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Characteristics of chain-forming cells in *Gyrodinium impudicum* using fluorescent ConA

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Cells of the dinoflagellate *Gyrodinium impudicum* form characteristic chains, which are associated with sugar accumulated on the cell surface. To resolve the relationship between chain-formation and cell surface sugar accumulation, confocal microscopy was used to observe sugar accumulation points in the vegetative cells and long chain-forming cells of *G. impudicum* cells treated with fluorescent-tagged ConA. In the stationary and exponential phases, both vegetative cells and chain-forming cells were similar to each other in fluorescent intensity. There was no evidence that chain-forming cells had a specific location for sugar accumulation on the cell surface. Most of the cells formed 2-cell chains one day after inoculation, but longer chains consisting of 4—8 cells increased markedly in 4 day and 8 day cultures. *Gyrodinium impudicum* chains usually consist of more than four cells, and chains of 8 or even 16 cells can be observed in mature cultures. Temperature played an important role in chain-formation of the cells, threshold temperature for the development of long chain-formation was 19 °C.

INTRODUCTION

The dinoflagellate *Gyrodinium impudicum* is morphologically similar to *Cochlodinium polykrikoides* (Cho *et al.*, 1998) and *Gymnodinium catenatum* (Frag a *et al.*, 1995). These orgnisms are difficult to differentiate under the light microscope when fixed with Lugols solution. *Gyrodinium impudicum* has not been reported to cause massive blooms in Korean coastal waters until recently (Kim *et al.*, 1997). One of the ecological characteristics of this species is chain-formation during intense blooms (Fraga *et al.*, 1995). Hoagland *et al.*, (1995) reported that extracellular polysaccharides produced by epiphytic diatom were associated with motility, adhesion to surfaces, habitat stabilization and colony formation.

The sugars on the cell surface may act as a signal of gamete recognition during fertilization (Kim and Fritz 1993; Kim *et al.*, 1996). It has been suggested that the stickiness between cells is caused by the secretion of mucus on the cell surface (Waite *et al.*, 1995). We found that this alga was stained by Alcian blue around the cell surface and showed a higher abundance of polysaccharides than any otherred tide causative phytoplankton collected from Korean coastal waters (Cho *et al.*, 1999a, b). Adhesion mechanisms are critical in the formation of biofilms and in cell aggregation, but the adhesion mechanism of cell binding remains unclear. Lectins can be used as a tool to monitor structural changes in cell membranes depending on various stimuli (Kim and Fritz 1993). Based on our previous reports, we hypothesize that polysaccharide produced by *G. impudicum* is associated with cell stickiness in the exponential phase and results in chain-formation. Therefore, we observed the formation of chains under the light microscope, and compared the localization of sugar accumulation points between vegetative cells and chain-forming cells treated with fluorescent ConA using confocal microscopy, and investigated characteristics of chain-forming cells under different environmental conditions.

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MATERIALS AND METHODS

Culture for experiments

Gyrodinium impudicum for this study was isolated from the waters off Tongyoung, Korea in 1997 and was cultured under standard conditions (Cho *et al.*, 1998). Culture conditions in temperature test were 16 °C, 19 °C, 22 °C, 25 °C at a light intensity of 100

mmol m^{-2} s^{-1}, 12:12 light/dark in a customized culture cabinet (BOD incubator, Japan prod.), grown in f/2-Si medium (Guillard and Ryther 1962) with antibiotic mixture (Hasui *et al.*, 1995). Salinity and pH tests were cultured from 20 to 35 psu (intervals of 5), and from 6.0 to 9.0 (intervals of 1.0), respectively. For the study of nutrient effects, cells were cultured without nitrogen ($NaNO_3$), phosphorus (NaH_2PO_4), trace metals ($MnCl_2$, $CoCl_2$, $CuSO_4$, $ZnSO_4$, Na_2MoO_4, $NaSeO_3$) and vitamins (B_{12}, biotin, thiamine) under the same culture conditions mentioned above. Chain-forming cells counted at 1, 4 and 8 day under an inverted Carl Zeiss MC 80 microscope in Multiwell tissue culture plates (Becton Dickinson) in quadruplicate. An initial cell density of 100—200 cells/mL was inoculated into filtered medium (0.2 μM AS 020). All experiments were conducted for 8 days.

Labeling and confocal laser scanning microscopy

Based upon the findings of Waites *et al.* (1995) and Cho *et al.* (1998), we selected FITC-labeled ConA from *Canavalia ensiformis*, having glucose- and mannose-binding specificity, to test our hypothesis that glucose and mannose residues on the cell surface are important factors for chain-forming cells. As suspension of lectin (Vector Lectin Kit, fluorescein FLK-2100, Vector Laboratories Inc., Burlingame, CA 94010) was prepared as described by Kim *et al.* (1995). Fluorescein isothiocyanate FITC-labelled lectin was added in 10 μL aliquots to about 10^3 cells on glass slides which were then incubated for 40 min at room temperature. The slide glasses were coated with 3% 3-aminopropyltriethoxy-saline solution. During the incubation, distilled water was added gently via filter paper (Whatmanto, GF/C) reduce the possible evaporation of lectin. The treated cells were mounted on siliconised glass slides and examined under a confocal microscope.

RESULTS

The observation of chain-forming cells

Most cells were chain-forming during the exponential growth phase. After cell division, each cell joined closely between one extra flagella at the posterior compartment and anterior portion of its neighbour (Figs. 1a—c), exceptionally long 4 cells formed to embed a temporary resting cyst (Fig. 1e). During cell division, chain-forming of 4 cells produced an unusually curved form of cell chain (Fig. 1f) compared with typical linear long chain of 8 cells (Fig. 1d) and 16 cells (Fig. 1g).

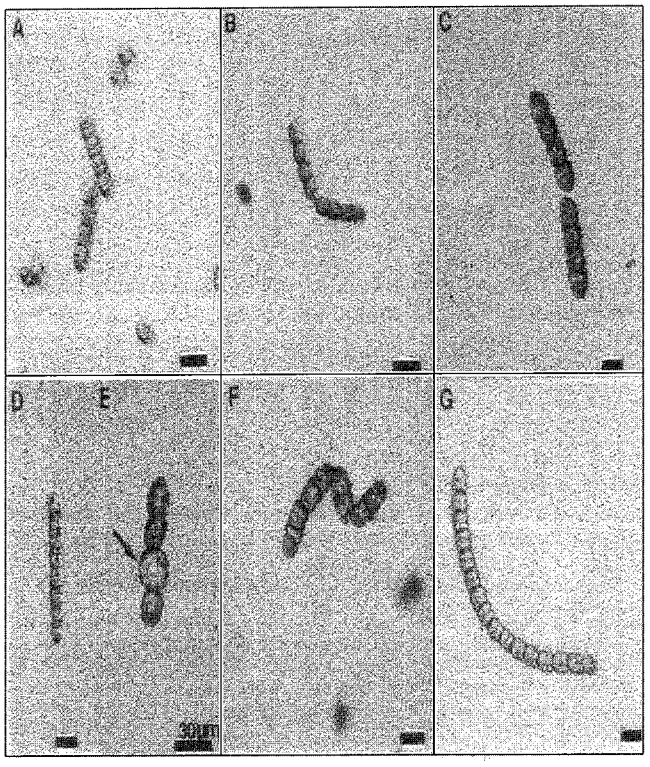

Fig. 1. Morphological characteristics on procedure of long chain of 8 cells in *Gyrodinium impudicum*. A: Two chains of four cells come together. B: The interconnection between the posterior side of the cell and antapical region of the anterior cell. C: Before observation of chain-formation of eight cells. D: Chain of eight cells. E: Unusually four-chain cell. Arrow marks embedded temporary cyst. F: Exceptionally changed feature of chain of eight cells. G: Long chain of 16 cells. (scale bar is applied to all micrographs)

Vegetative and chain-forming cells treated with fluorescent ConA

We have tested FITC-conjugated ConA lectin on a solitary cells and chain-forming cells (Fig. 2). Treated cells showed a fine fluorescent outline (Figs. 2a—b). Each vegetative cell had the same abundance of ConA labelling on the cell surface (Figs. 2c—d). In chain-forming cells, fluorescent ConA was abundant on the cell surface, in particular attached chain-formation point between cells (Figs. 2e—f).

The number of total chain-forming cells at different conditions

Table 1 showed the number of total chain-forming cells during incubation times and at different envi-

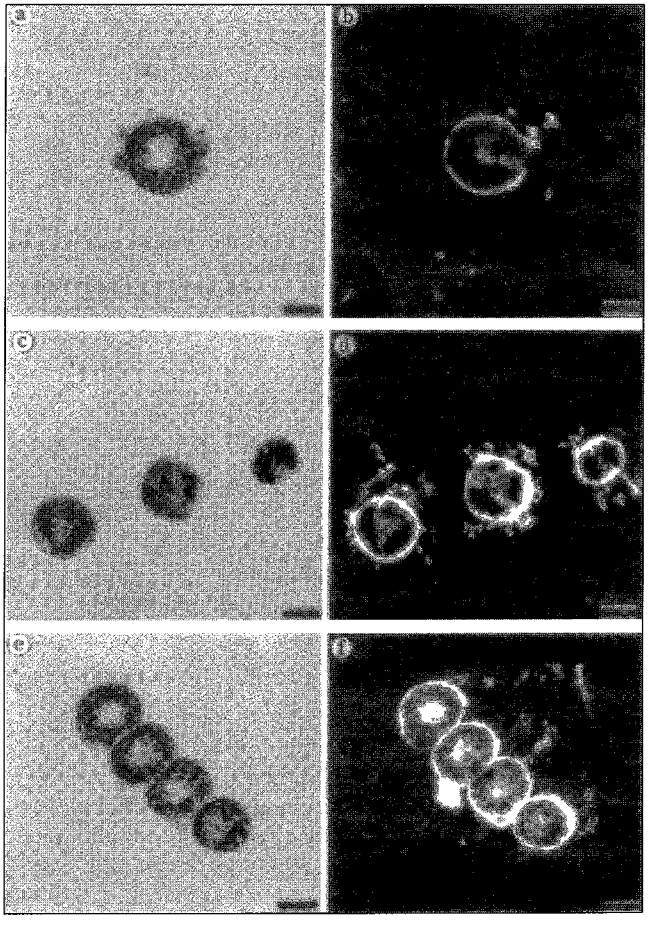

Fig. 2. The micrographs of vegetative cell of *Gyrdinium impudicum* in stationary phase under the light microscope (A) and confocal laser scanning microscopy (B). The observation of vegetative cell of *G. impudicum* in exponential phase under the light microscope (C) and confocal microscopy (D). The view of chain-forming *G. impudicum* in exponential phase under the light micrscope (E) and confocal microscopy (F). (scale bar is 20 μM)

ronmental conditions and deletion of each nutrient. pH and salinity had no significant effect on chain formation. After 4 days, long chains of 8 cells began to be observed but their abundance was quite different depending on temperature, (less than 5% at 16 °C) compared with 15−50% at 19 °C, 22 °C, 25 °C. At day 4 there was a high abundance of 4-cell chains compared with the initial day. On day 8, the number of long chains of 8 cells had increased markedly compared with day 4, the number of 2-cell chains had decreased compared with initial day and day 4, and long chains of 16 cells were observed.

DISCUSSION

Lectins are multivalent carbohydrate-binding proteins found in plants, bacteria, and marine invertebrates (Slifkin and Doyle 1990). They have been used in the purification of polysaccharides and glycoproteins and in a variety of biological applications, including cell separation, cellular localization of glycoconjugates, mitogenic stimulation of immune cells, identification of blood groups, and microorganism, and monitoring alterations on the surface of normal and neo-plastic cells. The range of sugar-specific binding properties of lectins can be used to map cell surface architecture and to monitor the moieties of carbohydrate on the cell membrane (Kim and Fritz 1993; Kim *et al.*, 1996). We expected to use this properly discriminate harmful marine microalgae in rapid HAB monitoring (Cho *et al.*, 1998).

Some studies have suggested that the carbohydrates on the cell surface have an ability to mediate cell-cell recognition events in red alga, and toxic *Alexandrium* sp. during sexual reproduction (Kim and Fritz 1993; Sawayama *et al.*, 1993). This implies that some species of unicellular macroalgae and microalgae modify the carbohydrate composition in their cell surface during their life cycle.

In case of diatom cells, cell surface sugar accumulation increases the stickiness of cells (Waite *et al.*, 1995). To find out the accurate point of sugar accumulation on the cell surface, we used a confocal laser scanning microscope. The location of sugar accumulation points on the cell surface was highly species-specific, and tight balls forming adhesive links between cells were observed. However, solitary cells in stationary phase labelled by fluorescent ConA showed a fine fluorescent outline, and vegetative cells and 4-cell chains in exponential phase were similar in fluorescent intensity to solitary cells in stationary phase. In particular no sugar accumulation points of chain-forming cells surface were noted (Figs. 2a−f). It is assumed that sugar residues on the cell sufrace are not strongly related to chain-formation of *Gyrodinium impudicum*. Waite *et al.* (1995) demonstrated that diatoms are able to exudate a great deal of polysaccharide to mucilage for cellular surface in order to increase the cell stickiness. *Gyrodinium impudicum* appears to be unable to excrete extracellular polysaccharides because it does not produce any mucilage. However, we found previously that *Gyrodinium impudicum* stains strongly with Alcian blue and the amount of polysaccharide in its cell wall is higher than in any other harmful microalge isolated from Korean coastal waters (Cho *et al.*, 1999a, b), suggesting that sugar moieties produced by *G. impu-*

Table 1. The number of total chain-forming cells in test wells according to elapsed times and at different environmental conditions including depletion of each nutrients

(unit: no./test wells)

Length-Chain	Day 1		Day 4			Day 8			
	2 (%)[1]	4 (%)	2 (%)	4 (%)	8 (%)	2 (%)	4 (%)	8 (%)	16 (%)
Control									
16 °C	35(74)	1(4)	27(37)	21(58)	1(5)	33(20)	42(52)	11(27)	0(0)
19 °C	39(61)	6(19)	75(29)	74(56)	10(15)	80(5)	202(24)	286(69)	4(2)
22 °C	66(61)	14(26)	83(25)	64(39)	30(36)	86(3)	374(28)	397(59)	36(11)
25 °C	62(55)	14(25)	92(16)	98(34)	73(50)	92(1)	395(13)	848(7)	235(30)
pH 6									
16 °C	33(80)	1(5)	33(62)	10(38)	0(0)	35(18)	55(57)	12(25)	0(0)
19 °C	21(48)	6(27)	73(32)	64(56)	7(12)	79(6)	219(36)	175(58)	0(0)
22 °C	53(58)	13(28)	93(25)	111(59)	16(16)	77(5)	289(35)	243(59)	4(2)
25 °C	53(68)	8(21)	92(19)	123(50)	39(31)	96(3)	414(26)	529(65)	25(6)
pH 7									
16 °C	24(88)	0(0)	25(33)	23(61)	1(5)	24(9)	60(47)	28(44)	0(0)
19 °C	41(67)	10(33)	50(28)	48(54)	8(17)	51(7)	135(40)	124(43)	0(0)
22 °C	65(70)	14(30)	77(23)	106(64)	11(13)	84(5)	197(23)	258(60)	28(12)
25 °C	62(72)	12(28)	105(22)	93(39)	47(39)	94(1)	567(18)	989(62)	151(18)
pH 8									
16 °C	25(91)	0(0)	23(32)	23(64)	1(5)	24(8)	88(56)	26(33)	1(2)
19 °C	38(86)	3(14)	64(31)	64(63)	3(5)	67(5)	241(39)	168(55)	0(0)
22 °C	78(76)	12(24)	79(26)	74(49)	18(24)	71(4)	323(32)	299(59)	13(5)
25 °C	62(70)	13(30)	85(15)	101(35)	71(49)	87(1)	603(20)	874(57)	170(22)
pH 9									
16 °C	29(96)	0(0)	39(46)	23(54)	0(0)	37(8)	91(39)	62(53)	0(0)
19 °C	38(90)	2(10)	46(25)	55(60)	7(15)	66(11)	153(49)	64(41)	0(0)
22 °C	57(66)	15(34)	75(26)	67(47)	19(26)	80(8)	184(37)	136(54)	2(1)
25 °C	52(68)	12(32)	88(19)	119(51)	36(30)	81(1)	559(20)	791(57)	144(20)
20 psu									
16	29(85)	0(0)	58(59)	20(41)	0(0)	37(8)	80(33)	56(46)	8(13)
19	33(89)	2(11)	54(22)	73(60)	11(18)	57(3)	28(32)	283(64)	1(1)
22	75(88)	5(12)	79(25)	81(52)	18(23)	79(4)	306(32)	296(61)	8(3)
25	58(69)	13(31)	73(12)	115(38)	76(50)	73(1)	892(32)	876(63)	28(4)
25 psu									
16	31(88)	0(0)	21(41)	15(59)	0(0)	21(10)	66(66)	12(24)	0(0)
19	38(59)	13(41)	78(22)	96(55)	20(22)	7(4)	364(36)	297(58)	7(1)
22	59(65)	16(35)	64(21)	79(53)	19(25)	64(2)	486(32)	497(66)	0(0)
25	57(69)	13(31)	80(10)	173(42)	98(47)	80(1)	933(23)	1345(66)	104(10)
30 psu									
16	20(82)	0(0)	30(45)	14(42)	2(12)	30(12)	63(52)	22(36)	0(0)
19	25(86)	2(14)	56(24)	74(63)	8(13)	56(3)	273(33)	258(63)	0(0)
22	81(74)	14(26)	98(27)	87(48)	23(25)	98(3)	494(32)	497(64)	6(1)
25	65(71)	13(29)	99(13)	169(44)	85(43)	99(1)	625(17)	1292(68)	130(13)
35 psu									
16	8(92)	0(0)	37(46)	22(54)	0(0)	37(11)	84(49)	35(41)	0(0)
19	19(76)	3(24)	55(24)	67(59)	10(17)	55(6)	249(53)	95(41)	0(0)
22	79(75)	13(25)	69(16)	92(46)	36(36)	69(4)	261(32)	229(56)	16(7)
25	61(35)	13(30)	129(17)	158(42)	75(40)	92(1)	366(11)	708(43)	362(44)

Table 1. Continued

Lengthchain	Day 1		Day 4			Day 8			
	2(%)[1]	4(%)	2(%)	4(%)	8(%)	2(%)	4(%)	8(%)	16(%)
$NaNO_3$[2]									
16 °C	8(50)	0(0)	23(37)	18(57)	1(6)	23(9)	64(49)	28(43)	0(0)
19 °C	37(50)	0(0)	33(26)	40(62)	4(12)	33(6)	179(65)	39(28)	1(1)
22 °C	44(35)	9(29)	59(17)	83(48)	30(34)	59(3)	295(29)	326(65)	7(2)
25 °C	46(41)	5(18)	68(13)	104(40)	61(46)	68(1)	457(15)	979(66)	135(18)
NaH_2PO_4[2]									
16 °C	16(50)	0(0)	44(59)	15(41)	0(0)	35(10)	76(45)	37(44)	0(0)
19 °C	39(44)	4(12)	46(25)	62(68)	3(6)	46(4)	267(49)	129(47)	0(0)
22 °C	70(34)	14(33)	70(21)	127(75)	4(4)	70(6)	424(77)	47(17)	0(0)
25 °C	70(31)	13(38)	46(11)	130(59)	33(30)	46(3)	756(83)	68(15)	0(0)
Minerals[3]									
16 °C	6(50)	0(0)	12(18)	28(82)	0(0)	12(13)	37(79)	2(9)	0(0)
19 °C	19(24)	10(51)	44(32)	44(65)	1(2)	44(6)	185(49)	86(45)	0(0)
22°C	66(29)	23(41)	53(20)	79(59)	14(20)	53(3)	181(19)	351(75)	7(2)
25 °C	56(25)	27(49)	75(14)	98(35)	71(51)	75(1)	412(16)	918(70)	82(12)
Vitamins[4]									
16 °C	32(50)	0(0)	24(29)	27(66)	1(4)	24(13)	53(57)	14(30)	0(0)
19 °C	58(44)	4(12)	68(29)	61(52)	11(18)	68(6)	196(33)	179(61)	0(0)
22 °C	70(34)	17(33)	53(17)	77(50)	26(33)	53(2)	360(31)	384(66)	1(1)
25 °C	55(31)	17(38)	65(12)	101(36)	74(52)	65(1)	1090(37)	843(58)	26(3)

[1]The abundance of chain-forming cells, [2]Deletion of each nutrient, [3]Deletion of $MnCl_2$, $CoCl_2$, $CuSO_4$, $ZnSO_4$, Na_2MoO_4, $NaSeO_3$, [4]Deletion of B_{12}, biotin, thiamine.

dicum are not responsible for chain-formation.

On the first day, most cells in this study (over 50%) formed 2-cell chains, with a significant decrease of these short chain at day 4 and day 8. At day 4 and day 8, the number of longer chains (4, 8, 16 cells) increased, indicating that *G. impudicum* changes its chain forming characteristics during the life cycle. Among environmental conditions and nutrients, higher temperature appeared to markedly increase the number of chain-forming cells, and the threshold temperature for the chain formation was 19 °C (Table 1).

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