Discovery of an Antarctic ascidian-associated uncultivated Verrucomicrobia that encodes antimelanoma palmerolide biosynthetic capacity

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Abstract

The Antarctic marine ecosystem harbors a wealth of biological and chemical innovation that has risen in concert over millennia since the isolation of the continent and formation of the Antarctic circumpolar current. Scientific inquiry into the novelty of marine natural products produced by Antarctic benthic invertebrates led to the discovery of a bioactive macrolide, palmerolide A, that has specific activity against melanoma and holds considerable promise as an anticancer therapeutic. While this compound was isolated from the Antarctic ascidian *Synoicum adareanum,* its biosynthesis has since been hypothesized to be microbially mediated, given structural similarities to microbially-produced hybrid non-ribosomal peptide-polyketide macrolides. Here, we describe a metagenome-enabled investigation aimed at identifying the biosynthetic gene cluster (BGC) and palmerolide A producing organism. A 74kb candidate BGC encoding the multimodular enzymatic machinery (hybrid Type I-trans-AT polyketide synthase-non-ribosomal peptide synthetase and tailoring functional domains) was identified and found to harbor key features predicted as necessary for palmerolide A biosynthesis. Surveys of ascidian microbiome samples targeting the candidate BGC revealed a high correlation between palmerolide-gene targets and a single 16S rRNA gene variant (R=0.83 – 0.99). Through repeated rounds of metagenome sequencing followed by binning contigs into metagenome-assembled genomes, we were able to retrieve a near-complete genome (10 contigs) of the BGC organism, a novel verrucomicrobius within the *Opitutaceae* family that we propose here as *Candidatus Synoicihabitans* palmerolidicus. The refined genome assembly harbors five highly similar BGC copies, along with structural and functional features that shed light on the host-associated nature of this unique bacterium.

Significance Statement

Palmerolide A has potential as a chemotherapeutic agent to target melanoma. We interrogated the microbiome of the Antarctic ascidian, *Synoicum adareanum,* using a cultivation-independent high-throughput sequencing and bioinformatic strategy. The metagenome-encoded biosynthetic machinery predicted to produce palmerolide A was found to be associated with the genome of a member of the *S. adareanum* core microbiome. Phylogenomic analysis suggests the organism represents a new deeply-branching genus, *Candidatus* Synoicihabitans palmerolidicus, in the *Opitutaceae* family of the Verrucomicrobia phylum. The Ca. *S.* palmerolidicus 4.29 Mb genome encodes a repertoire of carbohydrate-utilizing and transport pathways enabling its ascidian-associated lifestyle. The palmerolide-producer’s genome also contains five distinct copies of the large palmerolide biosynthetic gene cluster that may provide structural complexity of palmerolide variants.

Main Text

Introduction

Across the world’s oceans, marine benthic invertebrates harbor a rich source of natural products that serve metabolic and ecological roles in situ. These compounds provide a multitude of medicinal and biotechnological applications to science, health and industry. The organisms responsible for their biosynthesis are often not clear (1, 2). Increasingly, the products, especially in the polyketide class (trans-AT in particular), are found to be produced by microbial counterparts associated with the invertebrate host (3-5). Invertebrates including sponges, corals, and ascidians for example, are increasingly being recognized to harbor a wealth of diverse microbes, few of which have been cultivated (e.g., 6-8). Genomic tools, in particular, are revealing biochemical pathways potentially critical in the host-microbe associations (9). Microbes that form persistent mutualistic (symbiotic) associations provide key roles in host ecology, such as provision of...
metabolic requirements, production of adaptive features such as photoprotective pigments, bioluminescence, or antifoulants, and biosynthesis of chemical defense agents.

Antarctic marine ecosystems harbor species-rich macrobenthic communities (10-12), which have been the subject of natural products investigations over the past 30 years resulting in the identification of > 600 metabolites (13). Initially, it was not known whether the same selective pressures (namely predation and competition, e.g. (14)) that operate in mid and low latitudes would drive benthic organisms at the poles to create novel chemistry (15). However, this does appear to be the case, and novel natural products have been discovered across algae, sponges, corals, nudibranchs, echinoderms, bryozoans, ascidians, and increasingly amongst microorganisms (16) for which the ecological roles have been deduced in a number of cases (13, 17). Studies of Antarctic benthic invertebrate-microbe associations however, pale in comparison to studies at lower latitudes, yet the few studies that have been reported suggest these associations (i) harbor an untapped reservoir of biological diversity (18-21) including fungi (22), (ii) are host species-specific (23, 24), (iii) provide the host with sources of nitrogen and fixed carbon (25) and (iv) have biosynthetic functional potential (26, 27).

This study was specifically motivated by our desire to understand the biosynthetic origins of a natural product, palmerolide A, given its potent anticancer activity (28), that is found to be associated with the polyclinid Antarctic ascidian, Synoicum adareanum (Fig. 1 a and b). Ascidians are known to be rich sources of bioactive natural products (9). They have been found to harbor polyketide, terpenoid, peptide, alkaloid and a few other classes of natural products, of which the majority have cytotoxic and/or antimicrobial activities. In addition to palmerolide A, a few other natural products derived from Antarctic ascidians have been reported (29-31). Ascidian-associated microbes responsible for natural product biosynthesis have been shown to be affiliated with bacterial phyla including Actinobacteria (which dominates the recognized diversity), Cyanobacteria, Firmicutes, Proteobacteria (both Alphaproteobacteria and Gammaproteobacteria) and Verrucomicrobia in addition to many fungi (32, 33). Metagenome-enabled studies have been key in linking natural products to the organisms producing them in a number of cases, e.g. patellamide A and C to Cyanobacteria-affiliated Prochloron spp. (4), the tetrahydroisoquinoline alkaloid ET-743 to Gammaproteobacteria-affiliated Candidatus Endoeuteinascidia frumentensis (34), patellazoles to Alphaproteobacteria-affiliated Candidatus Endolissoclinium faulkneri (35), and mandelalides to Verrucomicrobia-affiliated Candidatus Didemnitutus mandela (36). However, this is most certainly an under-representation of the diversity of ascidian-associated microorganisms with capabilities for synthesizing bioactive compounds, given the breadth of ascidian biodiversity (37). These linkages have been yet to be investigated for Antarctic ascidians.

Palmerolide A has anticancer properties with selective activity against melanoma when tested in the National Cancer Institute 60 cell-line panel (28). This result is of particular interest, as there are few natural product therapeutics for this devastating form of cancer. Palmerolide A inhibits vacuolar ATPases, which are highly expressed in metastatic melanoma. Given the current level of understanding that macrolides often have microbial biosynthetic origins, that the holobiont metagenome has biosynthetic potential (26) and a diverse, yet persistent core microbiome is found in palmerolide A-containing S. adareanum (27) we have hypothesized that a microbe associated with S. adareanum is responsible for the biosynthesis of palmerolide A.

The core microbiome of the palmerolide A-producing ascidian S. adareanum in samples collected across the Anvers Island archipelago (n=63 samples; (27)) is comprised of five bacterial phyla including Proteobacteria (dominating the microbiome), Bacteroidetes, Nitrospirae, Actinobacteria and Verrucomicrobia. A few candidate taxa in particular, were suggested to be likely palmerolide A producers based on relative abundance and biosynthetic potential determined by analysis of lineage-targeted biosynthetic capability (genera: Microbulbifer, Pseudovibrio, Hoeflea, and the family Opitutaceae (27). This motivated interrogation of the S. adareanum microbiome metagenome, with the goals of determining the metagenome-encoded
biosynthetic potential, identifying candidate palmerolide A biosynthetic gene cluster (BGC)(s) and establishing the identity of the palmerolide A producing organism.

Results and Discussion

Identification of a putative palmerolide A biosynthetic gene cluster. Microbial-enriched fractions of S. adareanum metagenomic DNA sequence from 454 and Ion Proton next generation sequencing (NGS) libraries (almost 18 billion bases in all) were assembled independently, then merged, resulting in ~145 MB of assembled bases distributed over 86,387 contigs (referred to as CoAssembly 1; SI Appendix, Table S1). As the metagenome sequencing effort was focused on identifying potential BGCs encoding the machinery to synthesize palmerolide A, the initial steps of analysis specifically targeted those contigs in the assembly that were >40 kb, as the size of the macrolide ring with 24 carbons would require a large number of polyketide modules to be encoded. This large fragment subset of CoAssembly 1 was submitted to antiSMASH v.3 (38) and more recently to v.5 (39). The results indicated a heterogeneous suite of BGCs, including a bacteriocin, two non-ribosomal peptide synthetases (NRPS), two hybrid NRPS-Type I PKS, two terpenes, and three hybrid trans-AT-PKS hybrid NRPS clusters (SI Appendix, Table S2).

We predicted several functional characteristics of the BGC that would be required for palmerolide A biosynthesis which aided our analysis (see (40) for details). This included evidence of a hybrid nonribosomal peptide-polyketide pathway and enzymatic domains leading to placement of two distinct structural features of the polyketide backbone, a carbamoyl transferase that append a carbamate group at C-11 of the macrolide ring, and an HMGCoA synthetase that inserts a methyl group on an acetate C-1 position of the macrolide structure (C-25). The antiSMASH results indicated that two of the three predicted hybrid NRPS trans-AT-Type I PKS contained the predicted markers. Manual alignment of these two contigs suggested near-identical overlapping sequence (36,638 bases) and, when joined, the merged contig resulted in a 74,672 Kb BGC (Fig. 1c). The cluster size was in the range of other large trans-AT PKS encoding BGCs including pederin (54 Kb; 41), leinamycin (135.6 Kb; 42) as well as a cis-acting AT-PKS, jamaicamide (64.9 Kb; 43). The combined contigs encompassed what appeared to be a complete BGC that was flanked at the start with a transposase and otherwise unlinked in the assembly to other contiguous DNA. The cluster lacked phylogenetically informative marker genes from which putative taxonomic assignment could be attributed.

The antiSMASH results suggested that the BGC appears to be novel with the highest degree of relatedness to pyxipyrrylone A and B (encoded in the Pyxidicoccus sp. MCy9557 genome (44), to which only 14% of the genes have a significant BLAST hit to genes in the metagenome-encoded cluster. The ketosynthase (KS) sequences (13 in all) fell into three different sequence groups (40). One was nearly identical (99% amino acid identity) to a previously reported sequence from a targeted KS study of S. adareanum microbiome metagenomic DNA (26). The other two were most homologous to KS sequences from Allochromatium humboldtianum, and Dickeya dianthicola in addition to a number of hypothetical proteins from environmental sequence data sets.

Taxonomic inference of palmerolide A BGC. Taxonomic attribution of the BGC was inferred using a real time PCR strategy targeting three coding regions of the putative palmerolide A BGC spanning the length of the cluster (acyltransferase, AT1; hydroxymethylglutaryl Co-A synthase, HCS, and the condensation domain of the non-ribosomal peptide synthase NRPS, Fig. 1c) to assay a Synoicium microbiome collection of 63 samples that have been taxonomically classified using Illumina SSU rRNA gene tag sequencing (27). The three gene targets were present in all samples ranging within and between sites at levels from ~7 x 10^1 – 8 x 10^5 copies per gram of host tissue (Fig. 2a). The three BGC gene targets co-varied across all samples (r^2 > 0.7 for all pairs), with the NRPS gene copy levels slightly lower overall (mean: 0.66 and 0.59 copies per ng host tissue for NRPS:AT1 and NRPS:HCS respectively, n=63). We investigated the relationship between BGC gene copies per ng host tissue for each sample and palmerolide A levels determined for the same samples using mass spectrometry, however no correlation was found (R<0.03, n=63; (27)).
then assessed the semi-quantitative relationship between the occurrence of SSU rRNA amplon sequence variants (ASV, n=461; (27)) and the abundance of the three palmerolide A BGC gene targets. Here, we found a robust correlation (R=0.83 – 0.99) between all 3 gene targets and a single amplon sequence variant (ASV15) in the core microbiome (SI Appendix, Fig. S1). This ASV is affiliated with the Opitutaceae family of the Verrucomicrobiun phylum. The Opitutaceae family ASV (SaM_ASV15) was a member of the core microbiome as it was detected in 59 of the 63 samples surveyed at varying levels of relative abundance and displayed strong correlations with the abundances of BGC gene targets (Fig. 2b, r² =0.68 with AT1, 0.97 with HCS, and 0.69 with NRPS, n=63 for all). The only other correlations R > 0.5 were ASVs associated within the “variable” fraction of the microbiome, e.g., one low abundance ASV was present in 24 of 63 samples (SI Appendix, Fig. S1).

This result supports the finding of Murray et al. (27) in which gene abundance and natural product chemistry do not reflect a 1:1 ratio in this host-associated system. Neither the semi-quantitative measure of ASV copies nor the real time PCR abundance estimates of the three biosynthetic gene targets correlated with the mass-normalized levels of palmerolide A present in the same samples. As discussed (27), this is likely a result of bioaccumulation in the ascidian tissues. This result provided strong support that the genetic capacity for palmerolide A production was associated with a novel member of the Opitutaceae, a taxonomic family with representatives found across diverse host-associated and free-living ecosystems. Although the biosynthetic capacity of this family is not well known (45), recent evidence (36) suggests this family may be a fruitful target for cultivation efforts and natural product surveys.

Assembly of the palmerolide BGC-associated Opitutaceae-related metagenome assembled genome (MAG). With metagenomes some genomes come together easily – while others present compelling puzzles to solve. Assembly of the pal BGC-containing Opitutaceae genome was the result of a dedicated effort of binning contigs, gene searches, additional sequencing of samples with high BGC titer, and manual, targeted assembly. Binning efforts with CoAssembly 1 did not result in association of the pal BGC with an associated metagenome assembled genome (SI Appendix text). Therefore, a further round of metagenome sequencing using long read technology (Pacific Biosciences Sequel Systems technology; PacBio) ensued.

The 16S rRNA gene ASV occurrence (27) and real time PCR data were used to guide S. adarenum sample selection for sequencing. Two ascidian samples (Bon-1C-2011 and Del-2B-2011) with high Opitutaceae ASV occurrences (ASV_015; > 1000 sequences each) relative abundance of ~ 13.3-15.3 % compared to an overall average of 1.3 ± 2.77% across the 63 samples respectively, (27)) and high BGC gene target levels (> 6.9 x 10⁵ and > 2.0 x 10⁵ for the NRPS respectively; SI Appendix, Table S3) were selected for PacBio sequencing. This effort generated 28 GB of data that was used to create a new hybrid CoAssembly 2 which combined all three sequencing technologies. Similar to the assembly with the Mycale hentscheli-associated polyketide producers (46), the long-read data set improved the assembly metrics, and subsequent binning resulted in a highly resolved Opitutaceae-classified bin (SI Appendix, Fig. S2, Table S4). Interestingly however, the palmerolide BGC contigs still did not cluster with this bin, which we later attributed to binning reliance on sequence depth.

We used PacBio circular consensus sequence (CCS) reads to generate and manually edit the assembly for our Opitutaceae genome of interest. The resulting 4.3 MB genome (Fig. 3a) had a GC content of 58.7% and was resolved into a total of 10 contigs. Five of the contigs were unique and the other five contigs represented highly similar repeated units of the pal BGC (labeled pal BGC 1, 2, 3, 4, and 5) with broken ends resulting in linkage gaps. Nucmer alignment of contigs to the longest palmerolide-containing BGC revealed a long (36,198 kb) repeated region that was shared between all 5 contigs with some substantial differences at the beginning of the cluster and only minor differences at the end, indicating 3 full length, and 2 shorter palmerolide BGC-containing contigs (Fig. 1 and 3). This was consistent with coverage estimates based on read-mapping that suggested lower depth at the beginning of the cluster (Fig. 3b). BGC 1 and 3 are nearly identical (over 86,135 bases) with only 2 single nucleotide polymorphisms (SNPs) and an additional 1,468 bases in BGC1 (237 bases at the 5’ end and 1231 bases at the 3’ end). BGC 4 is 13,470 bases
shorter than BGC1 at the 5’ end, and 5 bases longer than BGC 1 at the 3’ end. Alignment of the
real time PCR gene targets to the 5 pal BGCs provided independent support for the different lengths
of the 5 BGCs, as the region targeted by the NRP primers was missing in two of the pal BGCs,
thus explaining lower NRP: AT or NRP:HCS gene dosages reported above.

Interestingly, precedent for naturally occurring multi-copy BGCs to our knowledge, has only
been found in another ascidian (Lissoclinum sp.)-associated Opitutaceae, Candidatus
Didemmitutus mandela which have been linked to cytotoxic mandelalides (36). Likewise, we can
invoke a rationale similar to (36) that multiple gene clusters may be linked to biosynthesis of
different palmerolide variants, see Avalon et al. (40) for retro-biosynthetic predictions of these
clusters. Gene duplication, loss, and rearrangement processes over evolutionary time, likely
explain the source of the multiple copies. At present we do not yet understand the regulatory
controls, whether all five are actively transcribed, if there is a producing-organism function and how
this may vary amongst host microorganisms.

Phylogenomic characterization of the Opitutaceae-related MAG. The taxonomic relationship
of the Opitutaceae MAG to other Verrucomicrobiota was assessed using distance-based analyses
with 16S rRNA and average amino acid identity (AAI). Then it was classified using the GTDB-Tk
tool (47), and a phylogenetic analysis based on concatenated ribosomal protein markers.
Comparison of 16S rRNA gene sequences amongst other Verrucomicrobia with available genome
sequences (that also have 16S rRNA genes; SI Appendix, Fig. S3) suggests that the nearest
relatives are Cephaloticoccus primus CAG34 (similarity of 0.9138), Opitutus terrae PB90-1
(similarity of 0.9132) and Geminisphaera coliterminitum TAV2 (similarity of 0.9108). The
Opitutaceae-affiliated MAG sequence is identical to a sequence (uncultured bacterium clone Tun-
3b A3) reported from the same host (S. adareanum) in a 2008 study (26); bootstrapping supported
a deep branching position in the Opitutaceae family.

When characterizing the MAG using AAI metrics (average nucleotide identity, ANI, found
no closely related genomes) the closest genomes were environmental metagenome assemblies
from the South Atlantic TOBG_SAT_155 (53.08 % AAI) and WB6_3A_236 (52.71 % AAI); and the
two closest isolate type genomes were Nibrificoccus aquaticus str. NZ CP023344 (52.82 % AAI) and
Opitutus terrae str. PB90 (52.75 % AAI). The Microbial Genome Atlas (MiGA) support for the MAG
belonging in the Opitutaceae family was weak (p-values of 0.5). Attempts to classify this MAG using
GTDB-Tk (47) were hampered by the fact we have no real representative in the genome databases,
resulting in low confidence predictions at the species or genus levels (see the SI Appendix text for
details).

Verrucomicrobia exhibit free-living and host-associated lifestyles in a multitude of terrestrial
and marine habitats on Earth. We performed a meta-analysis of Verrucomicrobia genomes, with
an emphasis on marine and host-associated Opitutaceae, to establish more confidence in the
phylogenetic position of the Opitutaceae MAG. The analysis was based on 24 conserved proteins
– 21 ribosomal proteins and three additional conserved proteins (InfB, lepA, pheS). The diversity
of the Opitutaceae family, and of Verrucomicrobia in general, is largely known from uncultivated
organisms in which there are 20 genera in GTDB (release 05-RS95), 2 additional genera in the
NCBI taxonomy database, and numerous unclassified single amplified genomes (SAGs); in all,
only eight genera have cultivated representatives. Given the uneven representations of the 24
proteins across all (115) genomes assessed (MAGs and SAGs are often incomplete), we selected
a balance of 16 proteins across 48 genomes to assess phylogenomic relatedness across the
Opitutaceae (Fig 4). Here too, as seen with the 16S rRNA gene phylogenetic tree, the S. adareanum-
Opitutaceae MAG held a basal position compared to the other Opitutaceae genomes in the
analysis.

Opitutaceae-related MAG relative abundance estimates and ecological inference. The
relative abundance of Opitutaceae bin 8 was estimated in the shotgun metagenomic samples by
mapping the NGS reads back to the assembled MAG across the four S. adareanum samples
collected. This indicated varying levels of genome coverage in the natural samples, with the two
samples selected based on real time PCR-quantified high BGC copy number being clearly enriched
These levels are higher than estimates of relative abundance derived from the 16S rRNA gene amplicon surveys (estimated at 13.33 and 15.34 % respectively) for the same samples. This is likely a result of the single-copy nature of the ribosomal operon in Opitutaceae bin 8 vs. other taxa with multiple rRNA operon copies that could thus be over-represented in the core microbiome library (e.g., Pseudovibrio sp. str. PSC04-5.14 has 9 and Microbulbifer sp. is estimated at 4.1 ± 0.8 based on 9 finished Microbulbifer genomes available at the Integrated Microbial Genomes Database). All host S. adareanum lobes surveyed (n=63) in the Anvers Island regional survey contained high levels (0.49 – 4.06 mg palmerolide A x g⁻¹ host dry weight) of palmerolide A (27), and variable, yet highly concordant levels of the pal BGCs and 16S rRNA ASV levels (Fig. 2). Despite the natural population structure sampled here (four single host lobes), the bin-level sequence variation was low (ranging from 72-243 SNPs) when the PacBio reads were mapped back to the Opitutaceae bin 8 (Table 1). This suggests maintenance of a relatively invariant population at the spatial and temporal scales of this coastal Antarctic region while highlighting our limited understanding of the biogeographical extent of the S. adareanum-symbiont-palmerolide relationship across a larger region of the Southern Ocean.

Several questions remain with regard to the in situ function of palmerolide A (a eukaryotic V-ATPase inhibitor in human cell line assays (28)) in this cryohabitat: how and why is it bioaccumulated by the host? Overall, the study of natural products in high latitude marine ecosystems is in its infancy. This palmerolide producing, ascidian-associated, Opitutaceae provides the first Antarctic example in which a well-characterized natural product has been linked to the genetic information responsible for its biosynthesis. Gaining an understanding of environmental and biosynthetic regulatory controls, establishing integrated transcriptomic, proteomic, and secondary metabolome expression in the environment will also reveal whether the different clusters are expressed in situ. In addition to ecological pursuits, the path to clinical studies of palmerolide will require genetic or cultivation efforts. At present, we hypothesize that cultivation of Opitutaceae bin 8 may be possible, given the lack of genome reduction or of other direct evidence for host-associated dependencies.

**Candidatus Synoicihabitans palmerolidicus genome attributes.** The Antarctic ascidian, *Synoicum adareanum*, harbors a dense community of bacteria that has a conserved core set of taxa (27). The near complete ~4.30 Mbp Opitutaceae bin 8 metagenome assembled genome (Fig. 3) represents one of the core members. This MAG is remarkable in that it encodes for five 36-74 kb copies of the candidate BGCs that are implicated in biosynthesis of palmerolide A and possibly other palmerolide compounds. Intriguingly, this genome does not seem to show evidence of genome reduction as found in *Candidatus Didemnitutus mandela* (36); the other ascidian-associated *Opitutaceae* genome currently known to encode multiple BGC gene copies. This is the first *Opitutaceae* genome characterized from a permanently cold, ~1.8 - 2 °C, often ice-covered ocean ecosystem. This genome encodes one rRNA operon, 45 tRNA genes, and an estimated 5058 coding sequences. Based on the low (<92%) SSU rRNA gene identity and low (<54% AAI) values to other genera in the *Opitutaceae*, along with the phylogenomic position of the *Opitutaceae* bin 8, the provisional name “Candidatus Synoicihabitans palmerolidicus” (Ca. S. palmerolidicus) is proposed for this novel verrucomicrobiom. The genus name Synoicihabitans (Syn.o.i.ci.ha’bitans. N.L. neut. N. Synoicum a genus of ascidians; L. pres. part habitanis inhabiting; N.L. masc. n.) references this organism as an inhabitant of the ascidian genus Synoicum. The species name palmerolidicus (pal.me.ro.li’di.cus. N.L. neut. n. palmerolidum palmerolide; N.L. masc. adj.) designates the species as pertaining to palmerolide.

The GC content of 58.7% is rather high compared to other marine *Opitutaceae* genomes (ave. 51.49 s.d. 0.02, n=12), yet is ~ average for the family overall (61.58 s.d. 0.06, n=69; SI Appendix, Table S5). MetaERG includes metagenome assembled genomes available in the GTDB as a resource for its custom GenomeDB that new genomes are annotated against. This was a clear advantage in annotating the Ca. S. palmerolidicus genome as Verrucomicrobia genomes are widely represented by uncultivated taxa. Likewise, antiSMASH was an invaluable tool for *pal* BGC
identification and domain structure annotation. This formed the basis to derive a predicted stepwise mechanism of pal biosynthesis (40).

Ca. S. palmerolidicus genome structure, function and host-associated features. Beyond the pal BGCs, the Ca. S. palmerolidicus genome encodes a variety of additional interesting structural and functional features that provide a window into its lifestyle. Here we will only provide a brief synopsis. In addition to the repeated BGCs, three additional repeats with two nearly identical copies each (15.3 Mb, 17.0 Mb, 27.4 Mb) were identified during the assembly process (Fig. 3a, SI Appendix, Table S6). These coded for 20, 25 and 41 CDSs respectively, were in some cases flanked by transposase/integrase genes (both internal and proximal) and had widespread homology with Verrucomicrobia orthologs. The contents of the three repetitive elements were unique.

Annotations were assigned to a little more than half of the CDSs in the 15.3 Mb repeat in which to support xyllose transport, two sulfatase copies, two endonuclease copies and a MacB-like (potential macrolide export) periplasmic core domain were encoded. Xylan might be sourced from seaweeds (48) or the even ascidian as it is a minor component of the tunic cellulose (49). Related to this, an endo-1,4-beta-xylanase which has exoenzyme activity in some microorganisms (50) was identified elsewhere in the genome. Altogether, eight sulfatase copies were identified in this host-associated organism (four in the 15.5 Mb repeat elements). These may be involved in catabolic activities of sulfonated polysaccharides, and possibly as trans-acting elements in palmerolide biosynthesis (40). In addition to the MacB-like CDSs found in this repeat, 13 different MacB-homologs were present in the genome – none of which were associated with the pal BGCs (SI Appendix, Fig. S4). MacB is a primary component of the macrolide tripartite efflux pump that operates as a mechanotransmission system which is involved both in antibiotic resistance and antibiotic export depending on the size of the macrolide molecule (51). However, two additional elements required for this pump to be functional, an intramembrane MacA and an outer membrane protein TolC, were not co-located in the genome. MacA may be missing, as hits to two other verrucomicrobia-associated MacA CDSs were not identified using BLAST (Peat Soil MAG SbV1 SBV1_730043 and Ca. Udaeobacter copiosis KAF5408997.1; (52)). At least nine MacB CDSs were flanked by a FstX-like permease family protein; the genomic structure of which were quite complex including several with multiple repeated domains. Detailed transporter modeling is beyond the scope of this work, but it is likely that these proteins are involved in signaling of cell division machinery rather than macrolide transport (53).

Predicted CDSs in the 17.0 Mb repeat included sugar binding and transport domains, as well as domains encoding rhamnosidase, arabino-furanosidase, and other carbohydrate catabolism functions. About half proteins encoded in the 27.4 Mb repeat were unknown in function, and those characterized suggested diverse potential functional capacities. For example, a zinc carboxypeptidase (1 of 3 in the genome), multidrug and toxic compound transporter (Mate/NorM), and an exodeoxyribonuclease were identified.

The Ca. S. palmerolidicus MAG has a number of features that suggest it is adapted to a host-associated lifestyle, several of these features were reported recently for two related sponge-associated Opitutales metagenome bins (Petrosia ficiformis-associated bins 0 and 01, Fig. 4; (54)). These include identification of a bacterial microcompartment (BMC) ‘super locus’. Such loci were recently reported to be enriched in host-associated Opitutales genomes when compared to free-living relatives. The structural proteins for the BMC were present as were other conserved Planctomycyes-Verrucomicrobia BMC genes (55). As in the sponge Pectioria ficiformis metagenome bins, enzymes for carbohydrate (rhamnose) catabolism and modification were found adjacent to the BMC locus (SI Appendix, Fig. S5), in addition to the two that were found in the 27.4 Mb repeat. The genome did not appear to encode the full complement of enzymes required for fucose metabolism, though a few alpha-L-fucosidases were identified. Further evidence for carbohydrate metabolism was supported through classification of the genome using the CAZY database (56), including 7 carbohydrate binding modules, a carbohydrate esterase, 14 glycoside hydrolases, 6 glycosyl transferases and a polysaccharide lyase. In addition, three bacterial cellulases (PF00150, cellulase family A; glycosyl hydrolase family 5) were identified, all with a canonical conserved glutamic acid residue. These appear to have different evolutionary histories in which each variant
has nearest neighbors in different bacterial phyla (SI Appendix, Fig. S6) matching between 68% identity for Protein J6386 03765 to *Lacunisphaera limbophila*, 57.5% identity for Protein J6386 22340 with a cellulase from a shipworm symbiont *Alteromonadaceae* (*Terridinibacter* sp.), and 37.5% sequence identity to a Bacteroidetes bacterium. This suggests the potential for cellulose degradation – which is consistent with ascidians being the only animals known to produce cellulose where it acts as a skeletal structure (49). In addition to the BGCs, the enzymatic resources in this genome (e.g., xylan and cellulose hydrolysis) are a treasure trove rich with biotechnological potential.

Other indicators of host-association in the Opitutales include T-A domains, which were prevalent in the *Petrosia ficiformis*-associated bins 0 and 01 (54). The Ca. *S. palmerolidicus* genome encoded at least 22 TA-related genes including multiple MazG and AbiEii toxin type IV TA systems, AbiEii-Phd_YefM type II toxin-antitoxin systems, along with genes coding for PIN domains, Zeta toxin, RelB, HipA, MazE and MraZ. This analysis also resulted in identifying a putative AbiEii toxin (PF13304) with homology to SyrD, a cyclic peptide ABC type transporter that was present in all 5 BGCs (Fig. 1c; BLAST percent identity 52.7% to a *Desulfamplus* sp. homolog over the full length of the gene, and a variety of other bacteria including an *Opitutaceae*-related strain at similar levels of identity). These genes are encoded downstream of the BGC following the acyl transferase domains and precede the predicted trans-acting domains at the 3’ end of the BGC. Given the proximity adjacent to the primary biosynthetic gene clusters, this protein is a candidate for palmerolide transport. Further research is needed however to discern the details of Ca. *S. palmerolidicus*’ cellular biology, localization of palmerolide production, transport, and resistance mechanisms to the potent vacuolar ATPase as well as products made by others in the *S. adareanum* microbiome. Along these lines, in addition to the MatE (found in the 27.4 Mb repeat) two other multidrug export systems with homology to MexB and MdtB were identified.

Unlike in Ca. *D. mandela* (36), there does not appear to be ongoing genome reduction, which may suggest that the *S. adareanum*-Ca. *S. palmerolidicus* relationship is more recent, and/or that the relationship is commensal rather than interdependent. Likewise, we suspect that the pseudogene content may be high as several CDS appear to be truncated, in which redundant CDS of varying lengths were found in several cases (including the MacB). There is evidence of lateral gene transfer acquisitions of cellulase and numerous other enzymes that may confer ecological advantages through the evolution of this genome. Likewise, the origin of the *pal* BGCs and how recombination events play out in the success of this Antarctic host-associated system in terms of adaptive evolution (57), not to mention the ecology of *S. adareanum* is a curiosity. This phylum promises to be an interesting target for further culture-based and cultivation-free studies – particularly in the marine environment.

Together, it appears that the genome of Ca. *S. palmerolidicus* is equipped for life in this host-associated interactive ecosystem that stands to be one of the first high latitude marine invertebrate-associated microbiomes with a genome-level understanding – and one that produces a highly potent natural product, palmerolide A. This system holds promise for future research now that we have identified the producing organism and *pal* BGC. We still have much to learn about the ecological role of palmerolide A – if is it involved predation avoidance, antifouling, antimicrobial defense or some other yet to be recognized aspect of life in the frigid, often ice-covered and seasonally light-limited waters of the Southern Ocean.

Materials and Methods

**Sample Collection.** *S. adareanum* lobes were collected in the coastal waters off Anvers Island, Antarctica and stored at -80 °C until processing (SI Appendix, Table S1). See the SI Appendix text and (27) for details of sample collection, microbial cell preparation and DNA extraction.

**Metagenome sequencing.** Three rounds of metagenome sequencing were conducted, the details of which are in the SI Appendix text. This included an initial 454 pyrosequencing effort with a bacterial-enriched metagenomic DNA preparation from *S. adareanum* lobe (Nor2c-2007). Next,
an Ion Proton System was used to sequence a metagenomic DNA sample prepared from S. adareanum lobe Nor2a-2007. Then two additional S. adareanum metagenome DNA samples (Bon-1C-2011 and Del-2b-2011) selected based on high copy numbers of the palmerolide A BGC (see real time PCR Methods, SI Appendix text) and sequenced using Pacific Biosciences Sequel Systems technology.

**Metagenome assembly, annotation and binning.** Raw 454 metagenomic reads (1,570,137 single end reads, 904,455,285 bases) were assembled by Newbler (58) v2.9 (Life Technologies, Carlsbad, CA, flags: -large -rip -mi 98 -ml 80), while Ion Proton metagenomic reads (89,330,870 reads, 17,053,251,055 bases) were assembled using SPAdes (59) v3.5 (flags: --iontorrent). Both assembled datasets were merged with MeGAMerge (60) v1.2 and produced 86,387 contigs with a maximum contig size 153,680 and total contig size 144,953,904 bases (CoAssembly 1). To achieve more complete metagenome coverage and facilitate metagenome assembled genome assembly, a Circular Consensus Sequence (CCS) protocol (PacBio) was used to obtain high quality long reads on two samples Bon-1C-2011 and Del-2b-2011. The 5,514,426 PacBio reads were assembled with aforementioned assembled contigs (CoAssembly 1) on EDGE Bioinformatics using wtdbg2 (61), a fast and accurate long-read assembler. The contigs were polished with three rounds of polishing by Racon (62) into a second Coassembly (CoAssembly 2) which has 4,215 contigs with a maximum contig size 2,235,039 and total size 97,970,181 bases. Lastly, A manual approach was implemented to arrive at assembly of the MAG of interest the details of which are described in the SI Appendix text.

The contigs from both co-assemblies 1 and 2 were submitted initially to the EDGE bioinformatics platform (63) for sequence annotation using Prokka (64) v1.13 and taxonomy classification using BWA (65) mapping to NCBI RefSeq (Version: NCBI 2017 Oct 3).

Bioinformatic predictions of natural product potential was performed using the antibiotics and secondary metabolite analysis shell (antiSMASH, bacterial versions 3.0, 4.0 and 5.0 (45, 70)). This tool executed contig identification, annotation and analysis of secondary metabolite biosynthesis gene clusters on both CoAssemblies 1 and 2 (> 1 kb and > 40 kb data sets). As most of our attention was focused on analysis the Ca. S. palmerolidicus assembled metagenome, we also used MetaERG (66) as the primary pipeline for metagenome annotation of the ten final contigs in addition to NCBI's PGAP pipeline. There were 5186 coding sequences predicted in the MetaERG annotation and 5186 in NCBI's PGAP annotation.

MaxBin (67) and MaxBin2 (68) were used to form metagenome bins for both CoAssembly 1 and 2. CheckM v1.1.11 (69) and v.1.1.12, and GTDB-Tk v.1.0.2 (47) were used to verify bin quality and taxonomic classification. See SI Appendix text for details. In order to assess the representation of assembled Opitutaceae genome across the 4 environmental samples used for metagenome sequencing (resulting from MaxBin2 binning of CoAssembly 2), we used BWA to map the CCS reads to each metagenome data set.

**Real time PCR.** Gene targets (non-ribosomal peptide synthase, acyltransferase, and 3-hydroxymethylglutaryl coenzyme A synthase) were selected at different positions along the length of the candidate BGC. SI Appendix, Table S7 lists the primer and the GBlocks synthetic positive control sequence. Metagenomic DNA extracts from a large S. adareanum sample set (n=63 S. adareanum lobes from 21 colonies), all containing high levels of palmerolide A (27), were screened with the real time PCR assays on a Quant Studio 3 (Thermo Fisher Scientific, Inc.; see SI Appendix text for details of controls and analysis).

**Phylogenomic analyses.** A phylogenomic analysis of the assembled Opitutaceae MAG was conducted based on shared ribosomal RNA and ribosomal proteins amongst 46 and 48 reference genomes respectively, out of 115 genomes in total, mined from various databases (NCBI, GTDB and IMG) for uncultivated and cultivated microorganisms identified in the Verrucomicrobia phylum (SI Appendix, Table S8). The details of these analyses are described in the SI Appendix text. In addition, we used MiGA (NCBI Prokaryotic taxonomy and the environmental TARA Oceans.
(Tully) databases; accessed August 2020) and GTDB-Tk (ver. 1.3.0) tools for MAG taxonomic classification.

Phylogenetic analysis of the MacB CDS sequences were retrieved from MetatERG annotated Ca. S. palmerolidicus contigs and homologs were retrieved from the NCBI based on BLAST results. Maximum likelihood analysis was conducted on 994 aligned (MUSCLE) positions using RAxML v.8.2.12 using the PROTGAMMALG model and 550 bootstrap replicates. For the phylogenetic analysis of the cellulase CDS, homologs were retrieved from the NCBI based on BLAST results resulting in 19 sequences and 496 aligned positions (ClustalOmega) was also conducted using RAxML v.8.2.12 under the PROTGAMMALG model of evolution with 1000 bootstraps.

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Figure 1. Palmerolide A, a cytotoxic, macrolide with anti-melanoma activity is found in the tissues of *Synoicum adareanum* in which a candidate biosynthetic gene cluster has been identified. (A) *S. adareanum* occurs on rocky coastal seafloor habitats in the Antarctic; this study focused on the region off-shore of Anvers Island in the Antarctic Peninsula. (B) Palmerolide A, is the product of a hybrid PKS-NRPS system in which biosynthesis begins with a PKS starter unit followed by incorporation of a glycine subunit by an NRPS module. Subsequent elongation, cyclization and termination steps follow. Two additional features of the molecule include a methyl group on C-25.
and a carbamate group on C-11. (C) Five repeats encompassing candidate palmerolide biosynthetic gene clusters were identified. The BGC (in blue) is defined as starting with the NRP unit and ending at the carbamoyltransferase (green). Candidate palmerolide A biosynthetic gene cluster BGC4 was identified from initial metagenome library assemblies. The other four clusters were identified following a third round of sequencing, assembly and manual finishing. Primary BGC coding sequences (CDS) and a conserved tailoring cassette are in blue. Light blue CDS are an ATP transporter with homology to an antibiotic transporter, SyrD. All black CDS are repeated amongst the BGCs. Orange CDS are transposase/integrase domains. Gray CDS are unique, non-repeated; and in BGC2 and 5, the unique CDS encode transposases, distinct from the predicted amino acid sequences of those in orange. The red lines associated with Contig 9 indicate targeted quantitative PCR regions.
Figure 2. Abundances of real time PCR-targeted coding regions in the candidate pal biosynthetic gene cluster in Antarctic ascidian samples. (A) Gene copies estimated for three targeted coding regions (Acyltransferase, AT1; 3-hydroxy-methyl-glutaryl coenzyme A synthase, HCS; and the condensation domain of a non-ribosomal peptide synthase; NRPS) in the candidate palA biosynthetic gene cluster surveyed over 63 DNA extracts derived from microbial cell preparations enriched from the Antarctic ascidian Synoicum adareanum. Nine samples were collected at each of seven sites: Bon, Bonaparte Point; Del, Delaca Island; Jan, Janus Island, Kil, Killer Whale Rocks; Lag, Laggard Island, Lit, Litchfield Island; Nor, Norsel Point (27). (B) Relationship between gene copy number for the three gene targets and the 16S rRNA gene ASV occurrences of Opitutaceae-related ASV_15 across a 63 S. adareanum microbial DNA sample set. * indicates samples Bon-1C-2011 and Del-2b-2011 that were selected for PacBio sequencing.
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Figure 3. Genome maps of assembled MAG, Candidatus Synoicohabitans palmerolidicus and evidence of multi-copy biosynthetic gene clusters. (a) The 4,297,084 bp gene map is oriented to dnaA at the origin. One possible assembly scenario of the Ca. S. palmerolidicus genome is shown as the order of the contigs and palmerolide BGC’s are not currently known. In addition to the 5 BGCs, three other internally repetitive regions were identified (15.3, 17.0 and 27.4 kb). The genes and orientation are shown with blue and tRNA indicated in red. (b) To demonstrate depth of coverage outside and inside the BGC regions, CCS reads from sample Bon-1C-2011 and Del-2b-2011 were mapped to a 167.6 Kb region. The profile extends 40 kb into the genome on either side of the BGC where depth of coverage averages 60 fold, while in the BGC depth of coverage varies across the BGC given differences in cover across the BGC, the highest cover is 5X, or ~300 fold, supporting the finding of 5 repeats encoding the BGC.
Figure 4. Maximum likelihood phylogenomic tree showing 48 verrucomicrobia genomes
Phylogenomic relationship of Candidatus Synicochabits palmerolidicus (Opitutaceae bin 8)
with respect to other mostly marine, and host-associated verrucomicrobia subdivision 4 and
other genomes. The tree is based on 16 concatenated ribosomal proteins (5325 amino acids)
common across 48 Verrucomicrobia genomes. Distance was estimated with RAxML with 300
bootstrap replicates. Symbols designate environmental origins of the organisms: free-living are
represented by circles: light blue - marine, green– freshwater, red – hydrothermal mud, brown –
soils. Host-associated taxa from marine systems with blue diamonds, and from terrestrial systems
with black diamonds.
Table 1. Metagenomic reads from 4 different samples were mapped back to the Ca. S. palmerolidicus MAG.

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