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**Off-axis Symbiosis Found: Characterization and Biogeography of Bacterial
Symbionts of *Bathymodiolus* Mussels from Lost City Hydrothermal Vents**

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Summary

Organisms at hydrothermal vents inhabit discontinuous chemical "islands" along mid-ocean ridges, a scenario that may promote genetic divergence among populations. The 2003 discovery of mussels at the Lost City Hydrothermal Field provided a means of evaluating factors that govern the biogeography of symbiotic bacteria in the deep-sea. The unusual chemical composition of vent fluids, the remote location, and paucity of characteristic vent macrofauna at the site, raised the question of whether microbial symbioses existed at the extraordinary Lost City. And if so, how did symbiotic bacteria therein relate to those hosted by invertebrates at the closest known hydrothermal vents along the Mid-Atlantic Ridge (MAR)? To answer these questions, we performed microscopic and molecular analyses on the bacteria found within the gill tissue of *Bathymodiolus* mussels (Mytilidae, Bathymodiolinae) that were discovered at the Lost City. Here we show that Lost City mussels harbour chemoautotrophic and methanotrophic endosymbionts simultaneously. Furthermore, populations of the chemoautotrophic symbionts from the Lost City and two sites along the MAR are genetically distinct from each other, which suggests spatial isolation of bacteria in the deep-sea. These findings provide new insights into the processes that drive diversification of bacteria and evolution of symbioses at hydrothermal vents.

37 **Introduction**

38 Recent evidence suggests that microbial populations in spatially and chemically
39 fragmented habitats exhibit geographic structure (Whitaker et al., 2003; Papke et al.,
40 2003) rather than being distributed ubiquitously as previously hypothesized (see Finlay
41 2002; Fenchel 2003). The patchy mosaic of populations in heterogeneous environments
42 restricts gene flow, while promoting genetic differentiation and local adaptation (Slatkin
43 1987). Due to the heterogeneous nature of hydrothermal vent environments,
44 chemosynthetic bacteria inhabiting vents probably have geographically structured
45 populations as well. If so, this would have direct implications for how topographic
46 features of the seafloor, deep-ocean currents, and chemically variable environments
47 impact the evolution and diversity of bacteria, the origin and evolution of bacteria-vent
48 invertebrate symbioses, and the assemblage of hydrothermal vent communities.

49 The fragmented distribution of deep-sea hydrothermal vents lies in stark contrast
50 to the uniform conditions of the marine abyssal zone (Tunnicliffe, 1988, 1991;
51 Tunnicliffe and Fowler, 1996; Van Dover, 2000). Discrete hydrothermal vent fields are
52 comparable to islands, distributed in a spatially, chemically, and temporally patchy
53 chain along the deep-sea ridges and remote, off-axis sites (Tunnicliffe, 1988, 1991;
54 Tunnicliffe and Fowler, 1996; Tunnicliffe et al., 1998; Van Dover et al., 2002).
55 Differences between ridges in geography, tectonic activity, age of spreading center, and
56 connectedness of ridge segments likely play a major role in regulating gene flow among
57 populations (Vrijenhoek, 1997; Van Dover et al., 2002; Hurtado et al., 2003), the
58 distribution of vent macrofauna (Van Dover, 1995; Tunnicliffe and Fowler, 1996;
59 Juniper and Tunnicliffe, 1997), and the composition of ecological communities
60 (Tunnicliffe, 1991). For example, 'fracture zones' (Fig. 1) likely inhibit dispersal of

larvae by separating ridge segments that are undergoing independent volcanic evolution (Van Dover et al. 2002). Though associations between chemosynthetic bacteria and their invertebrate hosts provide the basis for macrofaunal production at deep-sea hydrothermal vents, almost nothing is known about the distribution of genetic variation in the symbionts and how population structure of bacteria affects ecological interactions and the evolution of symbioses at vents.

Most dominant vent macrofauna host endosymbiotic bacteria for the capture of chemical energy, yielding a constant food source in this stochastic environment (Fisher, 1990; Cavanaugh et al., 2005; Stewart et al., 2005). Typically, the host provides the symbionts with simultaneous access to oxygen and reduced compounds, and the bacteria, in turn, supply the host with fixed carbon generated from C₁ compounds. Along the Mid-Atlantic Ridge (MAR), vents are inhabited by two species of mussels, *Bathymodiolus azoricus* and *B. puteoserpentis* (Mytilidae; Bathymodiolinae), that host chemoautotrophic (energy source: reduced compounds such as H₂S; carbon source: CO₂) and methanotrophic (energy and carbon source: CH₄) γ -Proteobacteria within their gill tissue (Cavanaugh et al., 1992; Distel et al., 1995; Nelson et al., 1995; Fiala-Médioni et al., 2002). The unique capacity of some bathymodioline mussels to house dual endosymbionts permits the host to utilize multiple compounds for energy acquisition, allowing colonization of diverse environments (Distel et al., 1995; Fiala-Médioni et al., 2002; DeChaine and Cavanaugh, 2005).

The off-axis location, shallow depth (~800 m), distinct chemical environment, and scarcity of known symbiont-hosting invertebrates at the Lost City Hydrothermal Field (LC) suggested the possibility that mussels discovered there (*B. aff. azoricus*, T. Shank unpublished data), host endosymbionts that are different from those on the MAR.

For example, the vent fluids of the off-axis Lost City are relatively cool (10-90°C), alkaline ($\text{pH} \geq 10$), methane-rich (0.13-0.28 mmol kg⁻¹), and lie in stark contrast to the acidic, sulfide-rich effluent of the MAR vents (200-360°C, $\text{pH} = 3-5$) that are ~15 km east and 2200 m deeper (Kelley, et al., 2001, 2005). The abundance of methane and hydrogen and low availability of H₂S (due to the high pH of the fluids, given the pK_a of H₂S = 7.04; Budavari 1996) at the LC suggested that mussels found therein might host primarily methanotrophs, and not thioautotrophs, which would be novel given that all known vent mussels in the Atlantic Ocean host dual symbionts. Furthermore, the apparent remoteness of the Lost City provided a unique setting to resolve whether symbiotic bacterial populations at hydrothermal vents are ubiquitous or structured. Answers to these questions provide a foundation for understanding the forces that promote genetic divergence among symbiont populations and thus determine the biogeography and evolution of bacteria.

The objectives of this study were to 1) determine if the bathymodioline mussels inhabiting the LC hosted symbiotic bacteria and 2) how the symbionts were related, both phylogenetically and demographically, to those hosted by mussels on the MAR. First, we employed transmission electron microscopy (TEM) to determine the presence and morphology of putative symbionts within the Lost City mussel gill tissue and then resolved the relationship of the Lost City symbionts with known symbiotic and free-living bacteria using sequence data from the conserved 16S rRNA gene (Woese, 1987). The bathymodioline chemoautotrophs from the Lost City, and from two MAR fields, Lucky Strike and Snake Pit, were selected for additional population genetic analyses because: 1) bathymodiolines apparently acquire their chemoautotrophic symbionts from the environment each generation (Won et al., 2003), and thus serve as sampling vessels

of the free-living bacterial population, 2) the chemoautotrophs only have one ribosomal RNA operon, precluding concerns over non-orthologous genetic variation (Won et al., 2003), and 3) chemoautotrophs are more widespread among invertebrate hosts at hydrothermal vents than methanotrophic symbionts (Cavanaugh et al., 2005), thus permitting broad genetic comparisons. An intraspecific phylogeny and the demographic history of each chemoautotroph population (defined by location) were inferred from sequence data of the rapidly evolving 16S-23S rRNA internal transcribed spacer (ITS; Antón et al., 1998). By employing both conserved and highly variable markers, the phylogenetic position of Lost City mussel symbionts was resolved at two scales, within the γ -Proteobacteria and among populations of chemoautotrophs hosted by vent-endemic mussels along the northern MAR.

Results

Characterization of the Lost City symbiosis

This characterization, which constitutes the first description of a symbiosis from the LC, revealed two morphologically distinct Gram negative bacteria in the mussel bacteriocytes, gill epithelial cells specialized for housing symbiotic bacteria (Fig. 2a). As in other vent symbioses, the bacteriocytes were separated by symbiont-free intercalary cells. Vacuoles within a bacteriocyte harbored either several coccoid bacteria ($\sim 0.3 \mu\text{m}$ in diameter) or a single, large bacterium ($\sim 1.5\text{-}2.0 \mu\text{m}$) exhibiting intracytoplasmic membranes typical of type I methanotrophs (Fig 2b). Based on the characteristics of endosymbionts in other bathymodioline mussels (Cavanaugh et al., 1992; Fiala-Médioni et al., 2002; Robinson et al., 1998), the small and large bacteria were inferred to be chemoautotrophs and methanotrophs, respectively.

Phylogenetic analyses of 16S rRNA sequence data corroborated the TEM observations of dual endosymbionts in the Lost City mussels. First, sequence alignments revealed that the symbiont phylotypes from the Lost City (Genbank accession numbers A and B) were identical to two phylogenetically distinct lineages of γ -Proteobacteria, a chemoautotroph and a methanotroph, previously found in both *B. azoricus* and *B. puteoserpentis* on the MAR. The presence of both phylotypes in the gill tissue of these two MAR mussel species has been verified through *in situ* hybridization with phylotype-specific probes (Distel et al., 1995; Duperron et al., 2005). In our analyses, chemoautotrophic and methanotrophic symbionts of the mussels formed separate, well-supported monophyletic clades that were nested with chemoautotrophs of vent-endemic vesicomysid clams and free-living methanotrophs, respectively (Fig. 3). This finding demonstrates the tight ecological and historic specificity of the interaction between the mussels and two distinct subsets of the γ Proteobacteria. While both Bayesian and maximum parsimony analyses inferred similar tree topologies, the deep relationships among the mussel methanotroph, mussel and clam chemoautotroph, and other symbiont clades remain uncertain (for $\alpha = 0.05$). Finally, the occurrence of both symbiont phylotypes across the 2-3 mussel species demonstrated a lack of host fidelity and implied that both methanotrophs and chemoautotrophs were acquired from the local environment.

All mussel individuals harboured several, distinct chemoautotrophic symbiont ITS-genotypes (Genbank accession numbers X through Y). Twenty-four percent of the ITS-genotypes from Lucky Strike and 38% from Snake Pit were shared among host individuals within each of those localities (Fig. 4). In contrast, the two Lost City host mussels had no chemoautotroph ITS-genotypes in common with each other, possibly

owing to the small population size of mussels at that location. The occurrence of multiple, geographically restricted chemoautotroph ITS-genotypes within an individual host reinforced the contention that each individual mussel acquired its symbionts from the local, free-living bacterial community as shown by the distribution of 16S rRNA phylotypes in this study and previous analyses of ITS variation (Won et al., 2003).

Biogeography of bathymodioline chemoautotrophic endosymbionts

Analyses of ITS sequence data showed that the chemoautotrophic symbionts of bathymodioline mussels were not distributed ubiquitously, but rather exhibited population structure associated with geographic location. This finding, which contrasts with the observed ubiquity of the 16S rRNA phylotype (above and Duperron et al., 2005), underscores the need to use highly variable markers in analyses at the population level. The genetic variation in the ITS region (1.05 % average pair-wise sequence divergence) permitted resolution of evolutionary relationships among populations of chemoautotrophic symbionts at hydrothermal fields. Two distinct ITS-clades of chemoautotrophs were separated by 13 nucleotide substitutions: the *Bathymodiolus puteoserpentis* (Snake Pit) symbionts and the *B. azoricus* - *B. aff. azoricus* clade, which included symbionts from both Lucky Strike and the Lost City (Fig. 4). Furthermore, the overall estimates of θ (= 6.9) and T (= 1.6) from MDIV imply that the northern and southern populations of *Bathymodiolus* chemoautotrophic symbionts in the north Atlantic (as defined by the ITS-clades) are large and historically have been separated from one another.

Our genetic analyses revealed that populations of chemoautotrophic symbionts inhabiting different hydrothermal vent fields were isolated and experienced independent

demographic histories. First, populations of chemoautotrophs at the Lost City and Lucky Strike were more genetically diverse, as estimated by θ based on the number of segregating sites (W) and the average pair-wise nucleotide diversity (π) for haploid genomes, than the population at Snake Pit (Table 1). Moreover, Tajima's D tests of neutrality suggest that the populations at the Lost City and at Lucky Strike have been demographically stable, whereas the symbionts at Snake Pit likely experienced a population bottleneck (a reduction in population size followed by rapid population growth; Table 1). We cannot rule out, however, the possibility of a selective sweep for Snake Pit symbionts, because Tajima's D does not effectively differentiate between population processes and selection (Tajima 1989). Finally, based on F_{ST} estimates of isolation, our analyses revealed genetic divergence among populations of chemoautotrophic symbionts at all study locations, irrespective of host species (Table 2) or distance between sites (no isolation-by-distance, $p = 0.9$).

Discussion

Though the LC lies distantly off-axis and has a novel chemical environment (Kelley, et al., 2001, 2005), mussels in the genus *Bathymodiolus* found at the Lost City host dual symbionts, a methanotroph and a chemoautotroph, with identical 16S rRNA phylotypes as those along the Mid-Atlantic Ridge (MAR). This result is unexpected given the paucity of H_2S (due to the high pH) in the effluent of Lost City vents (D. Butterfield pers. comm.) and raises the question of whether the chemoautotrophs are using sources of energy other than sulfur compounds, such as hydrogen that is abundant at the vent fluids. Indeed, alternate energy sources may be used by many symbionts, as only *B. thermophilus* found along the Eastern Pacific Rise have been shown to use

sulfur (Belkin et al. 1986; Nelson et al. 1995). The discovery and characterization of the Lost City bathymodioline symbionts, in light of the diversity of chemical environments inhabited by mussels, underscores the ecological and evolutionary stability of the dual symbiosis.

The occurrence of single phylotypes, for both the chemoautotroph and the methanotroph, across different host species demonstrated that neither of the symbiont types was host species-specific. A similar lack of host-species fidelity was shown for the chemoautotrophic endosymbionts of hydrothermal vent tubeworms that were inferred to be environmentally transmitted (Feldman et al., 1997; Nelson and Fisher, 2000; reviewed in Cavanaugh et al., 2005). Our analyses revealing the broad distribution of symbiont phylotypes across multiple host species suggest that *both* methanotrophs and chemoautotrophs of mussels in the northern Atlantic are acquired from the environment, rather than being transmitted from mother to offspring each generation as for the closely related chemoautotrophs of another vent bivalve, *Calymene magnifica* (Cary and Giovannoni, 1993). This finding implies that mussels acquire symbionts from the local community when they colonize a site and has implications for local adaptation of symbionts to that environment.

Chemoautotrophic symbiont populations hosted by bathymodioline mussels were inferred to be isolated from each other because no ITS-genotypes were shared among the three hydrothermal fields. Since *B. azoricus* individuals at the Broken Spur hybrid zone on the MAR (just south of the Lost City) harboured symbionts from both northern (*B. azoricus*) and southern (*B. puteoserpentis*) ITS-clades (Won et al., 2003), it is unlikely that the host organisms affected the distribution of ITS genotypes, though the host may have selected for certain bacterial phylotypes from the local free-living

population. Because the chemical environment of the Lost City is drastically different from both vent sites on the MAR (Kelley et al. 2001), and symbiont populations from Lost City and Lucky Strike are closely related phylogenetically while those from the two MAR sites are not, the chemical environment may not be a large factor in governing the distribution of bathymodioline symbionts. Rather, geography probably played a major role in generating isolation among populations.

The three sites in this study are separated by fracture zones, depth, distance, and deep ocean currents, all of which have been implicated as dispersal barriers that could promote genetic divergence among populations (Van Dover et al., 2002). Though the distance between the Lost City and Lucky Strike to the north (1253 km) is greater than the distance between the Lost City and Snake Pit to the south (832 km), the Lost City chemoautotrophs cluster with those in the north (Fig. 4). Thus, we inferred that distance did not have as large an effect on isolation as did topographic features that likely influence deep-ocean currents. For instance, the two ITS-clades (Fig. 4) are geographically separated by many transform faults that offset the spreading axis, including the ~6000 m deep Atlantis Fracture Zone, just to the south of the Lost City and the Kane Fracture Zone just north of Snake Pit (Fig. 1). Smaller fracture zones, such as the Oceanographer Fracture Zone to the north, are apparently not as strong of barriers to dispersal, but this remains to be evaluated. Thus, understanding the biogeographic history of bacteria that inhabit hydrothermal vents provides an empirical basis for and an independent means of assessing models of deep-ocean currents.

Isolation among hydrothermal vent fields has likely led each population of chemoautotrophic symbionts to experience independent demographic histories, as inferred through differences in θ , Tajima's D tests of neutrality, and the high levels of

isolation estimated by F_{ST} . Because the 16S-ITS-23S spacer is mostly comprised of seemingly functionless regions (Antón et al., 1998), the possibility that selection caused the observed patterns of genetic variation is unlikely. Rather, the demographic history of a symbiont population may depend on the tectonic activity at the site, which, in addition to supplying the bacteria with reduced compounds for energy production, could decimate the population in an intense eruption. For example, we inferred that the population of chemoautotrophic symbionts at Snake Pit was unstable, while the other two populations were at equilibrium. A long-lived hydrothermal vent field, such as the Lost City (Früh-Green et al., 2003) may maintain a heterogeneous and stable population of chemoautotrophs, while shorter-lived or more eruptive sites may generate greater fluctuations in population size and thus reduce genetic diversity.

Our findings fit with the biogeographic model for macrofauna larvae outlined by Van Dover et al. (2002), which states that the greater degree of faulting along slow-spreading ridges (e.g., the MAR) should serve to isolate populations. Since symbionts are acquired from the local environment each generation, the host likely plays little role in determining the distribution of genetic variation in bacterial populations among locations. Rather, the strong divergence between northern and southern symbiont populations and the lack of isolation-by-distance among localities demonstrated that geographic barriers to dispersal, such as faulting, depth, and other topographic features of the seafloor, divide bacterial populations. Also, though off-axis sites may be remotely located, they may be connected (or have a historic connection) via deep-ocean currents with sites along the ridge, as indicated by the Lost City populations clustering with those of Lucky Strike. We conclude that topography is a major influence on the distribution of diversity among populations of symbiotic bacteria at hydrothermal vents,

and that additional research is needed to clarify how differences among ridges in tectonic activity, geography, and physical oceanography have impacted the population structure of symbiotic bacteria and at what scale.

Resolving how populations of bacterial endosymbionts are structured has important implications for microbial biogeography, bacterial diversity and evolution, the origin and evolution of prokaryote-eukaryote symbioses, and the ecology and evolution of life at deep-sea hydrothermal vents. First, studies in microbial biogeography have revealed that limits to gene flow might yield geographic structure within microbial taxa (Papke et al. 2003; Whitaker et al. 2003; Kirchman et al., 2005). Population subdivision implies an increased potential for local adaptation and lineage diversification. Until now, genetic structure and potential for local adaptation in chemosynthetic endosymbionts have remained uncertain. Environmentally transmitted endosymbionts are expected to respond to abiotic selective forces in the environment as well as experience gene transfer with the hydrothermal vent free-living bacterial community. This will not only impact the genetic diversity of symbionts, but may ultimately affect the fitness of the invertebrate host. This and future studies on the biogeography of symbionts inhabiting deep-sea hydrothermal vents, including comparisons with the free-living bacterial community, host biogeography, and among-site variation in environmental factors, will provide a basis for understanding the processes responsible for the diversification of bacteria and symbioses on this planet.

Experimental Procedures

Specimen collection

Mussels were collected using DSV Alvin from the off-axis Lost City hydrothermal vent field (30°07.40'N, 42°07.24'W; 800 m deep) and from the Lucky Strike (37°17.26'N, 32°16.50'W; 1693 m deep) and Snake Pit (23°22.10'N, 44°56.91'W; 3492 m deep) vent sites on the MAR (Fig. 1). Specimens were preserved for ultrastructural analysis or stored at -80°C. Symbiont-bearing gill tissue was fixed, embedded, and examined by transmission electron microscopy (Distel et al., 1995). DNA was extracted from the frozen gill tissue of the two Lost City mussels, 20 individuals of *Bathymodiolus azoricus* from Lucky Strike and 20 of *B. puteoserpentis* from Snake Pit with DNeasy Tissue Extraction Kits (Qiagen, Valencia, CA).

Genetic sampling and analyses

To resolve evolutionary relationships, the symbiont(s) 16S rRNA gene was amplified using the universal bacteria primers 27f and 1492r (Weisburg et al., 1991), from multiple specimens of the three vent sites, gel purified (Qiagen Gel Extraction Kit), and cloned (TOPO TA Cloning Kit; Invitrogen Corp., Frederick, MD). Thirty-two clones per host population were analyzed (16 for each of the two mussels from the Lost City and two clones for each of 16 mussels at both the Lucky Strike and Snake Pit sites). The legitimacy of point mutations in all unique phylotypes was evaluated using ARB (Ludwig et al., 2004) by assessing complementary base pairing on the 16S rRNA secondary structure and by following the sequence conservation rule (Acinas et al., 2004).

To estimate within- and among-population genetic variation, sequence data from the polymorphic 16S-ITS-23S region of the chemoautotrophs was used. The marker was

amplified using two symbiont-specific primer combinations: Sym-ITS-830F and Sym-ITS-23SR; Sym-ITS-1322F and Sym-ITS-23SR (Won et al., 2003). The former primer set was used to confirm symbiont species identification, because it yielded an 1800 nucleotide sequence including approximately 600 bp of 16S rRNA. The latter pair provided the ITS sequences for population genetic analyses. Ninety clones from the Sym-ITS-1322F and Sym-ITS-23SR amplicons were sequenced from each of the two Lost City mussels. For both the Lucky Strike and Snake Pit populations, 143 clones were sequenced from 20 host individuals.

For each locus, forward and reverse strands were cycle sequenced using the M13 primer pair, the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Atlanta, GA), cleaned with Performa DTR 96-well Std. Plate Kit (Edge BioSystems, Gaithersburg, MD), and sequenced on an ABI 3730 Gene Analyzer. Sequences were edited in Sequencher 4.1.2 (Gene Codes Corp.), aligned in ClustalX (Thompson et al., 1997), and alignments were manually edited in MacClade 4.0 (Maddison and Maddison, 2003).

The phylogenetic relationships among the 1303 bp portion of the 16S rRNA phylotypes from this and previous studies of bathymodioline symbionts (with *Escherichia coli* as an outgroup; see Table 3 for Genbank accession numbers) were inferred using maximum parsimony in PAUP 4.10b (Swofford, 2003) and Bayesian posterior probabilities implemented with MrBayes v3.0b4 (Huelsenbeck and Ronquist, 2001). Maximum parsimony trees were generated on PAUP 4.10b (Swofford, 2003), with heuristic searches, random sequence addition with 100 replicates, and TBR branch swapping. Significance was determined from a 1000 replicate bootstrap analysis using the same search parameters. From the Bayesian analysis, using four-chain Metropolis-

coupled Markov chain Monte Carlo (MCMCMC) analysis, a consensus tree of 11,000 post burn-in sampled trees was generated in PAUP 4.10b (Swofford, 2003). Both Bayesian and parsimony analyses yielded similar inferences of evolutionary history.

Intraspecific phylogenies were inferred from the ITS sequences for the chemoautotrophic symbionts using parsimony implemented in the TCS software package (Clement et al., 2000) and a combination of Bayesian and maximum likelihood analyses. Eighty-five nucleotides of tRNA-Ala and 77 bp of tRNA-Ile occurred within the 16S-ITS-23S sequence. The 1200 bp of ITS included five indels at positions 361-364, 542-543, 711, 937-939, and 966-982, which were each transformed into a single polymorphic position (Widmer and Baltisberger, 1999). After converting the indels to one base substitution each, 48 polymorphic sites were described for the remaining 1178 bp, of which 36 sites were parsimony informative. For the Bayesian analysis, post burn-in trees were imported into PAUP 4.10b (Swofford, 2003) and sorted to choose the maximum likelihood tree. The parsimony and maximum likelihood trees were similar and the few differences did not affect any conclusions.

Within-population genetic variation and among-population genetic differentiation were estimated to test the relationships between the Lost City chemoautotrophic endosymbionts and the two populations on the MAR. All measures were averaged across individuals from the population to account for potential PCR bias. First, an overall measure of genetic diversity for haploid genomes ($\theta = 2Ne\mu$) for all populations and the amount of genetic divergence (T) between northern and southern clades (see Results) were estimated using MDIV (Nielsen and Wakeley 2001) assuming the HKY finite sites model and running the coalescent simulations three times for each species to evaluate convergence for each parameter. Within-population genetic diversity

was estimated based on the average pair-wise nucleotide diversity (θ_{π}) and the number of segregating sites (θ_W) for haploid genomes (e.g., Herbeck et al., 2003). In addition, both estimators of θ should be equivalent in a population at equilibrium that is evolving neutrally. Tajima's D was used to compare the two estimators of θ and examine whether populations were at equilibrium (Tajima, 1989). To test whether or not populations of bacterial symbionts were isolated, the mean pair-wise differences and degree of differentiation (F_{ST}) among locations were estimated (e.g., Whitaker et al., 2003). Isolation-by-distance was tested (Rousset, 1997), with distances among sites as follows: Lost City to Lucky Strike (1253 km), Lost City to Snake Pit (832 km), and Lucky Strike to Snake Pit (2037 km). All analyses were performed using Arlequin 2.0 (Schneider et al., 2000).

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References

- Acinas, S. G., V. Klepac-Ceraj, D. E. Hunt, C. Pharino, I. Ceraj, D. L. Distel, M. F. Polz. 2004. Fine-scale phylogenetic architecture of a complex bacterial community. *Nature* **430**: 551-554.
- Antón, A. I., A. J. Martínez-Murcia, F. Rodríguez-Valera. 1998. Sequence diversity in the 16S-23S intergenic spacer region (ISR) of the rRNA operons in representatives of the *Escherichia coli* ECOR collection. *J. Mol. Evol.* **47**: 62-72.
- Belkin, S., D. C. Nelson, and H. W. Jannasch. 1986. Symbiotic assimilation of CO₂ in two hydrothermal vent animals, the mussel *Bathymodiolus thermophilus* and the tubeworm *Riftia pachyptila*. *Biol. Bull.* **170**: 110-121.
- Budavari, S. 1996. *The Merck Index - An Encyclopedia of Chemicals, Drugs, and Biologicals*. Whitehouse Station, NJ: Merck and Co., Inc., p. 823.
- Cary, S. C. and S. J. Giovannoni. 1993. Transovarial inheritance of endosymbiotic bacteria in clams inhabiting deep-sea hydrothermal vents and cold seeps. *Proc. Natl. Acad. Sci. USA.* **90**: 5695-5699.
- Cavanaugh, C. M., C. Wirsén, and H. J. Jannasch. 1992. Evidence for methylotrophic symbionts in a hydrothermal vent mussel (Bivalvia: Mytilidae) from the Mid-Atlantic Ridge. *Appl. Environ. Microbiol.* **58**: 3799-3803.
- Cavanaugh, C.M., Z.P. McKiness, I.L.G. Newton, and F.J. Stewart. 2005. Marine chemosynthetic symbioses. In M. Dworkin et al., Eds., *The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community*, Springer-Verlag, New York.
- Clement, M., D. Posada, K. A. Crandall. 2000. TCS: a computer program to estimate gene genealogies. *Molec. Ecol.* **9**: 1657-1659.

- 416 DeChaine, E. G. and C. M. Cavanaugh. 2005. Symbioses of methanotrophs and deep-
 417 sea mussels (Mytilidae: Bathymodiolinae). In J. Overmann, Ed., *Molecular*
 418 *Basis of Symbiosis*, Sinauer Assoc. In press.
- 419 Distel, D., H. K. Lee, and C. M. Cavanaugh. 1995. Intracellular coexistence of
 420 methano- and thioautotrophic bacteria in a hydrothermal vent mussel. *Proc.*
 421 *Natl. Acad. Sci. USA.* **92**: 9598-9602.
- 422 Duperron, S. C. Bergin, F. Zielinski, Z. P. McKiness, E. G. DeChaine, M. Sibuet, C. M.
 423 Cavanaugh, and N. Dubilier. 2005. A dual symbiosis shared by two
 424 bathymodioline mussels (Bivalvia: Mytilidae) from the Mid-Atlantic Ridge.
 425 *Environ. Microbiol.* In review.
- 426 Feldman, R., Black, M., Cary, C., Lutz, R., and R. Vrijenhoek. 1997. Molecular phylogenetics
 427 of bacterial endosymbionts and their vestimentiferan hosts. *Molec. Mar. Biol. Biotech.*
 428 **6**: 268-277.
- 429 Fenchel, T. 2003. Biogeography for bacteria. *Science* **301**: 925-926.
- 430 Fisher, C. R. 1990. Chemoautotrophic and methanotrophic symbioses in marine
 431 invertebrates. *Rev. Aqua. Sci.* **2**: 399-436.
- 432 Fiala-Médioni, A., Z. McKiness, P. Dando, J. Boulegue, A. Mariotti, A. Alayse-Danet,
 433 J. Robinson, and C. Cavanaugh. 2002. Ultrastructural, biogchemical, and
 434 immunological characterization of two populations of a new species of Mytilid
 435 mussel, *Bathymodiolus azoricus*, from the Mid-Atlantic Ridge: evidence for a
 436 dual symbiosis. *Mar. Biol.* **141**: 1035-1043.
- 437 Finlay, B. J. 2002. Global dispersal of free-living microbial eukaryote species. *Science* **296**:
 438 1061-1063.

- 439 Fröh-Green, G. L., D. S. Kelley, S. M. Bernasconi, J. A. Karson, K. A. Ludwig, D. A.
 440 Butterfield, C. Boschi, and G. Proskurowski. 2003. 30,000 years of
 441 hydrothermal activity at the Lost City vent field. *Science* **301**: 495-498.
- 442 Herbeck, J. T., D. J. Funck, P. H. Degnan, & J. J. Wernegreen. 2003. A conservative
 443 test of genetic drift in endosymbiotic bacterium *Buchnera*: Slightly deleterious
 444 mutations in the chaperonin groEL. *Genetics* **165**: 1651-1660.
- 445 Huelsenbeck, J. P. & Ronquist, F. 2001. MrBayes: Bayesian inference of phylogenetic
 446 trees. *Bioinformatics* **17**, 754-755.
- 447 Hurtado, L. A., m. Mateos, R. A. Lutz, and R. C. Vrijenhoek. 2003. Coupling of bacterial
 448 endosymbiont and host mitochondrial genomes in the hydrothermal vent clam
 449 *Calymene magnifica*. *Applied Environ. Microbiol.* **69**: 2058-2064.
- 450 Juniper, S. K. and V. Tunnicliffe. 1997. Crustal accretion and the hot ecosystem. *Philos. Trans.*
 451 *R. Soc. Lond. A* **355**: 450-474.
- 452 Kelley, D. S., J. A. Karson, D. K. Blackman, G. L. Fröh-Green, D. A. Butterfield, M.
 453 D. Lilley, E. J. Olson, M. O. Schrenk, K. K. Roe, G. T. Lebon, P. Rivizzigno,
 454 and the AT3-60 Shipboard Party. 2001. An off-axis hydrothermal vent field near
 455 the Mid-Atlantic Ridge at 30°N. *Nature* **412**: 145-149.
- 456 Kelley, D. S., Karson, D. K., Fröh-Green, G. L., Yoerger, D. R., Shank, T. M.,
 457 Butterfield, D. A., Hayes, J. M., Schrenk, M. O., Olson, E. J., Proskurowski, G.,
 458 Jakuba, M., Bradley, A., Larson, B., Ludwig, K., Glickson, D., Buckamn, K.,
 459 Bradley, A. S., Brazelton, W. J., Roe, K., Elend, M. J., Delacour, A.,
 460 Baernasconi, S. M., Lilley, M. D., Baross, J. A., Summons, R. E., and S. P.
 461 Sylva. 2005. A serpentinite-hosted ecosystem: The Lost City Hydrothermal
 462 Field. *Science* **307**: 1428-1434.

- 463 Kirchman, D. L., Dittel, A. I., Malmstrom, R. R., and M. T. Cottrell. 2005. Biogeography of
464 major bacterial groups in the Delaware estuary. *Limnol. Oceanog.* **50**: 1697-1706.
- 465 Ludwig, W., O. Strunk, R. Westram et al. 2004. ARB: a software environment for
466 sequence data. *Nucleic Acid Res.* **32**: 1363-1371.
- 467 Maddison, D. R. and W. P. Maddison. 2000. *MacClade 4*. Sinauer Assoc. Inc.
468 Sunderland, MA.
- 469 Nelson, D. C. and C. R. Fisher. 2000. Absence of cospeciation in deep-sea
470 vestimentiferan tubeworms and their bacterial endosymbionts. *Symbiosis*. **28**: 1-
471 15.
- 472 Nelson, D. C., K. D. Hagan, and D. B. Edwards. 1995. The gill symbiont of the
473 hydrothermal vent mussel *Bathymodiolus thermophilus* is a psychrophilic,
474 chemoautotrophic, sulfur bacterium. *Mar. Biol.* **121**: 487-495.
- 475 Nielsen, R., and J. W. Wakeley. 2001. Distinguishing migration from isolation: and
476 MCMC approach. *Genetics* **158**: 885-896.
- 477 Papke, R. T., N. B. Ramsing, M. M. Bateson, and D. M. Ward. 2003. Geographical
478 isolation in hot spring cyanobacteria. *Environ. Microbiol.* **5**: 650-659.
- 479 Robinson, J. J., M. F. Polz, A. Fiala-Médioni, and C. M. Cavanaugh. 1998.
480 Physiological and immunological evidence for two distinct C-1-utilizing
481 pathways in *Bathymodiolus puteoserpentis* (Bivalvia: Mytilidae), a dual
482 endosymbiotic mussel from the Mid-Atlantic Ridge. *Mar. Biol.* **132**: 625-633.
- 483 Rousset, F. 1997. Genetic differentiation and estimation of gene flow from F-statistics
484 under isolation by distance. *Genetics* **145**: 1219-1228.

- 485 Schneider, S., D. Roessli, and L. Excoffier. 2000. *ARLEQUIN, a Software Package for*
 486 *Population Genetics Data Analysis, Version 2.0*. Genetica and Biometry
 487 Laboratory, Univ. of Geneva. Geneva, Switzerland.
- 488 Slatkin, M. 1987. Gene flow and the geographic structure of natural populations.
 489 *Science* 236: 787-792.
- 490 Stewart, F. J., I. L. G. Newton, and C. M. Cavanaugh. 2005. Chemosynthetic
 491 endosymbioses: adaptations to oxic-anoxic interfaces. *Trends Microbiol.* **13**:
 492 439-448.
- 493 Swofford, D. L. 2003. *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other*
 494 *Methods)*. Sinauer Associates, Sunderland, MA.
- 495 Tajima, F. 1989. The effect of change in population size on DNA polymorphism.
 496 *Genetics* **123**: 597-601.
- 497 Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997.
 498 The ClustalX windows interface: flexible strategies for multiple sequence
 499 alignment aided by quality analysis tools. *Nucl. Acid Res.* **24**: 4876-4882.
- 500 Tunnicliffe, V. 1988. Biogeography and evolution of hydrothermal-vent fauna in the eastern
 501 Pacific Ocean. *Proc. R. Soc. Lond. B* **233**: 347-366.
- 502 Tunnicliffe, V. 1991. The biology of hydrothermal vents: Ecology and evolution. *Oceanogr.*
 503 *Mar. Biol. Annu. Rev.* **29**: 319-407.
- 504 Tunnicliffe, V. and C. Fowler. 1996. Influence of sea-floor spreading on the global
 505 hydrothermal vent fauna. *Nature* **379**: 531-533.
- 506 Tunnicliffe, V., A. G. McArthur, and D. Mchugh. 1998. A biogeographical perspective of the
 507 deep-sea hydrothermal vent fauna. *Advances in Marine Biology* **34**: 353-442.
- 508 Van Dover, C. L. 1995. Ecology of Mid-Atlantic Ridge hydrothermal vents. In: Parson,

- 509 L. M., C. L. Walker, and D. R. Dixon (eds.). *Hydrothermal Vents and Processes*. *Geol.*
 510 *Soc. Spec. Publ.* **87**: 257-294.
- 511 Van Dover, C. L. 2000. *The Ecology of Deep-sea Hydrothermal Vents*. Princeton Univ. Press.
 512 Princeton, N. J.
- 513 Van Dover, C. L., C. R. German, K. G. Speer, L. M. Parson, and R. C. Vrijenhoek.
 514 2002. Evolution and biogeography of deep-sea vent and seep invertebrates.
 515 *Science* **295**: 1253-1257.
- 516 Vrijenhoek, R. C. 1997. Gene flow and genetic diversity in naturally fragmented
 517 metapopulations of deep-sea hydrothermal vent animals. *J. Heredity* **88**: 285-293.
- 518 Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane. 1991. 16S ribosomal
 519 DNA amplification for phylogenetic study. *J. Bacteriol.* **173**: 697-703.
- 520 Whitaker, R. J., S. W. Grogan, and J. W. Taylor. 2003. Geographic barriers isolate
 521 endemic populations of hyperthermophilic archaea. *Science* **301**: 976-978.
- 522 Widmer, A. and M. Baltisberger. 1999. Extensive intraspecific chloroplast DNA
 523 (cpDNA) variation in the alpine *Draba aizoides* L. (Brassicaceae): haplotype
 524 relationships and population structure. *Molec. Ecol.* **8**: 1405-1415.
- 525 Woese, C. R. 1987. Bacterial evolution. *Microbiol. Reviews* **51**: 221-271.
- 526 Won, Y., S. J. Hallam, G. D. O'Mullan, I. L. Pan, K. R. Buck, and R. C. Vrijenhoek.
 527 2003. Environmental acquisition of thiotrophic endosymbionts by deep-sea
 528 mussels of the genus *Bathymodiolus*. *Appl. Env. Microbiol.* **69**: 6785-6792.
 529

529 Table 1. Summary statistics for ITS sequences from the three populations of
 530 chemoautotrophic symbionts.

531

532	Location	Host Species	θ_{π} (sd)	θ_w (sd)	Tajima's D
533	Lucky Strike	<i>B. azoricus</i>	2.4 (1.4)	2.8 (1.0)	-0.63
534	Lost City	<i>B. aff. azoricus</i>	2.3 (1.4)	2.5 (0.8)	-0.23
535	Snake Pit	<i>B. puteoserpentis</i>	0.7 (0.6)	1.9 (0.7)	-1.67*

536

537 Estimates of genetic diversity (θ) based on the average pair-wise sequence divergence
 538 (π) and number of segregating sites (W) are shown, with standard deviations (sd).

539 Estimates of Tajima's D are given for each population (*denotes $p < 0.01$).

540

540 Table 2. Pair-wise comparisons of populations of chemoautotrophs hosted by
 541 bathymodioline mussels.

542

543 Mean pair-wise differences

544 Location	Lucky Strike	Lost City	Snake Pit
--------------	--------------	-----------	-----------

545 Lucky Strike		1.2	11.7
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546 Lost City	0.34		12.4
---------------	------	--	------

547 Snake Pit	0.89	0.89	
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548 Population pair-wise F_{ST}

549

550 Mean pair-wise differences for chemoautotroph ITS sequences are shown above the
 551 diagonal, and population pair-wise F_{ST} values are given below. All F_{ST} values are
 552 significant.

553

553 Table 3. List of bacteria and Genbank accession numbers used to generate the 16S
 554 rRNA phylogeny for γ -Proteobacteria (Fig. 3).

555	Environment	Species	Genbank accession no.
556	Free-living bacteria		
557		<i>Achromatium oxaliferum</i>	L48227
558		<i>Beggiatoa alba</i>	L40994
559		<i>Escherichia coli</i>	J01695
560		<i>Halomonas elongata</i>	X67023
561		<i>Hydrogenovibrio marinus</i>	D86374
562		<i>Methylobacter whittenburyi</i>	X72773
563		<i>M. capsulatus</i>	L20843
564		<i>M. luteus</i>	AF304195
565		<i>M. vinelandii</i>	L20841
566		<i>Methylomicrobium agile</i>	X72767
567		<i>M. pelagicum</i>	L35540
568		<i>Methylomonas methanica</i>	AF150806
569		<i>M. rubra</i>	AF150807
570		<i>Pseudomonas mendocina</i>	AF232713
571		<i>Rhabdochromatium marinum</i>	X84316
572		<i>Thiocystis gelatinosa</i>	Y11317
573		<i>Thiomicrospira thyasirae.</i>	AF16046
574			
575	Chemoautotrophic symbionts		
576	Host Taxonomy	Host species	

577	Phylum Annelida		
578	Oligochaeta	<i>Inanidrilus leukodermatus</i>	U24110
579		<i>Olavius loisae</i>	AF104472
580	Vestimentifera	<i>Escarpia spicata</i>	U77482
581		<i>Lamellabrachia columna</i>	U77481
582		<i>Ridgeia piscesae</i>	U77480
583		<i>Riftia pachyptila</i>	M99451
584	Phylum Mollusca		
585	Bivalvia		
586	Lucinidae	<i>Codakia orbicularis</i>	X84979
587		<i>Lucina nassula</i>	X84980
588		<i>Lucinoma aequizonata</i>	M99448
589	Mytilidae	<i>Bathymodiolus</i> aff. <i>brevior</i>	DQ077891
590		<i>B. puteoserpentis</i>	U29163
591		<i>B. azoricus</i> - <i>puteoserpentis</i>	AM083974 and this study
592		<i>B. septemdierum</i>	AB036709
593		<i>B. thermophilus</i>	M99445
594		<i>B. sp.</i> Gabon Margin	AJ745718
595		<i>B. sp.</i> Juan de Fuca	Z. McKiness unpub. data
596	Thyasiridae	<i>Thyasira flexuosa</i>	L01575
597	Vesicomyidae	<i>Calyptogena elongata</i>	AF035719
598		<i>C. fossajaponica</i>	AB044744
599		<i>C. phaseoliformes</i>	AF035724
600		<i>C. kilmeri</i>	AF035720

601		<i>C. magnifica</i>	AF035721
602		<i>C. pacifica</i>	AF035723
603		<i>Ectenogena extenta</i>	AF035725
604		<i>Vesicomys gigas</i>	AF035726
605	Phylum Nematoda		
606	Desmodoridae	<i>Laxus</i> sp.	U241110
607			
608	Methanotrophic symbionts		
609	Host Taxonomy	Host species	
610	Phylum Mollusca		
611	Bivalvia		
612	Mytilidae	<i>B. puteoserpentis</i> M	U29164
613		<i>B. azoricus</i> - <i>puteoserpentis</i> M	AM083950 and this study
614		<i>B. childressi</i>	U05595
615		<i>B. japonicus</i>	AB036711
616		<i>B. platifrons</i>	AB036710
617		<i>B. sp.</i> Gabon Margin M	AJ745717
618			

Figure Legends

Figure 1. Map of study sites and the Mid-Atlantic Ridge (MAR). The collection sites, including the Lost City, Lucky Strike, and Snake Pit vent fields, are designated by white circles and labelled. The MAR and its dominant fracture zones (F. Z.) are highlighted by black lines. Several fracture zones relevant to the discussion are also labelled to emphasize the geographically discontinuous nature of the MAR.

Figure 2. Transmission electron micrographs of endosymbionts within the gill tissue of a Lost City mussel. A. Chemoautotrophic (C) and type I methanotrophic (M) symbionts within the apical portion of two bacteriocytes (bc) separated by a symbiont-free intercalary cell (ic). Scale bar = 2 μm . B. Higher magnification of the two symbiont morphotypes; note intracytoplasmic membranes of the type I methanotroph. Scale bar = 0.5 μm .

Figure 3. Phylogeny of chemoautotrophic and methanotrophic endosymbionts hosted by bathymodioline mussels and free-living γ -Proteobacteria, inferred from 16S rRNA gene sequences (1303 nucleotides). Posterior probabilities from 11,000 bayesian trees are shown above branches (significant ≥ 95) and bootstrap values based on 1000 maximum parsimony replicates are given below the branches. The two phylotypes in this study (*B. azoricus* - *B. puteoserpentis*) are boxed in gray and lettered (C and M) for chemoautotrophs and methanotrophs, respectively. Furthermore, the two clades that

include mussel symbionts are boxed and labelled (C and M). All symbiotic bacteria are labelled 'symbiont' while free-living bacteria are designated by taxonomic name alone.

Figure 4. Parsimony network inferred from 1200 nucleotides of *rrn* internal transcribed spacer (ITS rRNA) genotypes from chemoautotrophic symbionts of *Bathymodiolus* mussels collected from the Lost City, Lucky Strike, and Snake Pit hydrothermal fields. ITS-genotypes are shown as circles, with size indicating relative frequency. Shading denotes location and the distribution of genotypes within the host mussel population as follows: Lucky Strike (black = genotypes found in >1 host individual, black checkerboard = genotypes restricted to only one host individual), Lost City (gray, no symbionts were shared between the two host individuals), and Snake Pit (white = genotypes found in >1 host individual, gray checkerboard = genotypes restricted to only one host individual). Lines connecting genotypes are one nucleotide difference. Small black dots represent unsampled, hypothetical ancestors. LC1 and LC2 designate genotype clades from the two individual Lost City mussels. Finally, the northern and southern 'clades' are boxed and labelled N and S, respectively.

655 Figure 1.

656

657

657 Figure 2.

658

659 Figure 3.

661 Figure 4.

Host Mussel	Genotype occurrence		Genotype Frequency						
	1 mussel	>1 mussel	1-2	3-5	6-10	11-15	15-20	21-50	51+
Lucky Strike <i>B. azoricus</i>	⊕	●	○	○	○	○	○	○	○
Lost City (LC) <i>B. aff. azoricus</i>	●	n/a							
Snake Pit <i>B. puteoserpentis</i>	⊕	○							
Unsampled	•								

662

663