the reproductive processes happening prior to encystment (von Stosch, 1973; Pfiester, 1975; Coats et al., 1984; Litaker et al., 2002; Parrow et al., 2002). However, such methods are hardly applicable for experimental culture or field studies aiming to clarify factors that induce gamete formation, the incidence of sexuality in a population, or gamete recognition and mating behavior, where large numbers of samples have to be generated and analyzed. Much of our present knowledge of quantitative aspects of sexual reproduction and encystment has been derived indirectly from cyst data (Anderson and Lindquist, 1985; Heiskanen, 1993; Ishikawa and Taniguchi, 1996; Sgrosso et al., 2001; Olli and Anderson, 2002; Godhe et al., 2001). Such data, however, allows only limited conclusions about timing, regulation and extent of sexuality prior to encystment.

In other eukaryotic unicellular organisms such as ciliates or the green alga *Chlamydomonas,* sexual differentiation has been shown to be accompanied by specific biochem-

ical changes of the cell (Goodenough, 1991; Görtz et al., 1999). Gametic cells, for example, excrete pheromones that mediate recognition of gametes or complementary mating types (Miyake, 1981; Luporini et al., 1995; Starr et al., 1995). Furthermore, the gamete cell wall often contains specific chemical structures to facilitate adhesion and fusion of gametes (Vacquier, 1998). In *Chlamydomonas* as well as in yeast, specific membrane proteins are expressed when cells come in contact during mating (Kurvari et al., 1998). A specific lytic enzyme has been described in gametes of *Chlamydomonas reinhardtii* that leads to digestion of the cell walls during fertilization. Its presence has been successfully used to differentiate gamete cells from vegetative cells of this species (Matsuda et al., 1990).

Glycoproteins with sugar residues that provide structural variety are recognized as another group of cell wall molecules that play a role in sexual reproduction of many organisms (Wassarman, 1987). Their complementary structures on the cell surface are lectins—glycoproteins with defined carbohydrate binding sites whose multimeric structure gives them the ability to agglutinate cells or form glycoconjugates similar to antigen–antibody interactions. Together with the carbohydrate receptors, lectins function as a highly specific recognition system (Sharon and Lis, 1989). Diverse taxa share this common chemical mechanism for gamete/mate interaction, including unicellular algae (Wiese et al., 1983) as well as macrophytes (Bolwell et al., 1979; Kim and Kim, 1999; Schmid et al., 1994), ciliates (Watanabe et al., 1981), or harpacticoids (Kelly and Snell, 1998). Lectin inhibition studies have indicated that glycoproteins are also involved in the fertilization process of the dinoflagellate *Alexandrium catenella* (Sawayama et al., 1993).

Specific binding patterns of FITC-conjugated lectins have been used to characterize carbohydrate properties on the cell surface of dinoflagellates in relation to taxonomic position or the physiological status of cells. Glycoconjugate composition appears to be species specific (Costas et al., 1993; Cho, 2003) and lectin analyses have furthermore been applied to discriminate between toxic and non-toxic strains of a single species (Costas et al., 1995; Rhodes et al., 1995; Cho et al., 2001). Aguilera and González-Gil (2001) reported variability of lectin binding depending on cell cycle progression. This was a first indication that glycoprotein moieties of dinoflagellate cells can change with physiological conditions.

Our objective was to test whether cells of the marine dinoflagellate *Scrippsiella lachrymosa* possess specific carbohydrate properties during sexuality and encystment that could potentially be used to detect sexual stages. The binding patterns of nine FITC-conjugated lectins complementary to different sugar residues were analyzed on cells of this species throughout the time course of an encystment experiment. *S. lachrymosa* is a coastal dinoflagellate that forms cysts with a calcareous surface layer comprised of irregularly shaped small crystals (Lewis, 1991). The isolate used in this study has been reported to encyst with high synchrony and an exceptional cyst yield and the thick walled cysts are considered to be true hypnozygotes (Olli and Anderson, 2002). *S. lachrymosa* should thus be a suitable model organism for studying encystment and sexual processes leading to hypnozygote formation. Because cyst formation is triggered by nutrient deprivation, we also examined the effects of nitrogen and phosphate limitation on lectin binding and a possible correlation between nutrient stress and the activity of an amino-sugar cleaving enzyme.

2. Materials and methods

2.1. Encystment experiments

A culture of *S. lachrymosa* Lewis (Olli and Anderson, 2002) was routinely grown in *f*/2 Si medium at 15 °C, 200 μmol photons m^{-2} s^{-1} and a 14:10 h light/dark cycle. Encystment was triggered by transferring cells from exponential growth phase into medium with reduced (*f*/8) levels of NO_3^- (220 μM). A multi-tube approach was used to monitor encystment dynamics and lectin binding. For each sampling point and treatment, replicate 50-ml borosilicate tubes containing 25 ml of the respective medium were inoculated with exponentially growing cells to obtain an initial cell concentration of 500 cells ml^{-1}. All tubes were incubated for 3 weeks at 15 °C, 200 μmol photons m^{-2} s^{-1} and a light/dark cycle of 14:10 h. Samples for cell counts and lectin labeling assays were collected every other day beginning on the day of inoculation (day 0). Each tube was briefly shaken on a vortex mixer to loosen cyst deposits on the bottom and distribute cells and cysts homogeneously in the tube. Duplicate subsamples of 2 ml were then taken for iodine staining and subsequent microscopic cell and cyst counts. From each of four replicate experimental tubes additional 14 ml of cell suspension were preserved with formalin (5% final concentration) in 15-ml centrifuge tubes for later application of lectins.

2.2. Lectin labeling

Samples were labeled with FITC-conjugated lectins (Vector Laboratories, Burlington, CA; Table 1), according to the methods of Aguilera and González-Gil (2002). Briefly, culture samples were centrifuged for 10 min at 5000 rpm and washed once in 0.02 M phosphate buffer solution, PBS (pH 7.45) to remove formalin. Cells were then resuspended in 2 ml PBS. At this point the contents of the four replicate centrifuge tubes were combined and aliquots (one for each lectin and a negative control) of 125 μl were distributed into separate 0.5-ml Eppendorff tubes. Lectins were then added at 100 μM final concentration and samples were incubated for 1.5 h at room temperature in the dark. Cells were washed twice with PBS to remove excess stain and analyzed thereafter in a Zeiss Axioskop epifluorescence (FITC filter set) microscope at 200 × magnification for quality and localization of lectin staining. The percentage of fluorescing cells was determined

Table 1
Lectins used in the present study and their complementary carbohydrates

Lectin	Abbreviation	Specificity
Concanavalin A	Con-A	Glucose, mannose
Jacalin	Jacalin	Galactose
Ulex europaeus agglutinin	UEA	Fucose
Wheat germ agglutinin	WGA	*N*-Acetylglucosamine
Lycopersicon esculentum lectin	LEL	*N*-Acetylglucosamine
Viccia villosa lectin	VVA	*N*-Acetylgalactosamine
Phaseolus vulgaris lectin	PHA-E	Oligosaccharides
Galanthus nivalis lectin	GNL	Mannose
Sambucus nigra lectin	SNA	Sialic Acid

from the proportion of positively and negatively labeled cells. Light micrographs were taken using a Zeiss Axioplan 2 imaging microscope equipped with a Zeiss Axiocam MRm digital camera.

2.3. Flow cytometric analysis

For analysis of relative fluorescence intensity of WGA labeled cells, a second time course experiment was conducted. *S. lachrymosa* cells were incubated in *f*/8 NO_3^- or *f*/2 medium for a period of 18 days. Samples from both treatments were collected every other day and processed as described above. After application of WGA, samples were transferred into plastic vials, mixed properly and run on a Becton Dickinson FACS Calibur flow cytometer. At least 5000 cells were analyzed. Scatter plots of green fluorescence data (FITC signal), red fluorescence data (chlorophyll) and side scatter (SSC) in relation to forward scatter (FSC) were acquired. By gating the chlorophyll fluorescence during data acquisition, we were able to prevent collection of signals that were not derived from intact cells. Fluorescence intensity of WGA was measured as the mean *Y* value of all data points appearing on the FITC/FSC plot. Negative controls were run to detect auto-fluorescence on the green emission channel. These auto-fluorescence measurements were subtracted from the mean fluorescence values of the labeled samples. The resulting values are presented as relative fluorescence units per cell.

2.4. Comparison of WGA labeling, nutrient status and carbohydrate recovery

To investigate the possibility of a correlation between nitrogen limitation of *S. lachrymosa* and amino-sugar expression on the cell surface we compared binding of WGA, a lectin that is complementary to *N*-acetylglucosamine, in cells from three treatments of different nutrient conditions: (a) nitrate-deplete *f*/2 Si (50 μM NO_3^-), (b) phosphate-deplete *f*/2 Si (1 μM PO_4^{3-}), and (c) nutrient-replete *f*/2 Si medium. Exponentially growing cells were inoculated into two sets of triplicate test tubes containing 25 ml of the respective medium and incubated under standard conditions. Growth was monitored daily by measuring *in vivo* fluorescence on a Turner fluorometer. Shortly after the onset of stationary phase in the nutrient-replete cultures, cells were harvested from all treatments. Ten millimeters of each sample was immediately processed for WGA labeling experiments. To detect differences in the cellular nutrient status C/N elemental ratios were determined. Therefore, the remaining volume of each test tube, containing approximately 5 to 10×10^4 cells, was filtered on a pre-combusted 25-mm GF/F filter. Filters were placed in Petri dishes and dried overnight at 60 °C. CHN analyses were conducted on an Elemental Analyzer Carlo Erba EA 1108.

To further test whether recovery of N-starved cells was reflected by specific changes in WGA binding patterns, the second set of replicates was spiked with nutrients after cells had become limited. When cells had entered stationary phase, a 10-ml subsample was removed from each tube for initial WGA staining and microscopic analysis. The remainder of the cell suspension was spiked at the same time with NO_3^- or PO_4^{3-}, respectively, to *f*/2 concentrations and placed back into the incubator. After 4 days, when cells had resumed growth and entered exponential phase, tubes were harvested and cells were examined for

WGA staining. The proportion of labeling was compared between nutrient-deficient and recovered (nutrient-replete) cells.

2.5. Enzyme assay

We used an enzyme assay adapted from Sakai et al. (1998) to evaluate the activity of an exochitinase in relation to WGA binding. Correlation of such an enzyme with glycoprotein properties would be an indication that amino sugars hydrolyzed from cell surface glycoproteins potentially serve as an alternative source of nitrogen during N-starvation. Another nutrient depletion experiment, similar to the one described above, was performed, again using (a) *f*/2 concentrations of NO_3^- or PO_4^{3-}, (b) 50 μM NO_3^- and (c) 1 μM PO_4^{3-}as experimental treatments. Cells were harvested every other day until several days into stationary phase for cell counts and enzyme assays. Approximately 40,000 cells were collected in a pellet by centrifuging the respective culture volume for 10 min at 5000 rpm. After aspiration of the residual media, cells were resuspended in 100 μl sterile filtered 0.1 M KPO_4 buffer (pH 7) and transferred to sterile Eppendorf tubes. A total of 100 μl of freshly prepared 1 M *p*-nitrophenyl-*N*-acetyl-B-D-glucosaminide (pNP-$(GlcNAc)_2$) was added as the substrate for the enzyme assay and the mixture was incubated at 60 °C for 1 h. Samples were centrifuged at 5000 rpm to pellet cells and the supernatant was pipetted into disposable cuvettes containing 1 ml of 0.2 M Borax solution (pH 10) which terminated the enzyme reaction. Absorbance of the samples was determined spectrophotometrically at 400 nm. The concentration of *p*-nitrophenol released by the enzyme reaction was calculated using an extinction coefficient of 17,700 cm^{-1} M^{-1}. Enzyme activity of the cells was determined from differences between a control, containing only substrate and buffer without cells, and the actual sample. A preparation of cells without substrate served as an additional control.

3. Results

3.1. Encystment and lectin labeling

In the low nitrogen treatment, cysts were first observed on day 6 (Fig. 1) when cells were in mid exponential growth phase. Most of the cysts, however, were formed only between day 12 and day 16, after the cultures had entered stationary phase. Assuming that cysts are produced sexually by fusion of two gametic cells, 42% of the cells (2*n* cysts/*n* cells + 2*n* cysts) had encysted by the end of the experiment on day 18, when cyst numbers had increased to 6×10^3 ml^{-1}. The transition to cysts was also reflected in a decrease of motile cell numbers from 24×10^3 ml^{-1} maximum to 17×10^3 ml^{-1} final concentration. Cell numbers in the *f*/2 control increased exponentially until day 14 reaching $>40 \times 10^3$ ml^{-1}. Encystment was negligible in this treatment. Apart from a few occasional observations, cysts were not usually found in the samples.

All lectins tested in this study labeled cells of *S. lachrymosa* positively in the –N as well as in the control treatment (Fig. 2). Binding patterns, however, varied considerably

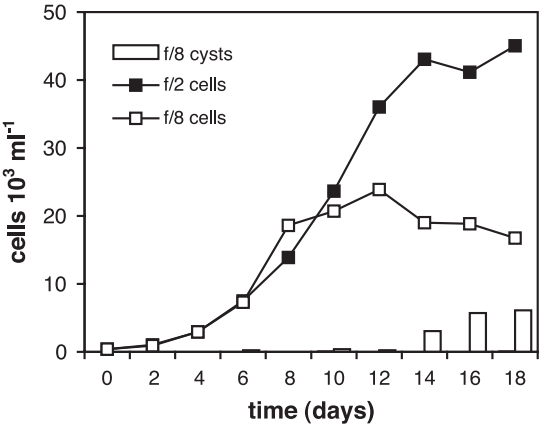

Fig. 1. *S. lachrymosa* encystment experiment. Time-course of cell growth and cyst production. Filled squares: cell numbers in nutrient replete control, empty squares: cells numbers in encystment cultures (*f*/8 nitrogen concentrations). Cyst concentrations are represented by bars. Values represent means of duplicate counts.

among lectins, physiological state of the cells and treatment. The complementary glycoproteins of Con A, Jacalin, WGA and VVA were commonly present on the cell surface, resulting in high percentages of positively labeled cells ranging from 70–100% at the beginning to 20–70% at the end of the experiment. A general decrease in labeling was observed around days 8–12 in cells from the N-deplete treatment, being particularly pronounced in the *N*-acetylglucosamine-binding WGA. Cells with positive binding of Con A, Jacalin and VVA decreased also in the *f*/2 control at that time, however changes were less pronounced than in the *f*/8 treatment. UEA, LEL, PHA-E and SNA labeling was mostly low (less than 20%) and scattered throughout the experiment. GNL was the only lectin that showed increasing labeling percentages with progressing time in both −N and control treatments, and here again the number of labeled cells remained lower in the −N treatment compared to the control.

None of the lectins appeared to be specific for the −N (encystment) treatment, that might have yielded a potential marker for an encystment stage. Even UEA, LEL, and PHA-E, which began to bind to cells on day 8 shortly before encystment set in, were detected in both the encystment and the control treatment. Furthermore, their labeling percentages did not correspond to the yield of produced cysts, which could be considered an indication of their specificity. All lectins were detected on all size classes and cell types, vegetative cells at the beginning of the experiment, fusing cell pairs, as well as large cells, presumably planozygotes, on the days preceding encystment.

Microscopic analysis of the labeled cells revealed differences in the localization and quality of fluorescent staining among the nine lectins (Fig. 3). The target glycoproteins of Con A seem to be coating the outermost layer of the cell wall with a fine film that can easily rupture (Fig. 3A). The negative imprint of the plate pattern that becomes visible on an empty theca suggests that glycoprotein structures are different in the zones between the plates (Fig. 3B). Also galactose residues, the complementary structure of the Jacalin lectin, seem to be located mainly on the outer theca as empty thecae were always brightly stained

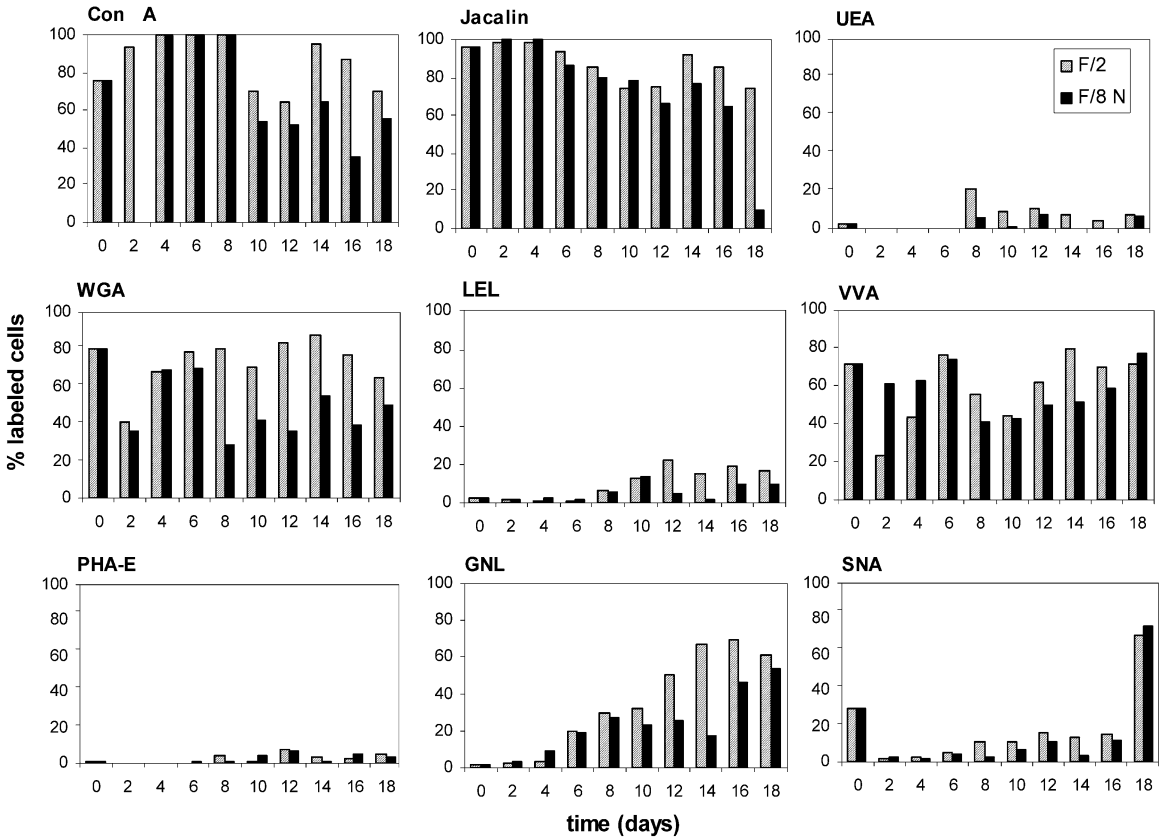

Fig. 2. Binding patterns of FITC labeled lectins in *S. lachrymosa* cultures during encystment. Percentages of positively labeled cells in nutrient replete control culture (hatched bars) and nitrogen deplete encystment treatment (filled bars).

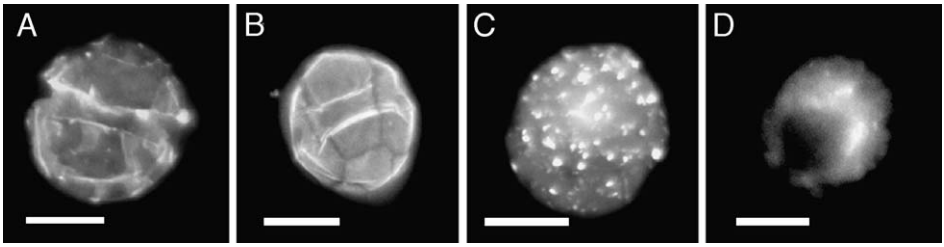

Fig. 3. Fluorescence light micrographs of FITC labeled *S. lachrymosa* cells. (A) Con A on an intact cell, (B) Con A on an empty theca, (C) WGA and (D) GNL binding patterns. Scale bars = 10 μm.

with this lectin, in contrast to cells that had recently shed their thecae that usually did not label at all. With WGA, LEL, VVA, SNL and PHA-E, staining appeared as localized, more or less bright spots covering the cell or parts of it (e.g., Fig. 3C). Empty thecae did not show fluorescence signals, thus, we assume that the target sugars of these lectins are located on an internal layer of the complex cell wall underneath the plate-containing amphiesma. In some cells, WGA seemed to concentrate in the sulcal area of the cells, but this binding pattern could not be related to a particular cell type or time interval during the encystment experiment. GNL was the only lectin that apparently penetrated into the cell, binding to intracellular mannose residues (Fig. 3D). The quality of staining did not differ between nutrient-replete and nutrient-deplete cells.

3.2. Nutrient conditions and WGA labeling

Flow cytometric measurements of relative WGA fluorescence were performed to relate lectin binding throughout the growth curve of nutrient-replete vs. deplete cultures in a

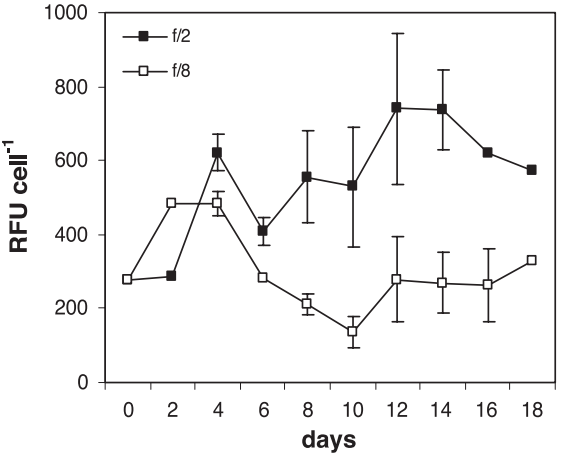

Fig. 4. Flow cytomentric measurements of WGA fluorescence (mean relative fluorescence units, RFU) on cells of *S. lachrymosa* from the *f*/2 control (white squares) and low NO_4^{3-} (filled squares) treatment. Error bars = S.D. ($n = 3$).

Table 2
Cell numbers, C/N molar ratios and WGA labeling percentages in cultures of *S. lachrymosa* harvested in mid exponential (*f*/2) and early stationary growth phase (50 μM NO_3^-, 1 μM PO_4^{3-}) from experimental treatments with different nutrient concentrations

Treatment (NO_3^-, PO_4^{3-} concentrations)	Cells 10^3 ml^{-1} (mean, $n=2$)	C/N ($\pm$ S.D., $n=3$)	% WGA labeled cells ($\pm$ S.D., $n=3$)
f/2	22.4	5.81 (0.04)	73.70 (11.29)
50 μM NO_3^-	8.0	23.19 (1.33)	46.42 (4.07)
1 μM PO_4^{3-}	4.7	9.27 (0.51)	24.28 (0.37)

more quantitative way than the +/− microscopic evaluation allowed. The results plotted in Fig. 4 show that *N*-acetylglucosamine residues on the cell surface decreased in the treatment containing reduced nitrate concentrations. A sharp drop in WGA labeling intensity occurred in exponential growth phase, where relative fluorescence decreased from initial values of 480 RFU $cell^{-1}$ on day 4 to a minimum of 130 RFU $cell^{-1}$ on day 10. In comparison, RFU values in the f/2 control remained high at 400–740 RFU $cell^{-1}$ throughout the experiment.

A comparison of WGA labeling and C/N ratios in nutrient replete cultures, N-limited and P-limited cells, respectively, revealed that the reduction in cell surface amino-sugar residues was not specific to N limitation but seemed to be a general response of cells to nutrient deficiency and growth limitation (Table 2). Lectin binding was observed in only 24% of the P limited cells in contrast to 46% of the N-limited cells and 74% of control cells, although cellular C/N ratios were highest in the N-limited culture (Table 2). Growth limitation was most severe in the P limited culture, where cell concentrations reached only a maximum of 5×10^3 cells ml^{-1} compared to 8×10^3 cells ml^{-1} in the –N treatment and 22×10^3 cells ml^{-1} in the control treatment.

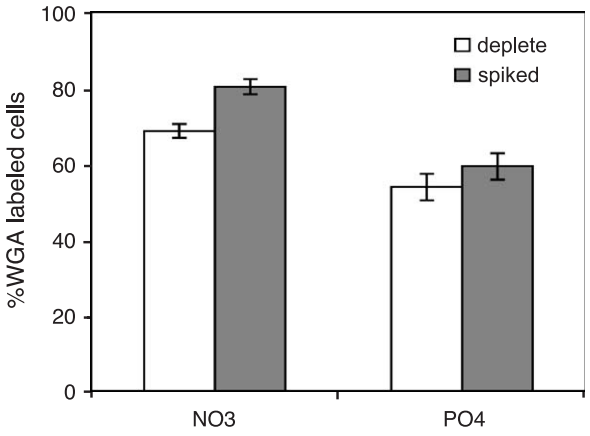

Fig. 5. Recovery of nutrient-limited cultures after N and P additions, respectively. White bars represent the percentage of WGA labeled growth limited cells prior to nutrient addition and grey bars show labeling percentages 4 days after spiking when cells had resumed exponential growth.

The recovery of cells from nitrogen starvation (Fig. 5) was reflected by a significant increase ($p < 0.05$, *t*-test) in the percentage of WGA labeled cells from 69% to 80% 4 days after spiking with excess nitrate. An increase in labeling was also observed in P-limited cultures spiked with PO_4^{3-} (Fig. 5). It was, however, not significant ($p > 0.05$, *t*-test).

The results of the enzyme assay indicated that nitrate starvation does not increase activity of amino-sugar cleaving enzymes in *S. lachrymosa*. No significant increase in absorption compared to a negative control was detected in any treatment at any time (data not shown).

4. Discussion

The aim of this study was to examine the glycoconjugate moieties of *S. lachrymosa* cells during cyst formation triggered by nutrient depletion. A variety of sugar residues were detected on the cells, most commonly mannose, glucose, *N*-acetylglucosamine and *N*-acetylgalactosamine, but occasionally also fucose, sialic acid and oligosaccarides in low amounts. A clear correlation between binding patterns of the complementary lectins and life cycle processes was not revealed by these binding studies, but the results do provide important insights to the dynamics of carbohydrate properties during growth, nutrient limitation and cyst formation. Our findings confirm that the glycoconjugate composition of dinoflagellate cells can vary depending on the physiological state of cells. The conspicuous decrease in labeling of lectins complementary to amino sugars that was observed in the low nitrogen treatment suggests a link between the nitrogen status of a cell and expression of surface carbohydrates. Labeling experiments with phosphate-limited cells showed that the decrease in WGA-complementary amino-sugar residues is not specific for N-stress, but appears to be a general response to nutrient limitation.

4.1. Lectins and sexual reproduction

The functioning of glycoconjugates in sexual reproduction can be mediated either by qualitative or quantitative changes in their composition. Mating type or gamete-specific carbohydrate structures may be synthesized during gametogenesis or, alternatively, a non-specific cell wall glycoprotein may increase in quantity and concentrate in areas where cell contact is established. It has been demonstrated for several organisms that specific glycoconjugate patterns are associated with sexual processes. Localized specific binding of Con A, WGA and SBA (soy bean agglutinin) on the surface of gametes has been reported from fertilization studies on Ceramiaceae (Kim and Kim, 1999). Also gametes of *Ectocarpus siliculosus* (Phaeophyceae) possess specific membrane glycoproteins that bind to WGA (Schmid et al., 1994), and Watanabe et al. (1981) demonstrated a concentration of Con A receptors in the conjugation regions of mating ciliate cells. Contrary to our expectations, such qualitative or quantitative specific glycoconjugate patterns were not observed during encystment of *S. lachrymosa*.

The observed changes in carbohydrate composition were either not specific to the encystment treatment (Con A, Jacalin, UEA, PHA-E), or did not coincide with the

assumed timing of the gamete stage (WGA, LEL, VVA, GNL and SNA). We cannot exactly define the interval when gametes were abundant because notably smaller cells, suggested to be gametes by Olli and Anderson (2002) did not occur in significant amounts, and mating cell pairs were rarely seen throughout the experiment. We assume that gametes, when present, had their peak in the first half of the experiment. Most studies on sexual reproduction of dinoflagellates report that mating begins hours to a few days after exposure to encystment conditions and may last hours to weeks (von Stosch, 1973; Pfiester, 1975; Coats et al., 1984; Litaker et al., 2002). In *Scrippsiella trochoidea*, gamete formation and fusion takes several days (Xiaoping et al., 1989) and it is likely that the closely related *S. lachrymosa* reproduces in a similar manner.

The reduction in labeling of Con A, WGA, LEL, VVA, GNL and SNA in the encystment treatment was most probably not related to sexuality. It happened (except for WGA) only during the second half of the experiment, when cysts were already abundant, suggesting that mating was over and planozygotes had formed. Furthermore, when WGA fluorescence was examined with the flow cytometer, the drop in labeling intensity was significant although no cysts had been formed in that experiment, despite the nutrient depletion. This indicates that the reduction of glycoproteins in the N-depleted encystment treatment reflected nutrient stress rather than sexual differentiation.

Although encystment was not as rapid and complete in our experiment as described in Olli and Anderson (2002), a final cyst yield of 42% in the −N treatment suggests a significant incidence of sexual reproduction. Provided that cysts were formed sexually by fusion of two gametes, a considerable number of gametes thus must have been present at some point in our samples. Thick walled dinoflagellate cysts have been generally considered to be hypnozygotes, i.e. sexual products (Dale, 1983; Pfiester and Anderson, 1987). However, recent life cycle investigations have provided evidence of asexually produced resting stages with a complex cyst wall (Litaker et al., 2002; Parrow et al., 2002). If we hypothesize that the cysts produced in our experiment were not sexual, this might explain the lack of specific glycoconjugate properties and other indications of sexual reproduction such as mating cell pairs or distinct size classes.

Alternatively, sexuality-specific sugar residues might not have been detected by the limited choice of common lectins used in this study. Carbohydrate residues may appear in highly specific configurations and require a particular complementary lectin (Sharon and Lis, 1989). Furthermore, lectins may contain sites specific for non-carbohydrate ligands that may be critical for the recognition functions of the lectin (Barondes, 1988). Thus, negative lectin binding or missing lectin inhibition is not necessarily evidence for lack of specific glycoconjugate structures. In order to detect highly specific carbohydrate–lectin recognition systems, both complementary molecules should be purified and characterized by protein labeling and fractionation methods and their function should be confirmed in respective inhibition experiments (Kim et al., 2003).

Despite their apparent non-specificity, the tested glycoconjugates might still have a function in sexual reproduction. Sawayama et al. (1993) demonstrated in simple inhibition experiments that the Con A complimentary glucose and mannose residues play an essential role in mating of *A. catenella* by mediating recognition and agglutination of gametes. Presumably, multiple carbohydrate–lectin systems on the cell surface are

involved in these processes, possibly a combination of common agglutination structures and more specific recognition molecules (Kim and Kim, 1999).

4.2. Lectin binding and nutrient limitation

Nutrient depletion is commonly used to induce cyst formation in the laboratory. This makes it difficult to determine whether the observed biochemical response to this manipulation is in fact genuinely associated with sexual differentiation or is instead a reflection of nutrient stress that may also occur in asexual, nutrient-starved cells. As discussed earlier, the changes in lectin binding properties shown in this study were most probably related to nutrient limitation of the cells. Our findings on cells of *S. lachrymosa* suggest that cellular nutrient status can considerably affect the amount and composition of cell surface-bound carbohydrates, similar to what has been described for extracellular carbohydrates produced by phytoplankton cells (Myklestad, 1995). According to this author, N and P limitation leads to a reduction in carbohydrates containing these elements, which is in agreement with our observed drop of surface amino-sugar residues on *S. lachrymosa* cells in nitrate-depleted cultures. The reduction was consistent with all of the three tested amino-sugar/lectin conjugates, although it was most evident with WGA labeling.

Flow cytomentric measurements of WGA fluorescence intensity highlighted the possibility of a correlation between reduced WGA labeling and nitrogen stress. Interestingly, the response to low ambient nitrogen concentrations was reflected much earlier in the decrease of WGA binding than in cell number. A reduction of surface amino-sugar residues might be an early reaction of cellular metabolism to a limited availability of inorganic nitrogen. It is thus possible that cell surface amino sugars are among the first molecules for which synthesis ceases or decreases when nitrogen becomes limiting. It could also be hypothesized that cell surface amino-sugar residues could serve as an alternative source of nitrogen when hydrolyzed from the glycoprotein as suggested by Oliveira et al. (1980) for the green algae *Dunaliella*. The uptake of dissolved free amino sugars has been mainly reported from pelagic marine bacteria (Riemann and Azam, 2002), but has also been proposed to be a strategy of phytoplankton to cope with limiting inorganic nitrogen concentrations (Antia et al., 1991; Berg et al., 2002). Such a strategy would require the action of an amino-sugar cleaving enzyme. We were not able to detect increased activity of such an enzyme in nitrogen-starved cells of *S. lachrymosa* with the method we chose. This may indicate that *S. lachrymosa* does not directly scavenge cell wall amino sugar residues. However, as little information is available to date on the mechanisms of amino-sugar utilization, we cannot exclude alternative mechanisms that make those nitrogen atoms available to the cell.

Cell surface WGA glycoconjugates also decreased under phosphate limitation. As phosphate limitation usually also affects the nitrogen metabolism of the cell, this response might be a reflection of secondary N stress. However, our data are conflicting. While the percentage of WGA labeling was lower in the −P treatment than for −N, C/N ratios were higher in the −N treatment, suggesting a higher degree of N-stress. This indicates that reduction of *N*-acetylglucosamine residues is not directly related to the

nitrogen status of the cell and therefore is probably not a specific response to N stress. When comparing cell numbers, C/N ratios and WGA labeling percentages of the three experimental treatments (Table 2), it becomes obvious that WGA binding correlates better with cell number than with C/N ratios. Growth appears to be limited most in the –P treatment where, in turn, we found the lowest percentage of WGA labeling. This suggests that glycoprotein expression in nutrient limited cells might also be related to events that regulate cell division and thus cell growth. When growth is limited, by nutrient depletion for example, cells do not divide any more, but are arrested in the cell cycle. Aguilera and González-Gil (2001) reported that the proportion of WGA-labeled cells of two dinoflagellate species dropped to nearly 0% after cell division, the complementary glycoproteins recovering many hours later. It is possible that the *N*-acetylglucosamine molecules on *S. lachrymosa* cells have similar properties, and that the decrease in WGA labeling we observed are due to cell cycle inhibition as a result of nutrient limitation. However, the results of the "recovery" experiments in which the limiting nutrient was spiked into the cultures are not consistent with this scenario, as glycoproteins recovered more successfully in the –N treatment than in the –P treatment, although cells resumed growth equally in both treatments (data not shown). Apparently, the relationship between amino-sugar expression and nutrient limitation is more complex than we can assess at this point.

4.3. General remarks

Fluorescence-tagged lectins have been repeatedly used as probes to differentiate among dinoflagellate species and strains (Costas et al., 1993, 1995; Rhodes et al., 1995; Cho et al., 2001; Cho, 2003) and are suggested as tools that may potentially aid species identification in harmful algal monitoring programs. Most of these studies were conducted on cultures from exponential growth phase and assume that glycoprotein properties on the cell wall differ among species and strains, but remain stable independent of the physiological state of the cells. Together with the results of Aguilera and González-Gil (2001), our findings emphasize that a lectin-based taxonomic analysis must be interpreted in the context of ambient conditions and the physiological status of the cells that are of interest. For field studies this requires additional assessments, for example using physiological probes (Dyhrman and Palenik, 2001). The potential variability of lectin binding with physiological condition may therefore restrict the application of lectin probes in monitoring studies, or may require that studies similar to those reported here are conducted to characterize the extent of that variability.

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