Characterization of the Microbial Community and Polyketide Biosynthetic Potential in the Palmerolide-Producing Tunicate *Synoicum adareanum*

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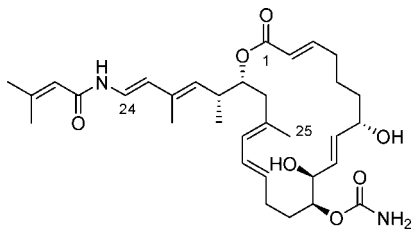
Palmerolide A (**1**) is a macrolide isolated from the Antarctic tunicate *Synoicum adareanum* that is of interest due to its potential as an antimelanoma drug. Biosynthesis is predicted to occur via a hybrid PKS-NRPS pathway within *S. adareanum*, but the identity of the palmerolide-producing organism (host or putative host-associated microorganism) has not been established. Microscopic observation revealed a dense microbial community inside the tunicate, and evidence from 16S rRNA gene DGGE profiles and clone library sequences suggests that the bacterial community has moderate phylogenetic complexity. The alpha and gamma classes of Proteobacteria account for ~75% of the cloned 16S rRNA genes, and the majority of these sequences are affiliated with the genera *Pseudovibrio* and *Microbulbifer*. DNA sequences encoding type I PKS ketosynthase (KS) domains were detected by PCR. The *S. adareanum* KS sequences, which affiliate with the trans-AT clade, are similar to portions of PKS proteins that lack integrated acyltransferase domains in pathways for generating bioactive polyketide compounds, including bryostatin, leinamycin, and pederin.

Synoicum adareanum is a circumpolar colonial tunicate (Polyclinidae family) commonly found in the frigid (−1.5 to +1.0 °C) coastal waters near Palmer Station, Antarctica. Analysis of the natural product component of this tunicate for a chemical ecology study revealed several classes of compounds, including ecdysteroids[1] and macrolide polyketides, exemplified by palmerolide A (**1**).[2] Interestingly, palmerolide A was shown to exhibit potent cytotoxicity toward melanoma cells (LC_{50} of 18 nM vs the UACC-62 cell line) when tested against the National Cancer Institute (NCI) 60 cell line panel.[2] The likely mode of action is inhibition of vacuolar ATPase proton pumps,[2] which are highly expressed in metastatic cancer cells,[3−5] where they modulate pH. V-ATPases are also the target of several other anticancer molecules, including salicylihalamide A,[6,7] bafilomycin A_1,[8] and oximidines.[9]



Palmerolide A (**1**)

Spectroscopic analysis of palmerolide A revealed a number of interesting structural features, such as the carbamate and vinyl amide groups. Such groups have been observed in polyketide molecules that originate in bacteria,[10,11] leading us to hypothesize that palmerolide A biosynthesis occurs by a hybrid polyketide synthase (PKS)/nonribosomal peptide synthetase (NRPS) pathway in bacteria living in association with *S. adareanum*. In this hypothesis (Figure 1), biosynthesis of the linear carbon backbone is initiated by NRPS-catalyzed incorporation of an amino acid such as glycine (Gly)[12] and/or valinylglycine (Val-Gly).[13] Subsequently the macrocyclic portion of the molecule could be generated by a series of PKS-catalyzed steps analogous to the biosynthetic pathway for epothilones in the myxobacterium *Sorangium cellulosum*,[14] among others.

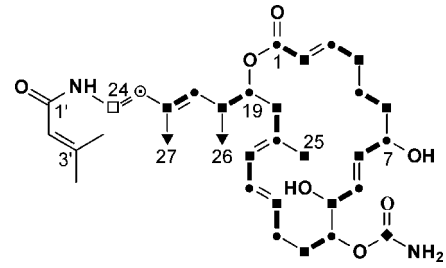


Figure 1. Hypothesized biosynthetic origin of palmerolide A: (■) acetate C2; (●) acetate C1; (—) intact acetate unit; (▼) *S*-adenosylmethionine methyl group; (□) glycine C2; (⊙) glycine C1; (♦) carbamoyl phosphate carbon. Unmarked carbons of the amide portion may derive from either the NRPS pathway (e.g., valine) or PKS. C25 of palmerolide A, found on a C1 acetate position, could originate from acetate via an HMG-CoA synthase-type methyl transferase.[75,76]

Additional modification of the linear or macrocyclic C24 carbon skeleton could be envisioned to install the carbamate function, as has been observed for geldanamycin.[15,16] Thus far, the identity of the putative palmerolide-producing bacterium is not known.

Marine invertebrates, especially sponges,[17] corals,[18] bryozoans,[19] and tunicates,[20] are well-established sources of bioactive natural products,[21] including a rich variety of compounds with anticancer properties, some of which are in clinical or preclinical trials.[22] The strong legacy of marine natural product discovery is hindered by the difficulty of obtaining bioactive molecules in sufficient quantities without the need for recurrent harvests, especially where difficult to access organisms, such as marine invertebrates, are concerned.[23] However, it is now clear that some of these compounds are produced by bacterial symbionts.[23] In these cases, one possibility for alleviating the supply problem is to isolate a pure culture of the bacterium that produces the bioactive molecule.

Invertebrate-associated bacteria often lack pure culture representatives.[24] That many bacteria are refractory to isolation in pure culture has been thoroughly documented,[25] and in some instances the explanation pertains to metabolic codependencies between organisms.[26] Attempts to isolate the desired invertebrate-associated microorganisms have often failed. To circumvent this problem, culture-independent alternatives have been developed, which entail extracting DNA from environmental samples and then cloning

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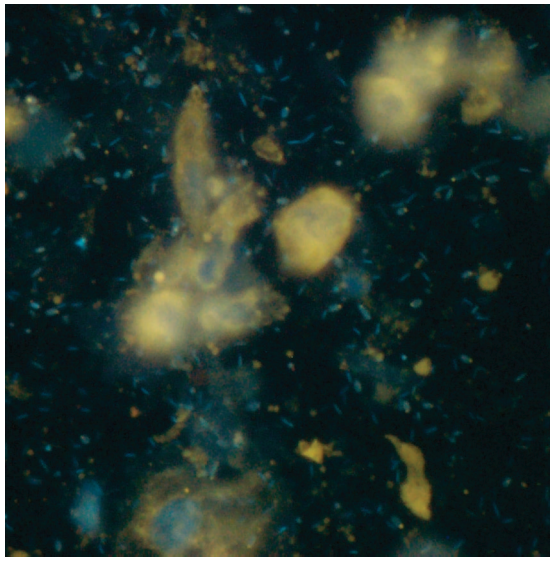

Figure 2. *S. adareanum* tissue homogenate (outer section) stained with 4′,6-diamidino-2-phenylindole (DAPI) and visualized under 1000× magnification with an epifluorescence microscope. The small blue-colored cells are bacteria.

fragments of DNA large enough to contain either partial or entire clusters of biosynthetic genes. The enormous potential of this approach was validated in several recent studies in which biosynthetic gene clusters for bryostatin,[27] onnamide,[28] and patellamide[29] were cloned from the DNA of as-yet-uncultured bacterial symbionts. There are at least four potential technical challenges with this approach: (1) separation of host and bacterial cells and/or DNA, (2) screening enough cloned DNA fragments to detect the desired biosynthetic gene cluster, (3) identification of the correct genes, and (4) expression of the cloned genes to generate sufficient quantities of the desired product. Successful implementation of either strategy (culturing or cloning) could provide a plentiful supply of a bioactive molecule from a microbial source, as well as the opportunity to improve the yield by optimization of conditions that enhance the growth rate of the bacterium or expression of genes in the biosynthetic pathway, which can be a daunting challenge requiring innovative solutions.[23]

Similarly, tunicate extracts are an impractical long-term source of palmerolide A. The chemotherapeutic potential of palmerolide A warrants investigation of its biological origin, which may eventually yield a more prolific source for future testing. The only current sources of palmerolide A are the tunicate itself and through chemical synthesis.[30,31] Since the tunicate inhabits Antarctic waters, harvesting or culturing *S. adareanum* would be impractical in the long term. Likewise, due to the challenging implementation of the synthetic route to palmerolide A, this is not currently a reliable route to a sustainable supply. Revelations that some marine natural products originate from microorganisms (not the invertebrate host) require that microbial communities associated with natural product producers are better understood. Here we present the first description of the *S. adareanum*-associated bacterial community and evidence for polyketide biosynthetic potential through detection of polyketide synthase (PKS) genes, specifically ketosynthase domains of modular type I PKS enzymes.

Results and Discussion

Identification of Bacteria Associated with *Synoicum adareanum.* Microscopy revealed a dense community of microorganisms associated with *S. adareanum*, in which both cocci and rod-shaped bacteria were observed (Figure 2). To identify the bacteria and examine the diversity of the community, denaturing gradient gel electrophoresis (DGGE) of amplified 16S rRNA gene

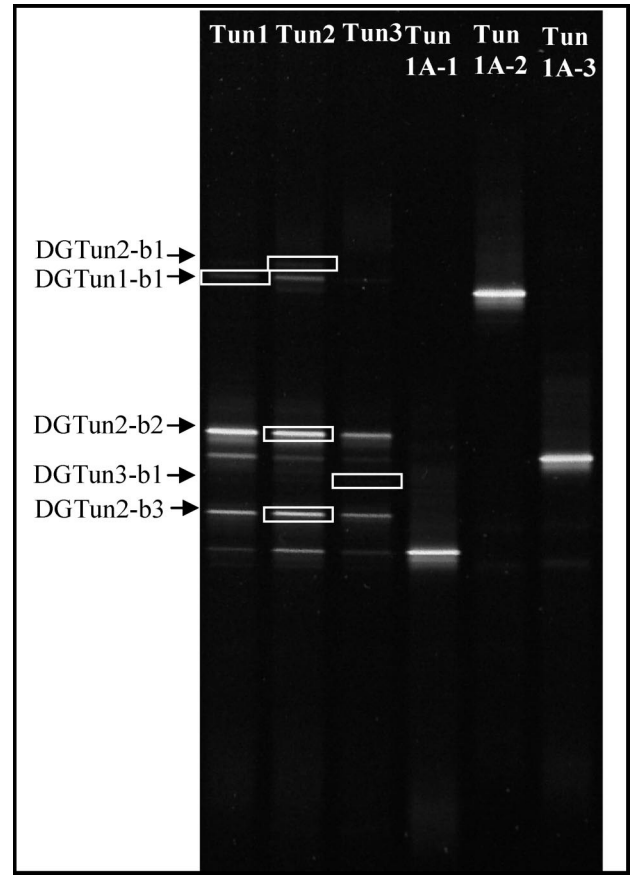

Figure 3. DGGE profiles of bacterial rRNA genes amplified from *Synoicum adareanum* partitioned in three sections, Tun1 (outer), Tun2 (middle), and Tun3 (inner), following removal of the outer 2 mm of tissue. Boxes indicate bands that were excised and identified (Table 1). Tun1A-1, Tun1A-2, and Tun1A-3 are cloned fragments with known sequences that were run to identify comigrating bands in the Tun1, Tun2, and Tun3 lanes. Their identification is also shown in Table 1.

fragments was performed. The DGGE profiles for three sections of a tunicate sample were similar to each other, which is suggestive of a homogeneous distribution of bacteria throughout the tunicate (Figure 3). Each profile included approximately six bands, and these correspond to members of the Proteobacteria, Bacteroidetes, and Verrucomicrobia phyla (Table 1).

In the 16S rRNA gene clone library ($n = 72$ sequences), 12 distinct sequence types were detected, and these correspond to Gammaproteobacteria (3 types; 16.7, 12.5, and 1.4% of the clone library), Alphaproteobacteria (3 types; 33.3, 9.7, and 1.4% of the clone library), and Flavobacteria (2 types; 11.1 and 4.2% of the clone library), as well as Betaproteobacteria, Actinobacteria, Verrucomicrobia, and candidate phylum TM7 (one type of each). Proteobacteria, Bacteroidetes, and Verrucomicrobia sequences accounted for 97% of the cloned genes, with the remaining sequences assigned to either the Actinobacteria or candidate phylum TM7. The alpha and gamma classes of Proteobacteria were especially highly represented in the clone library (collectively, ~75% of the cloned 16S rRNA sequences). In multiple cases, nearly identical sequences (>99% nucleotide identity) that differ by a few base pairs were observed in the *S. adareanum* clone library; these may reflect closely related strains or species of bacteria, or variation between operons in a single genome.

A BLAST search revealed that the *S. adareanum*-associated bacteria are most closely related to bacteria from marine environments, including bacteria known to be tunicate- or sponge-associated (Table 2). Several members of the *S. adareanum* microbial

Table 1. DGGE Results: Bacterial 16S rRNA Gene Sequences Identified in *Synoicum adareanum* Tissue Sections (sequenceIDs correspond to the bands in Figure 3)

tunicate microbial sequenceID	tunicate section	phylum or class of nearest neighbor	nearest neighbor sequence[a]	% identity
DGTun2-b1	1, 2	Bacteriodetes	uncultured Bacteroidetes bacterium clone SBI04_197 [Bacteroidetes 'AGG58' cluster; Arctic Ocean bacterioplankton]	94%
DGTun1-b1	1,2,3	Bacteriodetes	uncultured *Polaribacter* sp. clone SBI04_16 [bacterioplankton in Arctic Ocean]	100%
Tun1A-2	2	Verrucomicrobia	uncultured bacterium clone I1ANG45 [nidamental glands of squids]	93%
DGTun2-b2[b]	1,2,3	γ-proteobacteria	*Pelagiobacter variabilis*[c] [produces pelagiomicins]	97%
Tun1A-3	1,2,3	γ-proteobacteria	*Microbulbifer cystodytense* C1 [polysaccharide-degrading marine bacterium]	97%
DGTun3-b1	2, 3	γ-proteobacteria	*Shewanella* sp. 7051 [East Pacific Ocean deep sea sediments]	93%
DGTun2-b3[b]	1,2,3	α-proteobacteria	*Pseudovibrio ascidiaceicola F423* [tunicate isolate]	98%
Tun1A-1	1,2,3	γ-proteobacteria	uncultured bacterium 16S rRNA gene, clone OHKB9.63 (deep sea)	96%

[a] Nearest neighbor sequences identified by running BLAST against the GenBank "nr" database. [b] Bright DGGE band. [c] *Pelagiobacter variabilis* and *Microbulbifer* sp. are closely related bacteria, and several of the neighbor sequences (~95% identity) are *Microbulbifer* sp.

Table 2. Identification of 16S rRNA Sequences from *S. adareanum*-Associated Bacteria

	BLAST match[a]		
clone name	phylum (class)	name, source, GenBank accession no.	% identity
Tun3b.H11	Actinobacteria	uncultured actinobacterium clone NH10_01, Pacific Ocean, DQ372838	99
Tun3b.A1[b]	Bacteroidetes (Flavobacteria)	uncultured *Polaribacter* clone Arctic97A-15, Arctic Ocean, AF354620	99
Tun3b.B4[b]	Bacteroidetes (Flavobacteria)	uncultured Bacteroidetes clone C319a-R8C-A1, estuary sediment, AY678503	94
Tun3b.A11[b,c]	Proteobacteria (Alphaproteobacteria)	*Pseudovibrio ascidiaceicola* strain F10102, tunicate (*Botryllidae* sp.), AB210280	98
Tun3b.D6[b,c]	Proteobacteria (Alphaproteobacteria)	alpha proteobacterium strain CRA 5GI, marine sponge, AY562563	98
Tun3b.E2[c]	Proteobacteria (Alphaproteobacteria)	uncultured alpha proteobacterium clone 131720, whale carcass, AY922224	98
Tun3b.G11	Proteobacteria (Betaproteobacteria)	*Nitrosomonas* sp. clone BF16c57, enrichment culture, AF386746	96
Tun3b.A10[b,c]	Proteobacteria (Gammaproteobacteria)	*Microbulbifer* sp. strain SPO729, marine sponge, DQ993341	97
Tun3b.B6[b,c]	Proteobacteria (Gammaproteobacteria)	*Microbulbifer* sp. strain KBB-1, Pacific Ocean, DQ412068	92
Tun3b.F5[b,c]	Proteobacteria (Gammaproteobacteria)	Alteromonadales bacterium strain G-He12, EF554911	90
Tun3b.A3[b]	Verrucomicrobia	uncultured bacterium clone I1ANG45, squid (*Idiosepius pygmaeus*), AJ633978	95
Tun3b.E7	TM7	uncultured bacterium clone SBR2013, sludge, AF269000	90

[a] BLAST was run against a database of Ribosomal Database Project (release 9.56)[77] sequences longer than 1200 bp. [b] Sequence has ≥97% identity to a DGGE sequence described in Table 1 [c] Phylogenetic placement is shown in Figure 4

community are closely related to bacterial strains that have been isolated and characterized in pure culture, such as *Pseudovibrio ascidiaceicola*, *Microbulbifer maritimus*, and *Polaribacter glomeratus*. In contrast, a few *S. adareanum*-associated bacteria belong to lineages mostly represented by environmental sequences, and these bacteria could be challenging to isolate. For example, candidate phylum TM7 is a minimally characterized lineage of bacteria frequently detected in environmental 16S rRNA gene surveys.[32−34] With the exception of partial genome sequences generated through amplification of genomic DNA from individual cells,[35,36] the biology of TM7 bacteria remains largely unknown because numerous attempts to isolate pure cultures have failed.

A 16S rRNA phylogenetic tree (Figure 4) shows the relationship of *S. adareanum*-associated bacteria to other Alphaproteobacteria and Gammaproteobacteria. Some of the *S. adareanum*-associated bacteria are related to *Pseudovibrio* sp., including *P. ascidiaceicola*,[37] *P. japonicus*,[38] and *P. denitrificans*[39] (Figure 4A). The genus *Pseudovibrio* is within the Alphaproteobacteria, and members of this genus are generally associated with marine invertebrates, including tunicates,[37] sponges,[40−43] and corals,[44] although a few studies have reported *Pseudovibrio* rRNA sequences from free-living bacteria in seawater.[45,38,39] In sponges, recent evidence suggests that *Pseudovibrio* cells are vertically transmitted between generations via the sponge larvae,[40] although the nature of the symbiotic relationship is not known. Interestingly, *Pseudovibrio*-related sequences comprised a large portion (~43%) of the *S. adareanum* 16S rRNA clone library, suggesting a potential selective enrichment of these bacteria in the internal environment of *S. adareanum*.

Various *Microbulbifer*-related sequences were detected in the *S. adareanum* 16S rRNA clone library (Figure 4B). Bacteria in the genus *Microbulbifer* tend to be moderately halophilic, and they are often isolated from marine samples[46,47] and occasionally marine invertebrates.[48] *Microbulbifer* sp. were originally discovered in marine pulp mill effluent enrichment cultures,[49] and these bacteria are able to hydrolyze complex polysaccharides.[49] One way to rationalize the abundance of *Microbulbifer*-like bacteria in *S. adareanum* is that ascidians are encased in a tunic comprised of cellulose[50] and some members of the *Microbulbifer* lineage are cellulolytic.[49,51]

The DGGE and 16S rRNA gene clone library sequencing results were congruent, and both methods showed that the *S. adareanum* microbial community includes members of the Proteobacteria, Bacteroidetes, and Verrucomicrobia phyla. In fact, the same sequences (and by inference, organisms) were detected by both methods, as evidenced by 100% nucleotide identity (over the entire length of the shorter sequence, i.e., the DGGE band) between clone library and DGGE band sequences. This was the case for four of the five DGGE bands, while the other sequence (DGTun3-b1) contains a few mismatches to a sequence from the clone library. Compared to DGGE analysis, additional bacterial sequences were detected in the clone library because of greater sequencing depth.

The information obtained by analyzing the *S. adareanum* microbial community will guide subsequent research, including aiding in culture media selection to generate a diverse collection of bacteria, which (if successful) would be a simple and direct way to identify the palmerolide-producing organism and capture the genes necessary for palmerolide biosynthesis. For example, armed with the knowledge that *Pseudovibrio*-like bacteria are associated with *S. adareanum*, previous studies suggest that these bacteria should grow on standard rich media, such as marine agar 2216[40,37] or polypepton/yeast extract (PY) agar[39] during incubation under aerobic conditions. Likewise, bacteria affiliated with the *Microbulbifer* lineage are generally isolated on marine agar[52,46] or yeast extract/tryptone/sea salt (YTSS) agar.[49] In contrast, other members of the community belong to lineages of bacteria that are fastidious and may require specialized growth conditions.

An alternative to culture-based strategies is the metagenomic approach.[28,53−55] Cloning a large biosynthetic gene cluster directly from a community of bacteria following cell separation from invertebrate tissue is theoretically plausible if the microbial assemblage is restricted to a few members[56] and the target bacterium

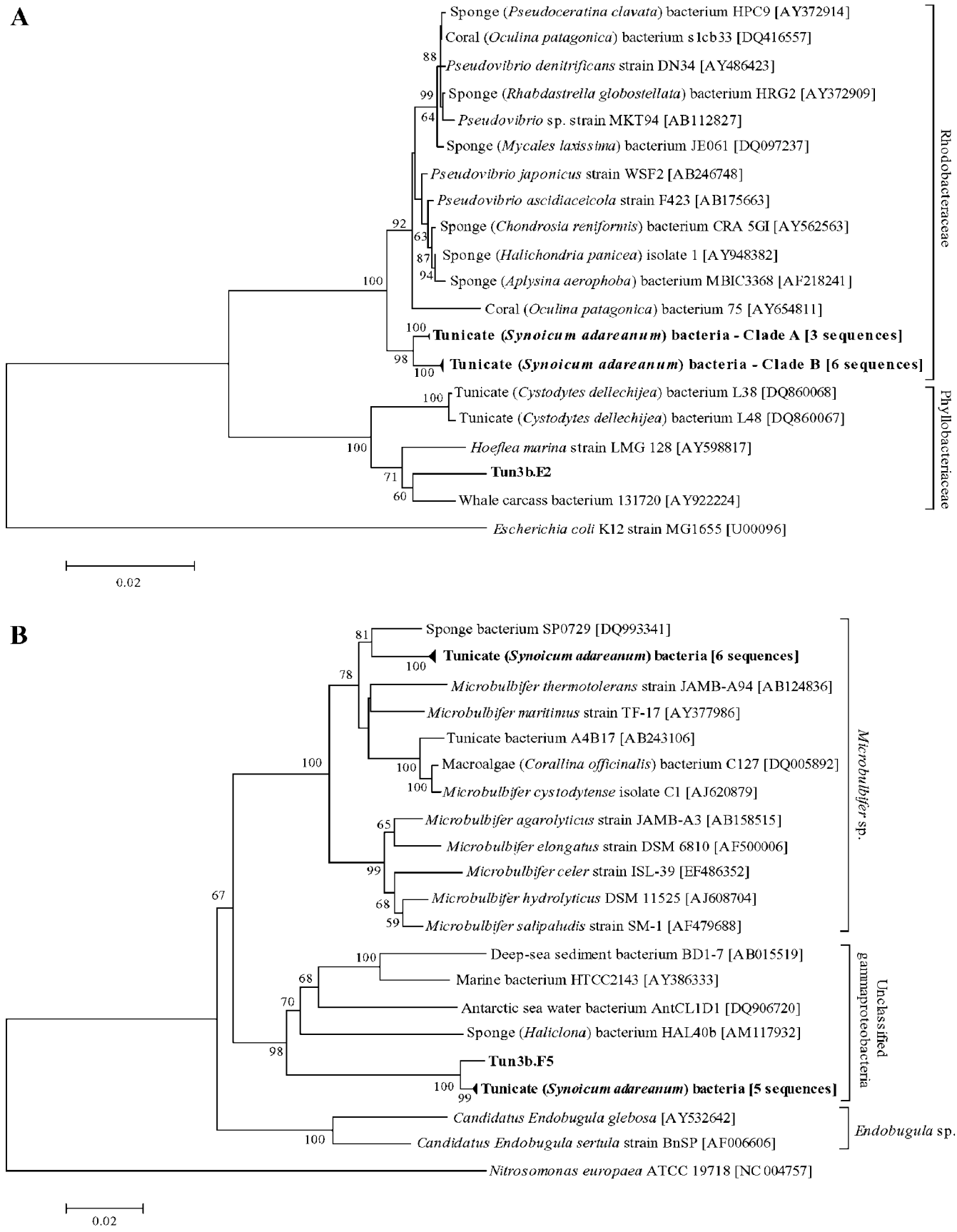

Figure 4. 16S rRNA neighbor-joining phylogenetic trees. (A) Alphaproteobacteria and (B) Gammaproteobacteria. Bold text indicates sequences amplified from *S. adareanum*.

is fairly abundant relative to other bacteria. In the case of *S. adareanum*, the complexity of the bacterial community appears moderate, and this would prove advantageous toward cloning a biosynthetic gene cluster if the palmerolide-producing cells are abundant, a conjecture supported by the fact that palmerolide A is abundant in the tunicate (~0.01% dry weight).[2]

Table 3. Sequence Characteristics and BLASTX Results for *S. adareanum* Ketosynthase Domains

			BLASTX match		
sequenceID[a]	length (bp)	gc %	name, organism, GenBank accession no.	% identity	natural product generated by BLAST match
Tun2pks.4	683	63	LnmJ, *Streptomyces atroolivaceus*, AAN85523	55	leinamycin[57]
Tun2pks.1	677	59	DszB, *Polyangium cellulosum*, AAY32965	68	disorazoles[58]
Tun2pks.5	686	58	BryB, symbiont bacterium of *Bugula neritina,* ABM63527	53	bryostatin[27]
Tun2pks.8	680	59	NRPS/PKS Ta1, *Myxococcus xanthus* DK 1622, YP_632115	54	myxovirescin(antibioticTA)[59,60]

[a] The table shows results for representatives of *S. adareanum* KS sequence type I through IV (Figure 5), respectively.

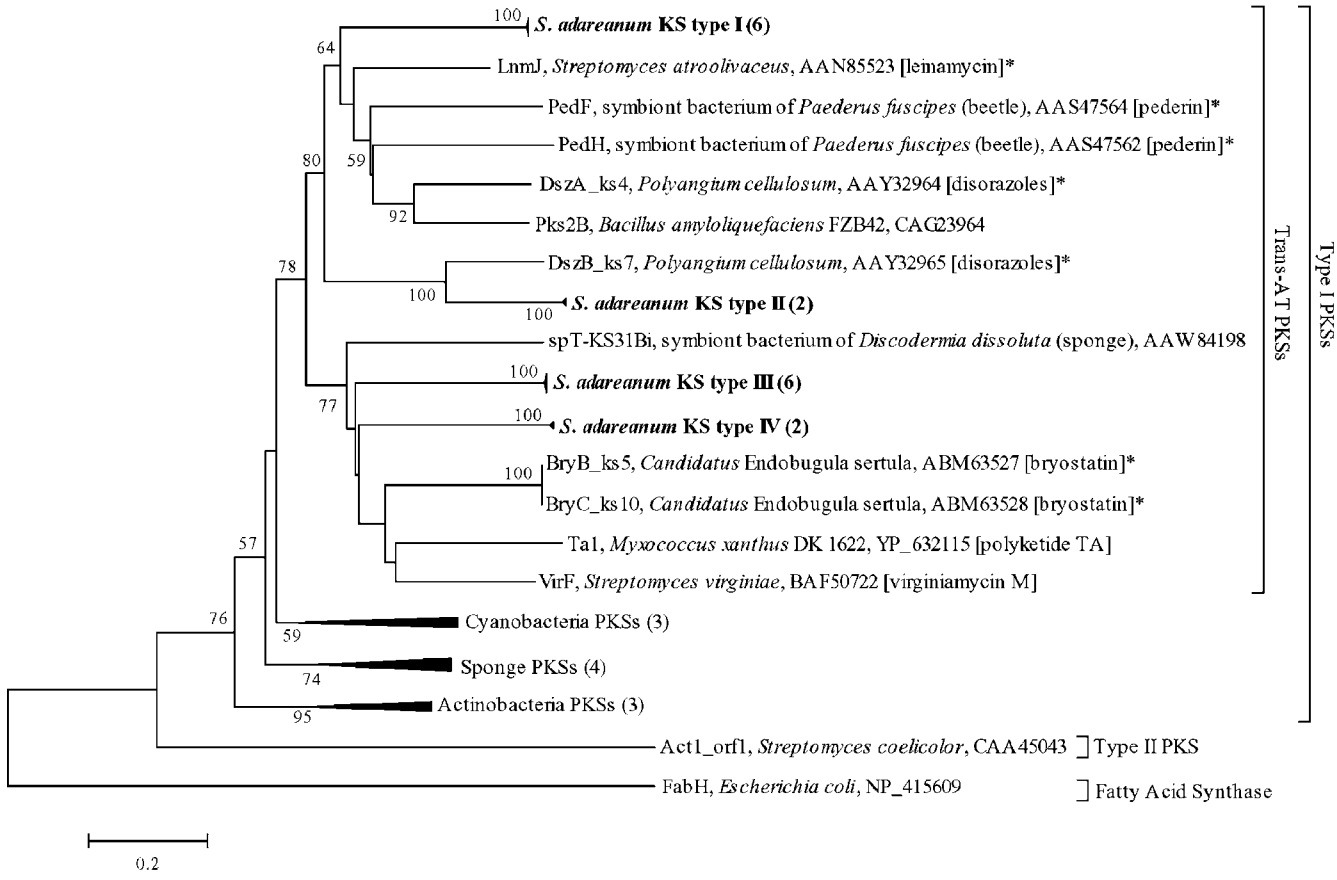

Figure 5. Neighbor-joining tree of type I PKS ketosynthase domain sequences. Bold text indicates sequences amplified from *S. adareanum* DNA. The number of sequences is indicated in parentheses next to each group. Natural product names are shown in brackets. Products with known anticancer activity are denoted by an asterisk.

Identification of PKS Ketosynthase Genes. To amplify conserved regions of genes that encode type I PKS ketosynthase (KS) domains, degenerate primers were used in PCR with DNA from *S. adareanum* and the associated microorganisms as the template. Gel electrophoresis revealed a fragment of the expected size (~700 bp) from each of the three tunicate sections, although the middle section had the strongest signal. This 700-bp band was gel-purified, cloned, and sequenced. A BLAST search revealed that the predicted amino acid sequences for 16 of the 23 cloned fragments have considerable sequence similarity to bacterial type I PKS enzymes that function during biosynthesis of natural products (Table 3). The *S. adareanum* KS sequences are closely related to proteins from (among others) *Streptomyces atroolivaceus*, *Polyangium* (*Sorangium*) *cellulosum*, *Myxococcus xanthus*, and a bacterial symbiont of *Bugula neritina*, which are organisms responsible for leinamycin,[57] disorazoles,[58] myxovirescin,[59,60] and bryostatin[27] biosynthesis, respectively. Additional confirmation that PKS ketosynthase sequences were isolated from *S. adareanum* was obtained by doing an RPS-BLAST against the COG database at NCBI, which revealed that the predicted amino acid sequences have the expected conserved domain (COG3321, polyketide synthase modules and related proteins). The observation that a few of the sequences were clearly not PKS genes indicates that the PCR conditions tested in this study were not stringent enough to prevent amplification of other types of genes from *S. adareanum* DNA, which presumably contain nucleic acid regions complementary to the primer sequences. Nevertheless, the majority of the PCR products were the desired type of gene (i.e., KS domains of PKS enzymes).

A phylogenetic analysis of ketosynthase sequences showed that the *S. adareanum* KS sequences are affiliated with the trans-AT clade of PKS enzymes (Figure 5). The defining characteristic of these proteins is the unusual domain architecture in which the acyltransferase (AT) portions are expressed as separate transcripts,[61] unlike typical "cis-AT" PKS enzymes, which include AT domains integrated into large multidomain proteins. The trans-AT clade contains KS sequences from a variety of bacteria, including *Streptomyces atroolivaceus*,[57] *Polyangium cellulosum*,[58] *Bacillus amyloliquefaciens*,[62] and bacterial symbionts of sponges (e.g, *Discodermia dissoluta*,[53] *Theonella swinhoei*,[28] and *Pseudoceratina clavata*[63]), a bryozoan (*Bugula neritina*),[27] and *Paederus* beetles.[61] The *S. adareanum* KS sequences ($n = 16$) cluster into four groups of closely related sequences, designated type I through IV.

Biochemical pathways for polyketide synthesis generally involve multiple gene products, where each PKS protein includes several domains. At the present time, it is not known whether the *S.*

adareanum KS sequence types catalyze steps in the same biosynthetic pathway or different pathways. The guanine + cytosine (gc) content of the *S. adareanum* KS nucleotide sequences ranged from 58 to 63% (Table 3), and this fairly narrow range is consistent with the possibility of genes that originate in a single organism. Corroboration of this could be achieved by cloning and sequencing a DNA fragment large enough to contain multiple KS domains from either a bacterial genome or a *S. adareanum* metagenomic library. The fairly close phylogenetic affiliation between *Microbulbifer* species and "*Candidatus* Endobugula sertula" (both bacteria are members of the Alteromonadales order in the Gammaproteobacteria) and the observation that the *S. adareanum* KS sequences are similar to bryostatin biosynthetic genes makes it tempting to speculate that some of the *S. adareanum* KS genes could originate in a member of the Gammaproteobacteria. However, the origin(s) of the genes remains unknown and the structures of palmerolide A and bryostatin are quite different. At present, it is not possible to establish a definitive link between the cloned *S. adareanum* KS genes and palmerolide A biosynthesis, nor is it possible to pinpoint a specific bacterium that harbors the genes.

Conclusions

The 16S rRNA gene data revealed a portrait of the bacterial diversity in a single *S. adareanum* colony, although the extent to which tunicate-associated bacterial populations vary is currently unknown. Those sequences with nearest relatives that are free-living bacteria may represent transient seawater-associated populations. Future studies will compare the composition of the bacterial assemblage across *S. adareanum* colonies to assess the potential for fluctuations in bacterial community composition and determine which members are persistently associated with *S. adareanum*. The associations between bacteria and invertebrates range from persistent obligate symbiosis to casual and transient associations. The extensive literature regarding sponge microbial diversity describes a wide range of associations, including some that are remarkably consistent across sponges collected at different geographic locations.[40,64,65] This type of data is not yet available for *S. adareanum* or other tunicates.

In summary, we have described the microbial community of *S. adareanum*, the original source of palmerolide A (**1**), as a baseline for culturing bacteria and metagenomic studies. In support of our hypothesis that palmerolide A may be produced by an associated microorganism, *S. adareanum* was found to harbor PKS genes, possibly of bacterial origin, that could encode a natural product such as palmerolide A. Subsequent studies to explore culturable *S. adareanum* bacteria are ongoing.

Experimental Section

***Synoicum adareanum* Sample Collection, Microscopy, and DNA Extraction.** *S. adareanum* samples were collected from ~30 m depth near Anvers Island (64°46′ S, 64°03′ W) on the Antarctic Peninsula and then stored at −80 °C to await further processing. After removing the outer 1−2 mm, which could contain surface-associated microorganisms, one tunicate was sectioned into three fractions, outer, middle, and inner portions (3 mm of each), using sterile technique. Subsamples of each section were homogenized with a sterile polypropylene pestle and inspected microscopically after staining with 4′,6-diamidino-2-phenylindole (DAPI), a fluorescent nucleic-acid stain. DNA was extracted from homogenized tissue sections according to a published protocol.[66]

Analysis of Bacterial Diversity in *Synoicum adareanum*. Two methods were used to assess the microbial diversity within *S. adareanum*: denaturing gradient gel electrophoresis (DGGE) and 16S rRNA clone library sequencing. DGGE of amplified 16S rRNA gene fragments was used to compare bacterial diversity between outer, middle, and inner sections of *S. adareanum* and identify bacteria in each fraction through sequencing excised bands from the gel. The primers (GC358f and 517r) and protocols for DGGE were described previously.[67] A subset of the bands were excised and sequenced. When bands of the same electrophoretic mobility occurred in multiple lanes, only one band was excised. To examine the microbial diversity more comprehensively, a 16S rRNA clone library was constructed by amplifying and cloning 16S rRNA genes. For simplicity, the clone library was constructed from a single section of the tunicate (middle portion). Briefly, PCR was performed with 27F (5′-AGAGTTTGATCCTGGCTCAG) and 1391R (5′-GACGGGCRGTGWGTRCA) primers, and the products were cloned into a TOPO TA cloning vector and transformed into One Shot TOP10 *E. coli* cells (Invitrogen, Carlsbad, CA). Prior to DNA sequencing, a Montage Plasmid MiniPrep$_{96}$ kit (Millipore, Billerica, MA) was used to extract plasmid DNAs, which were quantified with a PicoGreen assay (Invitrogen, Carlsbad, CA). Sequencing reactions were performed with BigDye chemistry (v.3.1) and run on an AB3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). The Ribosomal Database Project (RDP) Classifier tool[68] was used to assess the phylum-level composition of the bacterial community with 72 partial 16S rRNA sequences (average sequence length of 815 nucleotides) included in the analysis. The nucleotide sequence clustering program cd-hit-est[69] was used to identify groups of 16S rRNA sequences that are distinct at a 99% identity threshold. From the initial data, plasmids with nonidentical nucleotide sequences (one representative of each variant) were selected for additional sequencing with another vector primer to generate consensus 16S rRNA sequences that span the cloned region. Thirty-four consensus 16S rRNA sequences (>1300 bp) were assembled from paired reads using Sequencher 4.7 (Gene Codes, Ann Arbor, MI). Sequences were aligned using the NAST alignment tool[70] on the Greengenes[71] Web site (http://greengenes.lbl.gov), and phylogenetic trees were assembled by the neighbor-joining method (with 1000 bootstrap replicates, values above 50% are shown) using MEGA 4.0 software.[72]

PCR Amplification of Ketosynthase Fragments from *Synoicum adareanum* DNA. PCR primers and conditions that were described previously[53] were used to amplify partial ketosynthase genes from DNA extracted from *S. adareanum* tissue with the associated microorganisms. Briefly, 10 ng of DNA served as template for PCR with degenerate GC-biased PCR primers (i.e., degKS2F.gc (5′-GCSATGGAYCCSCARCARCGSVT) and degKSR5.gc (5′-GTSCCSGTSCCRTGSSCYTCSAC)), which anneal to conserved nucleotides in KS domains of type I PKS genes. The cycling conditions (i.e., 55 °C annealing temperature, 35 cycles) were identical to those used to amplify KS gene fragments from the marine sponge *Discodermia dissoluta*.[53] PCR products were analyzed by gel electrophoresis, and a QIAEX II gel extraction kit (Qiagen Inc., Valencia, CA) was used to purify a ~700-bp product, which was cloned according to a protocol supplied with the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). The resulting clone library was transformed into TOP10 *E. coli* cells (Invitrogen, Carlsbad, CA), and standard procedures were used to sequence the DNA from 23 clones. A sequence similarity search (BLASTX versus the NCBI "nr" database) was performed to confirm that KS domains were amplified and cloned. Other sequence characteristics (e.g., g+c content) were determined with BioPerl[73] scripts.

Phylogenetic Analysis of KS Domains. The predicted amino acid sequences of 16 KS sequences from *S. adareanum* were included in an amino acid sequence alignment along with various reference KS sequences from GenBank, and a phylogenetic tree was constructed by the neighbor-joining method (1000 bootstrap iterations, values above 50% are shown). The amino acid sequences were aligned using ClustalX 2.0.[74] Approximately 225 amino acid positions were included in the alignment. MEGA 4.0[72] was used to generate the phylogenetic tree.

GenBank Accession Numbers. Thirty-four 16S rRNA sequences (>1300 bp) and 16 PKS ketosynthase sequences were deposited in GenBank under accession numbers FJ169189−FJ169222 and FJ178415−FJ178430, respectively.

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