

A dual symbiosis shared by two mussel species, *Bathymodiolus azoricus* and *Bathymodiolus puteoserpentis* (Bivalvia: Mytilidae), from hydrothermal vents along the northern Mid-Atlantic Ridge

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Summary

Bathymodiolus azoricus and *Bathymodiolus puteoserpentis* are symbiont-bearing mussels that dominate hydrothermal vent sites along the northern Mid-Atlantic Ridge (MAR). Both species live in symbiosis with two physiologically and phylogenetically distinct Gammaproteobacteria: a sulfur-oxidizing chemoautotroph and a methane-oxidizer. A detailed analysis of mussels collected from four MAR vent sites (Menez Gwen, Lucky Strike, Rainbow, and Logatchev) using comparative 16S rRNA sequence analysis and fluorescence *in situ* hybridization (FISH) showed that the two mussel species share highly similar to identical symbiont phylotypes. FISH observations of symbiont distribution and relative abundances showed no obvious differences between the two host species. In contrast, distinct differences in relative symbiont abundances were observed between mussels from different sites, indicating that vent chemistry may influence the relative abundance of thiotrophs and methanotrophs in these dual symbioses.

Introduction

Mussels of the subfamily Bathymodiolinae (family Mytilidae) inhabit hydrothermal vents and cold seeps around

the world (Von Cosel, 2002). Their adaptive success in such challenging environments that are largely devoid of photosynthetic primary production and subject to rapid changes in geochemical regimes is due to their symbiotic association with chemosynthetic bacteria within their gills (Fisher, 1990; Cavanaugh *et al.*, 2005). Two phylogenetically and physiologically distinct types of Gammaproteobacteria occur as symbionts in bathymodioline mussels: small chemoautotrophic bacteria shown to oxidize sulfide in at least one species (Nelson *et al.*, 1995), and methanotrophic bacteria with typical stacked internal membranes that use methane both as an electron and a carbon source (Cavanaugh *et al.*, 1987; Fisher, 1993; Distel and Cavanaugh, 1994). Some bathymodioline mussels harbour only a thiotrophic or a methanotrophic symbiont, while others live in a dual symbiosis, with both types of bacteria co-occurring in the gill bacteriocytes (DeChaine and Cavanaugh, 2005). These symbioses enable their hosts to colonize sulfide and/or methane rich environments, in which the primary production of the symbionts ensures the nutrition of the host (Fisher *et al.*, 1987; Fisher and Childress, 1992; Distel and Cavanaugh, 1994; DeChaine and Cavanaugh, 2005).

Two species of bathymodioline mussels occur along the northern Mid-Atlantic Ridge (MAR) which are morphologically and genetically well differentiated (Craddock *et al.*, 1995; Maas *et al.*, 1999). *Bathymodiolus azoricus* occurs at the northernmost sites (Menez Gwen, Lucky Strike, Rainbow), while *Bathymodiolus puteoserpentis* is found at the two vent sites closest to the equator (Snake Pit and Logatchev), with a hybrid zone where both species co-occur at Broken Spur (O'Mullan *et al.*, 2001) (Fig. 1). These two mussel species harbour both thiotrophic and methanotrophic symbionts, based on transmission electron microscopy (TEM), and enzymatic and physiological assays (Cavanaugh *et al.*, 1992; Fiala-Médioni *et al.*, 2002). Studies using 16S rRNA sequence analysis and fluorescence *in situ* hybridization (FISH) confirmed the simultaneous presence of thiotrophic and methanotrophic phylotypes in gill cells of *B. puteoserpentis* from the Snake Pit site (Distel *et al.*, 1995). The relative abundance of thiotrophs and methanotrophs, and stable carbon isotope signatures of symbiotic gill tissue can vary both between

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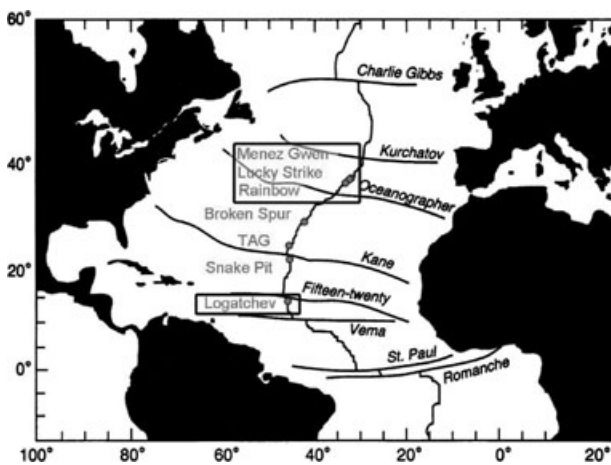


Fig. 1. Hydrothermal vent sites on the northern MAR. Sites where mussels were collected for this study are framed. *Bathymodiolus azoricus* is found at Menez Gwen, Lucky Strike, and Rainbow, *B. puteoserpentis* at Snake Pit and Logatchev, and both species co-occur at Broken Spur (no mussels occur at TAG).

mussels from a single site, as well as between mussels from different vent sites, indicating that the symbiosis may respond flexibly to differences in vent chemistry (Trask and Van Dover, 1999; Colaco *et al.*, 2002; Fiala-Médioni *et al.*, 2002).

In the present study, our aim was to determine whether *Bathymodiolus azoricus* and *B. puteoserpentis* share the same symbiotic 16S rRNA phylotypes. This was first shown by Won and colleagues (2003) for the thiotrophic symbiont in these two mussel species, but not investigated for the methanotrophic symbiont and not confirmed for either symbiont type with FISH. For this study, symbionts were examined in mussels collected from four vents along the northern MAR, *B. azoricus* from Menez Gwen, Lucky Strike, and Rainbow, and *B. puteoserpentis* from Logatchev. The 16S rRNA sequences from the mussel symbionts were compared with published symbiont sequences from *B. azoricus* collected at Rainbow (Won *et al.*, 2003) and *B. puteoserpentis* from Snake Pit (Distel *et al.*, 1995; Won *et al.*, 2003). FISH was used to confirm that the obtained sequences originated from symbionts in the gills and to examine whether there are differences in symbiont distribution and relative abundance between the two mussel species and between mussels from different vent sites.

Results and discussion

Symbiont diversity and phylogeny

The 16S rRNA genes of bacterial symbionts from *Bathymodiolus azoricus* and *B. puteoserpentis* specimens collected at the MAR vent sites Menez Gwen, Lucky Strike, Rainbow, and Logatchev were sequenced and compared

with published symbiont sequences from *B. azoricus* and *B. puteoserpentis* specimens collected at the MAR vent sites Rainbow and Snake Pit (Distel *et al.*, 1995; Won *et al.*, 2003) (Fig. 2). In both host species and at all four vent sites, all individuals contained two dominant bacterial phylotype groups in their 16S rRNA clone libraries. Within each phylotype group, sequences were highly similar with at least 99.7% sequence identity, while the difference between the two phylotype groups was at least 11%. Blast searches indicated one phylotype was more than 99% identical to the thiotrophic symbiont sequence of *B. puteoserpentis* from Snake Pit (Distel *et al.*, 1995; Won *et al.*, 2003) and *B. azoricus* from Rainbow (Won *et al.*, 2003), and the other to the methanotrophic symbiont of *B. puteoserpentis* from Snake Pit (Distel *et al.*, 1995). A third phylotype (AM083967) related to methanotrophic *Bathymodiolus* symbionts, but differing from the dominant methanotroph phylotype by 3%, was found in only two clones (out of 50) from a single Menez Gwen *B. azoricus* individual.

Identity matrices, critical visual examination of the full alignment of the dominant MAR mussel symbiont sequences, and comparison to all bathymodioline symbiont sequences in the database, showed that out of ~1500 nucleotide positions, there were 0–3 nucleotide substitutions in each thiotrophic symbiont sequence. The majority of the nucleotide substitutions in the thiotrophic sequences were unique and not found in other sequences or individuals. Only three nucleotide substitutions in a single thiotrophic sequence from a Rainbow mussel (AM083974) were shared with a phylotype identified as rare in Rainbow mussels by Won and colleagues (2003) (Table 1). In the methanotrophic sequences, there were 0–7 nucleotide substitutions in each symbiont sequence, of which the majority were unique. Shared substitutions only occurred at two nucleotide positions, with a dominant Logatchev phylotype showing one substitution to mussels from all other sites and a less common Logatchev phylotype showing two substitutions to all other methanotrophic symbiont sequences (Table 1). These substitutions are not typical for *B. puteoserpentis* symbionts, because at Snake Pit, the symbionts of this mussel species are identical to the dominant phylotype (Distel *et al.*, 1995).

Most of the unique nucleotide substitutions occurred in highly conserved regions of the 16S rRNA gene or resulted in miss-pairings of stem regions, indicating that these were caused by polymerase chain reaction (PCR) or sequencing error. In contrast, the shared nucleotide substitutions shown in Table 1 are assumed to be real because they were observed in more than one individual. However, these 2–3 nucleotide differences were not host specific, that is only in *B. azoricus* or *B. puteoserpentis* symbionts, and for the thiotrophs also not site specific. They can therefore not be used for biogeography analy-

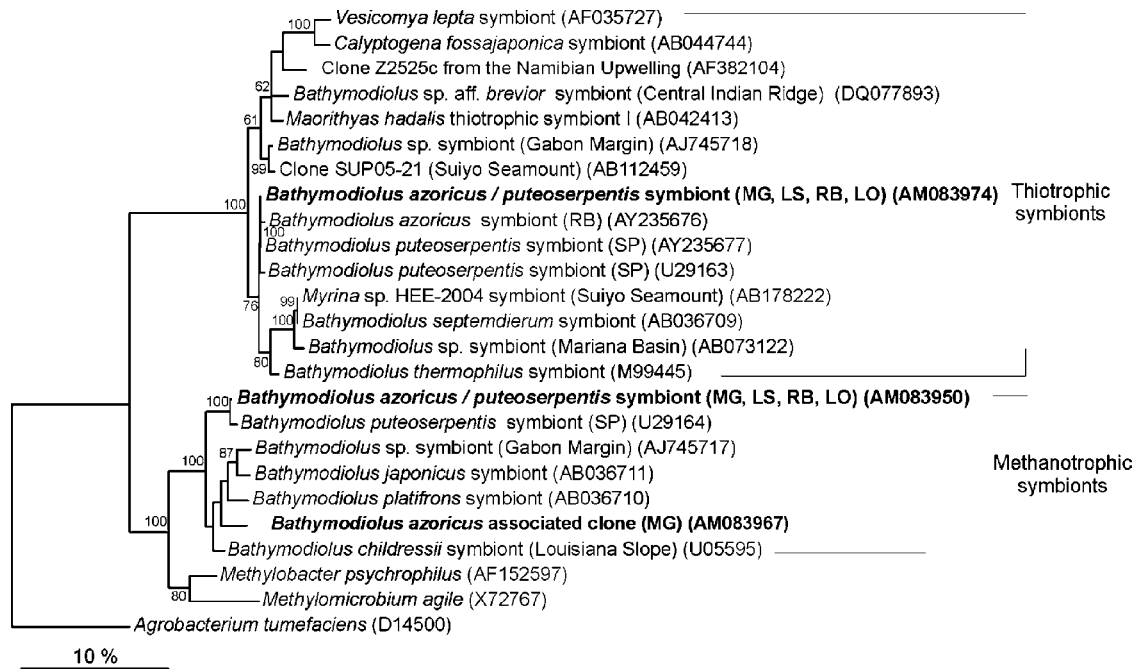


Fig. 2. Maximum likelihood tree based on 16S rRNA sequences from the thiotrophic and methanotrophic endosymbionts of bathymodioline mussels from the northern Mid-Atlantic Ridge (highlighted in bold) within the γ -Proteobacteria. *Agrobacterium tumefaciens* (α -proteobacteria) is included as an outgroup; 1309 nt positions were used; $L = -5906$. Bootstrap percentages obtained from 200 ml of replicates (values > 60 shown). Scale bar represents 10% estimated nucleotide substitution.

ses or to discern relationships between the symbionts of these two mussel species. Finally and most importantly, such a minimal degree of sequence divergence, namely only 0.1–0.2% is not phylogenetically informative and would not allow the reconstruction of a robust tree. We thus conclude that *Bathymodiolus azoricus* and *B. puteoserpentis* individuals collected from vent sites along the MAR share highly similar to identical 16S rRNA phylotypes of the two dominant thiotroph- and methanotroph-related symbionts.

One representative 16S rRNA sequence, with no unique nucleotide substitutions, was chosen for each of the two symbiont phylotypes and used for phylogenetic reconstruction (Fig. 2). The thiotrophic phylotype, together with the sequences previously published from *B. azoricus* and *B. puteoserpentis* (Distel *et al.*, 1995; Won *et al.*, 2003), falls within the larger group of mussel thiotrophic symbionts, and is related to a group of thiotrophic symbionts from hydrothermal vent mussels including *Bathymodiolus thermophilus* (bootstrap value 76). The

Table 1. Shared nucleotide substitutions in the thiotrophic and methanotrophic 16S rRNA phylotypes in *Bathymodiolus* mussels from the Menez Gwen (MG), Lucky Strike (LS), Rainbow (RB), and Logatchev (LO) vent sites.

Thiotroph	Nucleotide position ^a			No. of sequences (out of 19 total)	No. of individuals
	1014	1015	1262		
Dominant phylotype (MG, LS, RB, LO)	T	T	C	18	10
Rare phylotype (RB) ^b	A	A	T	1	1

Methanotroph	Nucleotide position ^a		No. of sequences (out of 17 total)	No. of individuals
	73	82		
Dominant phylotype (MG, LS, RB)	A	C	5	5
Dominant phylotype (LO)	G	C	9	4
Rare phylotype (LO)	A	T	3	2

The rare methanotroph-related sequence found in only a single mussel from MG is not included in this table as it shows more than 3% sequence divergence to all other MAR methanotrophic symbiont sequences.

a. Position based on *Escherichia coli* numbering.

b. These three differences are shared with the rare thiotroph phylotype identified by Won and colleagues (2003) from a Rainbow mussel specimen (AY235676).

methanotrophic *B. azoricus*/*B. puteoserpentis* phylotype belongs to the monophyletic group of methanotrophic symbionts from other *Bathymodiolus* species, but its phylogenetic relationship to these symbionts is unresolved (bootstrap values below 60). The rare methanotrophic sequence from a single *B. azoricus* individual from Menez Gwen clearly falls within the monophyletic clade of *Bathymodiolus* methanotrophic symbionts (bootstrap value 100), but is only distantly related to the dominant *B. azoricus*/*B. puteoserpentis* methanotroph.

The 16S rRNA gene does not provide enough resolution to distinguish between bacterial species at the strain or substrain level. Instead, regions with higher substitution rates such as the internal transcribed spacer (ITS) of ribosomal operons are more useful for resolving the phylogeny of very closely related bacteria (Roth *et al.*, 1998; Garcia-Martinez *et al.*, 1999). Indeed, for the thiotrophic symbionts of *B. azoricus* and *B. puteoserpentis*, Won and colleagues (2003) and DeChaine and colleagues (submitted) showed that the ITS differs within and between host specimens collected from the same MAR vent site as well as from different MAR vent sites. This indicates that MAR mussels harbour multiple strains of thiotrophic symbionts and unpublished data by S. Hallam (cited in Won *et al.*, 2003) suggests a similar heterogeneous mixture of methanotrophic symbionts in MAR mussels.

Distribution and relative abundance of thiotrophs and methanotrophs

Symbiont specific probes designed to target the dominant thiotrophic and methanotrophic 16S rRNA phylotype hybridized to their respective bacterial targets in the gills of *Bathymodiolus azoricus* and *B. puteoserpentis* (Fig. 3A–C). Both symbionts were detected in the apical part of the gill bacteriocytes, while the basal region and other parts of the gills including the ciliated ends of the filaments showed no signal. The rare methanotroph-related phylotype, found in the clone library from a single Menez Gwen individual, was not detected with FISH in Lucky Strike and Rainbow mussels, despite the design of four probes specific to this sequence. Unfortunately, no mussels from Menez Gwen were fixed for FISH analyses so that it remains unclear whether this phylotype originated from a gill symbiont that only occurs in Menez Gwen mussels.

The distribution of the symbionts in the gill bacteriocytes was similar in individuals from the same site, but differed between sites. In *Bathymodiolus azoricus* from Rainbow (Fig. 3B), thiotrophs occurred in the apical region exposed to the mantle fluid and methanotrophs were more basal within the bacteriocytes. Thiotrophic and methanotrophic symbionts were more evenly distributed within bacteriocytes of *B. azoricus* from Lucky Strike (Fig. 3A) and

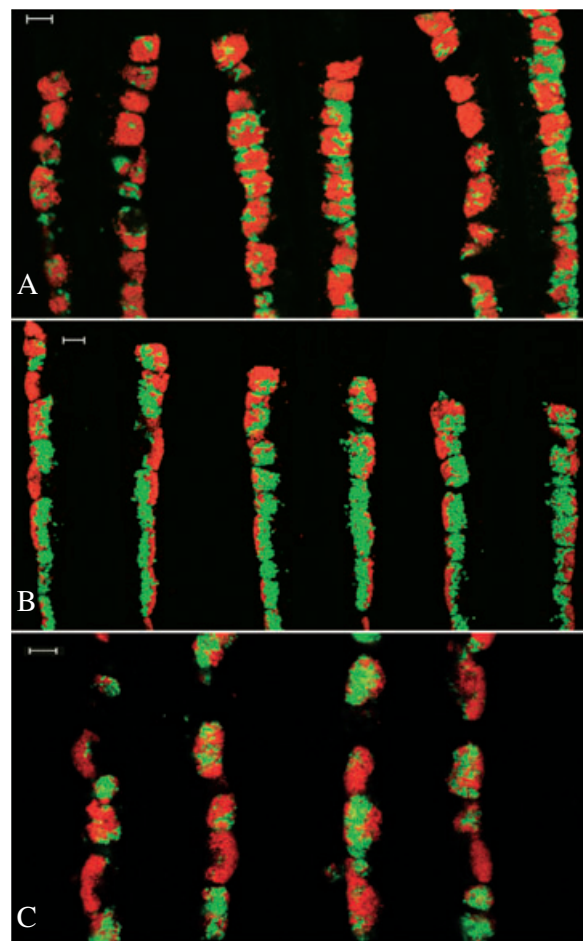


Fig. 3. Gill sections of *Bathymodiolus* specimens from the Mid-Atlantic Ridge hybridized with fluorescently labelled probes specific to the methanotrophic symbiont (shown in green) and the thiotrophic symbiont (shown in red). Three (A and B) or two (C) gill filaments are shown, with a cross section of the descending and ascending lamellae of each filament visible. The ciliated end of each filament is oriented towards the top of each figure. Scale bars = 10 μ m. A. *Bathymodiolus azoricus* from Lucky Strike. B. *Bathymodiolus azoricus* from Rainbow. C. *Bathymodiolus puteoserpentis* from Logatchev.

B. puteoserpentis from Logatchev (Fig. 3C). As with the distribution of the two symbionts, the relative abundance of the methanotrophs and thiotrophs was similar between mussels from the same site, but differed between vent sites. Methanotrophs were more abundant than thiotrophs in *B. azoricus* from Rainbow (Fig. 3B). In contrast, *B. azoricus* from Lucky Strike had more thiotrophs than methanotrophs (Fig. 3A), resembling *B. puteoserpentis* from Snake Pit (Distel *et al.*, 1995). The ratio of methanotrophs to chemoautotrophs in *B. puteoserpentis* from Logatchev (Fig. 3C) was intermediate between the two *B. azoricus* described above, but specimens of this population had more methanotrophs than the previously described *B. puteoserpentis* from Snake Pit (Distel *et al.*, 1995).

Table 2. Sulfide and methane concentrations and ratios in end-member fluids from the Lucky Strike (LS), Rainbow (RB), Snake Pit (SP), and Logatchev (LO) vent sites, and distribution and relative abundance of thiotrophic (SOX) and methanotrophic (MOX) symbionts in the gill cells of mussels based on qualitative FISH observations from this study (LS, RB, LO) and Distel and colleagues (1995) (SP).

Site	Species	H ₂ S (mM)	CH ₄ (mM)	H ₂ S/CH ₄	Distribution	Relative abundance
LS	<i>B. azoricus</i>	0.6–3.4	0.5–1.0	1.2–3.4	mixed	SOX > MOX
RB		1.0–2.5	2.2–2.5	0.45–1.0	MOX basal	SOX < MOX
SP	<i>B. puteoserpentis</i>	2.7–6.1	0.05–0.06	54–102	mixed	SOX > MOX
LO		0.8	2.1	0.38	mixed	SOX ≈ MOX

Chemical data are from Douville and colleagues (2002) and Desbruyères and colleagues (2000).

Symbiont distribution and relative abundance are clearly not host specific, as in both *B. azoricus* and *B. puteoserpentis* intraspecific differences were as distinct as those between the two species. A comparison of symbiont distribution and relative abundance to sulfide and methane concentrations in end-member fluids at the MAR vent sites is shown in Table 2. Symbionts were distributed evenly throughout the bacteriocytes at all sites except Rainbow, where methanotrophs were located in the basal and thiotrophs in the apical region of the gill cells. This distribution pattern was first observed in mussels from cold seeps on the Gabon continental margin and was hypothesized to reflect the high ratio of methane over sulfide in the seep environment (Duperron *et al.*, 2005). Thiotrophs were assumed to be more dependent on being close to the mantle fluids because of the low sulfide concentrations in the environment while methanotrophs were assumed to be able to inhabit the basal region because diffusive loss of methane through the bacteriocytes could be compensated for by the higher methane concentrations. At Rainbow, sulfide and methane concentrations were similar in end-member fluids, making the hypothesis used to explain the uneven distribution of the symbionts in the Gabon seep mussels unlikely for the Rainbow mussels. However, single measurements taken at one point and time may not reflect average concentrations over longer times.

The Rainbow site was also the only one at which methanotrophs were more abundant than thiotrophs, corresponding well with methane concentrations in end-member fluids, which were higher at Rainbow than at other vent sites (Table 2). Analogously, at the two sites with the highest sulfide concentrations and sulfide over methane ratios, Lucky Strike and Snake Pit, thiotrophs were more abundant than methanotrophs. At the Lucky Strike site, the higher abundance of thiotrophs was also observed in earlier studies (Trask and van Dover, 1999; Fiala-Médioni *et al.*, 2002) showing that the dominance of thiotrophic symbionts in the gills of these mussels has remained stable over time.

In summary, it appears as if vent chemistry affects the relative abundance of thiotrophs and methanotrophs, as suggested in earlier studies using TEM and stable isotope

analysis (Trask and van Dover, 1999; Colaco *et al.*, 2002; Fiala-Médioni *et al.*, 2002). In contrast, no clear effect of sulfide and methane concentrations on symbiont distribution was observed in this study. However, the relative concentrations of sulfide and methane in end-member fluids may not be representative of their ratios in diffuse fluids that deliver these energy sources to the mussels. For future studies, time series measurements of gradients in vent fluids taken at the mussel collection sites should be combined with quantitative studies of relative symbiont abundance, for example using rRNA slot blot hybridization with thiotroph- and methanotroph-specific probes. The northern MAR vent sites, with their distinct differences in vent chemistry, yet highly similar to identical *Bathymodiolus* symbiont phylotypes provide an ideal study site for gaining a better understanding of how gradients in energy sources at hydrothermal vents influence the nutritional response of mussels with dual symbioses.

Experimental procedures

Organisms and sampling sites

Bathymodiolus azoricus and *B. puteoserpentis* were collected from four hydrothermal vent sites located on the northern MAR (Fig. 1). *Bathymodiolus azoricus* specimens were collected from three sites: Menez Gwen (37°51'N, 31°31'W, 850 m depth) using the ROV *Victor* during the 2001 ATOS cruise (Ifremer), and Lucky Strike (37°17'N, 32°16'W, 1650-m depth) and Rainbow (36°13'N, 33°54'W, 2350-m depth) using the DSV *Alvin* during the 2001 AT5-2 cruise (Woods Hole Oceanographic Institution). *Bathymodiolus puteoserpentis* specimens were collected from the Logatchev site (14°45.19'N, 44°58.74'W, 3037-m depth) using the ROV *Quest* during the Meteor cruise Hydromar I, in February 2004.

Upon recovery, specimens were kept in insulated containers and chilled (4°C) seawater until dissection. Symbiont-containing gill tissues from two to four individuals per site were dissected, with one part frozen immediately in liquid nitrogen and stored at –20°C or –80°C for DNA extraction and another part fixed for FISH (see below).

DNA extraction and PCR amplification

Total DNA was extracted and nearly full 16S rRNA gene sequences were amplified from frozen gill tissues of three

individuals from Menez Gwen, two individuals each from Rainbow and Lucky Strike, and four individuals from Logatchev, using bacterial universal primers 8F and 1492R (Muyzer *et al.*, 1995) and the same protocol and cycling conditions as previously described (Duperron *et al.*, 2005). Four separate PCR reactions were run with 25 or 35 cycles each, and then pooled together prior to cloning.

Cloning and sequencing

For each specimen, the pooled PCR products were cloned and inserts of 15–100 positive clones were partially sequenced using primers 8F or 519F, yielding sequences of ~500 bp. Partial sequences were aligned and compared with BioEdit (Hall, 1997–2001), and potential chimeras, detected by visual inspection of the alignments and using Chimera-Check (Cole *et al.*, 2003), were eliminated from further analyses. Representative clones were selected and their inserts fully sequenced in both directions (2–5 full sequences per individual and symbiont type). Methanotroph-related sequences are registered at EMBL under Accession numbers AM083950–AM083967, thiotroph-related sequences under Accession numbers AM083968 to AM083986.

Sequence analysis

Full sequences were aligned with a database containing over 30 000 sequences using ARB (Ludwig *et al.*, 2004). Sequences from this study were aligned with symbiont sequences from the databases for MAR mussels and critically evaluated for nucleotide substitutions by assessment of position (e.g. stems vs. loops) and complementary base pairing in stems (EMBL Accession numbers: Align_000930 (methanotrophs) and Align_000931 (thiotrophs)). Related sequences were compared to determine whether differences were unique or shared. Phylogenetic trees were calculated with maximum likelihood using Treefinder (Jobb, 2005), with an optimized General Time Reversible model. Bootstrap values were obtained from 200 replicates using similar parameters.

Probe design

Specific oligonucleotide probes were designed for the two dominant 16S rRNA phylotypes obtained from *Bathymodiolus azoricus* and *B. puteoserpentis* using the PROBE_DESIGN tool of ARB, and their specificity tested against the ARB and GenBank databases. Both had at least one mismatch to all other sequences. Stringency and specificity of the Cy3- and Cy5-labelled probes (ThermoHybaid, Germany) was determined with the relevant reference symbiont or strain using a range of formamide concentrations (5–60%). References for the thiotroph probe BMARt-193 (CGAAGGTCCTCCACTTTA) and methanotroph probe BMARm-845 (GCTCCGCCACTAAGCCTA) were a *Bathymodiolus* sp. symbiont from Gabon margin (two mismatches; Duperron *et al.*, 2005) and *Xenorhabdus bovienii* (one mismatch; DSMZ strain 4766) respectively. Both probes hybridized specifically with their target at 30% formamide. Eub 338 (Amann *et al.*, 1990) and Non 338 (nonsense of Eub 338)

(Wallner *et al.*, 1993) were used as positive and negative controls. Four specific probes targeting the rare methanotroph-related phylotype found in a Menez Gwen *B. azoricus* specimen were designed: BazM2-845 (CAGGTTTAGTG GTGGAGC), BazM2-151 (ACGTGGTAAAACCACGC), BazM2-233 (CTGCGTTAGATTAGCTGG) and BazM2-160 (AAACCACGCTAATACCGC). As this rare phylotype was not found using these probes (see *Results and discussion*) their specificity was not further tested.

Fluorescence in situ hybridization (FISH)

Gill tissues of two individuals each from Rainbow and Lucky Strike, and of four individuals from Logatchev were fixed and prepared for FISH as described previously (Duperron *et al.*, 2005). Sections were hybridized using 100 ng of a given probe in 30 µl of hybridization buffer (0.9 M NaCl, 0.02 M Tris/HCl pH 8.0, 0.01% SDS, 30% formamide) for 3 h at 46°C in preheated chambers saturated with hybridization buffer. After hybridization, sections were washed for 15 min at 48°C with buffer (0.1 M NaCl, 0.02 M Tris/HCl pH 8.0, 0.01% SDS, 5 mM EDTA), air dried, and mounted in a non-fluorescent medium (Vectashield, Vector). Sections were examined using a confocal laser-scanning microscope (Zeiss Axioplan 2, Germany). Distribution and relative abundance of bacterial endosymbionts within gill tissues was estimated qualitatively by eye with only those observations described in *Results and discussion* that were consistent for all sections and all individuals examined.

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