Meta-omic Characterization of the Marine Invertebrate Microbial Consortium That Produces the Chemotherapeutic Natural Product ET-743

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Supporting Information

ABSTRACT: In many macroorganisms, the ultimate source of potent biologically active natural products has remained elusive due to an inability to identify and culture the producing symbiotic microorganisms. As a model system for developing a meta-omic approach to identify and characterize natural product pathways from invertebrate-derived microbial consortia, we chose to investigate the ET-743 (Yondelis) biosynthetic pathway. This molecule is an approved anticancer agent obtained in low abundance (10⁻⁴–10⁻⁵ % w/w) from the tunicate Ecteinascidia turbinata and is generated in suitable quantities for clinical use by a lengthy semisynthetic process. On the basis of structural similarities to three bacterial secondary metabolites, we hypothesized that ET-743 is the product of a marine bacterial symbiont. Using metagenomic sequencing of total DNA from the tunicate/microbial consortium, we targeted and assembled a 35 kb contig containing 25 genes that comprise the core of the NRPS biosynthetic pathway for this valuable anticancer agent. Metagenomic sequencing of total DNA from the tunicate/microbial consortium, we targeted and assembled a 35 kb contig containing 25 genes that comprise the core of the NRPS biosynthetic pathway for this valuable anticancer agent. Rigorous sequence analysis based on codon usage of two large unlinked contigs suggests that Candidatus Endoecteinascidia frumentensis produces the ET-743 metabolite. Subsequent metaproteomic analysis confirmed expression of three key biosynthetic proteins. Moreover, the predicted activity of an enzyme for assembly of the tetrahydroisoquinoline core of ET-743 was verified in vitro. This work provides a foundation for direct production of the drug and new analogues through metabolic engineering. We expect that the interdisciplinary approach described is applicable to diverse host-symbiont systems that generate valuable natural products for drug discovery and development.

One of the major challenges in gene and metabolic pathway discovery involves access to genomes from unculturable microorganisms. Efficient methods for accessing high quality samples of DNA from specialized ecological niches have enabled metagenomic sequencing, leading to discovery of new enzymes and in some cases partial assembly of unique genomes.1,2 Recent work to identify cellulases and other enzymes with bioenergy applications from the cow rumen microbial consortium established the promise of this approach. The potential for metabolic pathway assembly and deep annotation using next-generation sequencing motivated us to explore the seemingly inaccessible wealth of gene clusters for natural product biosynthesis derived from marine and terrestrial symbiont microbial consortia. Our effort has relied on the ready availability of the selected invertebrate-derived metagenomic source material, which enabled direct assembly of the target operon, and represents the development of a new strategy for secondary metabolite discovery and expansion of chemical diversity.

ET-743 (1) is a tetrahydroisoquinoline natural product with potent anticancer activity isolated from the tunicate Ecteinascidia.
ET-743 system.22 The tetrahydroisoquinoline pathways each consist of three nonribosomal peptide synthetase (NRPS) modules and a series of aligned tailoring enzymes. Each module contains three domains: adenylation (A), condensation (C), and thiolation (T) that combine the amino acid building blocks. Two of these pathways are initiated by an acyl-ligase (AL) and a T didomain. All three NRPS trimodules are terminated by a signature reductase domain (RE) that utilizes NAD(P)H to release the enzyme bound intermediate as an aldehyde. The final C domain in the saframycin pathway serves as a “Pictet-Spenglerase” to cyclize the activated intermediate.15 Recent efforts have shown that a fatty acid appended to the growing polypeptide on the NRPS T-domain is required to form the cyclic tri- and tetrapeptide tetrahydroisoquinoline core system.25 In considering a metagenomics discovery strategy, we reasoned that the ET-743 pathway would likely comprise in part an AL-T for initiation, three NRPS modules for elongation, and termination by an RE domain (Figure 1b, EtuA1-3).

Previous work directed toward identification of a producing organism and potential biosynthetic pathway assessed the phylogenetic diversity of bacterial species from E. turbinata as a source of ET-743 in the Mediterranean and Caribbean seas. A γ-proteobacterium Candidatus Endoecteinascidia frumentensis (AY054370) was identified as the most prevalent member from the tunicate microbial consortium at all collection sites,26,27 providing indirect evidence for a potential bacterial producer of the ET-743 anticancer agent. We considered a cloning-independent approach that would avoid technical barriers encountered...
when handling environmental metagenomic DNA samples and large clone libraries in order to gain direct access to the elusive gene cluster. Rapid advances in metagenomic and hologenomic sequencing technologies, as well as bioinformatic tools for contig assembly, indicated that this direct approach would provide facile access to the desired biosynthetic system derived from a host-symbiont community.

A key issue with metagenomic DNA derived from environmental samples and unculturable microorganisms is the lack of an in vivo genetic system to establish the identity of the biosynthetic pathway. This limitation can be overcome by in vitro characterization of heterologously expressed gene products. In vitro characterization provides a direct link between biosynthetic genes derived from field-collected samples and their corresponding metabolites, a key step toward understanding these complex systems. We also considered that metaproteomics would be an effective way to identify gene products in low abundance, particularly for samples consisting of multiple microbial species. Direct amino acid sequence evidence for predicted biosynthetic proteins can effectively link gene-based bioinformatics to in vitro biochemical function in diverse microbial symbiont-host systems.

Herein, we describe the identification and initial biochemical characterization of the ET-743 biosynthetic pathway from the host-symbiont community derived from E. turbinata. After confirming the presence of the tetrahydroisoquinoline secondary metabolites from the animal, metagenomic sequencing was conducted to identify the target biosynthetic genes. High-resolution mass spectrometry was then used to mine the metaproteome for the presence of the ET-743 biosynthetic pathway enzymes predicted from gene cluster sequence analysis. Finally, enzymatic activity for a key enzyme to form...
the tetrahydroisoquinoline core was verified in vitro with a model substrate to corroborate the identity of the metabolic pathway (Figure 1c). This knowledge enables a clear path for accessing ET-743 and new analogues through heterologous expression technologies and provides a general strategy for identification and characterization of host—symbiont-derived natural product systems.

RESULTS AND DISCUSSION

Secondary Metabolite Identification As a Starting Point for the “ET-743 Bacterial Symbiont Producer” Hypothesis. We confirmed that field-collected tunicate samples of E. turbinata from the Florida Keys contained ET-743 and related metabolites using high-resolution, high-mass accuracy, liquid chromatography-Fourier transform ion cyclotron resonance mass spectrometry (LC–FTICR–MS). Known biosynthetic precursors were identified from the tunicate by extracted ion chromatograms at ±20 ppm, including the M + H+ and (M – H2O) + H+ for ET-743 (1), ET-597 (19), ET-594 (21), and ET-583 (18) (Figure 2). Confirmation by LC–MS/MS was performed online with FTICR–MS and an ion trap-mass spectrometer (IT–MS). Since all four compounds identified had previously been characterized by MS/MS, assignment of product ions was straightforward and all four compounds identified had previously been characterized.

Confirmation by LC-FTICR

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Metagenomic Sequencing and Phylogenetics. Based on identification of ET-743 from E. turbinata, we prepared total holonomic DNA from fresh field-collected tunicate samples. This DNA was used to prepare a 16S rRNA gene amplicon library and a random shotgun fragment library for 454-based FLX pyrosequencing. Raw reads from the first shotgun sequencing run and an assembly of these data were filtered using relatedness of the translated protein sequences to the saframycin and safracin curated database. A cut-off of 1 × 10–4 was used. No significant classified populations were observed beyond the Class level except in α-proteobacteria (Rhodobacteraceae). % values represent abundance at each taxonomic level. Classiﬁcations with Subsystems pipeline (MG-RAST). Results from both sets were consistent, with ~40% of the classified sequences being of eukaryotic origin (mainly Ciona [sea squirt/tunicate]) and the remaining 60% being largely proteobacterial sequence (>90%) of which there were two major populations: β-proteobacterial (primarily Rhodobacteraceae, 78–85%) and γ-proteobacterial (10–17%) (Table 1 and Supplementary Table S2). 16S rRNA gene amplicon sequencing runs identified 30 variants but only three signiﬁcant ones (>1% of the total reads) (Table S2). No signiﬁcant classiﬁed populations were observed beyond the Class level except in α-proteobacteria (Rhodobacteraceae). % values represent abundance at each taxonomic level. Classiﬁed results <1% are not shown (Supplementary Table S2).

Table 1. MG-RAST Analysis of Raw Sequencing Reads and Assembly

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<th>total assembly</th>
<th>raw reads</th>
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<tr>
<td>classified by MG-RAST</td>
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<td>Eukaryota</td>
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</tr>
<tr>
<td>Bacteria</td>
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<tr>
<td>Proteobacteria</td>
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<tr>
<td>total</td>
<td>3,107</td>
<td>41,651</td>
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</table>

MG-RAST reads are classified by protein homology to a manually curated database. A cut-off of 1 × 10–4 was used. No significant classified populations were observed beyond the Class level except in α-proteobacteria (Rhodobacteraceae). % values represent abundance at each taxonomic level. Classiﬁed results <1% are not shown.

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These three variants account for >97% of the 16S rRNA gene sequencing reads (Figure 3). None of these three strains form a close phylogenetic relationship with S. lavendulae, M. xanthus, or P. fluorescens, pure culture producers of the three tetrahydroisoquinoline antibiotics whose pathways have been previously characterized.11

Table 2. 16S rRNA Gene Contig Identification

<table>
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<th>contig0016</th>
<th>contig00021</th>
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</thead>
<tbody>
<tr>
<td>Bacteria</td>
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<tr>
<td>Tenericutes</td>
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<td>Proteobacteria</td>
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<tr>
<td>Mollicutes</td>
<td>11%</td>
<td>γ-proteobacteria</td>
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<tr>
<td>Haloplasmatales</td>
<td>11%</td>
<td>Thiopicrobials</td>
</tr>
<tr>
<td>Haloplasma</td>
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<td>Thiopicrobials</td>
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<td>no. 16S reads</td>
<td>753</td>
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<tr>
<td>% 16S reads</td>
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<td>19%</td>
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<tr>
<td>Shotgun library match</td>
<td>read FS7DT</td>
<td>contig00422</td>
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</table>

A 454 16S rRNA gene amplicon library was assembled at an identity of 95%. Assembled contigs were submitted to the Ribosomal Database Project 16S Classifier. It should be noted that the generally accepted confidence threshold is 80%. % values represent a bootstrap confidence estimate calculated by the RDP Classifier. Only the three largest populations are shown (Supplementary Table S3).

Figure 3. Multiple sequence alignment tree. 16S rRNA gene sequences reported in previous E. turbinata analyses26,27 were aligned with 16S rRNA gene sequences representing the most abundant bacterial populations in our tunicate samples. A 16S rRNA gene-containing contig (00422) clusters with previously identified E. frumentensis.
We then sought to link the putative ET-743 35 kb biosynthetic gene cluster to the *E. frumentensis* 16S contig00422 by evaluating the codon usage bias. Bacteria typically do not employ synonymous codons equally, and this can be exploited as a unique marker.\(^34,35\) We performed a Relative Synonymous Codon Usage (RSCU) analysis using the annotated NRPS contig and contig00422 as well as ORFs identified in several contigs chosen at random. The RSCU score is the observed frequency of a codon divided by the frequency expected for equal usage of all synonymous codons, thereby making it a measure of nonrandomness. RSCU scores for each codon are similar between the genes on the contig bearing the presumed NRPS biosynthetic genes and the *E. frumentensis* 16S rRNA gene-containing contig00422 but vary compared to RSCU scores from genes located in the random contigs from the total assembly (Supplementary Figure S3). The extremely low GC content of the contig bearing the putative ET-743 NRPS genes (\(\sim 23\%\)) closely matches the GC content (26\%) of the contig bearing the 16S rRNA gene corresponding to *E. frumentensis*, providing another strong marker of genetic linkage. On the other hand, Rhodobacteraceae appear to have uniformly high GC content (54–70\%) according to current whole genome sequencing data, indicating that the contig containing NRPS genes is unlikely to be linked to this organism. The only fully sequenced and annotated tunicate genome, *Ciona intestinalis*,\(^36\) is 35\% GC (NZ_AABS000000000). To account for GC bias in codon usage, we included random genes from the low GC bacterium (\(\sim 29\%\)) *Clostridium botulinum* str. Okra. A comparison of the mean RSCU values for each codon revealed that only 12/60 values differed significantly \((p < 0.05)\) between the putative ET-743 NRPS and contig00422 genes, while 18/60 differed between the putative NRPS genes and random genes from *C. botulinum*. The significant differences between *C. botulinum* genes are most evident in the codons encoding isoleucine (AUU, AUC, AUA), lysine (AAA, AAG), aspartic acid (GAU, GAC), glutamic acid (GAA, GAG), and arginine (CGU, CGC, CGA, CGG, AGA, AGG). 49/60 codons differed significantly between the putative NRPS genes and random tunicate metagenome sequences. In addition to RSCU analysis we used the contig containing the 25 predicted ET-743 pathway genes in a correspondence analysis using *codonW* to generate a codon adaptive index (CAI). This index was then used as a reference for comparison with the same genes used in the RSCU analysis. Although all differed significantly from the NRPS contig CAI score \((p < 0.05)\), the *C. botulinum* CAI score and random gene CAI scores differed to a larger degree (Supplementary Figure S4). We also analyzed the contig bearing the NRPS genes and contig00422 with the Naïve Bayesian Classifier (NBC) tool, a composition-based metagenome fragment classifier that uses \(n\)-mer frequency profiles.\(^37\) NBC analysis based on 3- and 6-mer profiles results in high confidence classification of both contigs as...
γ-proteobacteria/Enterobacteriaceae. This same E. frumentensis 16S rRNA gene sequence has now been linked to E. turbinata collections from the Mediterranean, Caribbean, and Florida Keys. Taken together, these data strongly suggest that the sequence contig bearing NRPS module genes are derived from the same organism as contig00422 (E. frumentensis).

EtuA1, EtuA2, and EtuA3 Are Three Putative NRPSs with Catalytic Domains Bearing Predicted Amino Acid Specificity Motifs. Sequence analysis and deep annotation revealed that biosynthetic pathway architecture is noncollinear (as with SfaA-B) and is represented by EtuA3→EtuA1→EtuA2. Indeed, other pathway similarities are evident relative to the saframycin and safracin systems, including the architecture of the key NRPS modules central to the cluster with flanking open reading frames transcribed in both forward and reverse directions. The exact arrangement of other genes in the cluster is not fully conserved between these tetrahydrossoquinoline biosynthetic pathways. For example, the genes encoding tyrosine modification are closely linked in the safracin gene cluster but dispersed along the cluster in ET-743 and saframycin.

EtuA3 (AL-T-C-A-T) contains the AL-T starter module that is common to the safracin and safracin Mx1 metabolic systems. The role of this module was elucidated for the saframycin biosynthetic pathway, where acylation of the precursor is required for further chain extension, cyclization, and RE processing (e.g., Pictet–Spenglerase). The NRPS A-domain, based upon the amino acid specificity motif, was predicted to utilize cysteine (DLYNLSI, Supplementary Table S6) with 100% sequence identity to the top three cysteine A-domain sequence motifs. EtuA3 specificity is, therefore, unique to the Etu biosynthetic pathway and represents a unique feature compared to other characterized tetrahydrossoquinoline systems that all utilize alanine (DLFNNALT, Supplementary Table S6). EtuA1 (C-A-T) has the greatest homology to SaA module 1 by BLASTx; however, the protein sequence identity and similarity are relatively low (29/54) compared to the other NRPSs in the pathway. An A-domain selectivity motif cannot be identified in EtuA1. On the basis of structural analysis of ET-743, a glycolic acid unit is likely loaded and activated by the EtuA1 A-domain. Loading of hydroxy acids and formation of esters by NRPS modules have been characterized previously.39,40 This extender unit represents another key difference compared to characterized tetrahydrossoquinoline antibiotics, for which a conserved core motif (7/8 amino acid identity) is both predicted and observed to select glycine (Supplementary Table S6). EtuA2 (C-A-T-RE) contains the same A-domain specificity motif (DPWGLGLI, Supplementary Table S6) for the final NRPS module as all known tetrahydrossoquinoline biosynthetic pathways. As verified in the saframycin biosynthetic system,25 the EtuA2 homologue SfmC iteratively extends two 3-hydroxy-4-O-methyl-5-methyl-tyrosine (3H-4O-Me-5Me-Tyr) residues. The terminal EtuR2 RE domain serves as a key marker of the pathway and was examined biochemically to assess its activity in elaborating the tetrahydrossoquinoline core molecule (see below).

Non-NRPS Biosynthetic Genes. Pathway components that mediate production of essential cofactors or substrates are often encoded within biosynthetic gene clusters.23 EtuF1 and EtuF2 appear to represent subunits of an acetyl-CoA carboxylase. These enzymes transform acetyl-CoA to malonyl-CoA for fatty acid biosynthesis and may supply substrate for synthesis of the fatty acid for EtuA3 AL. EtuF3 appears to be a penicillin acylase.22 We propose that this key enzyme may act to release the predicted fatty acid modified intermediate of ET-743 after formation of the tetradepsipeptide and Pictet–Spengler cyclization (Figure 4) prior to further processing into mature intermediates that are isolable from the tunicate. ET-743 is derived from at least two units of the unusual amino acid 3H-4O-Me-5Me-Tyr. The intermediate may be generated through 3-hydroxylation, 4-O-methylation, and 5-methylation of tyrosine. EtuH1, an SfmD homologue, is predicted to hydroxylate tyrosine at the 3-position, whereas EtuM1, a SacF homologue, may be a SAM-dependent methyltransferase and a candidate for C-methylation at the S-position. SacF, an EtuM2 homologue, has been characterized in vitro as a catechol 4-O-methyltransferase.32 Biochemical studies in the saframycin pathway revealed that SfmD (EtuH homologue), SfmM2 (EtuM1 homologue), and SmfM3 (EtuM2 homologue) form a minimal unit for 3H-4O-Me-5Me-Tyr production from tyrosine.43 EtuO is an FAD-dependent mono-oxygenase that shows high similarity to SfmO2 and SacJ. EtuO may catalyze modification of the tetrahydrossoquinoline to produce the hydroxylated species based on previous work involving sacJ gene disruption (Figure 4, 17 and 18). In vitro biochemical characterization of this enzyme will require synthesis of an advanced biosynthetic intermediate to determine its precise activity.

Gene Products Involved in Regulation, Resistance, and Unknown Function. EtuT shows high similarity to drug transport proteins. Members of this superfamily are commonly present in natural product biosynthetic pathways and could serve as part of a resistance/export mechanism for ET-743.44 DNA processing enzymes such as EtuD1-3 are atypical in natural product biosynthetic pathways. We hypothesize that EtuD1-3 may have a role in repairing damage induced by ET-743 given its mechanism of action. EtuD1 appears to be a homologue of the TatD Mg2+-dependent DNase,45 while EtuD2 shows similarity to a DNA polymerase III subunit β, which has been characterized as part of the DNA−enzyme assembly complex. EtuD3 is a homologue of the Sβ′→3β′ exonuclease domain from DNA polymerase I. Three possible regulatory gene products EtuR1-3 have been identified in the biosynthetic pathway. EtuR1 has significant similarity (59%) to S29x, a protein previously shown to have a merA family transcriptional regulator. This class of regulators has been found in diverse classes of bacteria and responds to toxic effectors including heavy metals and antibiotics.32 EtuR3 resembles the TraR/DksA transcriptional regulator that functions as a DnaK suppressor protein. Three gene products in the ET-743 biosynthetic pathway could not be easily assigned to a possible role in the biosynthetic pathway. EtuU1 is related to a putative EtuP peptidase modulator of DNA gyrase, whereas EtuU2 appears to be a shikimate kinase I. EtuU3 is an unknown hypothetical protein. EtuN1, EtuN2, and EtuN3 appear to encode the three subunits of a Glu-tRNA synthetase.49 This enzyme forms correctly acylated Gln-tRNA by transamination of aberrant Glu-tRNA. The role of these genes in the ET-743 pathway is unknown. EtuP1 and EtuP2 form two components (E1 and E2) of a possible pyruvate dehydrogenase complex.
which catalyzes the transformation of pyruvate into acetate, but its function remains unclear in the Etu pathway. Other genes may be missing from the ET-743 biosynthetic pathway, for example, homologues of the proposed SAM recycling system and a putative N-methyltransferase in the saframycin biosynthetic pathway have not been identified.23

Proposed Scheme for ET-743 Biosynthesis. Our proposed scheme begins with assembly of the key subunit 3H-4-O-Me-SMe-Tyr (7) (Figure 4). This nonproteinogenic amino acid is likely formed by 3-hydroxylation of 4,4-O-methylation of 5, and 5-methylation of 6 catalyzed by EtuH1, EtuM2, and EtuM1, respectively. Next, the fatty acid CoA ligase of EtuA3 loads a fatty acid (8) onto the T domain. We presume that cysteine is N-acetylated and loaded by the C-A-T module of EtuA3 (9). Cysteine condenses with a T-loaded glycolate on EtuA1 to form the acetylated-depsipeptide (10). Based on Koketsu’s model,10 it is reductively released by the EtuA2 RE-domain as an aldehyde-depsipeptide (11) from the EtuA1 T-domain. Such a terminal domain “reach-back” model has been previously reported in natural product biosynthesis.49-50 EtuA2 loaded with 3H-4-O-Me-SMe-Tyr (7) is then condensed with 11 to form the cyclic aldehyde-tridepsipeptide (12) through the presumed Pictet–Spenglerase activity of the EtuA2 C-domain. Intermediate 12 is released from the EtuA2 T by the RE-domain activity as an aldehyde (13). In concert with Koketsu’s model it is proposed that EtuA2 catalyzes a second Pictet–Spengler reaction between another unit of 3H-4-O-Me-SMe-Tyr (7) and 13. The protein-bound tetradepsipeptide (14) is then reductively released to form aldehyde 15 that may undergo a further enzyme-catalyzed Pictet–Spengler reaction to form the fatty-acid-bound carboline pre-ET-743 (16). The penicillin acylase EtuF3 is then employed to cleave the fatty acid unit, which may serve to sequester substrate in the EtuA2 active site during repeated loading/release, forming pre-ET-743 (17). Proposed intermediates ET-583 (18), ET-597 (19), ET-596 (20), and ET-594 (21) have all been isolated and characterized,36 and all except ET-596 (20) have been confirmed by our secondary metabolite analysis (Supplementary Figure S3). We propose that pre-ET-743 (17) is hydroxylated by EtuO and acetylation and formation of the thiocarbonyl (20) form is catalyzed by the thiocarbonyl-bound enzyme. Proposed intermediate 20 has been identified in the RT-MS1 spectra (and MS2 for FTICR). Data sets were collected on LTQ-Orbitrap and 12T Q-FTICR mass spectrometers, with high-resolution/mass-accuracy MS1 spectra (and MS2 for FTICR). Data were processed in Trans Proteomic Pipeline51 with four distinct search engines (X! tandem, OMSSA, Inspect, and Spectrast) and the Peptide and Protein Prophet probability models with false discovery rates at the protein level of 0.6–0.9%. The database search consisted of a six-frame translation of the total metagenome assembly filtered to contain all possible polypeptides >60 amino acids in length. Sequence length-based cutoffs were utilized rather than ORF prediction due to the short length of many metagenomic contigs derived from the 454 sequencing. Filtering resulted in a 6-fold reduction in total sequence length versus the unfiltered six-frame translation. A 60 amino acid cutoff represents a 0.2% chance of any random sequence producing a translation without a stop codon appearing. On the basis of 235/16S rRNA gene sequences, the closest fully sequenced organisms to the four principle constituents of the assemblage were included to assign homologous proteins derived from genes that may have been incompletely sequenced in the metagenomic analysis (tunicate: Ciona intestinalis NZ_AABS000000000; α-proteobacteria: Ruegeria pomeroyi DSS-3 NC_003911; γ-proteobacteria: Coxiella burnetii RSA 331 NC_010115; unknown bacteria: Mycoplasma mycoides subsp. mycoides SC str. PG1 NC_005364). Reversed sequences for all proteins were included as decoys in the search database.

A total of 289 proteins were identified at a probability >95% from Interprophet pooled analysis of all four search engines prior to Protein Prophet analysis (Supplementary Tables S8 and S9). Three of the proteins identified were from the Etu
pathway with two identified by Orbitrap and one by FTICR and Orbitrap MS. The penicillin acylase EtuF3 was identified with two unique peptides, 3+ TIQHEIELSDIGPIINNLIQEN115-NQINKK (N115 = deamidated) and 2+ RPIELR, and the protein was identified in 3/4 search engines providing a total protein probability of 99.98%. The bacterial symbiont protein EtuR1 was identified with two unique peptides, 2+ GSNIHYDLENDHNDYEK and 3+ GSNIHYDLENDHNDYEK, identified by 3/4 search engines at the protein level with a combined protein probability of 100.00% (Supplementary Tables S9-S16). Identified Etu peptides were validated by comparison with synthetic peptide standards by LC elution time (±2 min on the same nano-LC system) and MS/MS fragmentation spectra (Figure 6). This synthetic peptide data strongly supports all Etu peptide and protein assignments from the metaproteomics data set. Detailed spectral information is provided (Supplementary Tables S20-S25, Supplementary Figure S5-S24, Tranche Proteome Commons). These three biosynthetic pathway proteins identified by multiple search algorithms and comparison with authentic standards suggest that ET-743 biosynthetic genes are expressed in the tunicate microbial symbiont assemblage.

Metagenomic and metaproteomic technologies enable powerful new approaches to gene, genome, protein and metabolic pathway discovery. Access to next-generation sequencing and development of bioinformatics tools is essential for deconvolution of the enormous databases generated from these technology applications. This study was motivated by the opportunity to identify and characterize an enormous range of host/symbiont derived natural product systems that have remained refractory to analysis. The inability to culture the vast majority of bacterial and fungal symbionts (outside of their natural host or environmental niche) that produce secondary metabolites has limited our access to a huge genetic diversity relating to untapped chemical resources for therapeutic and other industrial applications. This includes complex marine (e.g., sponge, tunicates, dinoflagellates)}
and terrestrial (e.g., plant-microbe, biofilm, insect-gut, human-gut) microbial consortia where the presence of large populations of diverse microorganisms and their corresponding genomes that bear natural product gene clusters remains unexplored. This new source of metabolic and chemical diversity will lead to important new basic knowledge and also contribute to ongoing drug discovery efforts against many disease indications. In order to initiate this meta-omic analysis, ET-743 was chosen as a model

Figure 6. Synthetic peptides as authentic standards to verify metaproteomics peptide assignments. (a) Total ion chromatogram for the standard peptide mixture on the LTQ-orbitrap. (b–g) Extracted ion chromatograms generated at ±0.1 m/z for each of the synthetic peptides in the mixture. Chromatograms are presented as time versus normalized abundance. Maximum abundance in each normalized total or extracted ion chromatogram is noted. *Denotes that the experimental retention time for doubly protonated tryptic LLDVGGGTAINAILAK was obtained on a different LC system with a different gradient and column, as compared to the authentic standard. In the case of all other synthetic standard versus experimental identifications the LC system and gradient were identical, although a different column was used. ◆ denotes the elution time of the experimental MS2 spectra assigned to each of the peptides. Peptide MS2 sequence coverage for metaproteomics versus authentic standard synthetic peptides (h–m). Only b and y ion assignments are shown, although other ions (e.g., a, b - H₂O, b - NH₃, y - H₂O, and y - NH₃) could also be assigned. Multiple bars indicate that a given fragment can be assigned to multiple charge states.
system because of the predicted genetic composition of core components of its biosynthetic pathway. This was based on the assumption of a highly conserved overall architecture from previously characterized pure culture bacterial-derived metabolic pathways for related tetrahydroisoquinoline natural products. Moreover, recent advances in next-generation sequencing and bioinformatic tools to assemble contigs from large metagenomic data sets and analysis of proteomic data to identify low-abundance proteins enabled the approaches described in this report. Although our model system choice was driven by the attributes of the ET-743 system (e.g., potent medicinal properties, complex structural and biosynthetic features), our overall strategy is not limited to symbiont-derived metabolic pathways that generate known natural products. In the future we anticipate that deep metagenomic sequencing will provide access to fully assembled genomes from uncharacterized DNA samples. Bioinformatic prediction of candidate secondary metabolic pathways and structures followed by MS-based network dereplication could then allow identification of the encoded natural product(s). Concurrent efforts can include proteomic analysis to confirm biosynthetic enzyme production. Finally, with fully assembled secondary metabolite pathways discovered from these approaches, heterologous expression and metabolic pathway engineering will then play a central role in harnessing the pathways to generate suitable quantities of molecules for drug discovery efforts.

In these studies, several approaches were taken to obtain evidence for identification of the ET-743 biosynthetic pathway and the corresponding producing microbial symbiont. First, the presence of the ET-743 natural product and intermediates were used as markers for the producing bacterium in the tunicate/microbial consortium. Second, codon usage similarity between the biosynthetic gene cluster and a contig containing a 16S rRNA gene sequence is consistent with E. frumentensis as the bacterial producer of ET-743. Direct functional analysis of a key biosynthetic enzyme confirmed its predicted catalytic assignment in the pathway. Finally, symbiont-derived expression of three ET-743 producer of ET-743. Direct functional analysis of a key biosynthetic enzyme confirmed its predicted catalytic assignment in the pathway. Finally, symbiont-derived expression of three ET-743 biosynthetic enzymes was confirmed by metaproteomic and bioinformatic analysis, enabling the direct correlation between natural product, the Etu gene cluster, and predicted biosynthetic proteins. This tiered strategy provides a general approach for future efforts to characterize orphan and target natural product biosynthetic systems from complex marine and terrestrial microbial assemblages including invertebrate-microbial symbionts, biofilm mats, and mammalian and insect gut consortia. The ET-743 biosynthetic system was selected as a model for this technological platform due to its importance as an approved chemotherapeutic agent.

The initial characterization of 25 putative ET-743 biosynthetic proteins will enable future efforts to confirm the function of individual enzymes by direct biochemical analysis. These in vitro findings will drive future efforts to engineer production of the ET-743 drug and related analogues. This work also provides the first key step toward supplying ET-743 and new congeners through heterologous expression in an amenable production host.

METHODS

See Supporting Information for detailed descriptions of experimental methodology.

E. turbinata Sample Collection. Tunicate specimens were collected in the Florida Keys, frozen on dry ice, and shipped overnight.

Secondary Metabolite Identification by LC–FTICR–MS and Confirmation by LC—MS/MS. Tunicate samples were deproteinized with MeOH, the protein was removed by centrifugation, and the supernatant was concentrated and analyzed on a C18 column. FTICR—MS was performed on an APEX-Qb by ESI in positive ion mode. A comprehensive peak list of possible ET-743 related metabolites was used for precursor ion selection. Data were processed in Data Analysis, and MS/MS spectra were interpreted manually. Metabolite peaks were detected over multiple samples and runs. Iontrap—MS/MS was performed as above and with an LTQ-Deca XP Ion trap MS. Data analysis was performed in Excalibur, and MS/MS spectra were interpreted manually.

454 and 16S rRNA Gene Library Construction and Sequencing Methods. Metagenomic DNA was extracted from frozen E. turbinata samples. DNA was used to prepare a 16S rRNA gene targeted amplicon library using primers and a random shotgun 454 FLX library. Sequencing was performed on a Roche/454 Life Sciences FLX Sequencer. A second shotgun library was prepared using the 454 Titanium upgrade.

NRPS Module Identification. Reads/contigs were filtered by protein homology to the saframycin, saframycin Mx1, and safracin NRPS genes characterized in S. lavendulae (DQ838002), M. xanthus (U24657), and P. fluorescens (AY061859) using BLASTx/tBLASTn searches. Primers were designed from the ends of filtered sequences, and PCR reactions were conducted based on the location of the BLAST hit on the reference sequences. Positive amplification reactions lead to extension of the contigs and further sequencing revealed the intervening DNA. Flanking sequence from high interest contigs was obtained by restriction-site PCR (RS-PCR).

Analysis of the E. turbinata Microbial Consortium by Metagenomic Sequence Analysis. Classification of the raw reads and total assembly was performed with MG-RAST. Sequences were classified by protein homology to a manually curated database. The 16S rRNA gene amplicon sequence data was analyzed by assembling the raw reads. The assembled contigs were submitted to the RDP. Gene-finding was performed on the NRPS contig, E. frumentensis 16S contig (contig00422), and random contigs from the total shotgun assembly.

EtuA2-RE and SfmC Cloning and Expression. The sfmC gene was amplified using genomic DNA of S. lavendulae NRRL 11002 as template. The PCR product was digested and cloned into pET-28a to generate pET28a-sfmC. The EtuA2-RE gene fragment was amplified via PCR using the metagenomic DNA mixture as template, digested, and cloned into pET-28a, to generate pET28a-RE. The N-His-tagged expression constructs were separately transformed into E. coli BL21 (DE3) +pRare and expressed and purified under standard conditions using Ni-NTA affinity chromatography.

Synthesis of Substrate (26 and 27) for EtuA2 RE Reactions. See Supporting Information.

Biochemical Reaction of EtuA2 RE-Domain and SfmC with the CoA Dipeptide Fatty Acid (26). The biochemical reaction of compound (26) to (27) was performed as described previously.25 Reactions took place in buffer with either no enzyme, EtuA RE-domain, or SfmC. Cofactors were then added from concentrated stocks. Compound 26 was then added in DMF followed by incubation overnight at RT. LC—FTICR—MS was performed to monitor the reaction products.

Metaproteomic Analysis of ET-743 Biosynthetic Gene Expression. Tunicate protein samples were precipitated with acetone then resolubilized, reduced, alkylated, diluted, and digested with trypsin. The sample was separated into 20 fractions using SCX chromatography. The 20 peptide fractions were analyzed once on an LTQ-Orbitrap XL interfaced with a nanoLC 2D system. Peptides were separated on a capillary column in-house packed with C18 resin after loading on a C18 trap column. LC eluent was introduced into the instrument via a chip-based nanoelectrospray source in positive ion mode. The LTQ-orbitrap
was operated in data-dependent mode. The 20 peptide fractions were also analyzed in duplicate on a Solarix 12T hybrid Q-FTICR interfaced with a nanoLC system. Peptides were separated on a capillary column packed in-house with C18 resin after loading on a C18 trap column. The FTICR was operated in data-dependent mode. All scans were collected in the profile mode, and peak picking was performed from the profile mode spectra. Bioinformatics analysis was performed with the Transproteomic Pipeline, and all assigned Etu peptides were validated by comparison with synthetic peptide standards.


Supporting Information. Detailed methods, additional figures, tables, and discussion. This material is available free of charge via the Internet at http://pubs.acs.org.

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