“*Candidatus Endobugula glebosa*,” a Specific Bacterial Symbiont of the Marine Bryozoan *Bugula simplex*

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The bryozoans *Bugula neritina* and *Bugula simplex* harbor bacteria in the pallial sinuses of their larvae as seen by electron microscopy. In *B. neritina*, the bacterial symbiont has been characterized as a gamma-proteobacterium, “*Candidatus Endobugula sertula*.” “*Candidatus E. sertula*” has been implicated as the source of the bryostatins, polyketides that provide chemical defense to the host and are also being tested for use in human cancer treatments. In this study, the bacterial symbiont in *B. simplex* larvae was identified by 16S rRNA-targeted PCR and sequencing as a gamma-proteobacterium closely related to and forming a monophyletic group with “*Candidatus E. sertula*.” In a fluorescence in situ hybridization, a 16S ribosomal DNA probe specific to the *B. simplex* symbiont hybridized to long rod-shaped bacteria in the pallial sinus of a *B. simplex* larva. The taxonomic status “*Candidatus Endobugula glebosa*” is proposed for the *B. simplex* larval symbiont. Degenerate polyketide synthase (PKS) primers amplified a gene fragment from *B. simplex* that closely matched a PKS gene fragment from the bryostatin PKS cluster. PCR surveys show that the symbiont and this PKS gene fragment are consistently and uniquely associated with *B. simplex*. Bryostatin activity assays and chemical analyses of *B. simplex* extracts reveal the presence of compounds similar to bryostatins. Taken together, these findings demonstrate a symbiosis in *B. simplex* that is similar and evolutionarily related to that in *B. neritina*.

Bugula is a bryozoan genus with a cosmopolitan distribution (15, 34). Members of this genus are colonial organisms that reproduce sexually by means of releasing free-swimming lecithotrophic (nonfeeding) larvae, which can settle, metamorphose, and develop into new colonies. One species, *B. neritina*, is a common fouling organism in temperate and tropical waters (34). By using transmission electron microscopy, Woollacott observed that the larvae of *B. neritina* harbored rod-shaped bacteria in the pallial sinus, a circular invagination of the larval surface, which is effectively sealed from the surrounding seawater (38). These extracellular symbionts were a monoculture of a gamma-proteobacterium, “*Candidatus Endobugula sertula*” (14), which has not been cultivated to date and has been implicated in the production of a family of polyketide metabolites called bryostatins (8). There are 20 known bryostatins in this family (21, 25), and while they all share a common macrocyclic bryopyran skeleton, they can be separated into two groups, chemotype O and M (9). The O chemotype is only found in *B. neritina* from California waters deeper than 10 m; the M bryostatins are found in all *B. neritina* populations that have “*Candidatus E. sertula*” (9, 22). Bryostatins 10 and 20 confer chemical defense to *B. neritina* by deterring fish from eating its larvae (20, 21). In addition to the ecological role of bryostatins, bryostatin 1 is being tested as an anticancer drug in humans (13, 37). Bryostatin 1 in humans modulates protein kinase C (PKC) activity (13), and the assay routinely used for bryostatin involves the competitive displacement of phorbol esters from PKC by bryostatin (32).

Macrolide polyketides like bryostatins are produced by type

I modular polyketide synthases (PKS) (17). These large polypeptides are comprised of modules that each contain several catalytic domains (17). One domain that is present in each module is the β -ketosynthase (KS) domain, which catalyzes the addition of extender units to the growing polyketide chain (4). Davidson et al. investigated the presence of PKS genes in the *B. neritina* “*Candidatus E. sertula*” symbiosis and presented evidence implicating the role of “*Candidatus E. sertula*” in producing bryostatins (8). *B. neritina* was partially cured of “*Candidatus E. sertula*” by antibiotic treatment, and bryostatin levels were also reduced (8, 21). A 300-bp PKS gene fragment, the 3' end of a KS domain, was amplified by PCR using degenerate primers, and it was present in all *B. neritina* populations and absent in other bryozoans tested (8). This fragment also hybridized to “*Candidatus E. sertula*” RNA in *B. neritina* larvae in in situ hybridization experiments. Additionally, denaturing gradient gel electrophoresis experiments demonstrated that “*Candidatus E. sertula*” was the only consistent bacterium uniquely associated with *B. neritina* (unpublished data).

The transmission of “*Candidatus E. sertula*” has not been thoroughly investigated, but it is thought to be vertical since “*Candidatus E. sertula*” is found in *B. neritina* larvae (14). Vertical transmission, the transfer of symbiont from parent to offspring, has been inferred in the symbioses of the bivalve *Solemya reidi* (6) and the shipworm *Bankia setacea* (33) based on the presence of symbionts in reproductive tissues such as eggs and larvae.

Other *Bugula* species, such as *B. pacifica* and *B. simplex*, also harbor bacteria in their pallial sinuses (38). We hypothesize that their symbionts are closely related to “*Candidatus E. sertula*” and may also have PKS genes that produce polyketides. These polyketides may function to protect host larvae from predation, in a manner homologous to bryostatins 10 and 20 in *B. neritina*. *B. simplex* is found on the coasts of the North

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TABLE 1. List of primers and probes used in this study

Primer name	Sequence (5' → 3')	<i>E. coli</i> position	Reference
Universal 27F	AGA GTT TGA TCM TGG CTC AG	8–27	19
Universal 1492R	TAC GGY TAC CTT GTT ACG ACT T	1492–1513	19
Egle95F	AAG TCG AGC GAG AAA GCC TT	59–92	This study
Endo240F	TGT TAT TTG ATG AGC CCG CGT T	219–240	This study
Endo1253R	CAT CAC TGC TTC GCA ACC C	1253–1271	This study
Egle240F	CGC TAT TTG ATG AGC CCG CGT T	219–240	This study
Egle264R	TTT ACC CCA CCA TCT AGC TA	245–264	This study
EndoMis	CAT CGC TGC TTC GCA ACC C	1253–1271	14
Eub338	GCT GCC TCC CGT AGG AGT	338–356	2
KSD1F	ACR TGI GCR TTI GTI CC	NA ^a	8
KSD1R	ICA YGG IAC IGG IAC	NA	8
BsimKSF	ACG GAT AAG CGT GAT TAC	NA	This study
BsimKSR	GCC AGG GCC GTA ATG TCG	NA	This study

^a NA, not applicable.

Atlantic (15). In Eel Pond in Woods Hole, Mass., it cooccurs with *B. stolonifera* and *B. turrita*, both of which apparently lack bacteria in their pallial sinuses (38). In this study, we sought to identify the bacteria in the pallial sinuses of *B. simplex* larvae and determine whether PKS genes and bryostatin-like polyketides were present in the organism.

MATERIALS AND METHODS

Sample collection and DNA extraction. *B. simplex* colonies were collected by hand from floating docks in Eel Pond during the summers of 2001 to 2003. *B. simplex* are most fecund during early summer. The colonies were either preserved in ethanol for DNA extraction or kept in running seawater with 12-h light-dark cycles for larval collection. Larvae were collected from reproductive adult colonies when they were first exposed to light during their light-dark cycle. The free-swimming larvae are positively buoyant and phototactic, and they were collected with Pasteur pipettes and rinsed once in seawater. Larvae were then preserved in ethanol for DNA extraction, in ethanol and methanol for bioassay and chemical characterization, or in 4% paraformaldehyde-morpholinepropane-sulfonic acid (MOPS) buffer (4% paraformaldehyde, 0.1 M MOPS, 0.5 M sodium chloride, pH 7.4) for fluorescence in situ hybridization (FISH). For fixation, the larvae were immersed in paraformaldehyde buffer for 2 h and then transferred to 70% ethanol and stored at –20°C. Larvae were also preserved in Trizol (Invitrogen, Carlsbad, Calif.) at –80°C for RNA extraction.

B. turrita and *B. stolonifera* colonies were also collected from the Eel Pond during the summers of 2001 and 2002. *B. neritina* colonies were collected off Scripps Pier, La Jolla, Calif., during the summer of 2001. They were either preserved in ethanol for DNA extractions and bioassay or in methanol for chemical characterization.

Species identification of each sample was performed using a combination of light microscopy under a dissecting scope and scanning electron microscopy. The samples were compared to species descriptions (15). *B. simplex* is easily distinguished from the other *Bugula* species in Eel Pond by its multiserial zooids.

Genomic DNA was extracted from the upper, minimally fouled portion of each colony by using a DNeasy DNA extraction kit (QIAGEN Inc., Valencia, Calif.) following the manufacturer's directions. DNA was also extracted from *B. simplex* larvae, which are nonfeeding; hence, it was expected that the extracted larval DNA would be less contaminated with DNA from environmental bacteria. The success of the extraction and the quality of the DNA was assessed by agarose gel electrophoresis. DNA from *B. neritina* separated from low-molecular-weight PCR inhibitors on agarose gels and was purified using a rapid gel extraction system kit (Marligen Biosciences Inc., Ijamsville, Md.) prior to PCR.

PCR and sequencing. Primers used in this study are listed in Table 1. The 16S rRNA gene was amplified from *B. simplex* larval DNA by using the following pairs of primers and *Taq* polymerase (Roche Applied Science, Indianapolis, Ind.): universal 27F-Endo1253R and Endo240F-universal 1492R. The Endo240F and Endo1253R primers were designed to amplify DNA from *Bugula* symbionts, and they each differ from Bn240F and Bn1253R (specific primers to "*Candidatus E. sertula*") (14) by one base pair. Gradient PCR was performed with annealing temperatures ranging from 54 to 68°C, and the PCR product from the highest annealing temperature was selected to be sequenced. PCR conditions were 30

cycles at 95°C for 50 s, 54 to 68°C for 1 min, and 72°C for 1 min and 20 s. The expected products for each primer pair were about 1,200 bp.

Degenerate primers (8) were used to amplify KS fragments of modular PKS in a touchdown PCR with *B. simplex* larval DNA as a template. PCR products were cloned using a TOPO TA cloning kit for sequencing (Invitrogen) following the manufacturer's instructions. Several colonies were picked, and colony PCR was performed using T3 and T7 primers to check for inserts of the correct size. PCR conditions used an initial denaturation at 95°C for 3 min, followed by 30 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 45 s. PCR products were sequenced, and nucleotide BLAST and translated BLAST searches were performed. ClustalW was used to align translated amino acid sequences (36).

Specific primers, BsimKSF and BsimKSR, were designed against the *B. simplex* KS sequence to perform a PCR survey of several *B. simplex* populations, *B. neritina*, *B. stolonifera*, and *B. turrita*. PCR conditions were 30 cycles of 95°C for 30 s, 65°C for 30 s, and 72°C for 45 s. A 16S rRNA PCR survey on these samples was also performed using Egle240F and Endo1253R as primers.

All PCR products were purified using either a MinElute PCR purification kit (QIAGEN Inc.) or a rapid PCR purification system kit (Marligen Biosciences Inc.) prior to sequencing. Sequencing was performed on an Applied Biosystems 3100 genetic analyzer to obtain at least twofold coverage of each PCR product.

Reverse transcription (RT)-PCR. RNA was extracted from *B. simplex* larvae with Trizol following the manufacturer's instructions with the following modifications: the larvae were immersed in Trizol at –80°C before use, and a plastic pestle was used to homogenize the larvae in Trizol. Approximately 50 µl of larvae was used for each RNA extraction.

The primers for RT-PCR, Egle95F and Egle264R, were designed considering 16S rRNA secondary structure (11). The reverse primer was designed to bind to an accessible region, and the forward primer was designed so that the length of the PCR product would be approximately 200 bp. The specificity of the primers was verified by performing a PCR on genomic DNA and sequencing the PCR product.

RNA (1.6 µg) was used for each reverse transcriptase reaction with Superscript-III (Invitrogen) following the manufacturer's directions. As a DNA contamination control, a duplicate reaction was set up with all the reagents except reverse transcriptase. Two microliters of the cDNA mixture was used for PCR, with Egle95F and Egle264R as primers. The PCR product was purified with a QIAGEN MinElute PCR purification kit and sequenced.

Phylogenetic analysis. The 16S rRNA gene sequence obtained from *B. simplex* was compared with other bacteria by using BLAST (1) and the Ribosomal Database Project (7). Sequences that matched closely were used in an alignment with the 16S rRNA sequence from *B. simplex*. Other gamma-proteobacteria, such as *Escherichia coli*, *Pseudomonas aeruginosa*, and the symbiont of the blister beetle *Paederus fuscipes*, were added to the alignment as well. *Rhodospirillum rubrum* was used as an outgroup. The sequences were assembled using Sequencher 4.0.5 (Gene Codes Corp., Ann Arbor, Mich.) and aligned by eye with secondary structure information (5) to give 1,353 bp of aligned sequences. Phylogenetic trees were constructed in PAUP* 4.0b10 (35) using neighbor-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) algorithms. For NJ analyses, a general time reversible (GTR) nucleotide substitution model with gamma distribution of rate variation across sites and a proportion of invariable sites (*I*) was used. The gamma shape parameter and *I* were estimated with ML. For MP analyses, transversions were weighted three times more than transitions

(based on ML estimations of the transition-to-transversion ratio), and a heuristic search of 100 repetitions with random addition of sequences was performed. NJ and MP bootstrapping were performed with 1,000 replicates. The evolution model for ML analyses was chosen by restricting the GTR-gamma-*I* model using the likelihood ratio test (18). MrBayes3 was used to calculate the Bayesian posterior probabilities of clades (30). Default priors were used, and likelihood settings corresponding to a GTR-gamma-*I* model were implemented. Four chains were run for 2 million generations, sampling every 50 generations. The first thousand trees were discarded before summarizing the analysis.

FISH. Cy5 5'-labeled Endo1253R and Eub338 probes (2) were used for FISH on fixed whole *B. simplex* larvae (Table 1). The Endo1253R probe was designed based on a sequence obtained from the 240F-1492R PCR product, and it was predicted to bind to an accessible site on the 16S rRNA molecule (11). A 1-bp mismatch probe (Endo1253Mis) was used to test the stringency of the hybridization. The Probe Match function in the Ribosomal Database Project (7) was used to check the specificity of the probe.

The Glockner et al. protocol for FISH was employed with slight modifications (12). Briefly, the fixed larvae were washed twice with phosphate-buffered saline (PBS) (20 mM sodium phosphate, 0.15 M sodium chloride), pH 7.4, and incubated in hybridization buffer with 5 ng of probe/ μ l at 46°C for 4 h. Thirty-five percent formamide buffer was used with the Eub338 probe, and 20% formamide buffer was used with the Endo1253R and Endo1253Mis probes. Fifty larvae were typically used for each hybridization condition, and hybridizations and washes were performed in 1.5-ml Eppendorf tubes in a water bath. Reagents were removed by pipetting, taking care not to remove larvae. The larvae were then incubated in washing solution twice at 48°C for 40 min each. Washing solution with 70 mM sodium chloride was used in the Eub338 hybridizations, and washing solution with 225 mM sodium chloride was used in the Endo1253R and Endo1253Mis hybridizations. The larvae were given a final rinse in PBS-Tween (PBS, pH 7.4, 0.1% Tween 20) and stored in this solution at 4°C in the dark until microscopy. Larvae were mounted on slides by pipetting with a 1-ml tip that was treated with SigmaCote (Sigma-Aldrich, St. Louis, Mo.) to prevent the larvae from sticking to the sides of the tip. VectaShield (Vector Laboratories, Burlingame, Calif.) was added to the larvae, which were examined under epifluorescence with a Cy5 filter (Set 41008; Chroma Technology Corp., Rockingham, Vt.). Images of larvae that had undergone hybridization were taken under the same exposure (4 s).

Bryostatin activity assay. Phorbol dibutyrate (PdBu) displacement assays were conducted on adult and larval *B. neritina* and *B. simplex* and adult *B. stolonifera* and *B. turrita* as described by Davidson et al. (8) with a few modifications. Reagents were obtained from sources in a previous study (8). Rat brain liposomes were prepared as described previously, and the same batch of liposomes was used for all assays. When the amount of sample was limited, the ethanol used to preserve the bryozoan for DNA extractions was used in the PdBu assay, since it contained metabolites from the bryozoan. Fresh ethanol was added to keep the bryozoan submerged. Two microliters of a 10-mg/ml ethanolic extract of *Bugula* sp. was added to 2 μ l of tritiated PdBu, 20 μ l of homogenized rat brain liposomes, and 156 μ l of 20 mM (pH 7.4) Tris buffer with 20 mg of bovine serum albumin/ml in 96-well Millipore MultiScreen-FB filter plates with 1- μ m-pore-size filters (Millipore, Billerica, Mass.). Samples were examined in replicate and at several dilutions using methanol as a diluent. The mixture was incubated at 30°C for an hour and then filtered to remove unbound PdBu and retain the liposomes. The filters were washed in 20 mM (pH 7.4) Tris buffer, and radioactivity in counts per minute (cpm) was measured using a scintillation counter. "Cold" (nontritiated) PdBu in place of bryozoan extract was used as a positive control, while 2 μ l of methanol was used as a negative control. The percentage of displacement was calculated using the following formula: (negative control cpm – sample cpm)/negative control cpm $\times$ 100. The 50% inhibitory concentration (IC₅₀) was the amount of extract required to displace 50% of the PdBu. A standard curve was generated with bryostatin 1, and the activity of the extracts was expressed in bryostatin 1 equivalents based on their IC₅₀s.

Chemical characterization. Methanolic extracts of *B. simplex* and *B. neritina* larvae were dried and partitioned between excess volumes of water and ethyl acetate. This step separates the salts and polar compounds from the rest of the metabolites, including most polyketides. The ethyl acetate fraction was dried and reconstituted at 10 mg/ml in methanol, and 5 μ l of this solution was injected into an Agilent 1100 series liquid chromatography-mass spectrometry (LC-MS) system (Agilent Technologies, Palo Alto, Calif.), with a photodiode array detector that scanned wavelengths between 210 and 400 nm and mass spectrometry set for detection in API-ES (positive) mode. Separation was achieved with a linear gradient of 10 to 100% acetonitrile to water over 30 min at a flow rate of 0.7 ml/min through an Agilent Hypersil ODS C18 analytical column. The fragmentor and capillary voltage were kept at 70 and 4,000 V, respectively, and the ion

source at 350°C. Nitrogen was used as a sheath gas at 13 liters/min. A mass range of *m/z* 100 to 1,000 was scanned in 0.1 min. A search for the masses of known bryostatins from both extracts was performed using ion extraction analyses.

Nucleotide sequence accession numbers. The 16S rRNA (accession number AY532642) and EgleKSa (accession number AY532643) gene sequences of "*Candidatus* Endobugula glebosa" have been deposited in GenBank.

RESULTS AND DISCUSSION

PCR and phylogenetic analysis of 16S rRNA sequence. By using a combination of universal and symbiont-specific 16S rRNA primers, we obtained 1,474 bp of sequence from *B. simplex* larval DNA. An annealing temperature of 60°C provided the necessary specificity for amplification. The sequence most closely matched "*Candidatus* E. sertula" in the BLAST and RDP-II databases.

Phylogenetic analysis showed that the symbiont of *B. simplex* clustered with "*Candidatus* E. sertula" by all phylogenetic methods implemented (Fig. 1). NJ and MP bootstrap values and the Bayesian posterior probability of this clade were 100%, 99%, and 1.00, respectively. The *B. simplex* symbiont shows a 95% similarity to "*Candidatus* E. sertula" over 1,395 bp, corresponding to *E. coli* positions 45 to 1477, and the *Endobugula* cluster is nested within the *Teredinibacter* clade, a group of bacterial symbionts in shipworms. These intracellular cellulolytic nitrogen-fixing symbionts are found in the gills, gonads, and eggs of shipworms, allowing shipworms to survive on a cellulose diet (33). The close relationship between bryozoan and shipworm symbionts is intriguing, since the hosts are phylogenetically unrelated and have different diets.

The close similarity between "*Candidatus* E. glebosa" and "*Candidatus* E. sertula" suggests that either the acquisition of symbiont occurred before the speciation of ancestral *Bugula* sp. or that *B. neritina* and *B. simplex* independently adopted similar bacterial symbionts. The first scenario is more parsimonious, especially taken together with the presumed vertical mode of symbiont transmission, since the symbionts are found in host larvae (14). The investigation of cospeciation in more *Bugula*-bacteria associations (three out of five *Bugula* species are known to harbor symbiotic bacteria [38]) should shed light on the evolutionary history of the symbiosis.

FISH. Figure 2a shows the location of the circular pallial sinus in a *B. simplex* larva. The symbiont-specific Endo1253R probe hybridized to long rod-shaped cells in the pallial sinuses of *B. simplex* larvae (Fig. 2d and f). The universal eubacterial probe also hybridized to similar cells with similar distribution and density (Fig. 2c and e). The negative control probe, Endo1253Mis, did not bind to any cells (Fig. 2b). This probe is a "*Candidatus* E. sertula"-specific probe (14), demonstrating that "*Candidatus* E. sertula", the symbiont of *B. neritina*, is not present in *B. simplex* larvae. The symbiont in *B. simplex* has a different morphology and distribution in the pallial sinus of the larva compared to "*Candidatus* E. sertula," unlike in previous reports (38). The *B. simplex* symbionts were elongated (with lengths ranging from 1 to 10 μ m), whereas "*Candidatus* E. sertula" were short rods. "*Candidatus* E. sertula" cells were evenly distributed, whereas the *B. simplex* symbionts are concentrated in discontinuous patches around the pallial sinus (Fig. 2c and d).

Transmission electron microscopy analysis has shown that *B. simplex* also harbors bacteria in its pallial sinus, but the

FIG. 1. A phylogenetic tree based on 16S rRNA of “*Candidatus E. glebosa*,” “*Candidatus E. sertula*,” and related gamma-proteobacteria with *R. rubrum* as an outgroup estimated using maximum likelihood. Bootstrap values and Bayesian posterior probabilities are shown on branches that had a bootstrap value higher than 50% or posterior probability of more than 0.50 (top, NJ bootstrap; middle, MP bootstrap; bottom, Bayesian posterior probability). –, a bootstrap value of less than 50%.

phylogenetic affiliation of the bacteria was previously unknown. We performed these FISH experiments on *B. simplex* larvae to verify that the 16S sequence obtained by PCR, and sequencing arose from the symbiont in the pallial sinus. Although 16S rRNA analysis shows that “*Candidatus E. sertula*” and “*Candidatus E. glebosa*” are closely related, their cell morphologies and distribution around the larval pallial sinus are different.

The name “*Candidatus Endobugula glebosa*” (“glebosa” meaning clumpy in Latin) is proposed for the *B. simplex* symbiont with the following description (24): “*Candidatus Endobugula glebosa*” [(γ -Proteobacteria) NC; G–; R; NAS (Gen-

Bank no. AY532642), oligonucleotide sequence complementary to the unique region of 16S rRNA 5'-CAT CAC TGC TTC GCA ACC C-3'; S (*Bugula simplex*, pallial sinus of larvae); MJ].

Insert sequence in “*Candidatus E. glebosa*” 16S rRNA. There is a 12-bp insertion in the “*Candidatus E. glebosa*” 16S rRNA gene between bases 186 and 191 (based on *E. coli* numbering). This insert is not found in “*Candidatus E. sertula*” and is not a PCR artifact. RT-PCR of *B. simplex*/“*Candidatus E. glebosa*” RNA indicates that this 12-bp insert is present in the mature rRNA molecule, unlike some alpha-proteobacteria symbionts that have intervening sequences in their 16S ribosomal DNA that are excised during rRNA maturation (3).

FIG. 2. (a) A bright-field micrograph showing the aboral surface of a *B. simplex* larva. (b to f) Epifluorescence micrographs of larvae after FISHs with Cy5-labeled probes. (b) Hybridization of a larva with the negative control (Mis1253) probe. No signal is observed in the pallial sinus. (c) Hybridization of a larva with Eub338, showing clumps of bacteria in the pallial sinus. (d) Hybridization of a larva with symbiont-specific Endo1253R, showing clumps of bacteria in the pallial sinus. (e) Close-up view of a hybridization of a larva with Eub338. (f) Close-up view of a hybridization of a larva with symbiont-specific Endo1253R. The location of the pallial sinus is indicated by brackets. Scale bar, 5 μm .

“*Candidatus* Blochmannia,” the gamma-proteobacterial symbionts of carpenter ants, also have putative intervening sequences in their 16S ribosomal DNA, but their excision has not been demonstrated (31). As in other cases (31), the G+C content of the insert sequence in the 16S rRNA gene of “*Candidatus* *E. glebosa*” (16.7%) is much lower than that of the remainder of the gene (53.7%). While possible base pairings to extend the stem region can be hypothesized (Fig. 3), the significance of this insert is unknown.

PKS genes. We obtained a PCR product of approximately 300 bp by using degenerate primers in a touchdown PCR and cloned it into a plasmid library. Colonies were picked and

sequenced with primers flanking the cloning site. One sequence, EgleKSa, made up 9 out of 12 clones sequenced. The other clones did not share homology with anything in BLAST searches. EgleKSa closely matches KSa from “*Candidatus* *E. sertula*,” the gene fragment believed to be part of the bryostatin-synthesizing cluster (8). It has 80% amino acid identity and 85% similarity with KSa in a 103-amino-acid span. The G+C content of the fragment from *B. simplex* is 48%, which is close to that of KSa (47%). This similarity is consistent with the possibility of a homologous PKS cluster in “*Candidatus* *E. glebosa*.” The next-closest match after KSa was the fourth module of PedI of the pederin cluster in the symbiont of

FIG. 3. 16S rRNA secondary structure of *E. coli*, "*Candidatus E. sertula*" secondary structure, and a possible RNA structure of the "*Candidatus E. glebosa*" insert sequence.

Paederus fuscipes (29) with 56% identity and 71% similarity. The amino acid alignment for these three sequences is shown in Fig. 4a.

In the previous study using these degenerate KS primers on *B. neritina* larvae, only one of the nine unique sequences obtained was consistently associated with all bryostatin-producing colonies, while the other eight KS sequences probably originated from environmental bacteria (8). We did not retrieve the other KS domains in the putative bryostatin cluster in "*Candidatus E. sertula*" by using this set of degenerate primers (unpublished data). Similarly, with these primers, we obtained only one KS sequence from *B. simplex*/*Candidatus E. glebosa*."

Just as the 300-bp KSa was used as a probe to isolate the 68-kbp putative bryostatin PKS cluster in "*Candidatus E. ser-*

tula" (16), EgleKSa isolated in this study could be used to retrieve the putative PKS cluster from "*Candidatus E. glebosa*." Sequencing of the entire cluster would reveal whether the PKS cluster is intact, as well as the similarities and differences of the "*Candidatus E. glebosa*" PKS to the putative bryostatin PKS, and it will provide clues into the nature of the "*Candidatus E. glebosa*" metabolite, whether it is another bryostatin (possessing the known bryopyran skeleton) or if it has a novel polyketide backbone.

PCR survey. By using specific 16S rRNA primers, the 16S rRNA gene of the symbiont "*Candidatus E. glebosa*" was amplified from a number of *B. simplex* adult colonies collected over 3 years (Fig. 4b). This sequence was absent in *B. neritina*, as well as the cooccurring *B. stolonifera* and *B. turrita*. Specific primers BsimKSF and BsimKSR amplified the fragment of expected size from several populations of *B. simplex* (Fig. 4c) and show the same amplification pattern as the "*Candidatus E. glebosa*" 16S gene survey. The consistent presence of "*Candidatus E. glebosa*" 16S rRNA and EgleKSa genes in several *B. simplex* populations strongly suggests that the symbiont is not a transient bacterium. This survey does not preclude the possibility of multiple symbionts in *B. simplex*, which occurs in other hosts. For example, at least four symbionts which are closely related to each other reside in the gills of shipworms (10). The analysis of a 16S rRNA clone library made from amplifying *B. simplex* DNA with universal primers, combined with FISH to localize the sequences, can help resolve this question (10).

Bryostatin activity and LC-MS. Various degrees of bryostatin activity have been measured using the PdBU assay on different samples of *Bugula* species (Fig. 4d and e). Figure 4d

FIG. 4. (a) Alignment of EgleKSa (accession number AY532643) with "*Candidatus E. sertula*" KSa (AF283572) and KS from module 4 of Pedl (Z14098). Conserved residues among three sequences have black backgrounds, and conserved residues between EgleKSa and "*Candidatus E. sertula*" KSa are in bold. (b and c) PCR survey of "*Candidatus E. glebosa*" 16S rRNA (b) and "*Candidatus E. glebosa*" KSa (c). Lanes 1 to 6 show amplification of both genes from various *B. simplex* populations (1, *B. simplex* larvae 2001; 2, *B. simplex* 2001 colony 1; 3, *B. simplex* 2001 colony 2; 4, *B. simplex* 2002 colony 1; 5, *B. simplex* 2002 colony 2; 6, *B. simplex* 2003 colony 1). Lanes 7 to 9 show lack of amplification from *B. neritina*, *B. stolonifera*, and *B. turrita*. (d) Representative PdBU assay plots of *B. simplex* (square) and *B. stolonifera* (triangle) extracts. The dashed line shows 50% displacement. (e) The graph shows bryostatin 1 equivalents in *B. neritina* (Bn) and *B. simplex* (Bs) adult and larval (Larv) extracts (IC₅₀ of bryostatin 1 is 1.2 ng).

shows a representative plot of percent displacement of PdBU for *B. simplex* and *B. stolonifera* extracts. The IC_{50} is converted to bryostatin 1 equivalents using the IC_{50} of bryostatin 1 (Fig. 4e). Although the PdBU assay is not necessarily specific for bryostatins, previous experiments have demonstrated that it is an accurate indication of bryostatin activity in bryozoans (8). Extracts of adults and larvae from *B. neritina* and *B. simplex* can displace PdBU from PKC (Fig. 4e), while extracts of the aposymbiotic and cooccurring *B. stolonifera* and *B. turrita* are inactive, supporting the hypothesis that *Endobugula* symbionts are involved in bryostatin production. *B. neritina* and *B. simplex* larval extracts have more bryostatin activity (when normalized by weight) than extracts of their respective adults (Fig. 4e), confirming the role of bryostatins in the chemical defense of larvae (21).

LC-MS analysis of the ethyl acetate fraction of *B. neritina* larval extracts showed peaks consistent with the bryostatins based on retention times, molecular masses, and UV spectra. The known bryostatins from *B. neritina* are relatively nonpolar and elute between 20 to 28 min. They have masses between 766 and 932 (see review in reference 25), and chemotype M bryostatins have a UV maximum at 230 nm (26). During the *B. neritina* reference run, ions tended to form sodium adducts $[M + Na]^+$. Ion extraction analyses showed that chemotype O bryostatins were absent, which was expected since the larvae were collected from shallow *B. neritina* populations, which contain only M bryostatins (9).

The ethyl acetate fraction of *B. simplex* larval extracts showed two peaks, Bs801 and Bs817, that resemble known bryostatins based on their UV spectra (Fig. 5c) but are distinct based on retention times (Fig. 5a and b) and molecular masses (Fig. 5d). Bs801, with retention time of 24.7 min, has a $[M + Na]^+$ m/z of 801 and has a UV spectrum consistent with type M bryostatins (Fig. 5). Though the mass of 778 ($M^+ - Na^+$) does not match known bryostatins, it falls within the correct range of their masses. Bs817, with a retention time of 27.4 min, has a $[M + Na]^+$ m/z of 817 and a UV spectrum consistent with type M bryostatins (Fig. 5). The molecular weight 794 ($M^+ - Na^+$) corresponds to either bryostatin 13 or 20, but the retention time of Bs817 does not match any peak in the *B. neritina* extract. The m/z of 817 in the *B. neritina* profile (Bryo817) matches retention times 25.5 and 26.5 min (Fig. 5b), which are probably bryostatins 13 and 20. The large peak at 27.7 min in *B. neritina*, Bryo831, has an m/z of 831 and is probably either bryostatin 10 or 18. Since Bs801 and Bs817 do not exactly match any known bryostatins but have bryostatin characteristics (approximate mass and correct UV spectrum), they are potentially new bryostatin-like polyketides. There is common peak in both *B. neritina* and *B. simplex* extracts at 28.4 min with m/z of 637 (Bn637 and Bs637; Fig. 5). This mass is lower than any known bryostatin, and its UV spectrum does not match that of bryostatins.

Extraction of 500 kg of *B. neritina* adult colonies was required for complete structure elucidation of bryostatin 1 (27). Thus, similar quantities of *B. simplex* may be necessary to determine the structures of the bryostatin-like molecules. Since *B. simplex* is less abundant than *B. neritina*, it may be a formidable task to collect sufficient quantities for chemical characterization of bryostatin-like molecules. Because PdBU results demonstrate that the larvae contain a higher level of bryostatin

FIG. 5. High-pressure liquid chromatograms of (a) *B. neritina* larval extract and (b) *B. simplex* larval extract showing putative bryostatin peaks (Bryo817 and Bryo831) and novel peaks (Bn637, Bs801, Bs817, and Bs637). (c) UV spectra of *B. simplex* peaks and a published spectrum of chemotype M bryostatin as a reference (26). (d) Retention times in minutes (R.T.), m/z values, presence of UV maximum at 230 nm, and provisional identification of novel peaks in *B. neritina* and *B. simplex*.

activity (21) (Fig. 5b), larvae may prove to be a more feasible source of novel bryostatin-like polyketides in *B. simplex*.

Bacterial symbionts in invertebrates typically provide their hosts with metabolic pathways that do not exist in the host. For example, in shipworms, the symbiont *Teredinibacter turnerae* digests cellulose and fixes nitrogen (33), and sulfur-oxidizing bacteria provide reduced carbon for bivalves (6). Another variation of this principle is that of bioactive metabolite symbiosis (16), in which the symbiont provides bioactive metabolites to their host for chemical defense. With this principle in mind, bacterial symbionts in invertebrates are being investigated as sources of bioactive metabolites that can be potential drugs (8, 23, 28). Since many obligate symbionts have not been cultivated, the biosynthetic gene clusters must be cloned and expressed in a heterologous host in order to produce the metabolite ex symbio (16, 28). Modular PKS from bacterial symbionts of invertebrates present an opportunity for combinatorial biosynthesis to produce novel compounds. Further investigations are needed to characterize bioactive metabolites in *B. simplex*/*Candidatus E. glebosa* and to clone the PKS cluster. More species of *Bugula* should be investigated to determine whether symbionts are present and related to the *Endobugula* clade and whether the symbionts produce bioactive metabolites to contribute to the defense of host larvae. These studies will aid in addressing cospeciation and the role of symbiosis in this genus.

In the *B. neritina*/*Candidatus E. sertula* system, "*Candidatus E. sertula*" resides in *B. neritina* and produces bryostatins to chemically defend host larvae (8, 21). The work presented here provides the following lines of evidence for a similar and evolutionarily related symbiosis in *B. simplex*/*Candidatus E. glebosa*: (i) "*Candidatus E. glebosa*" is similarly located in the larvae as "*Candidatus E. sertula*" is in *B. neritina*; (ii) "*Candidatus E. glebosa*" and "*Candidatus E. sertula*" form a monophyletic group; (iii) the PKS fragment isolated from *B. simplex*/*Candidatus E. glebosa* closely matches KSA from "*Candidatus E. sertula*"; (iv) "*Candidatus E. glebosa*" and its PKS fragment are consistently associated with *B. simplex*; (v) *B. simplex* extracts have bryostatin activity in the PdBU assay, similar to that of *B. neritina*; and (vi) compounds with retention times, masses, and UV profiles similar to those of known bryostatins are found in *B. simplex* extracts.

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