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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.
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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, mar-

ine 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

brevetoxin A

brevetoxin B

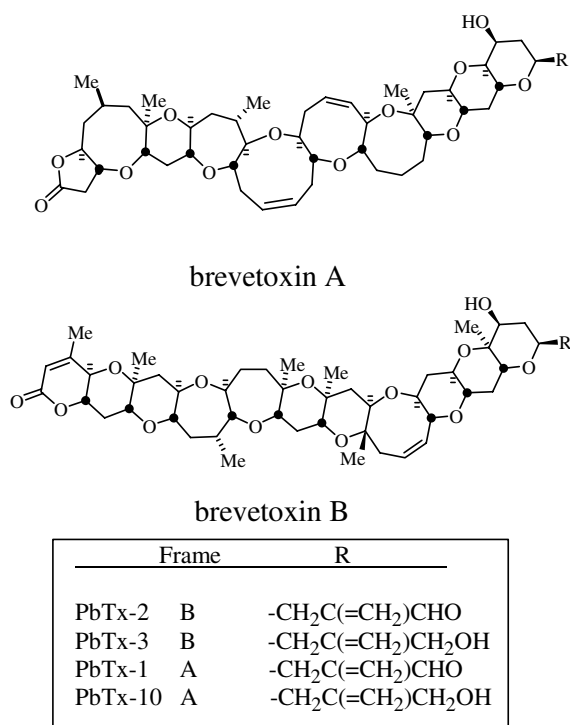|	Frame	R
PbTx-2	B	-CH₂C(=CH₂)CHO
PbTx-3	B	-CH₂C(=CH₂)CH₂OH
PbTx-1	A	-CH₂C(=CH₂)CHO
PbTx-10	A	-CH₂C(=CH₂)CH₂OH

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 aro-

matic 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-*

vis were analyzed by DGGE of the 16S rRNA gene to verify the variation of the bacterial population present in isolates derived from distinct geographical and temporal origins. The results of the DGGE analysis are shown in Fig. 2. The cultures CCMP2281 and JR1 are the same isolate and both are reportedly axenic. While the DGGE indicates that JR1 possesses a significantly less complex consortia of associated bacteria, the claim of axenicity should be viewed with some caution. We have in fact amplified and sequenced bacterial 16S ribo-

somal genes from CCMP2281. The DGGE analysis of JR1 indicates the presence of one major and two minor bands. The major band is also present in JR and CCMP2228, but is absent from CCMP2229 and CCMP718. CCMP2229 and CCMP718 both have one predominant associated bacteria (which are not the same). These bacteria are not present in CCMP2228, JR and JR1. Thus, while a few individual strains do share some common bands, there is not a single band that is common to all strains (Fig. 2). We therefore sought first to establish the presence of these PKS genes in all available isolates of *K. brevis* and their absence in associated bacteria or other marine protists. Six different cultures (or five distinct isolates) of *K. brevis* were examined by PCR or RT-PCR for the presence and expression of PKS genes. All strains tested positive for the presence of brevetoxin by ELISA (Naar et al., 2002). In addition to *K. brevis* isolates, flow sorted bacterial consortia from *K. brevis* CCMP718 and CCMP2229, five unique heterotrophic bacterial isolates from CCMP718 (data not shown) and a collection of 10 other species of marine protist, were screened by PCR using genomic templates.

Two sets of sequence-specific primers were designed from each of the four PKS related amplicons (1L a/b, 10L a/b, 6L a/b, 15 a/b, Table 1). Both sets of primers designed for AT2-10L yielded products of the expected size and T_m from all five isolates of *K. brevis* tested (CCMP718, 2228, 2229, 2281, JR and NOAA1) either by PCR using a genomic DNA template or by RT-PCR (Table 2). One primer set, specific for sequence AT2-15 (15a), failed to yield a product from CCMP718 or CCMP2228 using a genomic template or from
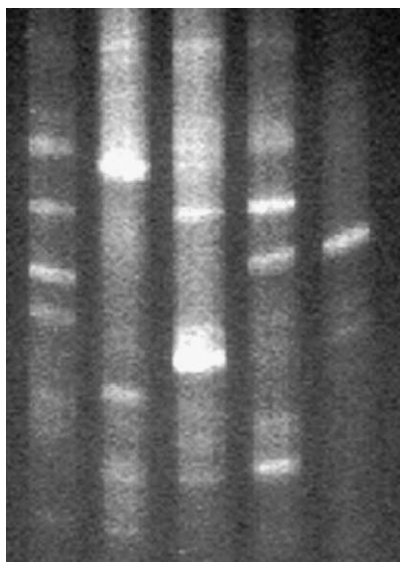

Fig. 2. DGGE analysis of 16S ribosomal gene amplified from different strains of *K. brevis*. The order of the lanes are (from left to right) CCMP2228, CCMP2229, CCMP 718, JR, JR-1 (see Section 4 for experimental details).

Table 1
Sequence-specific PKS primers

Primer set	Forward primer-3′ position	Reverse primer–3′ position	5′ → 3′ sequence	Product size (bp)	T_m (°C)
1La	AS11L-300		CAGCTTGTTCATCTTCACTTGTTGCCACTCAT		66
		AS11L-471	TTCACCGCGCACATACCCATCTG	203	63
1Lb	AS11L-357		CGACTGCGCAATTGCTGGTGGTGTA		66
		AS11L-504	TTGCAGATTCGAAAGGATGGTGAC	172	59
10La	AT210L-214		GGACAGGCAACCACCCATCAATCT		62
		AT210L-540	GCCGCGCAACGTACAAGTCACA	349	62
10Lb	AT210L-302		CGATTGGCAACGCAGAACTCA		58
		AT210L-471	CAAGCGCCATGGCACAAACAC	190	60
15a	AT215-42		ACCCGCAGCAACGTCTCCTCCTC		64
		AT215-410	GGTGACAGCATGCCAGCCTTACAA	391	62
15b	AT215-87		GAATGCTGCTGGTGTCCTCAAGAAG		60
		AT215-308	GCCAAGCGCACAGCAACAAGACC	246	64
6La	AT16L-135		CGTTGGCCAAGACAAGTGCG		59
		AT16L-236	CAGCCCGAGTGCATACGAGAT	121	56
6Lb	AT16L-263		GGCCGAGCTGCACAATGGATAC		60
		AT16L-531	AGAACCGCCAAGACTCACTAATG	290	54

Table 2
PKS PCR survey with *K. brevis* strains, other dinoflagellate, haptophyte, raphidophyte and bacterial templates

Microorganism	Primer set					
	10La	10Lb	15a	15b	6La	6Lb
K. brevis 718 (Wilson strain)	+[*]/+	+/+	–/–	+/+	–/+	–/+
K. brevis 2228	+[*]/+	+/+	–/+	+/+	+/–	–/+
K. brevis 2229	+/+	+/+	+/+	+/+	–/+	–/+
K. brevis 2281 (JR1)	+/+	+/+	+/+	+[*]/+	–/+	–/+
K. brevis JR	+/+	+/+	+/+	+/+	+/+[*]	–/+
K. brevis NOAA1	+	+/+	+	+	+	–/+
A. cartarae 1314	–/–	–/–	–/–	–/–	+[*]	–/–
A. operculatum 120	–/–	–/–	–/–	–/–	–	–/–
C. subsalsa 217	–/–	–/–	–/–	–/–	–	–/–
H. akashiwo 1870	–/–	–/–	–/–	–/–	–	–/–
P. hoffmannianum 683	–/–	–/–	–/–	–/–	–	–/–
P. lima (Baden)	–/–	–/–	–/–	–/–	–	–/–
P. micans 1591	–/–	–/–	–/–	–/–	–	–/–
P. parvum (Yasumoto)	–/–	–/–	–/–	–/–	–	–/–
Prymnesium sp. 1212	–/–	–/–	–/–	–/–	–	–/–
Symbiodinium sp. 831	–/–	–/–	–/–	–/–	–	–/–
K. brevis 718 sorted bacteria	–	–	–	–	+[*]	+
K. brevis 2229 sorted bacteria	–	–	–	–	+	+

For each primer set, the results are shown as PCR (genomic template) or PCR(genomic)/RTPCR.
[*] Sequenced.

CCMP718 cDNA. Nonetheless, a product of the expected size was amplified from all strains tested using the second AT2-15 primer set (15b) with both the genomic DNA or cDNA templates. Using a genomic DNA template, products were obtained from CCMP2228, JR and NOAA1, and primer set 6La. No product was obtained using CCMP718 or 2281 even though 2281 and JR are ostensibly the same strain and the amplicon was originally derived from CCMP718 using degenerate PKS primers. No product was obtained using the 6Lb primer set and genomic DNA. Surprisingly, these two primer sets (6La and 6Lb) yielded products of the anticipated size in all RT-PCRs with the single exception of CCMP2228 and primer set 6La. Neither primer set targeting AS1-1L yielded a product from either RT-PCR or genomic PCR, using any of the five *K. brevis* isolates including the Wilson strain (data not shown). This suggested to us that the original product (AS1-1L) was amplified from a bacterial contaminant, which is consistent with the placement of this sequence in the phylogenetic analysis (Snyder et al., 2003). The identities of the products amplified from genomic DNA were confirmed either by sequencing (indicated in Table 2) or were checked against the product amplified from a recombinant plasmid, which carried the target sequence, for identical restriction patterns using the enzymes *Hinc*II and *Sph*I (AT2-10L), *Hinc*II (AT2-15) or *Sph*I (AT1-6L). Each restriction site was predicted to occur only once in the amplified product. Melting curves of products amplified from cDNA were compared to those products amplified from the corresponding recombinant plasmid. Restriction patterns of all amplified products were consistent with the expected product with the fol-

lowing exception. Digestion of the products from the 10La primer set using *Hinc*II revealed two distinct banding patterns. Products amplified from CCMP2228, 2281 and NOAA1 gave the predicted band at ∼275 bp when digested with *Hinc*II which was also consistent with the positive control amplified from the recombinant plasmid. Products amplified from CCMP718, 2229 and JR each gave smaller bands at ∼200 bp. Sequencing of a representative for each of the 10La products and subsequent sequence analysis revealed a C → T point mutation which resulted in a second *Hinc*II digestion site in the CCMP718, 2229 and JR group, located ∼70 bp downstream of the previously identified *Hinc*II site.

2.2. Screening for PKS genes from other marine eukaryotes with specific primers

Sequence-specific primers based on each of the four sequences AS1-1L, AT1-6L, AT2-10L and AT2-15 were tested against cDNA and genomic DNA from a variety of dinoflagellates, raphidophytes and haptophytes (Table 2). The primer sets based on the AT2-10L and AT2-15 sequences failed to yield a product from any organism other than *K. brevis*, either from DNA or cDNA. However, the primer set (6La) based on AT1-6L consistently yielded a product of the anticipated size from *Amphidinium carterae*. Sequencing indicated that this product matched AT1-6L exactly.

2.3. Flow sorting PCR and DGGE

K. brevis strains (CCMP718 and CCMP2229) were subjected to flow cytometric cell sorting to separate

dinoflagellate cells from associated bacteria. Multi-parametric sort gate logic (Fig. 3) based on a combination of forward scatter, side scatter, pulse width and chlorophyll autofluorescence, was used to discriminate the bacterial and dinoflagellate populations from each other. Each targeted population (bacteria or dinoflagellate) was aseptically collected in sterile microfuge tubes for nucleic acid extraction and analysis and brevetoxin ELISA.

DNA was extracted from pre-sorted culture, as well as the dinoflagellate and bacterial fractions, and subsequently used in PCRs using degenerate PKS, and universal 18S rRNA, and 16S rRNA primer sets (Fig. 4A). The presorted material and the sorted dinoflagellate fraction yielded all three of the expected products, indicating that the bacteria were not successfully removed from *K. brevis*. However, the bacterial fraction yielded only PKS and 16S products, and failed to yield an 18S product. Therefore, we were able to obtain dinoflagellate-free bacteria. To ensure that the sorted bacteria were representative of the bacterial consortium associated with *K. brevis*, DGGE analysis was performed on the 16S ribosomal gene products amplified from CCMP718. While there are differences in relative intensities of the bands, the sorted dinoflagellate fraction and the sorted bacterial fraction appear to have the same number of bands. (Fig. 4B). In an effort to identify previously amplified PKS genes, the PKS product amplified from the bacterial fraction from CCMP718 using *degenerate* primers was cloned and 40 inserts were sorted into four groups by RFLP analysis using seven restriction enzymes (*Kpn*I, *Bam*HI, *Xba*I, *Xho*I, *Hpa*II, *Rsa*I and *Sph*I). A representative from each group (*K. brevis*: bac(3), bac(23), bac(28) and bac(30)) was sequenced and none of the sequences was found to match any of those previously amplified, including AT1-6L. However, one of the bacterial sequences K brev_bac(30), clustered with AT1-6L, AT2-10L and AT2-15 in the phylogenetic tree (vide infra). Dinoflagellate and bacterial fractions were analyzed by brevetoxin ELISA. While
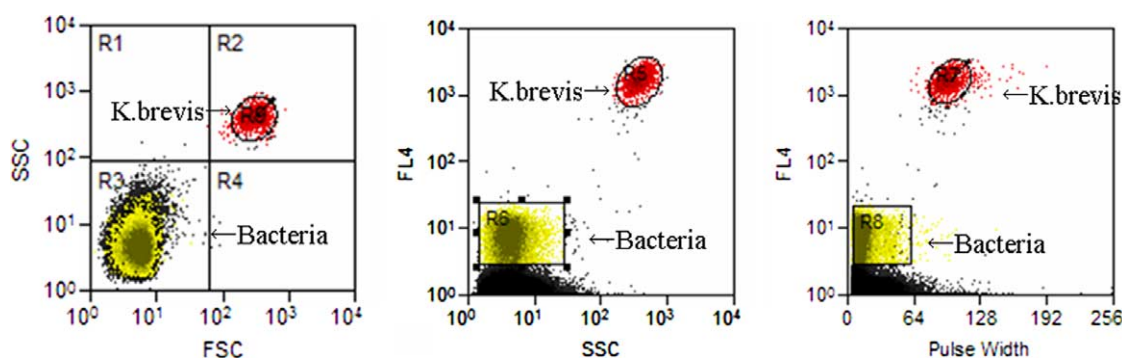

Fig. 3. Flow cytometric cell sorting gate logic for separation of bacterial and 6 dinoflagellate populations from a culture of *Karenia brevis* CCMP718. Dinoflagellate population (red) sorted by gates R9 + R5 + R7, and bacterial population (yellow) sorted by gates R3 + R6 + R8. SSC = side scatter, FSC = forward scatter, FL4 = Chlorophyll autofluorescence detection by 670/30 nm 670/30 nm bandpass. All excitation at 488 nm.
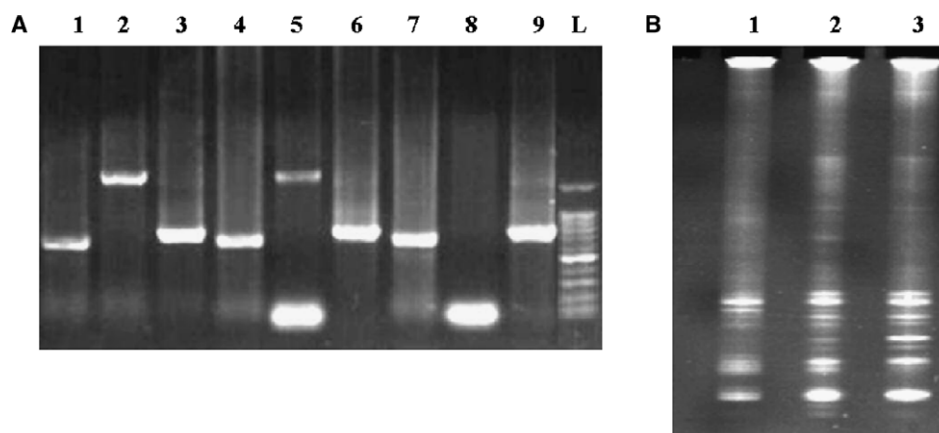

Fig. 4. PCR screening of flow-sorted bacterial and dinoflagellate fractions. (A) Screening for the presence of PKS, 18S rRNA and 16S rRNA genes from CCMP718. Products are in numerical order corresponding to PKS, 18S and 16S, respectively, for each of the following groups: 1–3 presorted *K. brevis*, 4–6 *K. brevis* fraction and 7–9 bacterial fraction. L refers to a 100 bp ladder (Promega). (B) DGGE analysis of 16S rRNA gene amplified from individual fractions. Lane 1, presorted *K. brevis*. Lane 2, *K. brevis* sorted fraction. Lane 3, sorted bacterial fraction.

the culture was strongly positive for brevetoxins, the bacterial fraction was negative and the dinoflagellate fraction tested weakly positive, but below the limit of quantitation (Fig. 5A). This suggested that brevetoxin is mostly present in the bulk media. Subsequent analysis of culture media from which *K. brevis* cells were gravity filtered supported this assumption (Fig. 5B).

Sequence-specific primers for PKS sequences AS1-1L, AT1-6L, AT2-10L and AT2-15 were tested against the sorted bacterial fractions from CCMP2229 and CCMP718 (Table 2). Although AT2-10L and AT2-15 were amplified from the total genomic DNA and genomic DNA from the sorted dinoflagellate fraction, neither was amplified from the bacterial fractions. One primer set (6La) yielded a product from the sorted bacterial fractions from both isolates of *K. brevis*. The 121-bp product from each strain was sequenced and found to match the AT1-6L sequence exactly.

The consistent amplification of the two PKS gene fragments (AT2-10L and AT2-15) from all strains of *K. brevis* and the absence of product from any other source, including flow sorted bacteria, bacterial isolates or any other protist, indicated that these two genes are clearly exclusive to *K. brevis*. However, the situation with AT1-6L appeared to be somewhat ambiguous. The sporadic amplification fragment from total genomic DNA from *K. brevis* culture, its presence in flow sorted bacteria and at least one other dinoflagellate, suggested that this sequence is derived from a bacterium which may be associated with marine eukaryotes. The amplification of this gene fragment from flow sorted bacteria from CCMP718 and CCMP2229 was somewhat surprising since this primer set failed to yield a product from these two strains when using total genomic DNA as the template, although cDNA from most isolates did yield a product. We speculate that flow sorting the bacterial consortium *or* the use of a cDNA template yields similar, positive result because each provides a template enriched in the target sequence when compared to total genomic DNA, as sorting removes dinoflagellate DNA and using a cDNA template eliminates unexpressed genes or structural DNA.

2.4. Phylogenetic analysis

Sequence and phylogenetic analysis was performed in order to infer a proper context for both dinoflagellate and the bacterial PKS/FAS-like gene sequences. Overall, although FAS sequences tend to show slightly higher similarities, KS sequences, even at the amino acid level, exhibit high sequence divergences (range = 0.04–0.86) which make definitive phylogenetic reconstructions tenuous using any algorithm (Kong et al., 2004; Lopez, 2003). Nevertheless, the amino acid based distance KS/FAS phylogeny in Fig. 6 yielded several expected groupings and had the same basic topology as the MP tree. For example, bacterial and eukaryotic PUFA (polyunsaturated fatty acid) PKS sequences (Metz et al., 2001) retrieved from GenBank, often formed a consistent clade with relatively high bootstrap support, and exhibited high divergences with dinoflagellate PKS sequences. Confirmed KS sequences from terrestrial sources were included as another reference and showed high bootstrap support for their own grouping. Other anonymous marine bacterial or fungal FAS sequences (e.g. K155, K618 and JL085) designated as outgroups appeared distinct from both PUFA and the *K. brevis* sequences within the single, major KS clade (which had 100% bootstrap support).

Phylogenetic analysis indicated that alveolate KS sequences from *C. parvum*, *Cryptosporidium hominus* and *K. brevis* formed a single, moderately supported group in spite of relatively high mean pairwise distances. However, maximum parsimony analysis yielded a similar topology, and bootstrap support for this alveolate clade increased to about 95% when the Kbrev_bac(30) sequence was omitted from the analysis.

Also similar to previous results (Snyder et al., 2003), *K. brevis* AT2-10L and AT2-15 exhibited high sequence similarity (0.755) to the exclusion of the more distant AT1-6L KS sequence. However, there were interesting differences in the KS active site motif even among these sequences: AT2-15 (CSGGL), AT2-10 (CSSGL) and AT1-6 (CSSSL). Furthermore, AT1-6L did not show any strong affinity for any bacterial
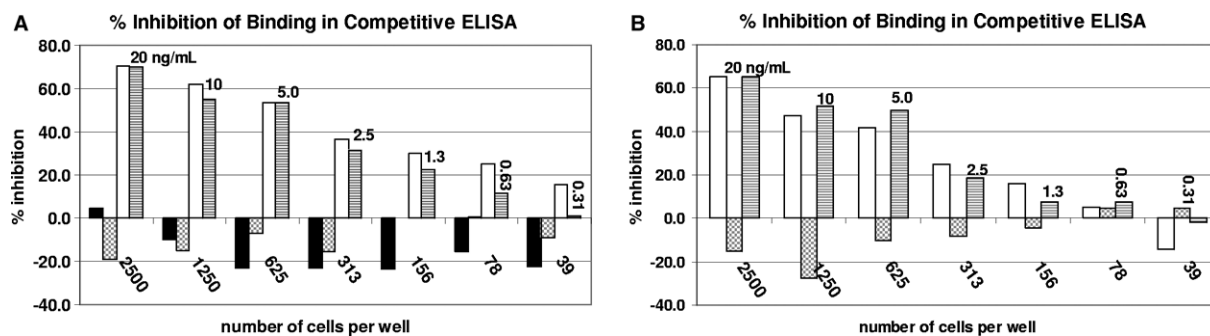

Fig. 5. (A) ■ sorted KB cells; ▨ sorted bacteria; □ whole KB culture, ▤ PbTx-3 standards. (B) □ KB filtrate, ▨ KB filtered cells, ▤ PbTx-3 standards. Numbers below the bars represent the number of *K. brevis* cells extracted, or an equivalent volume of culture.
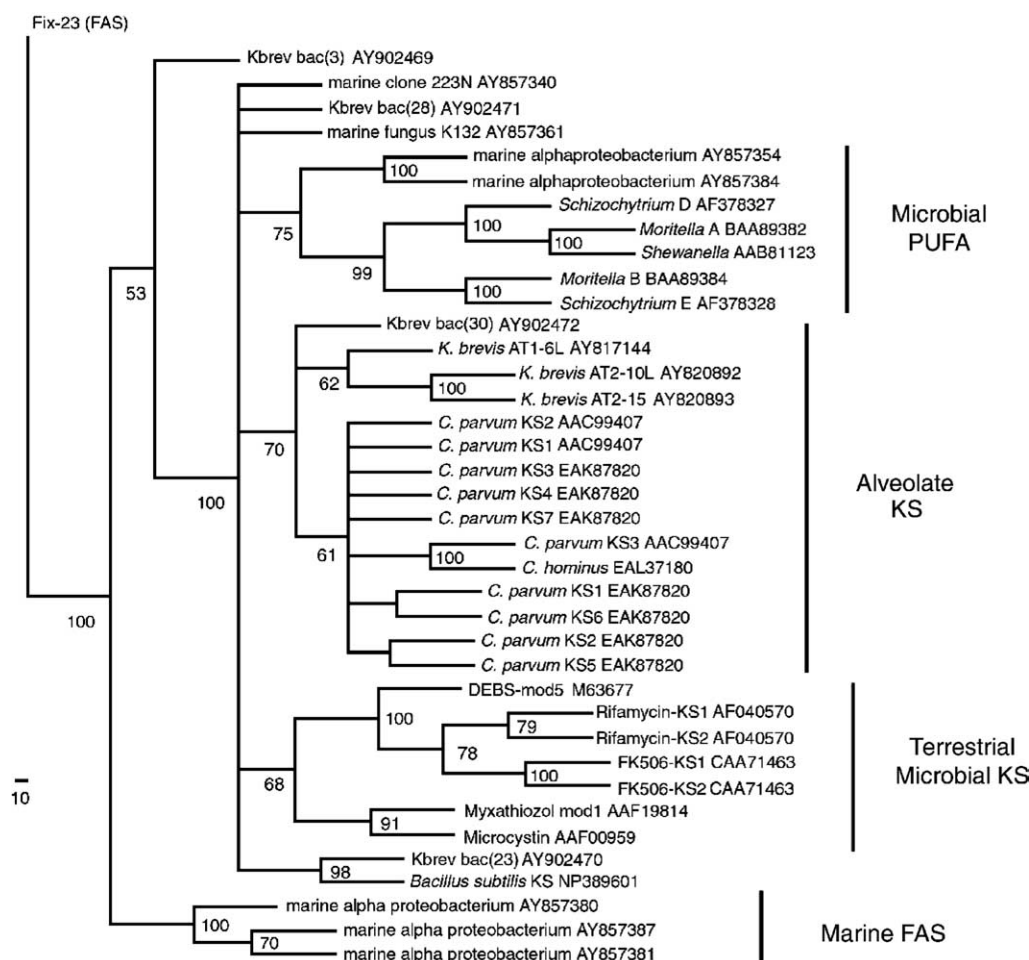
Fig. 6. Minimum evolution distance phylogeny of KS and FAS sequences generated with the neighbor-joining algorithm and mean pairwise amino acid distances. A total number of 350 amino acid residues from 39 PKS or FAS sequences were analyzed in PAUP* for this reconstruction. Only reference sequences are shown with their corresponding GenBank accession number. 500 bootstrap replicates were performed with the final bootstrap percentages (>50%) shown at the nodes, with well-supported clades indicated by name. K132, clone 223N, Kbrev_bac(28), Kbrev_bac(23) and *Bacillus* sequences are part of the major KS clade. The longest branch length of 0.34 was between the bifurcating node and 19E-*Schizochytrium*. The minimum evolution score of this tree was 8.46769, while the corresponding MP tree had a consistency index of 0.5845. The scale bar in the left corner represents the number of amino acid residue changes along each branch.

sequences, in spite of its amplification from sorted bacterial fractions. As a side note, the *C. parvum* sequences used here (AAC99407) had been originally designated as "FASs" (Zhu et al., 2000), but the current results indicate that these may be more closely related to PKS than FAS.

While *K. brevis* AT2-10L and AT2-15 KS exhibited high similarity with each other and to the *C. parvum* sequences to a lesser extent, the novel *K. brevis* associated bacterial sequences (Kbrev_bac) also displayed interesting results by their dispersion in several different parts of the tree. For example, Kbrev_bac(23) showed affinity with a *Bacillus subtilis* KS fragment (0.49 mean divergence), and Kbrev_bac(3) appeared to outgroup with FAS sequences. The sequence from Kbrev_bac(28) did not show clear affinity with any distinct KS clades, but

rather high identity (~0.97) to an unidentified *Prorocentrum mexicanum* symbiont (Moffit and Neilan, 2003) after a BLASTP search. Although bacterial Kbrev_bac(30) clustered with the three *K. brevis* sequences in several reconstructions, it did not show exceptionally high amino acid sequence conservation to any *K. brevis*, may have contributed to the overall lower bootstrap percentage (70%) for the alveolate KS group, and was only similar with some *C. parvum* sequences by having an alanine residue in the KS active site. Furthermore, a separate analysis of DNA base composition, another criteria for analyzing possible "horizontal gene transfers" (McInerney, 1998), did not yield unequivocal evidence of recent PKS gene transfers between *K. brevis* and its bacterial associates, such as Kbrev_bac(30) (Lopez, 2003).

2.5. Fluorescence in situ hybridization

To cross-validate the PCR results, FISH was used to detect the presence of specific AT1-6L or AT2-10L PKS target sequences in individual intact dinoflagellate and bacteria cells from the *K. brevis* culture CCMP718 (Fig. 7). The fluorochrome CY3 was chosen as the fluorescent label for the oligonucleotide probes, for a number of reasons, in spite of the fact that the emission signature for this dye is similar to the autofluorescence signature of chlorophyll. CY3 has been successfully used for FISH protocols with other algae, including dinoflagellates (Yokouchi et al., 2003). It is relatively easy to decolorize cells and reduce the background levels of chlorophyll autofluorescence to a degree that does not interfere with visualization of the probe. However, our primary reason for the choice of CY3 is that after fixation with paraformaldehyde (or other fixatives such as glutaraldehyde, etc.), *Karenia* cells showed a very strong green autofluorescence throughout the cell when illuminated with blue light and a strong blue autofluorescence with UV excitation. Our attempts to decolorize this

autofluorescence were unsuccessful and it masks the emission by all the common 488 nm excitation fluors we have tried, including FAM, FITC, and AlexaFluor-488; as well as most common UV probe fluorochromes.

Sorted dinoflagellate cells were fixed, permeabilized and probed by FISH with fluorescent-labeled oligonucleotide probes for: (1) AT1-6L PKS gene sequences; (2) AT2-10L PKS gene sequences; (3) universal eukaryotic 18S rRNA gene sequences and (4) negative control (using a "no-bind" probe of no significant similarity to known sequences).

In the case of sorted bacterial cell populations, the bacterial cells were fixed, permeabilized and probed by a combination of prokaryotic in situ PCR and fluorescent in situ hybridization (PI-PCR/FISH), using fluorescent-labeled oligonucleotide probes for: (1) AT1-6L PKS gene sequences; (2) AT2-10L gene sequences; (3) universal bacterial 16S rRNA gene sequences and (4) negative control ("no-bind" probe as above). Results from positive and negative control probes were as expected, with positive control probes indicating that the intact cells were capable of being labeled by FISH,
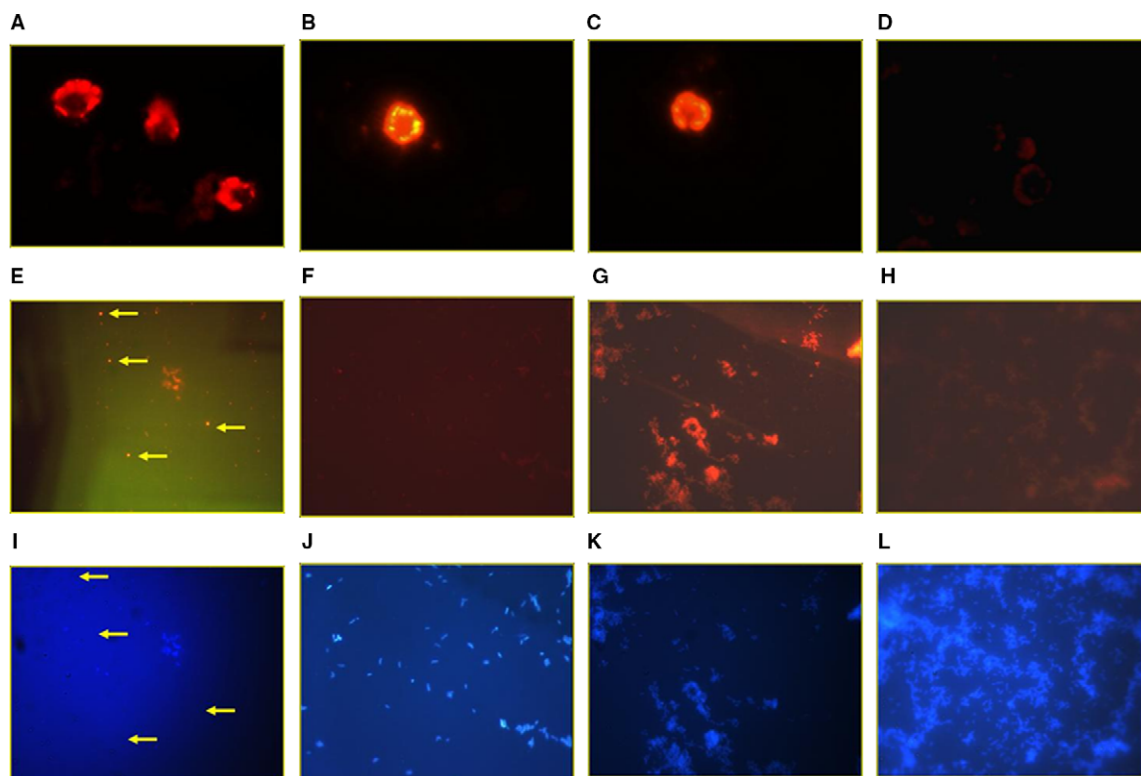

Fig. 7. Fluorescent in situ hybridization of *K. brevis* and its flow-sorted bacterial fraction. (A–D) FISH with whole fixed cells of the dinoflagellate *K. brevis* CCMP718, all at 400× total magnification and 2 s camera integration time. (E, F) PI-PCR/FISH with whole fixed cells of the bacterial population sorted by flow cytometry from *K. brevis* CCMP718, all at 1000× total magnification and 5 s camera integration time. (I–L) DAPI counterstain of the same images showed above in (E–F), respectively to show total bacterial population of the field of view, all at 1000× total magnification and 0.13 s camera integration time. CY3-labeled FISH probes were as follows: (A) *K. brevis* with AT1-6L PKS probe. (B) *K. brevis* with AT2-10L PKS probe. (C) *K. brevis* with universal 18S rRNA probe. (D) *K. brevis* with negative control "no-bind" probe. (E) Bacterial fraction with AT1-6L PKS probe. Arrows indicate putative positive cells. (F) Bacterial fraction with AT2-10L PKS probe. (G) Bacterial fraction with universal 16S rRNA probe. (H) Bacterial fraction with negative control "no-bind" probe.

and negative controls indicating that such labeling was not due to mere physical characteristics of the fixed cells or entrapment of the probe. The AT2-10L PKS probe exclusively labeled *K. brevis*, whereas the PKS probe for the AT1-6L sequence labeled both *K. brevis* cells and also labeled a small coccoid-type cell of bacterial size from the sorted bacterial fraction (Fig. 7). This AT1-6L hybridizing cell type appears to be in relatively low abundance with respect to the total bacterial population found in this *K. brevis* CCMP718 culture.

The positive FISH labeling with the AT1-6L probe for both dinoflagellate cells and some members of the bacterial population would appear to be consistent with the PCR results which find amplification of AT1-6L sequences from the sorted bacterial population of this culture. Likewise, the positive PKS probe results for the AT2-10L sequence appears consistent with the PCR results, in that this probe labeled the *K. brevis* cells, but did not label any cells from the sorted bacterial population. Thus both the PCR and the FISH independently support the conclusion that at least the AT2-10L PKS sequence is of dinoflagellate origin and is not found in the associated bacterial population.

In the case of whole cell labeling of *K. brevis*, some variation was seen in the localization of the probe within the cells, depending on the probe used. However, we must point out that the process of fixation, permeabilization and decolorization used here resulted in severe physical deformation of these dinoflagellate cells, as the cytoplasm and intracellular structures dehydrate and collapse around the nucleus. The resulting cellular morphology after such manipulations is highly variable even from the same batch of fixed unialgal culture. Also these cells were neither DNase nor RNase treated, although in other FISH experiments where nuclease control treatments were used (data not shown), it was determined that the fluorescent signal seen from these probes was predominately due to RNA. In general, the AT1-6L PKS probe appears to give a hybridization signal predominately from the cytoplasm collapsed around the nucleus, but the nucleus itself appears relatively dark. On the other hand, the AT2-10L PKS probe and the 18S rRNA probe both show signal throughout the cell, although the AT2-10L probe did show some increase in signal intensity from the cytoplasm periphery. This would seem to imply that the AT1-6L PKS probe signal observed in *K. brevis* is from endosymbiotic bacteria whereas the AT2-10L signal is from nucleic acid of the *Karenia* cell itself. While these results do not rule out the possibility that endosymbiotic bacteria caused (in part or whole) the AT2-10L probe signal, no labeling of the sorted bacterial population or of exogenous bacteria in the algal population was observed. Nonetheless, it is unwise at this point to over-interpret the localization pattern of the FISH labeling within the *K. brevis* cells, due to the severe and random deformation of the *Karenia* morphology caused by the fixation and permeabilization steps. For this work, FISH results were simply scored for sufficient retention of the probe at a particular stringency by the *Karenia* cells, and that the signal intensity was significantly greater than background and significantly greater than the no-bind negative control probe under similar conditions.

3. Conclusions

While the brevetoxins have been studied for more than 30 years, aside from the identification of precursors, little is known about their biosynthesis. To date, no gene or enzyme has been identified that is associated with the biosynthesis of a polyketide from any dinoflagellate. Further complicating the subject is the presence of associated bacteria in the unialgal cultures which have not been successfully eliminated. These uncultured bacterial symbionts can carry their own PKS biosynthetic repertoire. Determining the ultimate source of brevetoxin will require more extensive studies, such as isolating a complete PKS operon for comparative analyses. However, the large size of the dinoflagellate genome (Rizzo et al., 1982) and the lack of a general dinoflagellate transformation system discourage such studies. Nonetheless, advances in the genetics of polyketide biosynthesis have paved the way for studies of brevetoxin biosynthesis at the molecular level. This study expands on the discovery of putative PKS genes from *K. brevis* that are closely related to PKS genes from the protist *C. parvum*. One of these PKS encoding genes (AT2-10L) has been localized to *K. brevis*, both by fluorescence in situ hybridization and PCR screening of flow-cytometric sorted *K. brevis* associated bacteria. While not specifically analyzed by FISH, the AT2-15 sequence is likely also derived from *K. brevis* based on the PCR results, the high homology to AT2-10L (80% identical), and resulting phylogeny. Although *K. brevis*, like many dinoflagellates, produces significant amounts of polyunsaturated fatty acids, these genes are unrelated to PKSs responsible for PUFA production in other marine eukaryotes. While neither AT2-10L or AT2-15 have been linked to toxin production per se, this work does provide the first evidence of resident PKS genes in *K. brevis* or any other dinoflagellate and strengthens the hypothesis that the ultimate origin of the brevetoxins is indeed *K. brevis*.

4. Experimental

4.1. General procedures

4.1.1. Isolation of RNA and DNA

Dinoflagellate cells were isolated by centrifugation (3000*g* for 5 min, 20 °C). DNA was purified using a

Bio101 FastDNA kit (Qbiogene) and FastPrep instrument (Qbiogene) according to the manufacturer's instructions. Total RNA was purified by a modified guanidine thiocyanate method described for Gonyaulax polyhedra (Bae and Hastings, 1994), or using a Bio101 FastRNA kit (Qbiogene). The total RNA was treated with DNase (Promega) to remove any residual DNA. Nucleic acids were quantified spectrophotometrically using a μQuant (BioTek Instruments, Inc).

4.1.2. Cloning and sequencing of amplified fragments

Amplified DNA fragments were spun-purified from 1.2% agarose gels with Ultrafree-DA Millipore columns according to the manufacturer's instructions. Purified bands were cloned with the TOPO-TA kit and competent *Escherichia coli* (INVαF' or TOP10) cells (Invitrogen) according to the manufacturer's instructions. Both strands were sequenced in house on an ABI automated sequencer (Perkin Elmer) using the M13R and T7 primers or sequencing was performed by MWG Biotech using T3 or T7 primers. Translated DNA sequences were compared to protein databases over the Internet using the program Basic Local Alignment Search Tool (BLAST) (McGinnis and Madden, 2004).

4.1.3. RFLP analysis

The plasmid alkaline lysis minipreparation procedure was performed as described previously (Sambrook et al., 2001). For PKS products amplified from sorted bacteria, two of fifty microliters of each plasmid preparation were digested with each of seven restriction enzymes: *Kpn*I, *Bam*HI, *Xba*I, *Xho*I, *Hpa*II, *Rsa*I and *Sph*I (NEB). For 16S rRNA products amplified from cultured bacteria, a 1.5 kbp 16S product was amplified from 48 bacterial isolates using 20 ng DNA each and *Taq* polymerase (NEB) in a 50 μl reaction. Five microliters of each PCR was used for each of the four digestions using the enzymes *Hin*dIII, *Sau*3AI, *Hpa*II and *Rsa*I (NEB). Digestions were performed in a 20 μl reaction volume at the manufacturer's recommended temperature for one to three hours.

4.2. Dinoflagellate strains and culturing conditions

K. brevis (strains CCMP718, 2228, 2229, 2281, time and location of isolation may be found on the CCMP website: http://ccmp.bigelow.org/index.php), *A. carterae* (CCMP1314), *Amphidinium operculatum* (CCMP120), *Chatonella subsalsa* (CCMP217), *H. akashiwo* (CCMP1870), *Prorocentrum hoffmannianum* (CCMP683), *Prorocentrum micans* (CCMP1591), *Prymnesium parvum* (CCMP708) and *Symbiodinium* sp. (CCMP831) were obtained from the Provasoli-Guillard Center for the Culture of Marine Phytoplankton (CCMP) at the Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, ME. *K. brevis* strains JR, JR1

(which are identical to CCMP2281. CCMP2281 and JR1 are reportedly axenic) and NOAA1 (also known as Charlotte Harbor (Loret et al., 2002)) were obtained from the US EPA Gulf Ecology Division Laboratory in Gulf Breeze, Florida. *Prorocentrum lima* was obtained from the collection of Daniel Baden while at the University of Miami. *P. parvum* was obtained from the laboratory of Takeshi Yasumoto (Sendai, Japan). Cultures were maintained in variety of media: RE medium (*K. brevis* strains CCMP718, 2228, 2229, 2281, JR); f/2-Si *A. carterae* (CCMP1314) and *A. operculatum* (CCMP120)); Li-Si (*C. subsalsa* (CCMP217), *P. micans* (CCMP1591), and *H. akashiwo* (CCMP1870)); Prov 50 (*P. hoffmannianum* (CCMP683), *P. parvum* (CCMP708), *P. parvum* (Yasumoto), *Prorocentrum lima*, *Symbiodinium* sp. (*CCMP831*)). Cultures were maintained at 20–22 °C under constant illumination either from Cool White or Grow-Lux wide spectrum lamps (Guillard, 1975).

4.3. Flow cytometry and cell sorting

All flow cytometry analysis and cell sorting was performed on a DakoCytomation MoFlo high-speed cell sorter, equipped with a water-cooled tunable Argon Laser, 635 nm diode laser, sterile two-way and Cyclon™ single-cell sorting module, sterile sorting chamber with biohazard aerosol containment and evacuation module, cell-orienting nozzles, and a custom double-range pulse width analog-to-digital converter board. For the work reported here, all excitation was done at 488 nm, and sorting decisions were based on a multi-parameter combination of side scatter (SSC), forward scatter (FSC), pulse width and chlorophyll autofluorescence (FL4), as shown in Fig. 3. Cells were sorted under aseptic conditions into sterile microfuge tubes at a sheath pressure of 30 psi, with a sample differential of 2.5 psi, using the "sort-purify-1-drop" mode. Emission filters for FL4 (chlorophyll detection) = 670/30 nm. Purity of sorts was also monitored at time of sorting by epifluorescent microscopic observation of SYTO-9 stained subsamples of the sorted populations. Typically, 10^{6-7} *K. brevis* cells were sorted along with bacterial cells corresponding to the same volume of *K. brevis* culture, or 10^{7-8} bacterial cells.

4.4. PCR and RT-PCR

The DNA-free RNA was reverse-transcribed by M-MLV reverse transcriptase (Promega) using random hexamers according to the manufacturer's instructions. The following PCR conditions were typical: template cDNA (20 ng) or DNA (100 ng), 0.5 μM each primer, 3 mM MgCl$_2$, 1.25 U *Taq* (NEB or Genechoice) or *Pfu* (Stratagene). Degenerate PKS primers (forward:

5′-MGIGARGCIYTICARATGGAYCCICARCARM-G-3′, reverse: 5′-GGRTCNCCIARYTGIGTICCIGTI-CCRTGIGC-3′), specific PKS primers (Table 1), universal bacterial 16S rRNA primers (24f: 5′-GGAGAGTTT-GATCMTGGCT-3′, 1493r: 5′-ACGGYTACCTTGT-TACGACTT-3′) (Prokic et al., 1998) and universal eukaryotic 18S rRNA primers (forward: 5′-GGTTG-ATCCTGCCAGTAGTCATATGCTTG-3′, reverse: 5′-GA TCCTTCCGCAGGTTCACCTACGGAAACC-3′). The following standard PCR protocol was used to perform amplification reactions: 95 °C/5′, then 95 °C/20″, T_{anneal}/20″, 72 °C/t_{extend} × 30–40 cycles and a final 72 °C/5′ extension. For the sequence-specific PKS primers, $T_{anneal} = 60$ °C and $t_{extend} = 20$ s with 39 cycles. Genomic PCR and RT-PCR were performed on a Mastercycler gradient thermal cycler (Brinkmann, Eppendorf). Real-time RT-PCR was performed on a DNA Engine Opticon thermal cycler (MJ Research) with IQ SYBR Green Supermix reagent (Bio-Rad) according to the manufacturer's instructions. Melting curve analyses were performed between 65.0 and 90.0 °C; reading every 0.2 °C and holding for 1 s between reads.

4.5. Sequence and phylogenetic analysis

Previously described PKS and FAS sequences were retrieved from GenBank as reference sequences, such as DEBS-6 for erythromycin (M63677), rifamycin (AF040570), FAS Fix-23 – (X64131), myxothiazol MtaF from *Stigmatella aurantiaca* (AAF19814), McyD from *Microcystis aeruginosa* (AAF00959), FK506 from *Streptomyces* (CAA71463), pksM from *B. subtilis* (NP_389601), *C. parvum* (AAC99407; EAK87820 and T31307) and *C. hominis* (EAL37180); eicosapentaenoic acid biosynthetic (polyunsaturated fatty acid synthase – PUFA) sequences stemmed from *Schizochytrium* (AF378327–9), *Moritella marina* (BAA89382–4) and *Shewanella* (AAB81123). Novel marine microbe-derived PKS and FAS sequences were also included in phylogenetic analyses for reference and comparison: marine fungal strain K132 (AY857361), and eubacterial strains JL085 (AY857380), K155 (AY857387), K618 (AY857381), JL048 (AY857384), JL081 (AY857354) and 223N (AY857340) (Also see the HBOI Marine Microbial Database – http://www.hboi.edu/dbmr/dbmr_hbmmd.html) (Gunasekera et al., 2005). Protein sequences were aligned with ClustalX version 1.81 (Thompson et al., 1997), using the Gonnett series as the protein weight matrix. Default gap opening and gap extension penalties of 10.0 and 0.2 were adopted because conserved domains were aligned most accurately with few gaps. In the absence of a complete three-dimensional PKS crystal structure, these motifs assisted in the evaluation of the final multiple sequence alignment. Minor corrections on highly divergent regions were performed manually. Several regions were excluded from phylogenetic analysis due to ambiguous alignments or gap placements. Base composition and dGC content of DNA codon positions were determined using the program GCUA: General Codon Usage Analysis program (McInerney, 1998). Phylogenetic analyses of amino acid sequences using either maximum parsimony (MP) or minimum evolution (ME) neighbor-joining optimality criteria were conducted with PAUP* version 4.0b10 (Swofford, 2002) using the heuristic search option and the TBR branch-swapping algorithm. MP utilized random addition of taxa with ten repetitions. The ME analysis (Rzhetsky and Nei, 1992) was also carried out with PAUP* 4.0b10 using mean character distances. Robustness of the ME analyses was tested by bootstrapping with at least 500 replicates for each run (Felsenstein, 1985).

4.6. DGGE analysis of 16S rRNA gene

The 16S rRNA gene was amplified from 50 ng of genomic DNA purified from sorted bacteria, using the universal prokaryotic primer set (24f, 1393r). Cycling conditions were an initial 95 °C/5′, then 95 °C/30″, 55 °C/30″, 72 °C/1.5′ × 30 cycles and a final 72 °C/10′. The 1.5 kbp product was purified using the PCR Kleen Kit (Bio-Rad). One microliter of the purified 16S rRNA PCR was used as the template for a nested PCR using the primer set (GC-clamp forward, 5′-CGCCC-GGGGCGCGCCCCGGGCGGGGCGGGGGCACG-GGGGGAACGCGAAGAACCTTAC-3′, reverse, 5′-CGGTGTGTACAAGGCCCGGGAACG-3′, *E. coli* positions 984 and 1378, respectively) (Heuer et al., 1997). These primers were also used directly (1 μM each) with genomic DNA (100 ng) from *K. brevis* isolates (CCMP718, CCMP2228, CCMP2229, CCMP2281, JR) for comparison of bacterial populations. Cycling conditions: initial 95 °C/5′, then 95 °C/30″, 60 °C (−1 degree/cycle)/2′, 72 °C/2′ × 30 cycles and a final 72 °C/10′ extension. The resulting 450 bp product was purified using the PCR Kleen Kit (Bio-Rad). All PCRs performed in preparation for the DGGE analysis consisted of 50 μl final volume reactions and contained 2.5 U turbo *Pfu* DNA polymerase (Stratagene), 1× cloned *Pfu* reaction buffer (1.5 mM Mg^{2+} final) (Stratagene), 7% DMSO, 0.1 mmol each dNTP, 0.25 μM each primer.

Thirty microliters of each purified reaction (450 bp product) were mixed with five microliters of 3× loading dye, prepared from the standard 6× Blue/Orange loading dye (Promega) by dilution with an equal volume of 50% sterile glycerol. Twelve microliters of each sample (purified PCR product plus dye) were loaded onto the DGGE gel (8% polyacrylamide gel prepared with a gradient from 40% to 47.5% of urea/formamide). To enhance band sharpness, the top of the gel was overlaid with a comb-length layer of polyacrylamide containing no denaturant. After loading at room temperature the

DCode gel apparatus (Bio-Rad) was placed into the pre-heated running buffer (1× TAE, 60 °C) and electrophoresed at a constant 150 V for 5.5 h.

4.7. Brevetoxin ELISA

K. brevis cells from each of five strains (CCMP718, 2228, 2229, 2281 and JR) were counted using a Coulter Multisizer II (Coulter Scientific Instruments). The cultures (1.25×10^6 cells) were extracted with chloroform (20 ml), centrifuged and the organic layer was dried in vacuo. The dried crude extract was resuspended in methanol (1 ml), extracted with petroleum ether, centrifuged and the methanol layer was dried in vacuo. The crude extract was resuspended in 200 μl of methanol and dilutions were prepared according to the manufacturer's (HabLab, UNC Wilmington) instructions. Similarly prepared crude extracts from the flow sorted *K. brevis* CCMP718 dinoflagellate fraction (3×10^5 cells) and bacterial fraction (6×10^5 cells) were tested for the presence of brevetoxin by ELISA.

4.8. Isolation of heterotrophic bacteria from K. brevis

Bacteria were subcultured from *K. brevis* CCMP718 by inoculating marine agar (Difco) plates with aliquots (20 μl) of dinoflagellate culture that was diluted 10-fold. The plates were incubated at ambient temperature for three days. Fifty single colonies displaying a variety of morphologies were plated three times to ensure homogeneity of the isolates.

4.9. FISH and PI-PCR/FISH labeling of whole cells

All solutions and buffers were treated with diethyl pyrocarbonate (DEPC) and sterilized to prevent any potential nuclease contamination of samples. Dense dinoflagellate or bacterial suspensions, harvested by cell sorting (approx. 10^7 cells ml^{-1}), were fixed overnight with 4% paraformaldehyde at 4 °C, then washed with 1:1 Ethanol:1× PBS (Sinigalliano et al., 2003). For dinoflagellates, fixed cells were subsequently decolorized in 70% ethanol (Simon et al., 2000) with a slight modification: cells were washed in 2 mL of 70% EtOH, with changes of fresh EtOH every hour over a period of 4–5 h. This decolorization pre-treatment removed most of the chlorophyll and thus decreased autofluorescence background. Successive washes in 70% EtOH over a period of hours instead of days rapidly decreased the chlorophyll concentration significantly to the point where CY3-labeled probes could be visualized clearly against the reduced autofluorescent background. The fixed and decolorized cells were resuspended in 1:1 Ethanol:1× PBS, and then these cells were permeabilized by incubating them in the 50% EtOH at 80 °C for 20 min. In the case of bacterial cells, samples were treated the same except that no decolorization was done. After permeabilization, the pre-treated cells (10 μL) were spotted on gelatin subbed slides (0.3% gelatin with 0.03% chromium potassium sulphate), dried, then serially dehydrated by successive immersion of slides in 53% EtOH, 78% EtOH, and 98% EtOH, then allowed to dry. For bacterial samples, the cell spots on the slides were treated with lysozyme, washed, then the target signal was amplified prior to FISH by prokaryotic in situ PCR (PI-PCR) as previously described (Sinigalliano et al., 2003), except that primers specific for AT1-6L PKS genes, AT2-10L PKS genes, or universal 16S rRNA genes were used. FISH was then carried out with the cells immobilized on slides as described below. Frame-Seal™ incubation chambers (MJ Research, Inc. Watertown, MA, USA) were mounted around the periphery of the cell spot to serve as a reservoir for the hybridization mixture. The cell spots within the chambers were then overlain with a hybridization solution of 5X SSC with 0.1% Tween20, containing the respective CY3-labeled oligonucleotide probe at 5 μM final concentration (see below for sequence characteristics of probes used), then sealed with a plastic cover slip. Slides were incubated in a PTC-200 Thermal Cycler equipped with a Slide Chambers™ dual block alpha unit, and heated at 95 °C for 5 min, then held the at 55 °C hybridization temperature for 3 h. After hybridization, the chambers were removed from the slides and the slides were then washed three times for 10 min each in 50 mL of 0.5× SSC prewarmed to 55 °C. Slides were then counterstained with DAPI or Hoechst-33342 (1 μg mL^{-1}) and examined by epifluorescence microscopy (Leica DMLB microscope equipped for phase contrast and epifluorescence with a 100 W mercury lamp, and standard DAPI, rhodamine, and FITC filter sets). Images were acquired and digitized with a cooled color low-light CCD camera (Hammamatsu Model C5810, Hammamatsu Corp, Japan) and analyzed with the software package ImagePro Plus (Media Cybernetics, Silver Springs, MD, USA). The following probes were used in the FISH: The two PKS probes were AT2-10L 5′-[Cy3]-CGCCATGGC-ACAAACA-3′ and AT1-6L 5′-[Cy3]-TGATCTGG-GAGCCGCAA-3′. The universal 18S rRNA probe used was 5′-[Cy3]-ACCAGACTTGCCCTCC-3′ (Yokouchi et al., 2003), and the universal 16S rRNA probe was 5′-[Cy3]-GWATTACCGCGGCKGCTG-3′ (Karner and Fuhrman, 1997). The negative control "No-Bind" probe was 5′-[Cy3]-CCTAGTGACGCC-GTCGAC-3′ (Karner and Fuhrman, 1997). The following primers were used for the in situ PCR: For AT2-10L, the forward primer was 5′-CGCAACGTACAAGTCA-CATCTG-3′ and the reverse primer was 5′-CAC-CTGATGGCCGCTGTAAG-3′. For AT1-6L, the 6Lb set was used (Table 1). The primer pair for 16S amplification were universal 16S rRNA forward primer 27f 5′-AGAGTTTGATCMTGGCTCAG-3′ and universal

16S rRNA reverse primer 1392r 5′-ACGGGC-GGTGTGTGTRC-3′. *GenBank Accession Nos.*: AS1-1L [AY820891], AT1-6L [AY817144], AT2-10L [AY820892], AT2-15 [AY820893], Kbrev_bac(3) [AY902469], Kbrev_bac(23) [AY902470], Kbrev_bac(28) [AY902471] and Kbrev_bac(30) [AY902472].

Acknowledgments

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