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Molecular probe for identification of cysts of resting cyst of PSP-producer *Alexandrium tamarense* (Dinophyceae)

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Abstract

Identification of species within the toxin-producing genus *Alexandrium* is vital for biotoxin monitoring and mitigation decisions regarding shellfish industry. In particular, the discrimination of resting cysts of only *A. tamarense* from that of *Alexandrium* spp. is considerable important to fundamentally monitor and predict this species before vegetative cells occur in the nature. Fluorescent cTAM-F1 DNA probe was responsible to not only binding the activity of the vegetative cells in *A. tamarense*, but also to the resting cysts, which was treated with methanol after fixation and stained by primuline on the surface. The location of fluorescence in cultured vegetative cells and resting cysts was almost at the bottom of the nucleus. The optimal incubation temperature and time using *in situ* hybridization were 50~54℃ and 40~60 min, respectively, to penetrate the DNA probe into cell.

Key words – *Alexandrium tamarense, Alexandrium catenella,* biotoxin monitoring system, Chinhae Bay, DNA probe, red tide, resting cysts

Introduction

The genus *Alexandrium*, which consists of more than 20 species and some of species produce potent neurotoxins associated with paralytic shellfish poisoning (PSP), comprises similar morphology[5,8,10,19,21]. To clarify a better understanding of *Alexandrium*, molecular phylogenetic analyses using the sequences of the ribosomal small subunit[14], the ribosomal large subunit[17] and the internal transcribed spacers (ITS) ribosomal DNA[1] have been carried out in the last decade. Recently, Adachi *et al.*[2] developed fluorescent DNA probes discriminating *A. tamarense* (Lebour) Balech from *A. catenella* Whedon et Kofoid) Balech. Since then, one of these (cTAM-F1) has been applied to field samples in order to enumerate and identify *A. tamarense* and this method was found to be one of the newest and most promising method for the establishing reliable databases in Korean coastal waters[7]. A few research has been carried out trying to apply DNA probes to the resting cysts of *A. tamarense* in spite of the fact that they play an important role in the initiation of dinoflagellate blooms. Yamaguchi *et al.*[22] suggested that primuline-staining was a powerful new method for enumerating cysts in the natural environment. It is however impossible to differentiate between the resting cysts of *A. tamarense* and *A. catenella* using the primuline staining method. It is hypothesis that a combined staining method using both primuline and the cTAM-F1 DNA probe may be useful taxonomically for the identification of these species. In order to establish a monitoring program using the persistent

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resting cysts of *A. tamarense*, I applied both a fluorescent DNA probe (specific probe for *A. tamarense*) and primuline to the resting cysts of *A. tamarense* using whole cell hybridization.

Materilas and Methods

Cultures

The cells in this study were isolated from Chinhae Bay, Korea and established as *A. tamarense* AT-B in 1997. The culture was unialgal and maintained in f/2-Si medium[9] at 20℃ under a light intensity of 50 mol m-2 s-1 from white fluorescent tubes (12 L:12 D of light: dark cycle).

Cysts treatment

For isolation of the cysts, sediment samples were collected with a gravity core-sampler in Chinhae Bay. Samples were treated as described by Matsuoka *et al.*[11]. Briefly, samples were dried at 70℃ for 24 h and rinsed out with distilled water to remove salt. After then, 10% hydrochloric acid was added to remove calcium carbonate and silicate materials. Transferred into a beaker, sample was mixed with distilled water to make slurry. After sonication of the slurry for 30 seconds, it was sieved through 125 m and 20 m meshes. The isolated resting cysts were stored at 4℃ until analysis. *Alexandrium* cysts were identified following the primuline-staining method of Yamaguchi *et al.*[22]. Primuline-stained *Alexandrium* cysts exhibit an intense green fluorescence under a fluorescence microscope (Nikon Optiphoto) with blue-light excitation (450~490 nm).

DNA probes

In Situ hybridization with species-specific ITS ribosomal DNA targeted oligonucloeotide probes was applied to sediment samples, using the filtration methods[7, 13,18]. The probes used in this study were cTAM-F1 [probe specific to *A. tamarense*, 5-FluorePrime(Pharmacia) ATCGTACACAAACACAGCAC-3] and cCAT-F1 [probe specific to *A. catenella*, 5-FluorePrime (Pharmacia) GTTGCAGCAACTAGAGAAATG-3] as in Adachi *et al.*[2]. The fixed samples were obtained on 13 mm, 1.2 m pore size Isopore polycarbonate membranes (Millipore), placed in custom filter tubes. Once the cells had been captured on the filter, they were rinsed briefly with 1 mL hybridization buffer[13]. After filteration, 500 L of hybridization buffer was added to each tube to resuspend the cells. 12 L of the appropriate probe was added and the tubes were placed in a waterbath or a dry incubator at 50~54℃ for 40 to 60 min to hybridize. After hybridization, the cells were filtered and rinsed briefly with 1 mL of hybridization buffer to remove excess unbound probe. After filteration, the membranes were placed onto glass slides, 20 L of SlowFade Light (Molecular probes) were added and viewed using a Nikon Optiphoto microscope with a FITC optical filter set (emission 529 nm).

Results and Discussion

In Situ hybridization using the fluorescent cTAM-F1 probe labelled *A. tamarense*. Strong fluorescence was showed in the periphery of the cells (Fig. 1A). Cysts of *Alexandrium* spp. from the field samples was stained using primuline a bright green fluorescence emerged on the surface. The species-specific cTAM-F1 DNA probe reacted only with the resting cysts of *A. tamarense* and showed a strong fluorescence positioned similar to vegetative cells (Fig. 1B, C). Although a green fluorescence using primuline was visible from the outer of the cysts, no DNA probe binding was detected for any of the other *Alexandrium* cysts tested (Fig. 1D, E).

The biggest problem found during the present study, was to find out how the oligonucleotide probes could penetrate into cysts, as they possess solid cell wall. I found to permit optimal probes penetration by conducting incubation using varying incubation times and

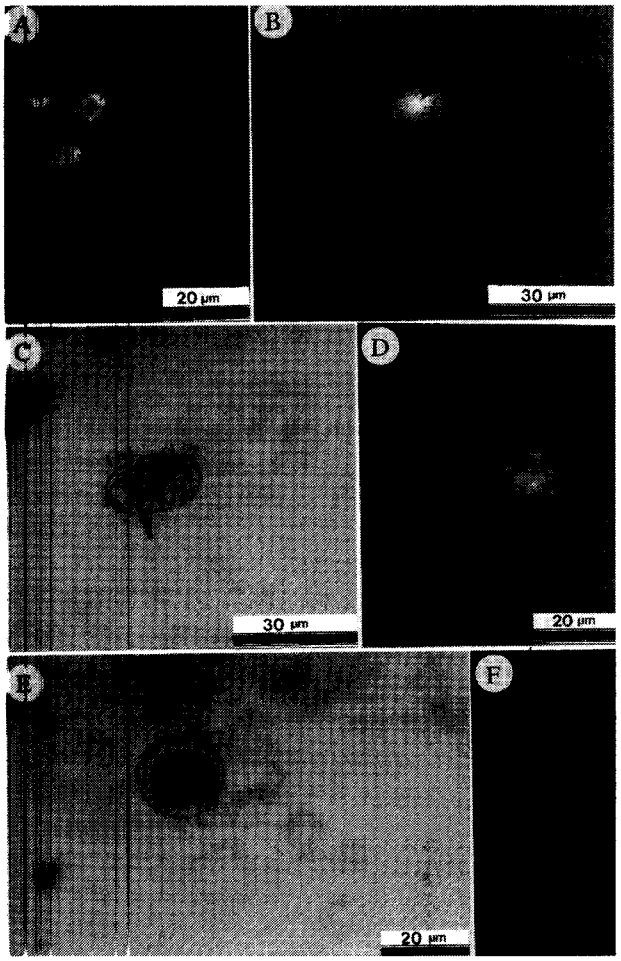

Fig. 1. Epifluorescence micrographs of vegetative cells of *A. tamarense* in the cultures and *Alexandrium* cysts in the nature. A: vegetative cells of A. tamarense hybridized with species-specific cTAM-FI probe. Arrow indicates the location of the label, B: application of cTAM-FI probe to resting cysts in *A. tamarense* collected from natural sediments in Chinhae Bay, Korea, with staining Primulin on the cell surface. Arrow indicates the location of the label, C: light microscopy of resting cysts of *A. tamarense* against Fig. B. Arrow indicates the location of the label, D: the use of Primulin stained against *Alexandrium* cysts, but no labeling of cTAM-F1 was shown. E: light microscopy of resting cysts of *Alexandrium* aganst Fig. D. F: control (no addition of cTAM-F1 and Primulin probes).

temperatures. It appeared that the fluorescence of cysts under hybridization reactions of 45℃ and incubation times of 1~2 h (data not shown) were less intense than when using 50~54℃ for 40~60 min (Fig. 1B). A recent study has suggested exploring alternative fixatives and methods for storing samples whole cell hybridization[13]. In addition, Yamaguchi *et al.*[22] demonstrated that methanol treatment after fixation with glutaraldehyde improved the labeling activity of cysts. *Alexandrium* cysts from field samples possess a rigid cell wall because of a sporopollenin-like polymer in the thin exospore and cellulose in the thick endospore[4]. This indicate that it may be difficult for DNA probes to penetrate into the cell wall, although the cysts are incubated for even quite a long time. Using FITC-conjugated lectins, which bind to specific sugars, has shown that *Alexandrium* cysts strongly bind ConA, indicating that the cell surface is rich in mannose and glucose residues (data not shown). Primuline reacts only with polysaccharides[6,20], suggesting that possibly *Alexandrium* cysts were rich in sugars before treatment with methanol. When the cysts are treated with methanol, DNA probes would expect to enhance significantly the penetration compared with untreated cysts. Consequently, it is assumed that reaction temperature and incubation times may play a important role in penetration of DNA probes into the cells. Alternative fixation processes and reaction conditions might show the cysts to be even more visible and penetrative in the further study.

The differentiation of *A. tamarense* from *A. catenella* in cultured vegetative stages is often difficult because of similar morphological features, as well as it is impossible to discriminate the resting cysts in nature. Due to recent advanced genetic studies, two species have found that is higher genetic variation and different phylogenetic relationship[1,2,14,16,17]. In addition, Adachi *et al.*[2] reported that the fluorescent DNA probe (cTAM-F1) was a useful tool for identifying *A. tamarense*. This study agrees well with Adachi *et al* results[2], in particular cCAT-F1 did not label either the cells or the the resting cyst of *A.*

tamarense (data not shown). Because cTAM-F1 correspond to staining the nuclear region and the fact that the amount of DNA remains constant during different growth stages, it is considered a more effective labeling than RNA probes, which are labeling the cytoplasm. Adachi *et al.*[2] reported that the location of fluorescence in the cells was at the bottom of the nucleus, similar to binding position in the present study.

In conclusion, cTAM-F1 probe is useful as a tool for discriminating the vegetative cells of *A. tamarense* from *A. catenella*, as well as resting cysts. In addition, this is able to apply the resting cysts in *A. tamarense* before the occurrence of vegetative cells and is expected to certainly encourage the prediction of *A. tamarense* of coming red tide in Chinhae Bay, where is a vigorous shellfish farming industry.

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초록 : 분자생물학적 방법을 이용하여 마비성 패류 독소를 생산하는 알렉산드륨 타마렌스 시스트 탐색

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알렉산드륨 적조생물 속에서 마비성 패류독소를 생산하는 종을 신속하게 동정하므로 패류양식의 독성 모니터링과 방제에 중요한 역할을 할 수 있다. 자연상태에서 영양세포가 출현하기 전 알렉산드륨 타마렌스의 휴면포자만을 신속하게 분리 동정한다는 것은 근본적인 마비성 패류독소 모니터링 및 예측에 큰 역할을 할 수 있다. cTAM-F1 DNA probe은 알렉산드륨 타마렌스의 영양세포 뿐만 아니라 primuline으로 염색하여 메타놀로 고정한 휴면포자에도 반응이 되었다. 영양세포와 휴면포자에 반응되는 DNA probe 위치는 핵내의 말단 부위에서 보였다. DNA probe가 세포내로 삽입되는데 가장 적합한 온도와 시간은 50~54℃, 40~60분이 좋았다.