Genetic Evidence Supports Secondary Metabolic Diversity in *Prochloron* spp., the Cyanobacterial Symbiont of a Tropical Ascidian†

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Didemnid family ascidians commonly harbor obligate cyanobacterial symbionts, *Prochloron* spp., which have been proposed to biosynthesize cyclic peptides. Here, it is shown that *Prochloron* spp. do indeed contain genes for nonribosomal peptide biosynthesis, although genes for cyclic peptide biosynthesis have not yet been characterized. A peptide synthetase-containing open reading frame of unknown function was cloned from the *Prochloron* symbionts of some didemnid ascidians, but not from others. These data indicate that *Prochloron* spp. have variable secondary metabolic potential.

Many of the natural products isolated from marine invertebrates share structural homology with compounds of microbial origin, leading to the hypothesis that the marine compounds are actually produced by bacterial or fungal symbionts and not by the animals themselves.[1] Although for the most part the origin of marine invertebrate natural products is unknown, in several cases the compounds have been traced to bacterial symbionts. D. John Faulkner maintained an active interest in this research area, leading his group in many studies of symbiosis in marine natural products. Under his guidance, brominated diphenyl ethers were found in cyanobacterial symbionts of the sponge *Dysidea herbacea*.[2] The potent polyketide cytotoxin swinholide A was discovered in a mixed unicellular bacterial fraction of the marine sponge *Theonella swinhoei*, while a hybrid peptide-polyketide was isolated from filamentous bacteria from the same sponge.[3] Several reviews of symbiont sources of marine natural products cover these and other advances.[4]

Evidence has been obtained that some sponge and bryozoan[5] compounds are likely produced by bacterial symbionts, but little is yet known about biosynthesis of natural products in ascidians. Many didemnid family ascidians contain near monocultures of the cyanobacterium *Prochloron* spp.[6] These ascidians also commonly contain cyclic peptides and other natural products (Figure 1). One such group of peptides, exemplified by patellamide A (Figure 1),[7] is very similar to compounds synthesized by cyanobacteria, leading to the hypothesis that *Prochloron* spp. makes patellamide-class peptides.[4d] Because of the compelling structural evidence, several research groups have investigated the source of these compounds by separating cells of the organism and examining the chemistry of each cell fraction.[8] Thus far, the data have been ambiguous, with one study asserting a cyanobacterial source.[8a] A later study by Salomon and Faulkner showed that peptides are found throughout the ascidian tunic, leading the authors to propose that the compounds are produced by ascidians, not by their symbionts.[8d] However, the authors noted that the data could also be consistent with bacterial production and secretion. Despite nearly 30 years of effort, *Prochloron* spp. still cannot be cultured outside of the host organism, compounding the difficulty of proving biosynthetic source.

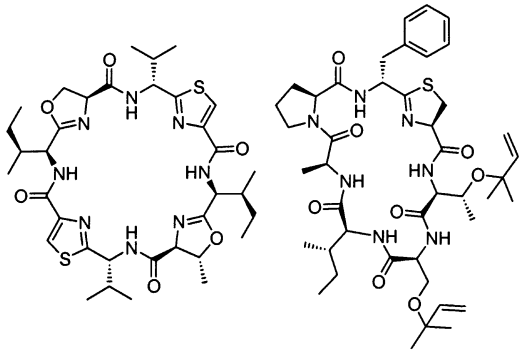

Figure 1. Patellamide A (left) and trunkamide (right) exemplify the patellamide- and mollamide-class cyclic peptides isolated from didemnid ascidians.

The symbiont origin hypothesis carries special weight with the highly modified peptides, since nonribosomal peptide synthetase (NRPS) genes have been characterized so far only in bacteria and fungi.[9] An NRPS-like gene has also been discovered in the genome sequence of *Drosophila*, but the NRPS likely does not synthesize a polypeptide.[10] Thus, for marine invertebrate peptides that appear to be synthesized by the nonribosomal route, there are four possibilities: (1) that the peptides are made by microbial symbionts using NRPS genes; (2) that, through lateral gene transfer, marine invertebrates have acquired NRPS genes; (3) that the peptides are made by an as yet uncharacterized biosynthetic pathway; or (4) that the peptides are synthesized by a dietary source.

Prochloron is an unusual cyanobacterium, containing both chlorophyll *a* + *b* and lacking phycobilins.[11] Plants have a similar pigment composition, but most cyanobacteria do not. The chlorophyll content and thylakoid structure place the microbe in a small, polyphyletic group of cyanobacteria known as the prochlorophytes, which share structural and chemical features with chloroplasts from plants.[12] *Prochloron* (reviewed in Lewin and Cheng[13]) is a large (~10 μm), single-celled organism, and thus unlike the filamentous forms commonly associated with natural product biosynthesis. Taxonomically, *Prochloron* is most closely related to *Synechocystis trididemni*, another didemnid ascidian symbiont, and free-living unicellular strains of *Synechocystis* spp.[14] The genome of *Synechocystis* PCC6803 has been sequenced, as have the genomes of several free-living prochlorophytes (e.g., *Prochlorococcus* spp.).[15] There

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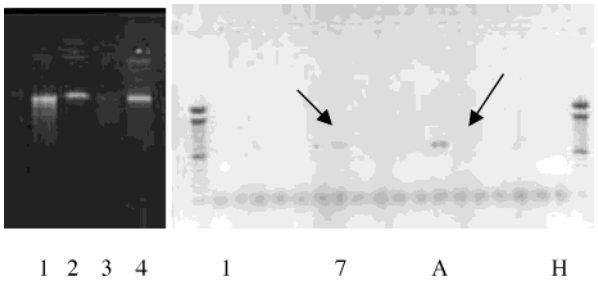

Figure 2. (Left) Gel electrophoresis (0.3% agarose) of DNA from Palau ascidians isolated using GenomicTip kit. Lane 1: λ DNA (50 kbp); Lane 2: *Prochloron* seagrass sample (10 μL/1.5 mL sample); Lane 3: *Prochloron* reef sample (10 μL/1.5 mL sample); Lane 4: 200 ng of T7 DNA (40 kbp). (Right) Arrayed library screen of Palau seagrass sample (480 colonies). A hit is present in column A, row 7.

Figure 3. DGGE (30−70% gradient) of *Prochloron* DNA preparations. Lane 1: Palau reef sample; Lane 2: Palau seagrass sample. Arrows indicate the bands excised and sequenced.

are no discernible NRPS, polyketide, or other natural product pathways in the genomes of these organisms, nor are their relatives known to make natural products. *Prochloron* is an extremely important nutrient source for its host ascidians through both photosynthesis and nitrogen fixation,[6] but thus far there has been no clear evidence to suggest that it is also involved in chemical defense or natural product synthesis.

In this study, a molecular biological approach was taken to identify nonribosomal biosynthetic genes within *Prochloron* spp. in order to assess the biosynthetic hypothesis that *Prochloron* make cyclic peptides. Evidence is presented that *Prochloron* spp. have the potential to make natural products and that biosynthetic genes vary between strains of *Prochloron*.

Results and Discussion

Lissoclinum patella and related ascidians containing *Prochloron* spp. were collected in the Republic of Palau in 2002 and in Papua New Guinea in 2003. *Prochloron* was readily purified from the host, and by light microscopy other bacterial strains could not be observed in preparations. Genomic DNA was purified immediately after *Prochloron* isolation, allowing >40 kbp genomic DNA to be obtained (Figure 2). Genomic DNA was also purified from *Didemnum* spp. from Papua New Guinea. These organisms contained a greater diversity of bacteria than other didemnids, and DNA extraction was more difficult due to a slimy exudate secreted by the animals. In all cases except for *Didemnum* spp., the presence of *Prochloron* could be readily confirmed by direct sequencing of a 16S rDNA PCR product obtained using universal bacterial primers.[16] Other bacteria were clearly present in the *Didemnum* spp., complicating the use of this technique.

The diversity of the Palau samples was explored with denaturing gradient gel electrophoresis (DGGE).[17] PCR products obtained with universal bacterial primers were run on a denaturing gel, with which separation depends on the nucleotide sequence of the PCR product (Figure 3). In both samples, a single major band was present and identified as *Prochloron* through sequencing. Some weak bands were also present in the reef sample, and their sequence was shown to be identical to the major band. These bands are probably caused by incomplete denaturation of the PCR product. As the DGGE results clearly show, *Prochloron* is the only bacterium present in appreciable numbers in the Palau DNA preparations. This is consistent with light microscopy studies, where only the characteristic *Prochloron* cell type was observed.

Fosmid libraries were constructed using DNA from several *Prochloron* samples, including both samples collected in Palau and PNG 03-001 and 03-017, from Papua New Guinea. Libraries contained average insert sizes of ~40 kbp. To facilitate screening by PCR, each library was picked into a 480-clone array, sufficient to cover a genome estimated to be 4 Mbp in size.[18] Libraries were screened using the chlorophyll *a* oxidase (*cao*) gene from *Prochloron* using exact match PCR primers. *cao* is found only in green plants and in three unrelated genera of cyanobacteria: the prochlorophytes *Prochlorococcus*, *Prochlorothrix*, and *Prochloron*.[19] As expected from genome and insert sizes, approximately 1−2 colonies containing the *Prochloron cao* were present in each library, thus validating the library.

Peptides of the patellamide and mollamide classes appear to be synthesized nonribosomally because they are cyclic and modified via heterocyclization or prenylation. Peptide cyclization via amide bonds is extremely rare outside of the NRPS family, although some plant cyclotides and a bacterial lantibiotic (ribosomally produced) are cyclized through amide bond formation by a non-NRPS mechanism.[20] Heterocyclization is also found almost solely among the bacterial NRPSs,[21] while modification by dimethylallylpyrophosphate is found in several natural product (but not ribosomal) pathways in bacteria, plants, and fungi.[22] Finally, didemnid peptides are replete with D-amino acids, which are usually associated with a nonribosomal process.[9] In particular, epimerization/heterocyclization events are associated with NRPS genes.[23] However, these precedents do not preclude a wholly novel biosynthetic mechanism for the patellamides and mollamides.

Because an NRPS-based biosynthesis of patellamides is probable on the basis of precedent, degenerate PCR primers were designed based on cyanobacterial NRPS sequences. These primers were used in PCR with *Prochloron* and whole ascidian DNA. While no products were obtained from the ascidian, products were obtained from purified *Prochloron* samples. The primers were then applied to the Palau *Prochloron* fosmid library from seagrass-inhabiting *L. patella*, leading to the isolation of a single clone containing an NRPS sequence, dubbed *prnA* for *Prochloron* NRPS A (Figure 2). The fosmid was partially sequenced, and several conserved NRPS features could be readily identified, including condensation, thiolation, and adenylation domains (Figure 4). An overlapping fosmid was also found in PNG 03-017 using PCR, but no other NRPS genes were found in >2000 clones from the four libraries.

The ~40 kbp *prnA*-containing fosmid was used for all sequencing reactions. Approximately 9.2 kbp of the *prnA* operon were sequenced, as were 1500 bp at both ends of the fosmid. *prnA* appears to be part of an operon containing at least five open reading frames (Table 1; Figure 4), although more orfs may be present since *orf1* is truncated at one end of the vector. *prnA* is the sole NRPS gene in

Table 1. Predicted orfs from the *prnA* Genomic Region

protein	amino acids	proposed function	sequence similarity	identity/similarity	accession #
Orf1	373	Fe-S oxidoreductase	AstB/ChuR/NirJ-related protein; *C. acetobutylicum*	24/45% (partial gene sequence)	AAK76881.1
Orf2	332	oxidoreductase	oxidoreductase; *C. acetobutylicum*	39/60%	AAK76880.1
Orf3	277	protein tyrosine sulfotransferase	Hypothetical; *Caenorhabditis elegans*	36/45%	T16350
PrnA	1643	NRPS C-T-C-A-T domains	NcpB; *Nostoc* sp. ATCC53789	variable; ~45/60%	AA023334
Orf5	101	nucleotidyltransferase	predicted nucleotidyltransferase; *Geobacter metallireducens*	44/60%	ZP_00090552.1

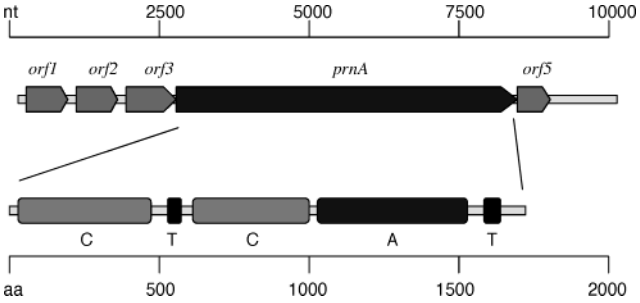

Figure 4. *prnA* operon (top) and predicted PrnA domain structure (bottom).

the cluster, since a neighboring *orf1*-containing fosmid did not contain an NRPS sequence, nor were other NRPSs found on the *prnA* fosmid. *prnA* was clearly cyanobacterial in origin, with approximately 45% translated sequence identity to NcpB from *Nostoc* sp. ATCC53789.[24] The sequence was also quite similar to a series of other NRPS predicted orfs from *Nostoc*, *Anabaena*, and *Lyngbya*, while sequences from other bacteria were much more distantly related. *prnA* had a GC content (41.1%) that reflected its *Prochloron* origin, and the cluster overall had a GC content of 41.7%.

The predicted PrnA protein contained all of the domains necessary for a functional NRPS, including all expected catalytic amino acid residues and condensation (C), adenylation (A), and thiolation (T) domains.[9] However, the protein exhibited an unsual domain order, C-T-C-A-T, while most proteins containing two C and T domains would follow the C-A-T-C-A-T canonical order. The unusual order is not due to a fosmid rearrangement, since PCR across the C-T-C-A region using *Prochloron* DNA yielded a product of identical size to the predicted *prnA* gene fragment. In fact, the C-T-C motif is found in other NRPS gene clusters, including the vibriobactin synthetase cluster from *Vibrio cholerae*.[25] In the *V. cholerae* cluster, the C-T-C motif follows an A domain, making *prnA* somewhat unique. To determine whether *prnA* could be involved in patellamide biosynthesis, the method of Challis et al. was applied to deduce substrate selectivity of the A domain.[26] Residues lining the amino acid binding pocket were DVWNIAAV, giving a closest match to the D,L-Phe-activating domain from GrsA[27] (DAWTIAAA; 63/88% identity/similarity). While the sequence is not close enough to definitively state that Phe is activated by PrnA, it is likely that the A domain selects Phe or other nonpolar amino acids. Thus, *prnA* could be involved in patellamide biosynthesis, despite the noncanonical domain order.

The predicted Orf1 and Orf2 appear to encode Fe-S and NADH oxidoreductases, respectively, and share homology with a broad variety of such reductases from diverse bacteria. The orfs are most closely related to an oxidoreductase couple from *Clostridium acetobutylicum*.[28] Orf3, a putative sulfotransferase, is most similar to a regulatory sulfotransferase from *Caenorhabditis elegans*, but it also shares identity with similar proteins from *Synechocystis* PCC6803, the closest relative to *Prochloron* for which a complete genome sequence is available. Similarly, although Orf5 is most closely related to a putative nucleotidyltransferase from *Geobacter metallireducens*, it also is homologous with several nucleotidyltransferases from *Synechocystis*, *Synechococcus*, and *Nostoc* cyanobacteria.

The *Clostridium* homologues of Orf1 and Orf2 are located on the megaplasmid pSOl1[28] in a genomic environment that also appears to encode a natural product biosynthetic pathway, although no NRPS gene is present in the region. *Clostridium* genes in the same operon as the oxidoreductases include polyketide synthase homologues, drug resistance proteins, and glucose-modifying proteins. The *prnA* clusters Orf1 and Orf2 share homology with several proteins known to reduce sugars in natural product pathways, and Orf5 could be involved in glycosyl transfer to a nucleotide.[29] Because of these features, it is tempting to speculate that the *prnA* cluster may be involved in the biosynthesis of a sulfated, glycosylated amino acid derivative. If this were true, it would preclude the involvement of this cluster in patellamide biosynthesis. Further work is underway to characterize this and other *Prochloron* gene clusters.

To investigate the presence of the *prnA* gene in other ascidians, PCR with exact match primers was employed using Palau and Papua New Guinea ascidian samples. Two different primer sets were used to yield products at approximately 3 kbp and 900 bp, reducing the possibility of false positives. In all organisms that contained *prnA*, both primer sets yielded strong hits of the appropriate size, while those without *prnA* were negative to both primer sets. The presence of the NRPS gene was found to be variable, with the gene occurring in some *Prochloron* strains but not others (Table 2).

To determine whether the cloned gene cluster could be correlated to the presence of cyclic peptides, crude organic extracts from the ascidians were analyzed by ESIMS (Figure 5). Didemnids from seagrass habitats, with the exception of a single organism, did not contain the cyclic peptides, while most reef didemnids contained either patellamide-like or mollamide-like compounds (Table 2). Specifically, *L. patella* from reef habitats contained patellamide relatives, one *Didemnum molle* sample contained mollamides, and patellamide derivatives were also isolated from a seagrass-containing sample. ^{1}H NMR was used to support MS data in some cases, and cyclic peptides corresponding to known compounds could be clearly identified by comparison to reference spectra. The presence of cyclic peptides in samples did not correlate with the presence of the cloned gene cluster. Thus, two possibilities present themselves. The gene cluster may not be responsible for synthesis of the cyclic peptides. Alternatively, the cluster may produce patellamide-like molecules. In this case, the presence of *prnA* in organisms that do not contain peptides would likely indicate gene regulation repressing biosyn-

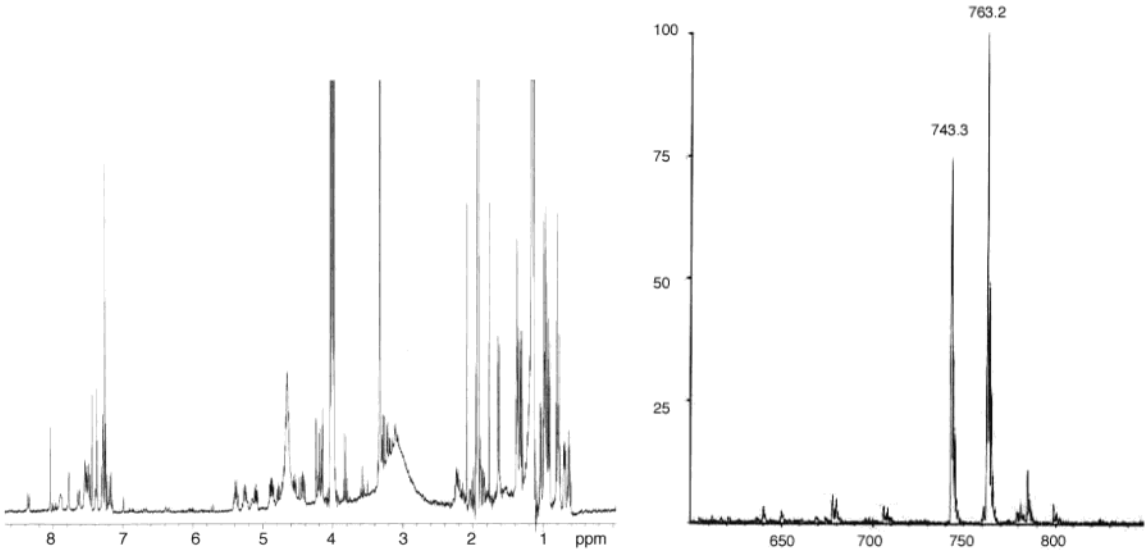

Figure 5. Utility of ESIMS screening in didemnid ascidian samples: Palau reef sample. (Left) ^{1}H NMR of crude organic extract in $CDCl_3$. The spectrum was compared to those for purified patellamides (data not shown), indicating characteristic NMR peaks at δ 7.0−8.5 and 4.0−5.5 ppm. (Right) ESIMS. *y*-axis: relative intensity; *x*-axis: *m*/*z* (amu). Major peaks at *m*/*z* = 743 and 763 correspond to patellamides A and C, respectively.

Table 2. *prnA* Gene Incidence and Chemistry in Palau and Papua New Guinea Ascidians

sample	ascidian[a]	chemistry[b]	16S rDNA[c]	*prnA*[d]
Palau reef	*L. patella*	patellamides	*Prochloron*	+
Palau seagrass	*L. patella*	none	*Prochloron*	+
PNG 03-001	*L. patella*	patellamides	*Prochloron*	−
PNG 03-002	*D. molle*	none	mixed	−
PNG 03-005	*L. patella*	patellamides	*Prochloron*	−
PNG 03-009	*D. molle*	none	mixed	−
PNG 03-011	*D. molle*	mollamides	mixed	−
PNG 03-012	*D. molle*	none	mixed	−
PNG 03-017	unidentified	patellamides	*Prochloron*	+
PNG 03-018	unidentified	none	*Prochloron*	+
PNG 03-019	unidentified	none	*Prochloron*	+
PNG 03-020	unidentified	none	*Prochloron*	−

[a] Taxonomy based upon external morphology. Unidentified samples are encrusting ascidians from a seagrass environment. [b] Based upon ESIMS: reported chemistry indicates compound class, not exact structure found. [c] Result of directly sequencing universal 16S rDNA PCR products. [d] Presence of *prnA* and neighboring genes determined by PCR using two specific primer sets (see text).

thesis. Peptide-containing ascidians that lack *prnA* would harbor related (but as yet unidentified) NRPS gene clusters. Further studies are underway to identify cyclic peptide biosynthetic genes in *Prochloron* and in the host ascidian.

The reported *prnA* gene represents the first NRPS identified in the prochlorophytes or in the *Prochloron* relatives, *Synechocystis* spp., despite four complete genome sequences available for these organisms.[15] It also represents the first natural product biosynthetic gene from obligate symbionts of marine ascidians. The presence of the genes in some strains of *Prochloron* but not others indicates that the organisms have the potential to produce secondary metabolites and that there is metabolic diversity among *Prochloron* spp.

Experimental Section

General Experimental Procedures. All solutions were autoclaved or sterile filtered (0.22 μm) prior to use. PCR tubes, water, and tips were exposed to UV light in a laminar flow hood for at least 1 h prior to use, and all PCRs were set up in a laminar flow hood. PlatinumTaq DNA polymerase (Invitrogen) was used for PCRs, and the Qiagen SpinKit and Qiaex gel extraction kit were used for plasmid minipreps and gel extractions, respectively. The pCR2.1TOPO kit (Invitrogen) was used to clone PCR products.

Collection of Ascidians, Purification of *Prochloron*, and DNA Extraction. All solutions and materials used to purify cells and DNA were first autoclaved and/or filter-sterilized (0.22 μm), and in some cases UV sterilized to remove any residual DNA contamination. After DNA purification, samples were handled only in a laminar flow hood using sterile technique with UV-treated pipets and aerosol-barrier tips. Ascidians that appeared to contain *Prochloron* spp. were collected in Palau at Omodes (seagrass environment) and near Blue Corner (reef environment) in 2002 and in Papua New Guinea near the city of Madang and Bagabag and Karkar islands in 2003. Prior to processing, Palau ascidians were kept alive in seawater in flow-through tanks at ambient seawater temperature for 0−24 h, using the facilities of the Coral Reef Research Foundation. In Papua New Guinea, ascidians were processed immediately. First, ascidians were rinsed with sterile artificial seawater to remove surface contaminants and pressed to release *Prochloron* cells. *Prochloron*-containing fractions were spun in a microcentrifuge, and the purity of the resulting pellets was assessed by light microscopy (Palau only). DNA was purified immediately using the Qiagen GenomicTip kit, and DNA was kept at 4 °C for up to 1 year without substantial degradation. Samples of *Prochloron* and whole ascidian tissue were also stored in RNALater, CsCl, various fixatives, and ethanol, or frozen for chemical analysis. DNA was analyzed for size and integrity by agarose gel electrophoresis using λ DNA, ligated λ DNA multimers, and λ *Hin*dIII ladders as standards. From ∼25 g wet weight of *L. patella*, ∼100 μL of pure *Prochloron* cells could be obtained, yielding ∼50−200 μg of DNA.

Chemical Analysis. Whole pieces of ascidian (∼1−25 g) were extracted in ethanol, and the ethanolic fractions were dried under Ar. The resulting aqueous phase was extracted three times with ethyl acetate and dried under Ar. Organic extracts were directly injected for ESIMS in the positive mode, and compounds were identified on the basis of mass using MarinLit. ^{1}H NMR spectroscopy (400 MHz, $CDCl_3$) was also used for some samples, and spectra were compared with those of known compounds.

Fosmid Library Construction. Libraries were constructed from purified *Prochloron* DNA from the following organisms: two Palau samples (seagrass and reef) and two Papua New Guinea samples (designated 03-001 and 03-017). Approximately 20 μg of DNA from each of the four samples were used with the EpiCentre CopyControl fosmid kit. The manufacturer's instructions were followed, except that DNA

was not sheared before being end-repaired, since it was already ~40 kbp in size. From each sample, 480 colonies were obtained and picked into five 96-well plates containing 150 μL of LB-chloramphenicol. Thirty microliters from each well were pooled into a single 96-well plate, while the remainder was stored as a frozen stock in 20% glycerol.

Fosmid Library Screening. The 96-well plate containing five clones per well was pooled into rows and columns, and fosmids were isolated from the resulting 20 fractions by miniprep. From each fraction, 1 μL of fosmid solution (<1 ng of fosmid DNA) was used in a 10 μL PCR. *cao* primers caof (5′-CAACCCCTATGCCTTTTGAA) and caor (5′-TAAACAAC-CCATGCTCCACA) were designed from the sequence available through GenBank (AB021314). The primers QVKProch (5′-CCTAATTCAATACGAAAACCACGAadyttnayytg) and YTS-Proch (5′-TGTATGTTATTTATACTTCTGGTTCTACTGGT-mrnccnaargg) were designed with the CODEHOP algorithm,[30] using an alignment of known cyanobacterial NRPS genes as the basis set and biasing for ~40% GC content. A 2 μM concentration of each primer was used for PCR with the cycle 94 °C, 4 min; then 35 cycles of 94 °C, 30 s; 53 °C, 30 s; 72 °C, 1.5 min; followed by 72 °C, 10 min. PCR for *cao* was performed in an identical manner, but with a 50 °C annealing temperature. Products were analyzed by agarose gel electrophoresis, and positive hits were validated by first subcloning (pCR2.1-TOPO kit, Invitrogen) and sequencing PCR products, then by subscreening individual clones. Libraries were also screened with NRPS primers AGGCHf (5′-GCCGGCGGCgcntaygtncc) and YTSProchr (5′-CACCTTTTGGTTTACCAGTAGAAccn-swngtrta).

***prnA* Gene Cluster.** The sequence has been deposited in GenBank, accession number (AY590470).

Fosmid Sequencing. A fosmid, designated 1A2 and containing *prnA*, was partially sequenced by cosmid walking from both ends and from the QVKProch/YTSProch PCR products.

16S rDNA Analysis. The universal bacterial 16S primers unifor (5′-TGCCAGCAGCCGCGGTA) and unirev (5′-GACGG-GCGGTGTGTACAA) were used,[11b] and the resulting products were gel extracted and directly sequenced using the unifor primer.

Denaturing Gradient Gel Electrophoresis (DGGE). DGGE was performed following established protocols[17] using the Bio-Rad DCODE system. Briefly, a 16S gene fragment was PCR amplified with universal primers 1055F and 1392R with GC clamp. PCR products were loaded onto the gradient gels with 30−70% denaturant. The gel was stained with SYBR Gold (Molecular Probes Inc.), and bands were visualized with UV illumination and excised from the gel. The gel matrix was mechanically disrupted and the DNA extracted into 20 μL of ddH_2O in an overnight incubation at 37 °C. One microliter was used as the template to reamplify the bands with the same primers for DNA sequencing.

PCR Analysis of *prnA* in Ascidian Samples. Dilutions (1×−1000×) of *Prochloron* DNA (approximately 1−10 ng per PCR) were used with the primer pairs Oxredf/CTCr (5′-GTGATGCAATTGATGCGG/5′-AGAGTGATTACAGGAGAC) and H1f/H1r (5′-TTACCCAAGAACTCTCAG/5′-CAAACACAAC-CGCCACTG). PCR was performed with the cycle 94 °C, 4 min; then 30 cycles of 94 °C, 30 s; 50 °C, 30 s; 72 °C, 1.5 min; followed by 72 °C, 10 min. Each PCR run contained a positive (1A2 fosmid) and negative (no DNA) control, and positive hits were evaluated by agarose gel electrophoresis. Samples giving products of the appropriate size with both primer pairs were considered to contain *prnA*.

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