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ORIGINAL ARTICLE

Characterization and Localization of a Hybrid Non-ribosomal Peptide Synthetase and Polyketide Synthase Gene from the Toxic Dinoflagellate *Karenia brevis*

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Abstract The toxic dinoflagellate *Karenia brevis*, a causative agent of the red tides in Florida, produces a series of toxic compounds known as brevetoxins and their derivatives. Recently, several putative genes encoding polyketide synthase (PKS) were identified from *K. brevis* in an effort to elucidate the genetic systems involved in brevetoxin production. In this study, novel PKS sequences were isolated from three clones of *K. brevis*. Eighteen unique sequences were obtained for the PKS ketosynthase (KS) domain of *K. brevis*. Phylogenetic comparison with closely related PKS genes revealed that 16 grouped with cyanobacteria sequences, while the remaining two grouped with Apicomplexa and previously reported sequences for *K. brevis*. A fosmid library was also constructed to further characterize PKS genes detected in *K. brevis* Wilson clone. Several fosmid clones were positive for the presence of PKS genes, and one was fully sequenced to determine the full structure of the PKS cluster. A hybrid non ribosomal peptide synthetase and PKS (NRPS-PKS) gene cluster of 16,061 bp was isolated. In addition, we assessed whether the isolated gene was being actively expressed using reverse transcription polymerase chain reaction (RT-PCR) and determined its localization at the cellular level by chloroplast isolation. RT-PCR analyses revealed that this gene was actively expressed in *K. brevis* cultures. The hybrid NRPS-PKS gene cluster was located in the chloroplast, suggesting that *K. brevis* acquired the ability to produce some of its secondary metabolites through endosymbiosis with ancestral cyanobacteria. Further work is needed to determine the compound produced by the NRPS-PKS hybrid, to find other PKS gene sequences, and to assess their role in *K. brevis* toxin biosynthetic pathway.

Keywords Genomics · Chloroplast DNA · Dinoflagellate · Cyanobacteria · Hybrid NRPS-PKS · *Karenia brevis*

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Introduction

Harmful algal blooms result from proliferation of microalgae to the detriment of public health and natural resources. Evidence of a recent increase in the number and intensity of harmful algal blooms has raised concern that toxic phytoplankton species may be replacing nontoxic species (Smayda 1990; Hallegraeff 1993; Anderson 1994; Boesch et al. 1997; Sellner et al. 2003). One of those organisms, the dinoflagellate *Karenia brevis*, is the causative agent of red tide blooms observed in Florida and has been occasionally transported by ocean currents to the Atlantic Sea cost (Tester and Steidinger 1997). Allelopathy is the main mechanism by which *K. brevis* appears to exhibit a competitive advantage over some sympatric phytoplanktoners (Kubanek et al. 2005). Indeed, *K. brevis* produces cyclic polyethers known as the brevetoxins, which are responsible for the harmful effects of red tides, including massive fish kills and marine mammal mortality, and affect public health through accumulation in seafood

but also by inhalation (Lin et al. 1981; Chou and Shimizu 1982; Golik et al. 1982; Baden 1983, 1989; Chou et al. 1985; Shimizu et al. 1986; Prasad and Shimizu 1989; Pierce et al. 1990; Baden et al. 1995; Crouch et al. 1995; Backer et al. 2003; Fisher et al. 2003; Magaña et al. 2003; Kirkpatrick et al. 2004). Additional compounds produced by *K. brevis* include brevenal (Bourdelais et al. 2005), hemibrevetoxin B (Prasad and Shimizu 1989), brevisamide (Satake et al. 2008), as well as fatty acids and phytopigments (Truxal and Baden 2007). Similar to many other dinoflagellates, *K. brevis* is a prolific producer of secondary metabolites, and new compounds are still being isolated from different clones of this organism.

The construction of dinoflagellate polyketides, such as brevetoxins and ciguatoxins, is achieved by the polyketide pathway and probably involves a polyketide synthase (PKS) with some additional functional segments (Shimizu et al. 2001; Shimizu 2003). PKSs are typically classified in three categories: types I, II, and III PKS (reviewed in Shen 2003). Type I PKS are large, multifunctional and highly modular proteins that use acyl carrier proteins (ACP) to activate acyl CoA substrates and channel the growing polyketide intermediates. Type II PKSs also use ACP but are aggregates of mono-functional proteins where each catalytic domain is located on a separate peptide. Finally, type III, also known as chalcone synthase-like PKSs, are smaller PKSs that do not present ACP domains and are involved in flavonoid biosynthesis in plants and melanin in bacteria (reviewed in Austin and Noel 2003). In dinoflagellates, PKS genes have been detected in *Amphidinium* sp. (Kubota et al. 2006) and *K. brevis* (Snyder et al. 2003; Monroe and Van Dolah 2008). The PKS fragments sequenced for *K. brevis* corresponded to the ketosynthase (KS) domain and presented homologies with existent and putative type I PKS, which are typically found in bacteria (Snyder et al. 2003; Monroe and Van Dolah 2008). Recently, Monroe and Van Dolah (2008) described a novel PKS structure based on a full-length PKS transcript that also presented significant homology to type I PKS but encoded for discrete catalytic domains (a structure more similar to type II PKSs).

Although molecular genetic studies of dinoflagellates are of critical importance to understanding the biosynthetic pathway and evolutionary history involved in toxin production, such studies are particularly scarce due to the inherent characteristics of these organisms. First, dinoflagellates have unusually large genomes that are generally considered to be haploid (Rizzo et al. 1982; Triplett et al. 1993; Plumley 1997; Santos and Coffroth 2003). In particular, the *K. brevis* genome consists of 121 chromosomes (Walker 1982). Second, marine algal toxins are unique, and the genes required for their synthesis are just being discovered (Snyder et al. 2003; Kubota et al. 2006). Third, toxin production and toxicity differ between geographical isolates, environmental conditions, and composition and abundance of other algal species (Steidinger 1993; Plumley 1997). Finally, the role that bacteria play in toxin synthesis is controversial, with at least one instance of autonomous toxin synthesis by a bacterium isolated from a toxic dinoflagellate (Kodama et al. 1988, 1990).

In this study, we attempted to further elucidate the biosynthetic pathways involved in the production of secondary metabolites in *K. brevis*. We first sequenced fragments of PKS genes from three clones of *K. brevis*, C6 Tomas, SP3, and Wilson, and we compared their phylogenetic relationship with other PKS genes retrieved from NCBI GenBank. Then, we constructed a fosmid library and fully sequenced one of the fosmids carrying a PKS gene. Finally, we determined whether the isolated gene was being actively expressed in *K. brevis* cultures and determined its location at the intracellular level.

Material and Methods

Samples

Three clones of *K. brevis* were analyzed: (1) The C6 Tomas clone was isolated by Dr. Carmelo Tomas from a bloom in Corpus Christi, Texas in 1999 (Tomas, personal communication); (2) the SP3 clone was isolated in 1999 by Suzanne Pargee during a bloom event in Texas (Magaña and Villareal 2006); (3) the Wilson clone was isolated near John's Pass, Florida in 1953 (CCMP records, Steidinger, unpublished data). Each culture contained only cells of a single clone grown in autoclaved NH-15 artificial seawater without the addition of any antibiotic. Cultures were maintained at 24°C with 24-h light. All cultures are available at UNCW Center for Marine Science upon request.

DNA Extraction and Amplification

Approximately 150 mL of each *K. brevis* culture was filtered through a 0.45-μm mesh filter (PALL Corporation). DNA was extracted from the filter using the PowerSoil™ DNA Isolation Kit (Mo Bio Laboratories). A nested polymerase chain reaction (PCR) protocol was developed using two different primer sets targeting the KS domain. The initial PCR amplification was performed using the primers KSDPQQF and KSHGTGR described by Piel et al. (2004) in a 25 μL total reaction volume with: 1.25 μL of each primer (10 μM), 0.5 μL dNTP's (2 mM), 2.5 μL 10× buffer, 2 μL $MgCl_2$, 0.4 μL Taq polymerase 5U, and 1 μL DNA. A touchdown PCR (95°C, 5 min; 95°C, 30 s; decrease from 60°C to 50°C, −1°C at a time 1 min; 72°C,

1 min) followed by a second round of 35 amplification cycles (95°C, 30 s; 55°C, 30 s; 72°C, 1 min), and a final extension at 72°C for 10 min. After the initial PCR, a nested PCR was performed using the primers PKSI (forward and reverse) described in Courtois et al. (2003). A single soak at 95°C for 5 min was followed by 35 amplification cycles (denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min) and a final extension at 72°C for 10 min. All DNA amplifications were performed in a Peltier PTC-200 gradient PCR (MJ Research).

The amplified products were cloned in *E. coli* using the TOPO® TA Cloning® Kit and One Shot® TOP10 competent cells (Invitrogen). A total of 32 positive colonies per sample were sequenced. The sequencing reaction was carried out with the BigDye TM terminator v. 3.1 using the plasmid primers, M13R and T7. Sequences were obtained on an ABI Prism 3100 automated sequencer.

Phylogenetic Analyses

Partial PKS sequences corresponding to the KS domain were aligned using Bioedit version 7.0.5.2 (Hall 1999) and ClustalX (Thompson et al. 1997). All sequences have been deposited in the GenBank (accession numbers listed in Table 1). To perform phylogenetic analyses and ensure a proper and unambiguous alignment, only nucleotide sequences that presented at least a 50% similarity with ours were retrieved from GenBank (Fig. 1). Relationships between KS sequences were established with the neighbor-joining (NJ) algorithm with Jukes–Cantor correction using Mega v 3.1 (Kumar et al. 2004). Confidence in the nodes was assessed by 10,000 bootstrap replicates (Felsenstein 1985).

Isolation and Characterization of a Hybrid NRPS-PKS Gene

DNA extracted from *K. brevis* Wilson's clone was sheared by sonication. The resulting DNA fragments were cloned into the CopyControlTM pCC1FOS fosmid vector following manufacturer instructions (Epicentre Biotechnologies). The ligated DNA was packaged into MaxPlax lambda (Epicenter) and transformed into the EPI300-T1 plating cells. Fosmid-containing *E. coli* colonies were selected on Luria–Bertani (LB) solid medium supplemented with chloramphenicol (15 μg/ml). A total of 3,840 clones were handpicked and transferred to ten plates of 384 wells each, representing less than 1% of *K. brevis* total genome (predicted to be 1×10^{11} bp; Monroe and Van Dolah 2008). The expression of the *trf*A gene was induced for amplification of the clones to high copy numbers.

Table 1 Localization, size, and predicted function of the different regions identified for the NRPS-PKS gene cluster isolated from *Karenia brevis*' clone Wilson 4D F3, based on CDD BLAST searches

Location		Size (bp)	Predicted function	Sequence similarity (best hit)	*E* value	Accession no.
Start	End					
903	1,231	328	Promotor	–	–	–
1,306	2,202	896	Condensation domain	*Acaryochloris marina*	1E−77	YP_001520888
1,984	3,957	1,973	EntF NRPS module	*Nostoc* sp.	2E−143	AAO23333
2,782	3,978	1,196	Amp-Binding enzyme	*Microcystis* sp.	3E−98	AAZ03550
4,291	4,482	191	PP-Binding	*Microcystis* sp.	6E−13	AAZ03552
4,783	5,673	890	Condensation domain	*Opitutus terrae*	1E−80	YP_001818851
5,458	7,461	2,003	EntF NRPS module	*Nostoc* sp.	5E−96	AAO23333
6,241	7,689	1,448	Amp-Binding enzyme	*Nodularia spumigena*	8E−61	ZP_01632061
7,756	7,953	197	PP-Binding	*Nostoc punctiforme*	2E−12	YP_001866472
8,108	9,019	911	Condensation domain	*Brevibacillus parabrevis*	3E−72	Q70LM6
8,801	10,807	2,006	EntF NRPS module	*Bacterium Ellin514*	1E−153	ZP_02967912
9,572	11,047	1,475	Amp-Binding enzyme	*Myxococcus xanthus*	1E−118	YP_631821
11,099	11,293	194	PP-Binding	*Nostoc punctiforme*	5E−12	YP_001866470
11,545	12,810	1,265	PKS (β-KetoacylSynthase)	*Nostoc punctiforme*	1E−148	YP_001866780
13,120	14,076	956	Acyl-transferase	*Bacterium Ellin514*	6E−78	ZP_02966379
14,986	15,579	593	KR multi-domain	*Herpetosiphon aurantiacus*	5E−26	YP_001546721
15,871	16,059	188	PP-Binding	*Nostoc punctiforme*	2E−13	YP_001866472
16,242	16,943	701	Predicted thioesterase	*Nostoc* sp.	3E−57	NP_486085

The promoter region was predicted based on an EMBOSS CpG analysis. The most similar sequence to each NRPS-PKS region, *E* value, and accession number were obtained by BLASTx searches.

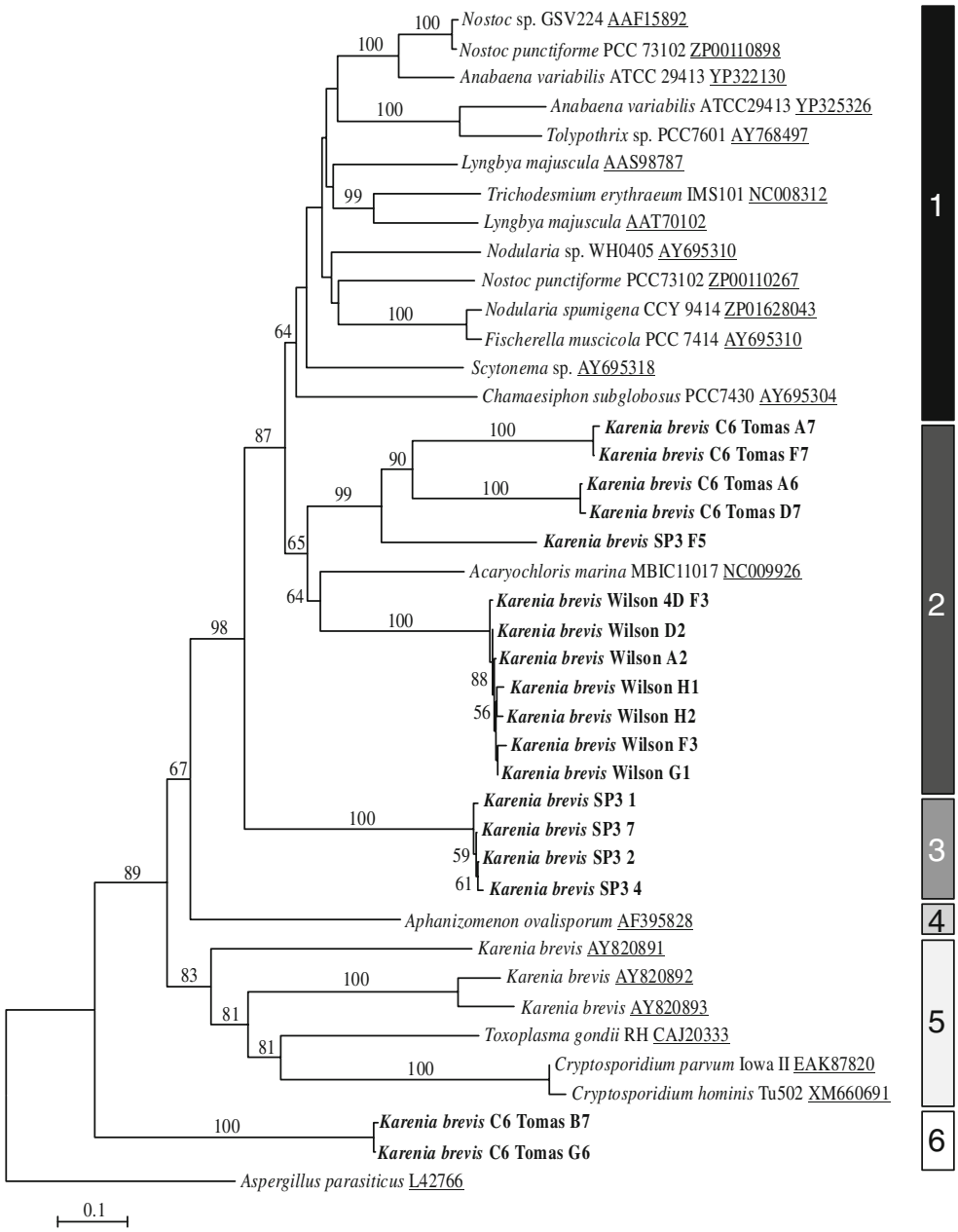

Fig. 1 Phylogeny of ketosynthase (KS) gene sequences from *Karenia brevis* obtained in this study (*bold*) and retrieved from NCBI GenBank. Labels on terminal nodes indicate the species and NCBI GenBank accession numbers. Tree topology was obtained from neighbor-joining (NJ) analysis. The six major clades obtained are also depicted (*1–6*). *Numbers above branches* indicate bootstrap support percentages (only when ≥50%)

To screen for the presence of PKS genes, copies of the ten libraries were pooled to one 384-well plate for initial PCR screening. Randomly selected wells were analyzed for the presence of PKS genes using the nested PCR procedure described above ("DNA Extraction and Amplification"). When a positive result was obtained, we analyzed the corresponding clones from the ten original libraries until the positive was found. Using this procedure, seven positive clones carrying a PKS gene were detected in our fosmid library, and one (Wilson 4D F3) was selected for subsequent analyses. Fosmids were extracted using the PerfectPrep plasmid Mini kit (Eppendorf) and re-suspended in 50 µL of elution buffer. The resulting purified fosmids were then digested using the restriction enzyme *HaeIII* (Promega) and cloned in *E. coli* using the Zero Blunt® TOPO® Cloning Kit (Invitrogen). Positive clones were sequenced and analyzed by the contig method using SeqMan Pro v. 7.2.1 (DNA*, Lasergene). The gene sequence was considered complete when no positive BLAST search hits were obtained for ~1,000 bp upstream and downstream from the gene cluster. The promoter region was predicted based on a CpG analysis (EMBOSS, Rice et al. 2000). Regions, conserved domains, and modules were annotated according to GenBank BLASTx and CDD BLAST results (Marchler-Bauer et al. 2007) and Artemis v. 10 (Rutherford et al. 2002).

RT-PCR and Gene Expression

K. brevis cells (Wilson clone) were grown and maintained in a 10-L batch culture containing autoclaved NH-15 artificial seawater and maintained at 24°C with 24-h light. After 5 weeks, approximately 150 ml of culture were filtered through a 0.45-μm mesh filter (PALL Corporation) and immediately transferred to a −70°C freezer. Half of a filter was homogenized in TRIzol® reagent (Invitrogen), and purified RNA was obtained using the Micro-to-midi RNA purification kit (Invitrogen), according to manufacturer instructions. RNA was re-suspended in 100 μL nuclease free water. Samples were DNAse-treated using DNase Amplification Grade I (Invitrogen). Purified RNA was quantified with the Quant-iT dsDNA HS Assay Kit on a Qubit fluorometer (Invitrogen). Two sets of primers were designed to test for the expression of the non-ribosomal peptide synthetase (NRPS) and PKS regions. The first set, NRPSExpF 5′-CGCTCATGT TCGATGCGTCCATTT-3′ (forward) and NRPSExpR 5′-TTGACCATTTCGACCTCAAGGCGT-3′ (reverse), was designed targeting a 500-bp region of the NRPS adenylation domain (amp-binding). The second set, PKSExpF 5′-ACAAGTTCGATGCCGGGTTCTTTG-3′ (forward) and PKSExpR 5′-ACATACGGCAACAAGAGAGGTGGA-3′ (reverse), was designed against a 654-bp region of the PKS KS domain. Reverse transcription and amplification was performed using DNAse untreated and treated samples (RNA-NT and RNA-T, respectively) and the SuperScript™ One-step reverse transcription PCR (RT-PCR) for long templates (Invitrogen). Total reaction volume was 25 μL with 12.5 μL of 2× reaction mix, 0.5 μL of each primer (10 μM), 0.5 μL RT/Platinium Taq polymerase, 10 μL of PCR grade water, and ~1 μg of RNA. A first incubation of 30 min at 50°C was followed by a single soak at 94°C for 2 min, 35 amplification cycles (denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 68°C for 1 min), and a final extension at 72°C for 5 min, in a Peltier PTC-200 gradient PCR. RT-PCR products were run in a 1% agarose gel and sequenced on an ABI Prism 3100 automated sequencer to verify fragment identity.

Gene Localization

Chloroplast Isolation

Intact chloroplasts were isolated using Sigma chloroplast isolation kit and modified as follows. One hundred milliliters of *K. brevis* culture were carefully filtred through a 0.45-μm mesh filter (PALL Corporation). Filters were cut in two and placed in separated tubs. Each half filter was grounded using a disposable pestle and 200 μl of 1× CIB buffer with BSA. Samples were centrifuged at 1,000 rpm for 20 min at 4°C. The recovered supernatant was centrifuged at 4,000 rpm for 20 min at 4°C to precipitate the chloroplasts. The supernatant was discarded and the pellet resuspend in 30 μl of 1× CIB buffer without BSA (Chl-NT). One of the samples was then stored at – 20°C. The other sample was DNAse treated (Chl-T) using DNase Amplification Grade I (Invitrogen), 5 μl buffer and 3 μl of the enzyme and incubated at room temperature for 15 min. The reaction was stopped by adding 3 μl of EDTA solution and centrifugation at 4,000 rpm for 20 min at 4°C. The supernatant was then decanted and the pellet resuspended in 200 μl of 1×CIB buffer without BSA.

Chloroplast DNA Extraction and Amplification

Chloroplast DNA was extracted using the Puregene kit (Gentra Systems) from both the DNAse untreated and treated samples (Chl-NT and Chl-T, respectively). To test for the presence of the hybrid NRPS-PKS gene in chloroplast DNA, we used the primers NRPSExpF and NRPSExpR and PKSExpeF and PKSExpR descrived above (gene expression section). In addition, another set of primers, KBB-TUBF 5′-ATGCGGATGAGTGCTTC TTGCTTG-3′ (forward) and KBB-TUBR 5′-AACATCT GTTGGGTGAGCTCTGGT-3′ (reverse), was designed to amplify a 298-bp segment of the nuclear gene β-tubulin (GenBank accession number EF455751; Yoon et al. 2008) and test for nuclear DNA contamination. Amplification for both genes was performed in a 25 μL total-reaction volume with 1.25 μL of each primer (10 μM), 0.5 μL dNTP's (10 mM), 2.5 μL 10× buffer, 2.5 μL $MgCl_2$, 0.5 μL Taq polymerase 5U, and 1 μL DNA. A single soak at 95°C for 5 min was followed by 35 amplification cycles (denaturation at 95°C for 30 s, annealing at 58°C for 30 s and extension at 68°C for 1 min) and a final extension at 72°C for 5 min, in a Peltier PTC-200 gradient PCR. A standard DNA extraction of *K. brevis* (Wilson clone) was used as positive control. PCR products were run in a 1% agarose gel and sequenced on an ABI Prism 3100 automated sequencer to verify fragment identity.

Results and Discussion

Phylogenetic Analyses

We obtained 18 unique sequences of the PKS KS domain from three different clones of the dinoflagellate *K. brevis*. After alignment and trimming, a final length of 689 bp was used for further analysis. All sequences have been deposited in the GenBank data base (Accession numbers FJ172508 to FJ172525). Six major clades appeared in the NJ tree and were supported by bootstrap values >50 (Fig. 1). The first clade (labeled 1) was comprised entirely

of cyanobacteria PKS sequence retrieved from GenBank. The second clade (2) grouped all sequences obtained for the Wilson clone (4D F3, H1, H2, D2, A2, F3, and G1), four out of six sequences obtained for C6 Tomas clone (A7, F7, A6, and D7), one out of five sequences from SP3 clone (F5) and another cyanobacteria sequence retrieved from GenBank (*Acaryochloris marina*). The third clade (3) was formed by the remaining four SP3 sequences (1, 7, 2, and 4), while clade 4 contained a single sequence from the cyanobacteria *Aphanizomenon ovalisporum*. Clade 5 was composed of the three *K. brevis* sequences obtained by Snyder et al. (2003) and the apicomplexa *Cryptosporidium* spp. and *Toxoplasma gondii* (the eukaryote group most closely related to dinoflagellates; Fast et al. 2002). Finally, clade 6 contained the two remaining sequences for the C6 Tomas clone (B7 and G6) and stood as an outgroup to the remaining *K. brevis* sequences.

Interestingly, all PKS gene sequences obtained in this study, except two, were more closely related to cyanobacterial PKS gene sequences than to other eukaryote sequences. Moreover, even when nucleotide sequences were translated to amino acids, thereby allowing for comparison with other *K. brevis* sequences that presented less than 50% similarity, only C6 Tomas B7 and C6 Tomas G6 sequences grouped with previously described *K. brevis* PKS gene sequences (maximum similarity 46%), while all other sequences grouped with prokaryotes. Horizontal gene transfer is the process of exchanging genetic material between distant-related species. This phenomenon played a major role in prokaryotic genome evolution, and recent data suggest that it also occurred frequently in unicellular eukaryotes, possibly at rates comparable to prokaryotic organisms (Andersson 2005). There are two types of gene transfer in eukaryotes (Andersson 2005). First, transfer of genes from organelles with an endosymbiotic origin (the mitochondrion and chloroplast) to the nucleus of the eukaryotic cell. Data to date suggest that massive plastid to nucleus gene transfer has indeed occurred in dinoflagellates (Delwiche 1999; Cavalier-Smith 2000; Keeling and Palmer 2001; Keeling 2004; Bachvaroff et al. 2004). Second, horizontal gene transfer may occur between unrelated species. For instance, the glyceraldehyde 3-phosphate dehydrogenase gene could have been transferred between a dinoflagellate and an euglenophyte (eukaryotic algae; Takishita et al. 2003). Our results suggest that gene transfer from cyanobacteria to at least some marine dinoflagellate species has occurred more than once, thus explaining the similarity found between part of the *K. brevis* PKS sequences obtained in this study and cyanobacteria sequences retrieved from GenBank.

Isolation and Characterization of a Hybrid NRPS-PKS Gene

The sequencing of *K. brevis* fosmid clone Wilson 4D F3 resulted in a fragment of 18,027 base pairs. The resulting gene cluster contained a hybrid NRPS-PKS of 16,061 bp identified based on BLASTx and CDD BLAST searches (GenBank accession number FJ172507). The promoter region was predicted based on a CpG analysis and did not present any known transcriptional element (Table 1). The NRPS portion of the gene was formed by three modules (Table 1, Fig. 2), each containing a condensation domain (C), an adenylation domain (A; amp-binding enzyme), and a peptidyl carrier protein domain (PP-binding). These three modules were followed by a PKS type I gene formed by a KS like region, an acyl transferase, a ketoreductase domain, an ACP (PP-binding), and a thiosterase.

The polycyclic ether compounds, macrolides, and other oxygenated alkyl compounds found in dinoflagellates are considered to be biosynthesized by the polyketide pathway (Shimizu 2003). However, other compounds such as DTX-5a, DTX-5b (Macpherson et al. 2003), and brevisamide (Satake et al. 2008) point toward the existence of mixed NRPS-PKS systems that use glycine as a source of nitrogen. Along with PKSs, NRPSs produce numerous and structurally diverse secondary metabolites, including branched cyclic structures and unusual and modified amino acids in small peptides (Dittmann et al. 2001). Both PKSs and NRPSs have a modular organization, with each module carrying all essential information for recognition, activation, and modification of one substrate into the growing

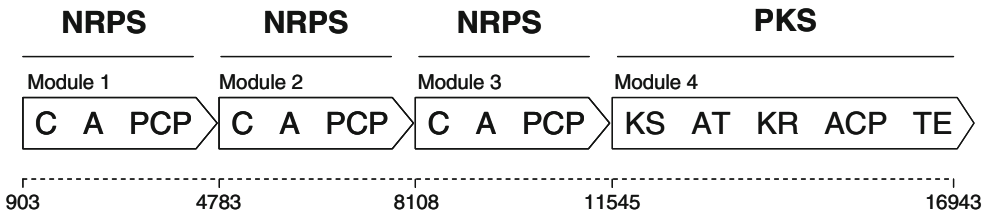

Fig. 2 Predicted domains for the isolated hybrid NRPS-PKS from *Karenia brevis*—Wilson clone. *C* Condensation, *A* adenylation, *PCP* peptidyl carrier protein, *KS* ketosynthase, *AT* acyl transferase, *KR* ketoreductase, *ACP* acyl carrier protein, *TE* thioesterase. The *dashed line* indicates relative base pair position

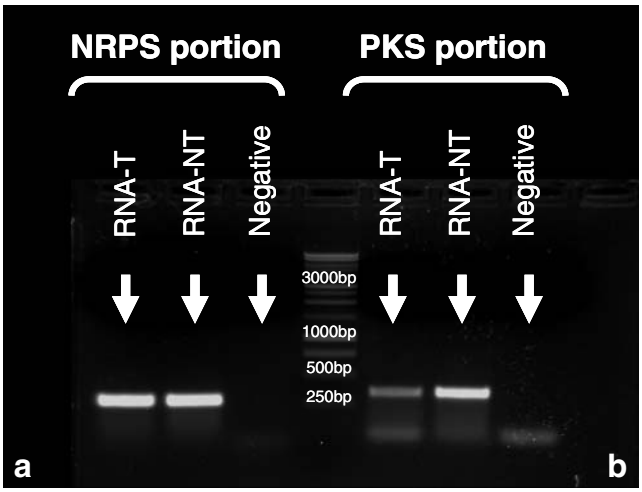

Fig. 3 RT-PCR products amplified from total RNA using specific primers against a 500-bp fragment of the NRPS Amp-binding domain (**a**) and a 654-bp fragment of the PKS KR multi-domain region (**b**). Results for both RNA treated with DNase amplification grade (RNA-T) and non-treated (RNA-NT) are shown. Negative refers to the negative control (no RNA added)

chain. PKSs and NRPSs are found in both prokaryotes and lower eukaryotes. PKS and NRPS can also form hybrid systems or clusters (NRPS-PKS), which have been often reported in bacteria and cyanobacteria (e.g., Dittmann et al. 2001; Chang et al. 2002; Piel 2002; Edwards et al. 2004) and has been identified here in a dinoflagellate for the first time.

Gene Expression and Localization

RT-PCR analyses with specific primers targeting both NRPS and the PKS regions of the isolated NRPS-PKS gene showed that both regions were actively expressed in *K. brevis* cultures (Wilson's clone; Fig. 3). Sequencing of the resulting amplicons confirmed sequence identity. A positive amplification for the presence of NRPS-PKS gene was also obtained when using chloroplast DNA. No amplification for β-tubulin was obtained, indicating that our chloroplast DNA extraction was not contaminated with nuclear DNA (Fig. 4). Moreover, we did not find the spliced leader sequence (Palenchar and Bellofatto 2006) typical of dinoflagellates PKSs in the NRPS-PKS gene cluster or in the ~1,000 bp upstream and downstream flanking regions (Lidie and Van Dolah 2007; Monroe and Van Dolah 2008). Therefore, the evidence indicated that the hybrid NRPS-PKS reported in this study was being actively expressed in the chloroplast of *K. brevis* cultures, although it did not exhibit the typical eukaryotic configuration.

Indirect evidence of the presence of biosynthetic genes in chloroplasts has been reported in other dinoflegellates. For instance, okadaic acid was localized immunocytochemically to the chloroplast of *Prorocentrum* (Zhou and Fritz 1994). However, the authors did not demonstrate whether the biosynthetic gene was also located in the chloroplast. In addition, several dinoflagellates have anomalous plastids derived from various endosymbionts, including green algae, diatoms, cryptophytes, and haptophytes (reviewed in Delwiche 1999, Tengs et al. 2000). Some of the information contained in these plastids may have been transferred to the dinoflagellate nucleus, while some may have remained in the plastid and still be expressed. The presence of cyanobacteria-like genes in marine dinoflagellate cells is even more intriguing when considering that cyanobacteria, rather than dinoflagellates, almost exclusively synthesize freshwater harmful algal bloom toxins (Carmichael et al. 1990; Carmichael 1992, 1994). Moreover, some toxins, like the saxitoxins and gonyautoxins, are produced by cyanobacteria in freshwater (Carmichael 1992; Falconer 1997) and by dinoflagellates or bacteria in marine systems (Plumley 1997). Recently, Laurent et al. (2008) reported

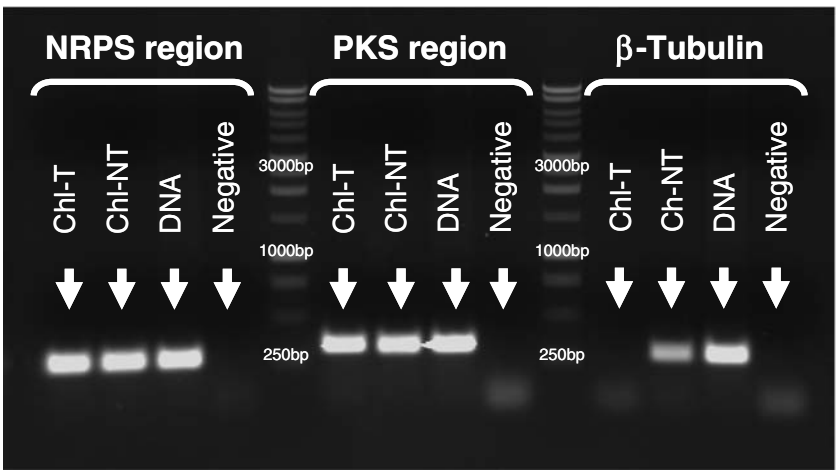

Fig. 4 PCR products amplified from chloroplast DNA (Chl DNA) using specific primers for the NRPS (**a**) and PKS (**b**) regions of the isolated gene cluster and the nuclear β-tubulin gene (**c**). Results for isolated chloroplasts treated with DNase amplification grade (Chl-T) before chloroplast DNA extraction and non-treated (Chl-NT) are shown. Amplification was also performed on standard DNA extractions (DNA) from *Karenia brevis* as a positive control. *Negative* refers to the negative control (no DNA added)

the biogenesis of paralytic shellfish toxins, neurotoxins, and ciguatoxin-like compounds by marine cyanobacteria in the genus *Hydrocoleum*. As mentioned above, gene transfer has often facilitated the acquisition of functions encoded in prokaryotic genomes by eukaryotic organisms (Adams and Palmer 2003; Timmis et al. 2004; Andersson 2005), and our results provided the first genetic evidence of gene transfer from an endosymbiotic, toxigenic cyanobacterium to a marine dinoflagellate species, *K. brevis*. The presence of ancestral cyanobacteria genes in dinoflagellates would explain both the similarity of some *K. brevis* PKS gene sequences to cyanobacterial genes and the ability of some marine dinoflagellates to produce compounds found in freshwater cyanobacteria, such as the saxitoxins.

Conclusion

Phylogenetic analysis of PKS KS domain sequences from three clones of *K. brevis* revealed three distinct clades. The first two clades grouped with cyanobacterial sequences, while the third clade was closer to the Apicomplexa. Further genomic analysis of the Wilson clone revealed a hybrid NRPS-PKS gene in *K. brevis*. The presence of NRPS-PKS systems in dinoflagellates was suggested by the chemical structure of some compounds produced by these organisms (e.g. Moore 2005; Satake et al. 2008). However, this is the first time a hybrid NRPS-PKS gene cluster has been directly isolated from a dinoflagellate. Furthermore, the NRPS and PKS regions were being actively expressed by *K. brevis* and were located in the chloroplast, suggesting that *K. brevis* has acquired at least some of its biosynthetic genes though endosymbiosis with cyanobacteria.

Future research needs to focus on determining the compound produced by the isolated NRPS-PKS gene and its chemical structure. This task may prove especially challenging as natural product structures derived from mixed PKS and NRPS biosynthetic systems involve complex protein–protein interactions that have yet to be fully understood (Chang et al. 2002). Further efforts to isolate other PKS and mixed NRPS-PKS genes are necessary in order to determine the role of these genes in toxin biosynthetic pathways and to understand the production of neurotoxins by *K. brevis*.

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