Boroned tartrolon antibiotic produced by symbiotic cellulose-degrading bacteria in shipworm gills

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Shipworms are marine wood-boring bivalve mollusks (family Teredinidae) that harbor a community of closely related Gammaproteobacteria as intracellular endosymbionts in their gills. These symbionts have been proposed to assist the shipworm host in cellulose digestion and have been shown to play a role in nitrogen fixation. The genome of one strain of Teredinibacter turnerae, the first shipworm symbiont to be sequenced, was sequenced, revealing potential as a rich source of polyketides and nonribosomal peptides. Bioassay-guided fractionation led to the isolation and identification of two macrodioloide polyketides belonging to the tartrolon class. Both compounds were found to possess antibacterial properties, and the major compound was found to inhibit other shipworm symbiont strains and various pathogenic bacteria. The gene cluster responsible for the synthesis of these compounds was identified and characterized, and the ketosynthese domains were analyzed phylogenetically. Reverse-transcription PCR in addition to liquid chromatography and high-resolution mass spectrometry revealed the transcription of these genes and the presence of the compounds in the shipworm, suggesting that the gene cluster is expressed in vivo and that the compounds may fulfill a specific function for the shipworm host. This study reports tartrolon polyketides from a shipworm symbiont and unveils the biosynthetic gene cluster of a member of this class of compounds, which might reveal the mechanism by which these bioactive metabolites are biosynthesized.

symbiosis | biosynthesis | natural products | acyl-transferase | cecum

Marine bivalve mollusks of the family Teredinidae (commonly known as shipworms) seem to rely on their gill symbionts to survive in their unusual environment. They comprise a diverse, cosmopolitan group that is well known for the ability to burrow into wood, causing damage to wooden ships and other manmade structures in marine and brackish waters (1). As in other animals that consume wood, it is thought that the shipworm’s microbial symbionts facilitate the degradation of lignocellulose, which otherwise is difficult for animals to digest (2, 3). Multiple genetically distinct but closely related Gammaproteobacterial symbionts exist in the gill (ctenidium) of shipworm species examined to date (4). Teredinibacter turnerae, a cultivated shipworm symbiont species, has been isolated from different shipworm hosts collected around the world (5). T. turnerae secretes lignocellulose-degrading enzymes thought to assist the host in wood decomposition. The symbiotic bacteria live in the gill of the shipworms, but cellulose is degraded in the digestive tract in a specific organ known as the cecum. Although the gut is an excellent habitat for microbes in most xylophagous organisms, the cecum in shipworms contains very few bacteria (6). This absence of microbes is striking, because cellulose digestion liberates glucose, which is an excellent nutrient source for microbes. The genome of one strain, T. turnerae T7901, was sequenced and revealed, in addition to genes that encode enzymes specific for lignocellulose degradation and nitrogen fixation, at least nine regions that encode enzymes for the biosynthesis of polyketides and nonribosomal peptides (7). We therefore hypothesized that some of the secondary metabolites produced by the shipworm symbiont T. turnerae might contribute to reducing the bacterial population in the cecum to prevent glucose scavenging. Moreover, secondary metabolites might play a significant role in microbial competition among symbionts in the gill. Here, we describe the polyketed tartrolons, antibiotics that are produced by T. turnerae and were detected in whole shipworm animals. These and other antibiotics from shipworm symbionts may help structure the symbiotic community, possibly even enabling the unique lignocellulose digestion strategy found in shipworms. This study reports the secondary metabolites identified from T. turnerae and their bioactivities, describes the biosynthetic gene cluster linked to them, and presents evidence that these metabolites are produced in the symbiotic state.

Results

Growth Conditions, Isolation, and Identification of the Antibacterial Metabolites. Growth of T. turnerae T7901 in modified shipworm basal medium (SBM) (8) with sucrose as the carbon source and with reduced inorganic phosphate was found to cause the highest antibacterial activity against Bacillus subtilis using a disk diffusion assay. T. turnerae was grown in a medium that optimized antibiotic production, and a combined liquid culture of 24 L was extracted (Supporting Information). Antibacterial activity-guided fractionation led to isolation of the previously reported tartron D (compound 1) and its boronated derivative tartron E (compound 2) (9) (Table 1 and Fig. 1). Although in the initial report of compound 1 the authors noted the presence of a boronated derivative that was related to compound 2, they did not purify or fully characterize the compound (Figs. S1–S5). Other boronated tartron derivatives were present in the extracts, as determined by MS (Fig. S6), but their quantities were too small to be characterized.

Bioactivities of Tartrolons. Compounds 1 and 2 inhibited B. subtilis in disk diffusion assays (Table S1). Furthermore, compound 2 was found to have significant antibacterial activity against Pseudomonas aeruginosa in addition to methicillin-sensitive and methicillin-resistant Staphylococcus aureus (Table 2). All T. turnerae


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strains tested were resistant (n = 3). Compound 2 also inhibited the growth of the marine organism Vibrio anguillarum in addition to one (BS02) of eight shipworm symbiont strains selected from our Ocean Genome Legacy (Ipswich, MA) culture collection (Table S1 and Fig. S7) representing three groups of phyllogenetically distinct symbiont strains (6). The minimum inhibitory concentration (MIC) of compound 2 against B. subtilis was determined to be 1 μg/mL (1.1 μM), but no inhibition was detected against Escherichia coli at a concentration >32 μg/mL (39 μM). Moreover, compound 2 did not show any inhibition activity against the fungus Candida albicans (Table S1). Compound 2 also possessed an IC_{50} of 2 μM against the breast cancer cell line MCF-7.

**Gene Disruption.** Nine secondary metabolite gene clusters were found during the analysis of the genome of T. turnerae T7901, including three encoding polyketide synthase (PKS) genes (7). Analyses of the tartrolon polyketide structure and the PKS regions led to the hypothesis that region 2 was responsible for tartrolon biosynthesis. For confirmation, mutant AH02 was constructed by disrupting the KS1 domain of trtD by a single-crossover recombination. High-resolution MS (HR-MS) and tandem MS (MS/MS) of the ethyl acetate fractions of both the wild type and the mutant were compared, showing absence of tartrolons in the mutant (Fig. 2). We therefore propose region 2 to be the tartrolon biosynthetic gene cluster, trt.

**Analysis of the trt Gene Cluster.** The trt cluster was analyzed and found to be ~50 kb in length and to contain 20 ORFs possibly involved in the tartrolon biosynthesis (Fig. 3). Of these ORFs, 10 (trtA–trtI) seem to be the core biosynthetic genes. These ORFs are organized in a single operon including PKS domains (Table 3). Genes in the cluster had the same orientation, except for trtA, -B, and -C at the 5’ end of the cluster. More than 42.5 kb of this region are formed by trtDEF, three large genes that encode trans acyltransferase (AT) type I PKSs (10). These three multimodular PKS ORFs contain 11 modules in addition to the loading module. The first PKS is trtD, which is most similar (amino acid similarity of 50%) to dfnG from Bacillus amyloliquefaciens involved in the biosynthesis of the macrolide difficidin (11, 12). trtD is followed by trtE, the largest ORF in this region, which is similar to an uncharacterized PKS from Clostridium cellulolyticum (Table 3). It also was found to have a 44% amino acid similarity to baeN, which is involved in the biosynthesis of baecillene from B. amyloliquefaciens FZB42 (12, 13). The third PKS ORF, trtF, was similar to a PKS from Paenibacillus polymyxa.

Other biosynthetic genes are also present, including two hypothetical acyltransferases, trtB, one of which could act as a proofreader (14); two putative oxidoreductases, trtGI, which appear to encode proteins for oxygenases, and a putative polyketide cyclase, trtJ (15), which may be involved in the cyclization of compound 1. In addition to the integrated thioesterase (TE) in trtF, which is expected to cause release of the elongated chain, a standalone type II TE, trtH, also is present and is proposed to cause regeneration of misprimed thiolation domains (16).

The flanking regions for the core biosynthetic genes trtA–I include 10 more genes that might be involved in the transcription or resistance of tartrolon (Fig. 3). TERTU_2194 and TERTU_2212 function as potential transcription regulators upstream and down-

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**Table 1. Tartrolons and structurally related compounds with their bacterial sources and reported biological activities**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Boron</th>
<th>Bacterial source</th>
<th>Biological activity</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boromycin</td>
<td>Present</td>
<td>Streptomyces antibioticus (terrestrial)</td>
<td>Antibacterial (58, 59)</td>
<td></td>
</tr>
<tr>
<td>Aplasmomycin</td>
<td>Present</td>
<td>Streptomyces griseus (marine)</td>
<td>Anti-HIV (27), Antiplasmodium (60)</td>
<td></td>
</tr>
<tr>
<td>Borophycin</td>
<td>Present</td>
<td>Nostoc linckia, Nostoc spongiaformae var. tenue (marine)</td>
<td>Antibacterial (48, 63)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>T. turnerae (marine)</td>
<td>Antibacterial (48, 63)</td>
<td></td>
</tr>
<tr>
<td>Tartrolon A</td>
<td>Absent</td>
<td>Myxobacterium Sorangium cellulosum (terrestrial)</td>
<td>Antibacterial (48, 63)</td>
<td></td>
</tr>
<tr>
<td>Tartrolon C</td>
<td>Present</td>
<td>Myxobacterium Sorangium cellulosum (terrestrial)</td>
<td>Antibacterial (48, 63)</td>
<td></td>
</tr>
<tr>
<td>Tartrolon  D</td>
<td>Absent</td>
<td>Streptomyces species (terrestrial)</td>
<td>Insecticidal activity (64)</td>
<td></td>
</tr>
<tr>
<td>Tartrolon E</td>
<td>Present</td>
<td>T. turnerae (marine)</td>
<td>Antibacterial (9)</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Fig. 1.** Chemical structure of tartrolons and structurally related compounds.
stream, respectively. TERTU_2194 is similar to the transcription factor LysR, whereas TERTU_2212 is similar to a putative Rho-independent transcription terminator (17). TERTU_2190 and TERTU_2191 encode possible aldolases that might be involved in the synthesis of the loading substrate. A transposase TERTU_2188 is encoded by a gene located upstream of the core genes, suggesting a possible lateral gene-transfer event. TERTU_2193 is a potential acyl carrier protein (ACP) phosphodiesterase required for the turnover of the ACP prosthetic group (18). Four ORFs with no predicted functions in the tartrolon biosynthesis are also present: TERTU_2189, TERTU_2195, TERTU_2209, and TERTU_2211. No transport-related coding sequences are present in the cluster or within the cluster’s neighboring genes.

Acylation of the trt trans-AT PKS Enzymes. The ACP domains of AT-les PKS modules are suggested to be trans-acylated by mono- or bifunctional AT, where AT-AT or AT-Oxy domain compositions are commonly seen (10). Of the two discrete ATs found in the trt cluster trtB, only trtB seems likely to be involved in loading trtDEF PKs. The gene trtB codes for a 287-amino acid hypothetical protein with high similarity (60% similarity, 46% identity) to the malonyltransferase domain of the malonyltransferase/oxido-reductase didomain enzyme DszD in Sorangium cellulosum. TrtB’s hypothetical malonyltransferase functionality is indicated by the presence of (i) the catalytic dyad Ser92-His201 (numbers from E. coli FabD), (ii) the prescribed active binding-site N-terminal motif (P/S/T) GQC, (iii) recently described key residues for malonyl-CoA substrate specificity in integrated and discrete AT systems, and (iv) the malonyltransferase-active site (GHSxxxR) (Fig. S8). Additionally, phylogenetic analysis has shown that trtB falls within the clade of discrete FAS-like PKS malonyltransferases (Fig. S8B) composed by discrete AT domains that hypothetically are involved or biochemically are proven to load malonyl-CoA units in a variety of trans-AT PKS systems (19).

**Evolutionary Rationale Supports the trtDEF Pathway Role in Tartrolons Synthesis.** Type I trans-AT PKS ketosynthase (KS) domains usually group according to their substrate specificity when analyzed phylogenetically (20). Here this evolutionary rationale was applied to analyze the tartrolon retrobiosynthesis and to investigate the presence of new clades of KS functionality. The amino acid sequence of 430 trans-AT PKS-derived KS domains, including the 11 KSs from the trtDEF PKS core in addition to 301 KSs with known substrate specificity, were aligned and subjected to phylogenetic analyses through three different methods. Maximum-likelihood (ML) reconstruction produced the most robust tree architecture. The ML tree topology was strikingly similar to the one seen in the phylogenetic analysis recently reported by Teta et al. (21) for the analysis of the elansolids (eb) gene cluster. A total of 33 clades were observed, adding five clades to the 28 previously observed by these authors. Three of these additional clades appear to resolve different substrate specificities than those reported previously (21). The clade named “double-bond 3” (DB3) grouped the domains RhiKS14 and MgsKS2, both of which receive olefinic substrates that are subject to ring formation by Michael addition-dependent processes (22). The two other clades directly reflect the addition of TrtKS domains into the phylogeny. The domain TrtKS1 grouped

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**Table 2. MIC antibacterial activities of tartrolon E against pathogenic bacteria**

<table>
<thead>
<tr>
<th>Strain tested</th>
<th>MIC in microgram per milliliter (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. aeruginosa</td>
<td>0.31 (0.36)</td>
</tr>
<tr>
<td>Methicillin-sensitive S. aureus</td>
<td>0.08* (0.095)</td>
</tr>
<tr>
<td>Methicillin-resistant S. aureus</td>
<td>1.25 (1.14)</td>
</tr>
</tbody>
</table>

*Lowest concentration tested.

**Fig. 2.** HR-MS and tandem MS spectra of the crude extracts of each of *T. turnerae* wild type (Upper) and region 2 mutant, AH02 (Lower). The high-resolution signal corresponding to compound 2 is present in the wild type but absent from AH02. The high-resolution mass of the main peak in the wild-type spectrum is 873.4174; this mass is absent in AH02. The mass of the main peak in the mutant AH02 is 873.5529, a mass which also is present in the wild type as a minor compound. In addition, the MS/MS fragmentation patterns of these peaks were different, confirming the absence of compound 2 in the disrupted mutant.
consistently with BryKS1 from the bryostatin biosynthetic gene cluster (bry) and with BT2KS1 from a Burkholderia thailandensis putative trans-AT PKS (GenBank accession no. ZP_02468762). This clade groups KSs receiving a D-lactate starter unit. The domains TrtKS2 and TrtKS11 grouped together with CorKS6, forming a clade of KSs for reduced substrates. This clade seems to resolve KSs specific for rare α-methylated reduced moieties, but new KS sequences with such specificity need to be analyzed in future studies to confirm this branching. All other TrtKSs, except for TrtKS6 and TrtKS7, could be associated unequivocally with clades. TrtKS6 and TrtKS7 grouped with KSs for various substrates in a phylogenetically unresolved area, also observed previously (21) and labeled as “mixed” clade (Fig. 4). In fact, TrtKS6 was most similar to RhiKS12 (58%), the phylogenetic grouping of which has been reported as inconsistent. The substrate of TrtKS6 also is unpredictable by colinearity, because of the nature of KS5, which is a nonextending KS that would deliver only the product of module four. In fact, KS5 grouped with nonextending KSs from aberrant bimodules denominated as type A (23) common in trans-AT PKSs. TrtKS7, on other hand, follows module 6 containing a ketoreductase as the only β-keto reduction domain and therefore can be associated with a β-hydroxylated substrate.

**Biosynthesis of Tartrolons.** Correlation of the bioinformatic analysis of the domains in each module of region 2 with that of the tartrolon chemical substructure, in addition to mutational anal-

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**Fig. 3.** Biosynthesis scheme of tartrolons from the trt cluster. Compounds 1 and 2 are color coded based on the corresponding domains. Plasmid pDMrg2KS was used to disrupt KS1. ACP, acyl carrier protein; AT, acyltransferase; cmr, chloramphenicol resistance gene; DH, dehydratase; GNAT, Gcn 5′-N-acetyltransferase; KR, ketoreductase; KS, ketosynthase; M, module; MT, methyl transferase; PKS, polyketide synthase; R, enoyl reductase; TE, thioesterase.
ysis, confirmed that region 2 is the gene cluster responsible for the biosynthesis of tartrolons. We propose a route to the biosynthesis of tartrolons (Fig. 3). Tartrolon biosynthesis starts by the loading of the three-carbon unit, α-lactate. A conjugated diene formed at C14-C17 is predicted to be formed by the KS2-KR-ACP-KS3-DH-ACP of modules 2 and 3 through a stuttering mechanism reported previously in the biosynthesis of other conjugated dienes such as kalimantacin C (24), chivosazole (25), and macroactin (26), and difucidin (11). The bimodule M4-M5 loads a saturated intermediate that is transferred by the nonextending Thioesterase; DH, dehydratase; GNAT, Gcn 5-acetyltransferase; MT, methyltransferase; PKS, polyketide synthase; TE, thioesterase.

Table 3. Genes and functions of region 2

<table>
<thead>
<tr>
<th>Protein</th>
<th>Gene name</th>
<th>No. of amino acids</th>
<th>Proposed function</th>
<th>Organism</th>
<th>Identity/similarity (I/S) (%)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>TERTU_2188</td>
<td>TERTU_2188</td>
<td>519</td>
<td>Transposase</td>
<td>Cellvibrio japonicus</td>
<td>68/81</td>
<td>YP_001983638.1</td>
</tr>
<tr>
<td>TERTU_2189</td>
<td>TERTU_2189</td>
<td>62</td>
<td>Hypothetical protein</td>
<td>Saccharophagus degradans</td>
<td>49/63</td>
<td>YP_526685.1</td>
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<tr>
<td>TERTU_2190</td>
<td>TERTU_2190</td>
<td>68</td>
<td>Hypothetical protein</td>
<td>Saccharophagus degradans</td>
<td>66/78</td>
<td>YP_526685.1</td>
</tr>
<tr>
<td>TERTU_2191</td>
<td>TERTU_2191</td>
<td>171</td>
<td>Hypothetical protein</td>
<td>Idiomarina loihiensis</td>
<td>86/94</td>
<td>YP_155995.1</td>
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<tr>
<td>TERTU_2193</td>
<td>TERTU_2193</td>
<td>234</td>
<td>ACP phosphodiesterase</td>
<td>Marinobacter adhaerens</td>
<td>80/91</td>
<td>ADP97201.1</td>
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<tr>
<td>TERTU_2194</td>
<td>TERTU_2194</td>
<td>290</td>
<td>Transcription regulator LyrR</td>
<td>Plesiocystis pacifica</td>
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<td>ZP_01910797.1</td>
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<tr>
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<td>549</td>
<td>Peptidase</td>
<td>Burkholderia thailandensis</td>
<td>46/69</td>
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<tr>
<td>TERTU_2198</td>
<td>trtA</td>
<td>196</td>
<td>Acyltransferase (GNAT)</td>
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<tr>
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<td>trtB</td>
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<td>Acyltransferase</td>
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<tr>
<td>TERTU_2200</td>
<td>trtC</td>
<td>236</td>
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<tr>
<td>TERTU_2205</td>
<td>trtG</td>
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<td>Oxygenase</td>
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<td>trtI</td>
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<td>Polyketide cyclase</td>
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<td>ZP_08490092.1</td>
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<td>TERTU_2209</td>
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<td>Hypothetical</td>
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<td>YP_001617495.1</td>
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<tr>
<td>TERTU_2211</td>
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<td>486</td>
<td>Glycoside hydrolase</td>
<td>Saccharophagus degradans</td>
<td>33/54</td>
<td>YP_527963.1</td>
</tr>
</tbody>
</table>

Size, proposed functions, source of closest homologs, and their accession number and percentage of identity and similarity are determined. AT, acyltransferase; DH, dehydratase; GNAT, Gcn 5-acetyltransferase; ACP, acyl carrier protein; KS, ketosynthase; KR, ketoreductase; MT, methyltransferase; PKS, polyketide synthase; TE, thioesterase.

Prevalence of Tartrolons in Different Strains of T. turnerae. To determine whether compound 2, the major tartrolon in T. turnerae, is produced only in T. turnerae T7901 or is widespread in the Teredinibacter clade, we examined 11 additional T. turnerae strains that were reported previously (5) from different Teredinidae host species from different environments. Cultures of the 12 T. turnerae strains were grown under the same conditions and extracted followed by HR-MS and MS/MS analyses. T. turnerae T7901, in addition to the other 11 strains, was analyzed under the same conditions. The analysis showed that compound 2 is present in at least 8 of the 12 strains tested (Table 4). However, strains that did not show the presence of tartrolons might contain a trt gene cluster within the genome that is silent (not expressed) under these growth conditions. Thus, we surveyed all the strains for the trt cluster using PCR.

DNA extraction of each of the T. turnerae strains followed by PCR amplification of eight locations in the PKS coding ORFs trtDEF yielded the expected products in most of the reactions for all tested strains (Table 4). The full set of expected amplicons was obtained for nine strains, reinforcing the idea that the trt gene cluster is prevalent in the Teredinibacter clade. However, the presence of these genes does not confirm the structural integrity of the cluster within the genome or the chemical identity of the product.

All but one of the positive MS samples showed strong PCR evidence of the trt cluster. The one exception, T8203, failed to amplify one fragment in trtF but was positive for all the others. In this case, a slight difference in the gene sequence may be present. Two samples (T6002 and CS30) that showed positive PCR amplification for all fragments in the three ORFs did not show the compound using MS analysis. These two strains, in addition to the two other strains that did not show the presence of compound 2 may contain either nonfunctional or silent trt clusters under the specified growth conditions. All the strains showed at least some evidence of genes related to trt. Overall, tartrolon production appears to be common among the examined T. turnerae strains.
**Expression Analysis of trtD in Culture.** Quantitative PCR (qPCR) was used to study the expression analysis of trtD in *T. turnerae* in medium with normal and low phosphate levels or with iron-starved conditions. The gene *trtD* was found to be overexpressed relative to the housekeeping gene *ftsZ* when *T. turnerae* was grown under the low inorganic phosphate condition (Fig. 5). This result is in agreement with previous reports (29) that low phosphate induces the production of secondary metabolites in microorganisms.

**Detection of Tartrolons in the Shipworm Host Using Expression Analysis and LC/MS and MS/MS.** To determine whether the *trt* cluster is expressed in vivo, reverse transcription PCR of the *trtDEF* PKS core was carried out on gill RNA from the teredinid shipworm *Lyrodus pedicellatus* and produced the expected amplification product (Fig. 64). In addition, qPCR was performed using specific primers targeting the *trtD* mRNA to study the expression of this gene in three *L. pedicellatus* shipworm individuals. This analysis revealed that *trtD* is expressed in the shipworm gills relative to the prokaryotic cell-cell division gene *ftsZ* (Fig. 6B).

To determine whether tartrolons can be detected in shipworms using HR-MS and MS/MS, whole *L. pedicellatus* shipworms were pooled and the organic fraction was extracted. This fraction was analyzed using HR-MS and MS/MS. The characteristic peaks of tartrolons were detected (Fig. 7), strongly suggesting the expression of tartrolons in vivo.

**Discussion**

Bioactive metabolite symbiosis (30) is a term used to describe a symbiotic relationship between organisms based on chemical compounds. Usually one of the organisms produces one or more secondary metabolites that provide a benefit to the host or have the potential of protecting the host or the rest of the community from environmental threats. Several examples have been reported recently. For example, a wide spectrum of nine antibiotics produced from a group of symbiotic actinobacteria seems to protect the host insect from fungal and bacterial pathogens (31). Another example comes from the leaf-cutting ants that protect their fungal food by a group of antibacterial and antifungal compounds produced by actinobacterial symbionts (32). Finally, the bryozoan, *Bugula neritina*, was found to harbor a Gammaproteobacterial endosymbiont that was proposed to be the true producer of bryostatins that protect the larvae against predators (33, 34). These examples provide evidence that secondary metabolites sometimes are important in symbiosis.

Although shipworm symbionts previously have been shown to contribute to nitrogen metabolism (35) in the host and have been proposed to contribute to lignocellulose digestion (7, 8), their potential function as producers of secondary metabolites has not been proposed or explored. Compound 1 and its boronated derivative compound 2 (Fig. 1) were isolated from one of these shipworm symbionts, *T. turnerae* T7901. Pérez et al. (9) identified compound 1 from a marine actinomycete species that is phylogenetically distant from the Gammaproteobacteria *T. turnerae*. These authors also reported the boronated derivative of this compound as a minor contaminant in their original sample, which we have dubbed “tartrolon E” (compound 2).

Tartrolons belong to a group of macrodilides with well-known pharmacological activities (Fig. 1 and Table 1). They are either natural or pseudodimers consisting of two polyketide chains joined as diesters. Members of this group are nearly identical in their C-terminal regions, differing primarily in oxidation state.

**Fig. 4.** ML-reconstructed tree of full-length unedited KS domains from trans-AT PKS enzymes. For clarity, known clades and clades not relevant to this cluster are collapsed. The KS domains are numbered according to the occurrence in the gene cluster starting from the 5' end, as previously established (20). The numbers in parenthesis indicate total number of KS domains in the clade, number of KS with known function and matching specificity/number of KS with unknown function/number of KS with known function and mismatching specificity, exactly as previously described (21). Alb, albicidin; Bae, bacilamide (*Bacillus amyloliquefaciens*); Bry and BryX, bryostatin; BRR and BB82, *Brevibacillus brevis* NBR 100599 clusters BB847_31930-32020 and BB47_39780-39920; BCR, *Bacillus cereus* BSGC 681 cluster; BT2, *Burkholderia thailandensis* M5MB43 cluster; Bat, batumin; BATR, *Bacillus atrobacter* 1942 cluster; BTP, *Bacillus thuringiensis* pongdichierensis BGSC 48A1 cluster; CACL, *Catenulipora acidiphila* DSM 44928; CC1, CC2, and CC3, *Clostridium cellulolyticum* H10 clusters Ccel_0858-0868, Ccel_2373-2386, and Ccel_0965-0980; Ch, chivosazol; Cor, corallopyronin; Dif, difffidron; Dz, dorazol; Els, elansolid; Etn, etnangien; GU, *Geobacter uraniireducens* Rf4; Kir, kirromycin; Lkc, lankacidin; Lnm, leniamycin; Mga, migratassin; MICAU, *Micromonospora aurantiaca* ATCC 27029 cluster; Mmp, mupirocin; Min, macrolactin; MSP, *Micromonospora sp.* ATCC 39149; Onn, onnamide; Ozm, oxazolomycin; Ped, pederin; Pel, *Peltigera membranacea* cluster; PPA, Pleioglyctis pacifica SR-1 cluster; Psp, pyruberin; Rhi, rhizoxin; SBI, *Streptomyces cinghengensis* BCW-1 cluster; Sor, sorangicin; SG, *Streptomyces griseus* NIPRC 13350 cluster; Ta, myxovirescin; Tai, thailandamide; Trt, tartrolon (T. *turnerae* T7901); Vir, virginiamycin M.

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**Image 0x1 to 19x816**

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and in chain length. The production of this class of compounds is not restricted to marine organisms; soil bacteria are capable of producing them as well (Table 1). Their biosynthesis is not phylogenetically restricted, because their production was found in actinobacteria, cyanobacteria, and Deltaproteobacteria, in addition to the Gammaproteobacterium *T. turnerae* (Table 1). The prevalence of tartrolons among different classes of bacteria suggests convergent evolution, a common ancestor, or lateral gene transfer among these species reflecting an important function that this class fulfills. Because of the close structural relationship, it is possible that the members of this family are biosynthetically related as well. Despite the isolation of several members of compounds that belong to the tartrolon class and the fact that they are produced from diverse bacteria, no genes involved in their biosynthesis have been reported. The potential starter unit, \( \delta \)-lactate, most probably is derived from pyruvate which originates from glycerol based on feeding studies of structurally related compounds (28, 36, 37). Analyses of domain composition of modular PKSs showed that TrtKS1, in addition to BT2KS1 from *B. thailandensis* and BryKS1 from *Candidatus* *E. sertula* (38), also is preceded by the same catalytic organization, making this KS group a new clade. The analysis of the amino acid alignments had shown that the motif AXAVI/LAN, presented in KSs recognizing acetyl and others nonacetyl starters (39), is replaced by DY/LYQIAN in the \( \delta \)-lactate–specific KSs. Two internal domains, TrtKS3 and TrtKS8, grouped within clades for starter units (Fig. 4). TrtKS3 appears as an out-group of the clade for olefinic starters, and TrtKS8 grouped into the main clade for acetyl- and nonacetyl-derived starters. Such grouping patterns suggest the possibility that the tartrolon biosynthetic route also releases other tartrolon derivatives as suggested by the detection of other boronated tartrolons in the crude extract of *T. turnerae* T7901 (Fig. S6).

*T. turnerae* is thought to play a major role in the shipworm symbiosis. It is cultivable in relatively simple conditions, and its genome contains information enabling this bacterium to have a facultative endosymbiotic or even a free-living lifestyle (7). To date, however, *T. turnerae* has been found exclusively in intracellular symbiotic association with its teredinid mollusk counterparts. The *ttr* gene cluster was found to be expressed in the shipworms as shown by qPCR and reverse transcription PCR, (Fig. 6) and as confirmed by the detection of the compound in the shipworm by HR-MS (Fig. 7). These results, together with the production of compound 2 by other *T. turnerae* strains isolated from different shipworms that live in various environmental conditions (Table 4), strongly suggests that the *ttr* cluster is not silent in vivo and has a potential role in the shipworm–microbial symbiosis.

Most members of this group of boronated polyketides are reported to have antibacterial activity. Both compounds 1 and 2 inhibited *B. subtilis*. Moreover, compound 2 inhibited the growth of the marine pathogen *V. anguillarum* and a shipworm *Bankia setacea* isolate, BS02, but not the eukaryote *C. albicans* (Table S1). This effect suggests that tartrolons could play a role in microbial competition in the shipworm system, possibly targeting opportunistic bacteria while sparing the host (Fig. 8). Compound 2 seems to possess a deterrent activity against certain members of the symbiotic community but not others, possibly thus maintaining a population of similar strains within distinct bacterioocytes. Fluorescence in situ hybridization analysis of shipworm sections showed that similar bacterial phylotypes seem to be located in specific bacterioocytes separate from other phylotypes (6). Another possibility arises from recent results (6) that show that the cecum, the wood-digesting organ of shipworms, unlike that of other most xylophagous animals, has few microbes. Antibacterial tartrolons produced by the shipworm symbionts in the gills might contribute to bacterial suppression in the cecum. This suppression could allow the host to maximize efficient uptake of the glucose liberated by the breakdown of lignocellulose (Fig. 8). The mechanism by which products of the symbionts in the gill could be translocated to the cecum is unknown.
A common feature of this group of compounds is their ability to bind boron. Boron exists in the form of borate or orthoborate and is known to play important roles in living organisms (40, 41) but is toxic at high levels (42, 43). Boronated tartrolons have a decreased permeability relative to unchelated borate and thus could play an important role in the transport of boron. Boron transporters have been reported from other living systems, suggesting that its transfer across the cell membrane is regulated by active transport (44, 45). No homologs for borate transporters were detected in the genome of T. turnerae T7901. Just as some microorganisms have evolved biosynthetic pathways to acquire iron in the form of siderophores, others that lack boron transporters might have evolved molecules to facilitate boron transport or even to exclude toxic levels of boron. Harris et al. (41) reported siderophores isolated from marine bacteria that bind boron more strongly than iron and suggested that these siderophores have a role in the detoxification of boron in the ocean. Given that the concentration of boron in the ocean is estimated to be 400 μM (46), organisms might have evolved a control mechanism either to decrease its toxicity or to make use of its abundance. In fact, the crystal structure of the universal quorum-sensing molecule AI-2 bound to its receptor was found to be in the deboronated form in terrestrial bacteria but in the boronated form in marine ones (47). Although boron can result from glass contamination in some cultures (48), such contamination is less likely to be the source of boron in marine organisms because of the high concentration of boron in the ocean and marine growth media. It also is possible that tartrolons serve multiple functions, acting as both as an antibacterial and as a boron transporter in the shipworm system.

In summary, we have isolated and identified two macrodiolides from the marine shipworm symbiont T. turnerae T7901, compound 1 and its boronated derivative compound 2, that were found to act as antibacterials. We identified a biosynthetic gene cluster that will shed more light on the biosynthesis of other active natural products in this class. The biosynthetic scheme was predicted based on phylogenetic analysis of the KS domains. Moreover, tartrolons were detected in the shipworm host and in other T. turnerae strains, strongly suggesting that it plays a role in the bioactive metabolite symbiosis of the shipworm.

Materials and Methods

Alignment and Phylogenetic Analyses of KSs. Sequence alignments were conducted in Clustal W (49). ML tree topology was inferred in PhyML 3.0 (50) using the LG model of sequence evolution (51) with Gamma-distributed rates across sites. Model choice was conducted in ProtTest 2.4 by the likelihood ratio test (52). Node support was accessed by the approximate likelihood ratio test (aLRT) statistic (53). The Bayesian tree was estimated in MrBayes 3.1 (54) with the WAG + G model of sequence evolution (55), which was applied previously (20). Bayesian inference in MrBayes is performed via the Markov chain Monte Carlo (MCMC) algorithm. Two independent MCMC
runs with four chains each were used to check convergence of parametric estimates. Chains were sampled every 100th cycle for 5,000,000 generations, yielding 50,000 analyzable samples in each run, of which 25% were discarded as burn in. Convergence was checked by the average SD of split frequencies and by the phylogenetic species recognition (PSR) factor in MrBayes. Additionally, we used the R package CODA (56) to test convergence using the Heidelberger–Welch test to estimate the effective sample sizes.

Prevalence of Tartrolons in Different T. turnerae Strains. Each strain was streaked on agar plates and then inoculated in 50-ml liquid cultures. The same temperature and SMB medium were used to grow all 12 strains to test for the prevalence of compound 2. For MS analysis; cells were centrifuged at 5,000 × g for 20 min, and sonicated in 50% (vol/vol) chloroform in methanol. All dry extracts were dissolved in the same volume of methanol and analyzed by HR-MS and MS/MS, and the region between m/z 800–930 was analyzed. In addition, MS/MS was used to fragment the characteristic peaks for compound 2. For PCR analysis, DNA was isolated from 12 T. turnerae strains using phenol and chloroform as reported previously (57). PCR reactions were performed with 10–100 ng of T. turnerae genomic DNA, using Platinum Taq DNA polymerase (Invitrogen). PCR was used to check the prevalence using primers targeting each of the three biosynthetic ORFs trdA, -E, and -F (Table 52).

Analysis of Different Shipworms for the Presence of Tartrolons Using MS. Individual shipworms of the species L. pedicellatus were extracted from the wood, on ice, using appropriate tools and sterile seawater. Shipworms were washed with sterile seawater and frozen rapidly at −80 °C. The samples were lyophilized and sonicated three times in 50% (vol/vol) chloroform in methanol using a Branson digital Sonifer. The combined organic fraction was concentrated under vacuum and partitioned between ethyl acetate and water. The ethyl acetate fraction was filtered over anhydrous sodium sulfate and dried under vacuum. This fraction then was dissolved in methanol before being injected into LC/MS with a reverse-phase column and mobile-phase water and methanol. Proper controls containing solvents were used to detect any contamination from solvents or instruments.

Other Experiments. Additional experiments are available in the Materials and Methods section of the supporting information.

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Fig. 8. Potential functions of tartrolon in the shipworm-microbial symbiosis. Tartrolon is proposed to participate in bacterial inhibition, either in the gills (right) by inhibiting certain bacterial phenotypes or in the cecum (left) by preventing microorganisms from scavenging glucose.


