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Localization of polyketide synthase encoding genes to the toxic dinoflagellate *Karenia brevis*

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Abstract

Karenia brevis is a toxic marine dinoflagellate endemic to the Gulf of Mexico. Blooms of this harmful alga cause fish kills, marine mammal mortalities and neurotoxic shellfish poisonings. These harmful effects are attributed to a suite of polyketide secondary metabolites known as the brevetoxins. The carbon framework of all polyketides is assembled by a polyketide synthase (PKS). Previously, PKS encoding genes were amplified from *K. brevis* culture and their similarity to a PKS gene from the closely related protist, *Cryptosporidium parvum*, suggested that these genes originate from the dinoflagellate. However, *K. brevis* has not been grown axenically. The associated bacteria might be the source of the toxins or the PKS genes. Herein we report the localization of PKS encoding genes by a combination of flow cytometry/PCR and fluorescence in situ hybridization (FISH). Two genes localized exclusively to *K. brevis* cells while a third localized to both *K. brevis* and associated bacteria. While these genes have not yet been linked to toxin production, the work describes the first definitive evidence of resident PKS genes in any dinoflagellate.

Keywords: *Karenia brevis*; Brevetoxin; Polyketide synthase; Dinoflagellate; Fluorescence in situ hybridization

1. Introduction

The planktonic marine dinoflagellate *Karenia brevis* blooms annually in the Gulf of Mexico and is most prevalent along the west coast of Florida. Historically, blooms have occurred primarily during the fall and winter months. However, over recent years the Florida red tide specifically and HABs (harmful algal blooms) in general appear to be more frequent, persistent and widespread (Chretiennot-Dinet, 2001; Hallegraeff, 1993). Associated with these blooms are massive fish kills, marine mammal mortalities, human poisonings due to the consumption of tainted shellfish and complaints of respiratory irritations among beach-goers (Kirkpatrick et al., 2004; Van Dolah et al., 2002). These effects are caused by the neurotoxic brevetoxins. The brevetoxins are a suite of polyether ladder type compounds which have two parent backbone structures, brevetoxin A and brevetoxin B, each with several side chain variants (Fig. 1). Brevetoxins act at the voltage-gated sodium channel by prolonging and lowering the threshold for channel opening, resulting in a dose-dependant depolarization of excitable membranes (Jeglitsch et al., 1998). The brevetoxins are representative of a larger family of dinoflagellate-derived polyketide toxins that pose a threat to human health through the consumption of

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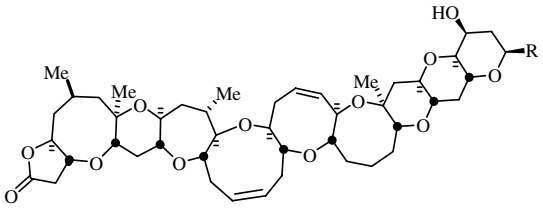
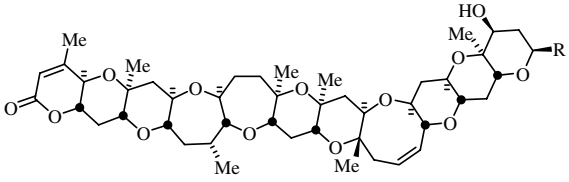


brevetoxin A



brevetoxin B

	Frame	R
PbTx-2	B	$-CH_2C(=CH_2)CHO$
PbTx-3	B	$-CH_2C(=CH_2)CH_2OH$
PbTx-1	A	$-CH_2C(=CH_2)CHO$
PbTx-10	A	$-CH_2C(=CH_2)CH_2OH$

Fig. 1. Structures of the polyether brevetoxins.

tainted seafood. These include ciguatoxin, okadaic acid and the related dinophysistoxins, pectenotoxins, yessotoxin and the azaspiracids, to name just a few.

Brevetoxin production may not be exclusive to *K. brevis*. Several raphidophytes, including *Chatonella antiqua*, *Chatonella marina*, *Fibrocapsa japonica* and *Heterosigma akashiwo* have all been reported to produce brevetoxin B in culture (Khan et al., 1995, 1996a,b, 1997). Furthermore, brevetoxins have been isolated from a fish-killing bloom in which the predominant organism was *Chatonella verruculosa* (Bourdelais et al., 2002). Raphidophytes are unarmored, photosynthetic marine eukaryotes which belong to the Stramenopiles, and are phylogenetically distinct from dinoflagellates, which fall within the Alveolates. The biogenic origin of the brevetoxins was established by two groups independently using stable isotope incorporation experiments (Chou and Shimizu, 1987; Lee et al., 1986, 1989). The head-to-tail incorporation of acetate units confirmed the polyketide origins of these compounds.

Over the past decade, numerous polyketide biosynthetic pathways have been cloned and expressed in heterologous hosts, offering unique opportunities for overexpression and/or manipulation (Pfeifer and Khosla, 2001). Three fundamental types of polyketide synthases have emerged (Shen, 2003). Type I are large multifunctional enzymes having several functional domains located within a single protein. Type II PKSs are multiprotein complexes of several individual enzymes found only in bacteria and typically produce aromatic polyketides. Type III PKSs are found only in plants, utilize unusual starter units and act on acyl Co-A thioesters. The complex structures of dinoflagellate-derived polyketides suggest that they are produced by type I modular synthases.

However, no biosynthetic pathway for a secondary metabolite from a dinoflagellate has been identified at the genomic level. The numerous challenges associated with molecular genetic studies of dinoflagellates (Plumley, 1997) no doubt serve as a deterrent to many researchers. Particularly daunting is the extraordinary size of the dinoflagellate genome (Rizzo et al., 1982). Furthermore, many polyketide toxin-producing dinoflagellates have not been maintained in culture axenically, raising the question of whether the ultimate origins of these secondary metabolites are dinoflagellates or their associated bacteria. A bacterial origin for dinoflagellate polyketide toxins has been the subject of much speculation (Rausch de Traubenberg and Lassus, 1991). However, no polyketide toxin-producing bacteria have been isolated from a dinoflagellate to date.

Recently, we reported the amplification of type I, but not type II, polyketide synthase genes from non-axenic cultures of marine dinoflagellates (Snyder et al., 2003). Using a degenerate primer set for the β-ketosynthase domain of type I PKS encoding genes, a product of the anticipated size was amplified by reverse transcriptase PCR (RT-PCR) from the Wilson strain of *K. brevis*. Four of the fifteen sequenced amplicons (AS1-1L, AT1-6L, AT2-10L and AT2-15) were PKS related. The amino acid sequences were subjected to a phylogenetic analysis, and three (AT1-6L, AT2-10L and AT2-15) branched into a clade with PKS encoding genes from the related protist *Cryptosporidium parvum*, a member of the Coccidia which is a sister clade to the Dinophyceae within the Alveolata (Zhu et al., 2002).

In part based on this analysis and the apparent methylation of the DNA, we surmised that these three sequences originated from *K. brevis* rather than from associated bacteria. Herein we report that based on PCR screening of dinoflagellate and bacterial fractions as well as fluorescence in situ hybridization, two of the four PKS amplicons localize exclusively to *K. brevis*, while a third localizes to *K. brevis* and a small population of the associated bacteria.

2. Results and discussion

2.1. Amplification of PKS genes from other K. brevis strains with specific primers

If the amplified PKS encoding genes were derived from *K. brevis*, presumably they should be detectable in all available isolates of the dinoflagellate. In addition, the bacterial consortia present in these isolates of *K. bre-*