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ScienceDirect
Harmful Algae 7 (2008) 495–503
HARMFUL ALGAE
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Development of a PNA probe for the detection of the toxic dinoflagellate *Takayama pulchella*

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Received 5 March 2007; received in revised form 9 November 2007; accepted 16 November 2007

Abstract

A peptide nucleic acid (PNA) probe was developed to detect the toxic dinoflagellate, *Takayama pulchella* TPXM, using fluorescent *in situ* hybridization (FISH) combined with epifluorescent microscopy and flow cytometry. The PNA probe was then used to analyze HAB samples from Xiamen Bay. The results indicated that the fluorescein phosphoramidite (FAM)-labeled probe (PNATP28S01) [Flu]-OO ATG CCA TCT CAA GA, entered the algal cells easily and bound to the target species specifically. High hybridization efficiency (nearly 100%) was observed. Detection by epifluorescence microscopy and flow cytometry gave comparable results. The fluorescence intensity of the PNA probe hybridized to *T. pulchella* cells was remarkably higher than that of two DNA probes used in this study and than the autofluorescence of the blank and negative control cells. In addition, the hybridization condition of the PNA probe was easier to control than DNA probes, and when applied to field-collected samples, the PNA probe showed higher binding efficiency to the target species than DNA probes. With the observed high specificity, binding efficiency, and detection signal intensity, the PNA probe will be useful for monitoring harmful algal blooms of *T. pulchella*.
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Keywords: Fluorescent in situ hybridization (FISH); Harmful algal blooms (HABs); Molecular probes; Peptide nucleic acid (PNA); *Takayama pulchella* TPXM

1. Introduction

Rapid and unequivocal identification of toxic species has become one focal point of toxic algal research (Rhodes et al., 1995; Cho and Costas, 2004; Hosoi-Tanabe and Sako, 2005, 2006). Different technologies have been developed to differentiate non-toxic from toxic algae and to monitor the development of algal blooms in coastal waters (Cho, 2003; Cho and Costas, 2004). These detection techniques include scanning electron microscopy (SEM), high performance liquid chromatography (HPLC), polymerase chain reaction (PCR), immunofluorescence, lectin probe, fluorescent in situ hybridization (FISH) (Simon et al., 1997; Anderson et al., 1999; Scholin et al., 1999; Hosoi-Tanabe and Sako, 2005, 2006), peptide nucleic acid (PNA) probes (Worden et al., 2000; Litaker et al., 2002; Worden and Binder, 2003; Hou and Huang, 2005; Connell et al., 2006) and protein markers (Chan et al., 2005). Of these detection protocols, molecular markers have proved particularly useful to separate closely related species (Adachi et al., 1994; Martin et al., 1996; Anderson et al., 1999; Scholin et al., 1999). Among them, the PNA probe is relatively new and its demonstrated high sensitivity and specificity (Litaker et al., 2002; Stender et al., 2002; Hou and Huang, 2005; Connell et al., 2006) render it a method of choice.

The PNA probe is an artificial synthetic DNA analog, in which the sugar phosphate backbone of the DNA helix is replaced with an uncharged structurally homomorphous pseudopeptide backbone (Nielsen et al., 1991). The synthetic backbone provides PNA probes with unique hybridization characteristics such as more rapid and stronger binding to complementary targets according to the Watson and Crick basepairing rules (Worden et al., 2000; Litaker et al., 2002; Stender et al., 2002; Worden and Binder, 2003; Connell et al., 2006). PNA probes have higher specificity than analogous DNA probes, because a single-base-pair mismatch is more thermally unstable in the former than in the latter (Kim et al., 1993). PNA probes also have higher hybridization efficiency (resulting in higher signal intensity) and target site accessibility due to the

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1568-9883/$ – see front matter © 2008 Published by Elsevier B.V.
doi:10.1016/j.hal.2007.11.006

uncharged nature of the PNA backbone (Nielsen et al., 1994). A PNA–RNA duplex exhibits higher thermal stability than the corresponding DNA–RNA duplex due to the lack of electrostatic repulsion between the target site and the PNA strand (Nielsen et al., 1991; Kim et al., 1993). The unnatural PNA backbone also means that PNA is not degraded by ubiquitous enzymes, such as nucleases and proteases (Nielsen et al., 1994; Stender et al., 2002). The unique chemical characteristics of PNA probes have been used over a broad range of conditions and in a variety of research and diagnostic applications (Litaker et al., 2002; Worden et al., 2000; Stender et al., 2002; Worden and Binder, 2003; Connell et al., 2006). However, utility of PNA probes for identifying and detecting harmful algae is still limited (Connell et al., 2006).

Takayama pulchella was first recorded in Japan as an ichthyotoxic dinoflagellate (Onoue et al., 1985). It was initially described as *Gymnodinium pulchella* (Larsen, 1994), but recently renamed as *T. pulchella* based on the sigmoid shape of the apical groove and distinct cytological features typical of *Takayama*, the newly erected genus (Salas et al., 2003). *T. pulchella* formed HABs in America and Australia in recent years, causing large numbers of fish kills (Larsen, 1994; Steidinger et al., 1998). A *Gymnodinium*-like algal bloom occurred in Xiamen Bay in 1986 with a density exceeding 10^7 cells/L (Zhang et al., 1988), and in 2003 with a density up to 10^7 cells/L. The *Gymnodinium*-like species was isolated from Xiamen Bay during the bloom in 2003, and identified as *T. pulchella* (TPXM) based on morphology and D1–D2 region of LSU (large subunit) rDNA and total ITS1-5.8S-ITS2 sequence (Gu et al., 2006). The vegetative cells were relatively small in size, ranging from 20–23 μm long to 14–20 μm wide. They were broadly oval, with a conspicuous and well-defined characteristic, counterclockwise sigmoid apical groove present on the epitheca. The nucleus was located in the epicone and most cellular cingula were not displaced, which was different from the type species of *Gymnodinium*. The same species bloomed again in Xiamen Bay in 2004 and 2005, indicating that *T. pulchella* is a key HAB species in Xiamen Bay. The 721-bp D1–D2 region of the 28S rRNA in the *T. pulchella* strain isolated from the 2004 blooms is 99–100% identical to reported sequences of the same species (GenBank accession numbers AY764178 and U92254). Understanding of population dynamics and regulating factors is still limited largely due to lack of a rapid and accurate method to detect and quantify this species. In this study, we designed a PNA probe and assessed its specificity and sensitivity for *T. pulchella* when applied to mixed and natural samples. We demonstrated that this probe, with FISH and flow cytometry detection techniques, was highly species specific and sensitive for the detection of this HAB species.

2. Materials and methods

2.1. Natural and culture samples

Natural samples collected from Xiamen Bay, China (24°29′N, 118°04′E), in May and June in 2004 and 2005 were concentrated using a plankton net with a mesh size of 10 μm (Hosoi-Tanabe and Sako, 2005, 2006; Hosoi-Tanabe, personal communication), and preserved with 0.75 mL formaldehyde (5%, v/v, final concentration = 1.9% formaldehyde). Samples were stored at 4 °C before processing. Samples were centrifuged at 3000 × *g* for 5 min at room temperature, the supernatant was aspirated, and the cell pellet resuspended in ice-cold methanol to extract chlorophyll and stabilize rRNA. Samples were required to stand in methanol for at least 1 h prior

Table 1
Microalgal species and strains tested in this study

Species	Taxonomy	Strains	Sources
Alexandrium tamarense	Dinophyceae	ATDH01	East China Sea
A. catenella	Dinophyceae	ACDH01	East China Sea
Alexandrium sp	Dinophyceae	ASPGX01	South China Sea
Alexandrium minutum	Dinophyceae	AMTW	Taiwan waters
Karenia mikimotoi	Dinophyceae	KMDH	East China Sea
	Dinophyceae	KMHK	Hong Kong waters
	Dinophyceae	KMBJ	South China Sea
Takayama pulchella	Dinophyceae	TPXM	Xiamen Bay
Akashiwo sanguinea	Dinophyceae	CCMP1740	Caribbean Sea
Gyrodinium instriatum	Dinophyceae	GIXM	Xiamen Bay
Prorocentrum donghaiense	Dinophyceae	PDDH	East China Sea
P. minimum	Dinophyceae	PMXM	Xiamen Bay
Scrippsiella trochoidea	Dinophyceae	STXM	Xiamen Bay
Skeletonema costatum	Bacillariophyceae	SCXM	Xiamen Bay
Pseudo-nitzschia sp	Bacillariophyceae	PNXM	Xiamen Bay
Chaetoceros sp	Bacillariophyceae	CHAXM	Xiamen Bay
Thalassiosira weissflogii	Bacillariophyceae	TWDH	East China Sea
Platymonas sp	Chlorophyceae	PLADH	East China Sea
Dunaliella salina	Chlorophyceae	DZDH	East China Sea
Dicrateria zhanjiangensis	Prymnesiophyceae	DZEC	South China Sea
Phaeocystis globsa	Prymnesiophyceae	PGDH	East China Sea
Emiliania huxleyi	Prymnesiophyceae	EHDH	East China Sea
Heterosigma akashiwo	Raphidophyceae	HADH	East China Sea

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