Evolutionary Relationships of “*Candidatus Endobugula*” Bacterial Symbionts and Their *Bugula* Bryozoan Hosts[∇]

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Ribosomal gene sequences were obtained from bryozoans in the genus *Bugula* and their bacterial symbionts; analyses of host and symbiont phylogenetic trees did not support a history of strict cospeciation. Symbiont-derived compounds known to defend host larvae from predation were only detected in two out of four symbiotic *Bugula* species.

Like several other marine invertebrates (7, 8), some bryozoans harbor proteobacterial symbionts (12, 15, 28, 29). Woollacott (28) observed bacteria in the pallial sinus of larvae of the bryozoans *Bugula neritina*, *Bugula simplex*, and *Bugula pacifica* under the electron microscope, whereas bacteria were absent in *Bugula turrita* and *Bugula stolonifera* larvae. *B. neritina* was subsequently found to be a complex of at least three sibling species, Shallow (S), Deep (D) and North Atlantic (N) (4, 18). The D and S sibling species of *B. neritina* contain a bacterial symbiont called “*Candidatus Endobugula sertula*,” the producer of bryostatins (3, 17), a class of defensive compounds which was shown to deter fish from eating *B. neritina* larvae in the S sibling species (16, 17). Bryostatins also have anticancer activity (20). Thus, “*Ca. Endobugula sertula*” confers chemical defense as a benefit to the bryozoan host (17). Neither “*Ca. Endobugula sertula*” nor bryostatins were detected in the N sibling species of *B. neritina* (17, 18). *Bugula simplex* also possesses a bacterial symbiont, “*Candidatus Endobugula glebosa*,” that is closely related to “*Ca. Endobugula sertula*.” This symbiont, which resides in the same location of the larva, is also associated with bryostatin activity (15).

The presence of the bacterial symbionts in larval tissue and their absence in the surrounding seawater are indicative of a vertical mode of transmission (26). Moreover, sympatric *Bugula* species do not appear to exchange symbionts (28). In general, vertically transmitted symbionts tend to cospeciate with their hosts, as evidenced by the aphid *Buchnera* (2) and ant “*Candidatus Blochmannia*” (23) examples. Topological congruence between the host and symbiont phylogenetic trees is usually used as evidence for cospeciation (9, 11). This study aimed to determine whether cospeciation could account for the pattern of symbiont distribution in the genus *Bugula* and whether the symbionts produce bryostatin-like molecules for host defense throughout the genus. If so, the symbiotic *Bugula*

species should form a monophyletic group whose topology is congruent with the tree of “*Ca. Endobugula*” symbionts, and bryostatin activity should be present only in symbiotic *Bugula* species.

Sample collection and DNA extraction. Bryozoan colonies were either collected by hand off floating docks or via scuba dive and were rinsed in sterile seawater. DNA was extracted from the samples immediately after collection, or the samples were preserved in 100% ethanol for the bryostatin assays. Genomic DNA, which comprises host and symbiont DNA, was extracted from the upper portion of each colony using the DNeasy DNA extraction kit (Qiagen, Inc., Valencia, CA) by following the manufacturer's directions. DNA from *B. neritina* was 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.

Symbiont 16S SSU rRNA gene sequencing and analysis. We used a combination of universal and specific primers (Table 1) to amplify the symbiont 16S small-subunit (SSU) rRNA gene from *Bugula dentata*, *B. pacifica*, *B. stolonifera*, *Bugula turbinata*, and *B. turrita*. Only the *B. pacifica* and *B. turbinata* samples resulted in a positive amplification (Table 2), indicating that of the five *Bugula* species tested, only those two contained “*Ca. Endobugula*”-related symbionts. These results are consistent with Woollacott's electron microscopy results for the species he examined.

These symbiont 16S SSU rRNA sequences were edited in Sequencher 4.7 (Genecodes Corp., Ann Arbor, MI) and aligned by eye with sequences from “*Ca. Endobugula sertula*” and “*Ca. Endobugula glebosa*” (symbionts of *B. neritina* and *B. simplex*, respectively) and other gammaproteobacteria using secondary-structure information (1), and a phylogenetic tree was constructed using 1,376 characters with the alphaproteobacterium *Rhodospirillum rubrum* as an out-group.

Maximum parsimony (MP), neighbor-joining (NJ), and maximum likelihood (ML) trees were constructed in PAUP* version 4.0b (D. L. Swofford, Sinauer Associates, Sunderland, MA). Transversions (T_v) were weighted twice the transitions (T_i) in parsimony analyses based on the $T_i:T_v$ ratio estimated by ML, and the evolution model used in NJ and ML analyses

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TABLE 1. List of primers used in this study^a

Primer	Sequence (5' → 3')	<i>Escherichia coli</i> or <i>Drosophila yakuba</i> position	Reference or source
27F	AGA GTT TGA TCM TGG CTC AG	8–27	12
1492R	TAC GGY TAC CTT GTT ACG ACT T	1492–1513	12
Endo240F	TGT TAT TTG ATG AGC CCG CGT T	219–240	15
Endo1253R	CAT CAC TGC TTC GCA ACC C	1253–1271	15
Bturb1253R	CCT CGC GGC TTA GCA ACC C	1253–1271	This study
COIF	GGT CAA CAA ATC ATA AAG ATA TTG	1490–1514	10
COIR	TAA ACT TCA GGG TGA CCA AAA AAT CA	2173–2198	10
MT16SF	CGC CTG TTT ATC AAA AAC AT	665–684	5
MT16SR	CCG GTC TGA ACT CAG AT	1201–1217	5

^a The first four primers in Table 1 amplify bacterial 16S SSU rRNA genes, and the remaining primers amplify eukaryotic mitochondrial DNA (COI and 16S LSU rRNA genes, respectively).

was chosen by restricting the general time-reversible (GTR) gamma I model using the likelihood ratios test. Nodal support of the tree topologies was calculated by bootstrapping the MP and NJ trees with 1,000 replicates and by Bayesian analysis (22). Bayesian posterior probabilities were obtained by Mr-Bayes in two ways: (i) by applying a 4×4 model of DNA substitution to all characters and (ii) by applying a doublet model to stem regions of the SSU 16S rRNA molecule. Default settings were used except for the rate prior, which was set to variable. One million generations were run with sampling at every 100th generation; the first 1,000 Bayesian trees were discarded as burn-in. All analyses, regardless of the phylogenetic method, demonstrate that the “*Ca. Endobugula*” symbionts form a well-supported clade among the gammaproteobacteria and are closely related to the cellulolytic symbionts of the shipworm mollusk *Lyrodus pedicellatus* (Fig. 1). We used RRTree (21) to compare the G+C contents and the evolutionary rates of the 16S rRNA genes of “*Ca. Endobugula*” symbionts to those of closely related free-living bacteria and found no significant difference for either parameter ($P = 0.44$ and $P = 0.12$, respectively).

Bryozoan host 16S LSU rRNA and COI gene sequencing and analysis. The bryozoan mitochondrial 16S large-subunit (LSU) rRNA and cytochrome oxidase I (COI) genes were amplified with universal primers (Table 2) as previously de-

scribed (5, 10) for all the collected bryozoans. The N sibling species was not tested, as specimens were not available. *B. dentata* did not yield a clean COI PCR product, so a *B. dentata* COI sequence from GenBank was used instead. The 16S LSU rRNA (409 bp) and the COI genes (507 bp) were used to determine the phylogeny of the host bryozoans, and these genes were chosen as they are routinely used to resolve bryozoan phylogenies at the species level (6, 14). The incongruent-length difference test (9) demonstrated that the two genes were not incongruent ($P = 0.56$) and can thus be concatenated for data analysis. The likelihood criterion estimated $T_i:T_v$ ratios of 2:1 and 3:1 for the 16S LSU rRNA and COI genes, respectively, and these estimates were used in MP reconstructions of the concatenated data set. Bayesian posterior probabilities were obtained in a manner similar to that described for the symbiont phylogeny with the following exceptions: a 4×4 DNA substitution model was applied to the 16S LSU rRNA partition, and a codon model was applied to the COI partition. Both MP and Bayesian analyses indicate that the symbiotic *Bugula* species do not form a monophyletic clade (Fig. 2). This observation was also supported by separate analyses of the two genes (data not shown). Using the Shimodaira-Hasegawa test (25) in PAUP*, we found that constraining the symbiotic *Bugula* into a monophyletic group could not explain the observed data as well as the consensus tree ($P = 0.003$). Even

TABLE 2. Gene sequences obtained in this study and deposited in GenBank

Species	Place and time of collection	Gene	Accession no.
<i>B. simplex</i>	Woods Hole, MA, June 2001	COI	AY633478
		Mitochondrial 16S rRNA	AY633942
<i>B. turrita</i>	Woods Hole, MA, June 2001	COI	AY633483
		Mitochondrial 16S rRNA	AY633950
<i>B. stolonifera</i>	Woods Hole, MA, June 2001	COI	AY633479
		Mitochondrial 16S rRNA	AY633943
<i>B. dentata</i>	Port Elizabeth, South Africa, March 2002	Mitochondrial 16S rRNA	AY633933
<i>B. turbinata</i>	Bamfield, British Columbia, May 2000	COI	AY633481
		Mitochondrial 16S rRNA	AY633947
<i>B. turbinata</i> symbiont	Bamfield, British Columbia, May 2000	Bacterial 16S rRNA	AY633930
<i>B. pacifica</i>	Friday Harbor, WA, July 2000	COI	AY633474
		Mitochondrial 16S rRNA	AY633938
<i>B. pacifica</i> symbiont	Friday Harbor, WA, July 2000	Bacterial 16S rRNA	AY633929
<i>B. neritina</i> , Shallow	La Jolla, CA, June 2000	Mitochondrial 16S rRNA	AY633934
<i>B. neritina</i> , Deep	Palos Verdes, CA, July 1997	Mitochondrial 16S rRNA	AY633935
<i>Watersipora</i> sp.	San Diego, CA, September 2003	COI	AY647167
		Mitochondrial 16S rRNA	AY647168

FIG. 1. Phylogenetic tree of “*Ca. Endobugula*” symbionts and closely related bacteria based on the 16S SSU rRNA gene with *R. rubrum* as an out-group estimated using MP analysis. Support for internal nodes is shown as follows (top to bottom): MP bootstrap, NJ bootstrap, Bayesian posterior probability for a 4×4 model, Bayesian posterior probability, and a 4×4 model for loops/doublet model for stems. Only values above 50% or 0.5 are shown. The scale bar represents 10 nucleotide changes.

though we can rule out the possibility of strict cospeciation (without host switching or symbiont loss) explaining the distribution of bacterial symbionts in *Bugula*, other evolutionary scenarios should be explored.

Cospeciation analyses. One possible scenario is the early acquisition of a symbiont followed by a subsequent loss of symbionts in some taxa. In this case, the symbiotic *Bugula* species tree should be congruent with the “*Ca. Endobugula*” tree. This does not appear to be the case (Fig. 2). We performed parametric bootstrapping (13) and found that the difference in host and symbiont topologies is not due to sampling

error ($P < 0.0001$), meaning that the host and symbiont truly have different evolutionary histories. We then reconstructed a possible cospeciation scenario in TreeMap 2.0 (19) by comparing the symbiotic host topology to that of its symbionts. The most parsimonious reconstruction (weighting cospeciations: host switching:symbiont loss as 0:1:1) required three cospeciation events and one host switch. One thousand random symbiont trees were generated, and we found that it was possible to arrive at the same reconstruction by chance ($P = 0.11$). The TreeMap analysis assumes that all nodes are strongly supported, and this is not the case for the node leading to *B.*

FIG. 2. Incongruent Bayesian host and symbiont phylogenetic trees based on mitochondrial COI and LSU 16S rRNA genes and bacterial 16S rRNA genes, respectively. Support for internal nodes is shown on the host tree as follows (top to bottom): MP bootstrap, Bayesian posterior probability for the combined data set, Bayesian posterior probability for the COI data set, and Bayesian posterior probability for the 16S LSU rRNA data set. Support for internal nodes is shown on the symbiont tree as follows (top to bottom): MP bootstrap, NJ bootstrap, and Bayesian posterior probability for a 4×4 model. Only values above 50% or 0.5 are shown. Bryostatin activity was detected in bryozoans whose names are in a bold font. The scale bar represents 0.1 substitutions per site.

pacifica. We thus swapped the positions of the *B. neritina* cluster and *B. pacifica* on the tree and performed the Shimodaira-Hasegawa test (25) to determine if the observed data could be explained equally well by this rearranged tree. The swapped tree was equally supported by the data ($P = 0.10$), indicating that the ambiguous placement of *B. pacifica* will make it hard to determine if the similarity between the host and symbiont topologies arose by chance.

Bryostatin activity. The bryostatin activities of various *Bugula* species were tested using the phorbol dibutyrate (PdBu) displacement assay, which tests for the ability of a compound, like a bryostatin, to displace PdBu from protein kinase C (24). Previous research has demonstrated activity in extracts of *B. neritina* and *B. simplex* (15); these extracts were used as positive controls. The assay was performed on adult extracts of *B. pacifica*, *B. turbinata*, *B. turrita*, *B. stolonifera*, and *B. dentata*, using the protocol described previously (15), and none of the tested extracts showed any bryostatin activity. One drawback of the PdBu displacement assay is that polyketides structurally and biosynthetically related to bryostatins but which possess altered functional groups will not be detected,

and these polyketides may still be able to function as defensive metabolites. These data suggest that *B. pacifica*, *B. turbinata*, *B. turrita*, *B. stolonifera*, and *B. dentata* may lack symbiotic chemical defense; however, direct feeding deterrence assays are required to confirm this inference.

In this study, we obtained symbiont 16S SSU rRNA gene sequences from *B. pacifica* and *B. turbinata*, and these sequences form a monophyletic group with the corresponding gene in “*Ca. Endobugula sertula*” (*B. neritina* symbiont) and “*Ca. Endobugula glebosa*” (*B. simplex* symbiont). Though the symbionts are very closely related, we do not currently have sufficient evidence supporting cospeciation. Alternative hypotheses include one in which “*Ca. Endobugula*” colonized their respective hosts independently or one in which rampant host switching masks a few cospeciation events. Some nodes of the host tree are poorly supported, and perhaps the use of additional genetic markers will clarify the position of *B. pacifica* on the tree; these additional data should make cospeciation analyses more robust. The absence of bryostatin activity detectable by the PdBu displacement assay in symbiotic *B. pacifica* and *B. turbinata* is surprising. One possible scenario is

that the bryostatin genes were present in the ancestral "Ca. Endobugula" species and were only retained in *B. neritina* and *B. simplex*, as these bryozoans have large and brightly colored larvae that need chemical protection (14). We postulate that "Ca. Endobugula" species might enhance the fitness of their hosts by perhaps playing either nutritional (26) or developmental (27) roles, which have been documented in other marine invertebrates. The addition of more species to the host tree, and the investigation of the presence of bacterial symbionts of these host species, will shed more light on the evolutionary history of the *Bugula*-*Ca. Endobugula* symbiosis and on the alternative roles that these symbionts might play in their host bryozoans.

Nucleotide sequence accession numbers. The nucleotide sequence accession numbers for the gene sequences obtained in this study and deposited in GenBank are listed in Table 2.

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REFERENCES

- Cannone, J. J., S. Subramanian, M. N. Schnare, J. R. Collett, L. M. D'Souza, Y. Du, B. Feng, N. Lin, L. V. Madabusi, K. M. Müller, N. Pande, Z. Shang, N. Yu, and R. R. Gutell. 2002. The comparative RNA web (CRW) site: an online database of comparative sequence and structure information for ribosomal, intron, and other RNAs. *BMC Bioinformatics* 3:2.
- Clark, M. A., N. A. Moran, P. Baumann, and J. J. Wernegreen. 2000. Cospeciation between bacterial endosymbionts (*Buchnera*) and a recent radiation of aphids (*Uroleucon*) and pitfalls of testing for phylogenetic congruence. *Evolution Int. J. Org. Evolution* 54:517–525.
- Davidson, S. K., S. W. Allen, G. E. Lim, C. M. Anderson, and M. G. Haygood. 2001. Evidence for the biosynthesis of bryostatins by the bacterial symbiont "*Candidatus Endobugula sertula*" of the bryozoan *Bugula neritina*. *Appl. Environ. Microbiol.* 67:4531–4537.
- Davidson, S. K., and M. G. Haygood. 1999. Identification of sibling species of the bryozoan *Bugula neritina* that produce different anticancer bryostatins and harbor distinct strains of the bacterial symbiont "*Candidatus Endobugula sertula*." *Biol. Bull.* 196:273–280.
- Dick, M. H., J. R. Freeland, L. P. Williams, and M. Coggeshall-Burr. 2000. Use of 16S mitochondrial ribosomal DNA sequences to investigate sister-group relationships among gymnolaemate bryozoans, p. 197–210. In A. Herrera Cubilla and J. B. C. Jackson (ed.), *Proceedings of the 11th International Bryozoology Association Conference*. International Bryozoological Association, Balboa, Republic of Panama.
- Dick, M. H., A. Herrera-Cubilla, and J. B. Jackson. 2003. Molecular phylogeny and phylogeography of free-living Bryozoa (Cupuladriidae) from both sides of the Isthmus of Panama. *Mol. Phylogenet. Evol.* 27:355–371.
- Distel, D. L., E. F. DeLong, and J. B. Waterbury. 1991. Phylogenetic characterization and in situ localization of the bacterial symbiont of shipworms (Teredinidae: Bivalvia) by using 16S rRNA sequence analysis and oligodeoxynucleotide probe hybridization. *Appl. Environ. Microbiol.* 57:2376–2382.
- Eisen, J. A., S. W. Smith, and C. M. Cavanaugh. 1992. Phylogenetic relationships of chemoautotrophic bacterial symbionts of *Solemya velum* Say (Mollusca: Bivalvia) determined by 16S rRNA gene sequence analysis. *J. Bacteriol.* 174:3416–3421.
- Farris, J. D., M. Kallersjö, A. G. Kluge, and C. Bult. 1995. Constructing a significance test for incongruence. *Syst. Biol.* 44:570–572.
- Folmer, O., M. Black, W. Hoeh, R. A. Lutz, and R. C. Vrijenhoek. 1994. DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotechnol.* 3:294–299.
- Hafner, M. S., and S. A. Nadler. 1988. Phylogenetic trees support the co-evolution of parasites and their hosts. *Nature* 332:258–259.
- Haygood, M. G., and S. K. Davidson. 1997. Small subunit ribosomal RNA genes and in situ hybridization of the bacterial symbionts in the larvae of the bryozoan *Bugula neritina* and proposal of "*Candidatus Endobugula sertula*." *Appl. Environ. Microbiol.* 63:4612–4616.
- Huelsenbeck, J. P., B. Rannala, and Z. Yang. 1997. Statistical tests of host-parasite cospeciation. *Evolution Int. J. Org. Evolution* 51:410–419.
- Hughes, R. N., A. Gómez, P. J. Wright, H. I. Moyano, J. M. Cancino, G. R. Carvalho, and D. H. Lunt. 2008. Molecular phylogeny supports division of the 'cosmopolitan' taxon *Celleporella* (Bryozoa; Cheilostomata) into four major clades. *Mol. Phylogenet. Evol.* 46:369–374.
- Lim, G. E., and M. G. Haygood. 2004. "*Candidatus Endobugula glebosa*," a specific bacterial symbiont of the marine bryozoan *Bugula simplex*. *Appl. Environ. Microbiol.* 70:4921–4929.
- Lindquist, N., and M. E. Hay. 1996. Palatability and chemical defense of marine invertebrate larvae. *Ecol. Monogr.* 66:431–450.
- Lopanik, N. B., N. Lindquist, and N. Targett. 2004. Potent cytotoxins produced by a microbial symbiont protect host larvae from predation. *Oecologia* 139:131–139.
- McGovern, T. M., and M. E. Hellberg. 2003. Cryptic species, cryptic endosymbionts, and geographical variation in chemical defences in the bryozoan *Bugula neritina*. *Mol. Ecol.* 12:1207–1215.
- Page, R. D. M., and M. A. Charleston. 1998. Trees within trees: phylogeny and historical associations. *Trends Ecol. Evol.* 13:356–359.
- Pettit, G. R. 1991. The bryostatins. *Fortschr. Chem. Org. Naturst.* 57:153–195.
- Robinson-Rechavi, M., and D. Huchon. 2000. RRTree: relative-rate tests between groups of sequences on a phylogenetic tree. *Bioinformatics* 16:296–297.
- Ronquist, F., and J. P. Huelsenbeck. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19:1572–1574.
- Sauer, C., E. Stackebrandt, J. Gadau, B. Holldobler, and R. Gross. 2000. Systematic relationships and cospeciation of bacterial endosymbionts and their carpenter ant host species: proposal of the new taxon *Candidatus Blochmannia* gen. nov. *Int. J. Syst. Evol. Microbiol.* 50:1877–1886.
- Schaufelberger, D. E., B. Alvarado, P. Andrews, and J. A. Beutler. 1990. Detection and quantitation of bryostatin 1 and 2 in *Bugula neritina* by combined high-performance liquid chromatography and ³H-phorbol dibutyrate displacement. *J. Liq. Chromatogr.* 13:583–598.
- Shimodaira, H., and M. Hasegawa. 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Mol. Biol. Evol.* 16:1114–1116.
- Sipe, A. R., A. E. Wilbur, and S. C. Cary. 2000. Bacterial symbiont transmission in the wood-boring shipworm *Bankia setacea* (Bivalvia: Teredinidae). *Appl. Environ. Microbiol.* 66:1685–1691.
- Visick, K. L., and M. J. McFall-Ngai. 2000. An exclusive contract: specificity in the *Vibrio fischeri*-*Euprymna scolopes* partnership. *J. Bacteriol.* 182:1779–1787.
- Woollacott, R. M. 1981. Association of bacteria with bryozoan larvae. *Mar. Biol.* 65:155–158.
- Zimmer, R., and R. M. Woollacott. 1983. Mycoplasma-like organisms: occurrence with the larvae and adults of a marine bryozoan. *Science* 220:208–210.