ORIGINAL ARTICLE

3D FISH for the quantification of methane- and sulphur-oxidizing endosymbionts in bacteriocytes of the hydrothermal vent mussel *Bathymodiolus azoricus*

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Dual endosymbioses involving methane- and sulphur-oxidizing bacteria occur in the gills of several species of mussels from deep-sea hydrothermal vents and cold seeps. Variations of total and relative abundances of symbionts depending on local environmental parameters are not yet understood, due to a lack of reliable quantification of bacteria in the host tissue. Here, we report the first attempt to quantify volumes occupied by each type of symbiont in bacteriocyte sections from a vent mussel, *Bathymodiolus azoricus*, using fluorescence *in situ* hybridization (FISH) coupled to three dimensional microscopy and image analysis carried out by a dedicated software, which we developed. Bacteriocytes from mussels recovered at different vent sites displayed significantly different abundances of bacteria. Specimens kept in aquaria at atmospheric pressure and exposed to an artificial pulse of sulphur displayed an increase in absolute and relative abundance of sulphur oxidizers within their bacteriocytes. Distributions of all measured parameters fitted normal distributions, indicating that bacteriocytes from a specimen tend to display similar behaviours. This study shows that symbiont volume quantification is tractable using 3D FISH, and confirms the impact of local environmental parameters on symbiont abundances.

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Introduction

Bacterial symbiosis is essential for the adaptation and survival of many metazoans inhabiting deep-sea chemosynthesis-based ecosystems, such as hydrothermal vents and cold seeps. Since the discovery of these ecosystems, a great diversity of symbiotic associations has been described (Cavanaugh *et al.*, 1981; Felbeck *et al.*, 1981; Cavanaugh, 1983; Childress *et al.*, 1986; Cavanaugh *et al.*, 1987; Fisher, 1990).

Among symbiont-bearing invertebrates, mytilid mussels are unique in that they display endosymbioses involving methane- and/or sulphur-oxidizing bacteria, making them good models to study multiple symbioses (Fiala-Médioni *et al.*, 1986; Cavanaugh *et al.*, 1987; Fisher *et al.*, 1993). Bacteria are localized within vacuoles in specialized epithelial cells of the gill, hence called bacteriocytes. Hosts are known to acquire organic matter from their symbionts by digesting them, although metabolites excreted by the bacteria could also provide the host with nutrients (Fiala-Médioni *et al.*, 1990). Symbiotic relationships play a major role in the nutrition of mussels (Fisher *et al.*, 1987; Fisher and Childress, 1992; Jahnke *et al.*, 1995; Pond *et al.*, 1998). Sulphur-oxidizing symbionts, when present, may also play a role in detoxication because

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their energy source, H_2S , is known to be extremely toxic for metazoans (Vismann, 1991; Childress and Fisher, 1992).

The hydrothermal environment is highly variable with regard to fluid composition and fluxes (Von Damm, 1995; Desbruyères *et al.*, 2001). It is still unknown how populations of symbiotic bacteria react to these variations. Studies on dual symbiotic mussels have shown that symbiont-relative abundances and contribution to the host diet can vary among specimens from different vent sites. This supports the hypothesis of a direct environmental effect on the symbiotic association as suggested by different authors (Pond *et al.*, 1998; Colaco *et al.*, 2002; Fiala-Médioni *et al.*, 2002; Salerno *et al.*, 2005; Duperron *et al.*, 2006). The main difficulty with these studies is that no reliable quantification of symbionts was performed. For example, manual counts on transmission electron micrographs, which were used in many studies, permit to investigate just one single plane of the overall volume of a bacteriocyte, which may not be representative of the distribution of symbionts in the whole bacteriocyte. To assess the effect of environmental parameters on symbiotic populations, the quantification of volumes occupied by each type of symbiont and estimation of bacterial densities in bacteriocytes appear to be a more satisfying approach. Confocal laser scanning microscopy yields high-quality three dimensional images that can be analysed using information toolboxes already available (Abramoff *et al.*, 2004; Daims and Wagner, 2007). Such tools have been successfully applied, in particular to the study of biofilms (Daims *et al.*, 2001; Kara *et al.*, 2007).

In the present study, we tested a method based on 3D fluorescence *in situ* hybridization (FISH) and image analysis to quantify populations of bacterial endosymbionts in bacteriocytes from *Bathymodiolus azoricus*. This mussel species was chosen because of its dual endosymbiosis involving sulphur and methane oxidizers, and because of its occurrence in various vent sites along the Mid-Atlantic Ridge (MAR). Furthermore, specimens from the shallow MAR vent site, Menez Gwen, can recover from decompression stress and survive for periods of months at atmospheric pressure, allowing the setup of experiments (Kadar *et al.*, 2005; Colaço *et al.*, 2006). To study the respective effects of natural environmental variations and of an artificial hydrogen sulphur pulse on the populations of symbionts, we investigated specimens collected at different vent sites as well as specimens kept in aquaria at atmospheric pressure and exposed for 6 h to an artificial pulse of hydrogen sulphur, followed by starvation. Bacterial symbionts were labelled by FISH, using specific probes, and 3D reconstructions of bacteriocyte sections were generated and analysed using a dedicated software. Acquired data were then analysed using robust statistical tools.

Materials and methods

Specimen sampling

Specimens of *B. azoricus* were collected using the ROV *Victor 6000* during the MOMARETO 2006 cruise to the MAR hydrothermal vents aboard the research vessel 'Pourquoi Pas?'. Mussels were sampled at Menez Gwen (MG, 37°50.654N 31°31.182W, 814 m depth) and from two different patches located at the base of the 'Tour Eiffel' chimney at Lucky Strike (LS1: 37°17.337N 32°16.529W, 1694 m depth; LS2: 37°17.347N 32°16.529W, 1692 m depth). Upon recovery, specimens were kept in chilled (4 °C), filtered sea water (0.22 µm). Gill tissues from three MG, three LS1 and three LS2 specimens were immediately fixed for FISH (see below). Four MG specimens were used for starvation experiments (see below).

Pulse/starvation experiment

Mussels were transferred on board to aquaria containing 4 l filtered sea water (0.22 µm) and kept for 24 h at 4 °C to allow recovery from collection stress. A sulphur pulse was then generated by adding 7 ml of a 20 mM Na_2S solution (final concentration 35 µM, pH 8). Additionally, 2 ml of the sulphur-containing solution was added every hour during a period of 6 h. Following the pulse, mussels were kept in filtered seawater, and water was replaced everyday. Two mussels were collected 6 h after the end of the pulse (MG6h), and two more 7 days after the end of the pulse (MG7d). Mussel gill tissues were then fixed for FISH (see below).

FISH

Upon recovery of mussel specimens, the anterior most part of their left gill was fixed in 2% formaldehyde (2–4 h, 4 °C), rinsed twice with filtered seawater and stored at –20 °C in 1:1 ethanol/filtered seawater. In the lab, gill tissue was dehydrated in increasing ethanol series and embedded in polyester wax (90% polyethylene glycol, 10% 1-hexadecanol). Sections of thickness 10 µm were cut using a microtome (JUNG, Heidelberg, Germany) and collected on Superfrost Plus slides (Roth, Karlsruhe, Germany). After wax removal in ethanol and rehydration in decreasing ethanol series, sections were hybridized for 3 h at 46 °C with 100 ng of probe in a buffer (0.9 M NaCl, 0.02 M Tris-HCl, 0.01% SDS and 20% formamide). After hybridization, slides were washed in 0.1 M NaCl, 0.02 M Tris-HCl, 0.001% SDS, 5 mM EDTA (15 min, 48 °C), air dried and mounted using a SlowFade medium (Invitrogen, Carlsbad, CA, USA) and a coverslip.

Three 16S rRNA-based specific probes were applied simultaneously on every section: the Cy3-labelled general *Eubacteria* probe EUB338 (Amann *et al.*, 1990), the Atto488-labelled probe ImedM (5'-ACCAGGTTGTCCCCCACTAA-3'), specific

for methanotrophic symbionts (ATTO-TEC GmbH, Siegen, Germany) and the probe BangT-642 (5'-CCTATACTCTAGCTTGCCAG-3'), labelled with Cy5 (Biomers.net, Ulm, Germany) and specific for thiotrophic symbionts (Duperron *et al.*, 2005, 2008).

Image acquisition and analysis

Three-dimensional fluorescence microscopy was performed using a BX61 microscope (Olympus Optical Co., Tokyo, Japan) equipped with the Optigrid system (QiOptic, Rochester, NY, USA). Image stacks were obtained by acquiring images on consecutive focal planes separated by 0.3 µm over the whole thickness of the sections using a 100× objective (NA 1.30). Three stacks, one for each fluorochrome, were produced from each field. Only bacteriocytes visible on all focal planes were analysed.

Image stacks were analysed using 'SymbiontJ', a software implemented in JAVA for this study and available as an ImageJ software plug-in (download at the following address <http://www.snv.jussieu.fr/~wboudier/softs/symbiontj.html>) (Abramoff *et al.*, 2004). The red channel corresponding to the EUB338 probe was binarized by applying an intensity threshold to distinguish between background fluorescence and probe signal, and used as a mask when processing other channels. The green channel corresponding to the ImedM probe (methanotrophs) was submitted to a low-pass filter in the frequency domain, to remove autofluorescence signal of the tissue. The blue channel corresponding to the BangT probe (thiotrophs) was processed by a Fourier Transform band-pass filter, adapted to enhance the bacterial objects from the background. Stacks were then segmented by binarization and the number of voxels belonging to the bacterial objects was computed. Total volume occupied by bacteria and respective proportions of each phylotype were computed for every bacteriocyte. From filtered images of the green channel, the volume of methanotrophic bacteria was calculated by using the ImageJ plug-in '3DObjectsCounter' (<http://rsb.info.nih.gov/ij/plugins/track/objects.html>) ($n > 30$ for each section). Their number was then estimated by dividing the mean volume of the overall methanotrophic bacterial fraction by the mean volume of a single methanotrophic bacterium. The same was carried out for sulphur-oxidizing symbionts, although isolated bacteria were less abundant and their size was not homogeneous. Estimated volume of sulphur oxidizers was thus not reliable, and was not further investigated.

Statistical tests

Density distributions were computed and normality was tested using the Kolmogorov–Smirnov test on each investigated variable (volume occupied by bacteria, percentages, bacterial volumes and number

of cells) (Campbell, 1967). Variance homogeneity was assessed using a Bartlett's test. Means of volumes occupied by bacteria were compared among bacteriocyte sections from mussel specimens corresponding to different natural (MG, LS1 and LS2) or experimental (MG, MG6h and MG7d) conditions using one-way analysis of variance and pairwise comparisons tests with correction for multiple testing. Homogeneity hypothesis was favoured when *P*-values were above 5%. All tests were realized using the statistical computing free software R (Ihaka and Gentleman, 1996).

Results

FISH

Gills of specimens collected from the three sampling locations, LS1, LS2 and MG, looked similar in their overall aspect and colour. Gills of MG specimens collected 6 h after the sulphur pulse were more brown than gills from any other sample. After 7 days, gills appeared more whitish. Hybridizations performed on 10-µm-thick transverse sections of gill filaments did yield unambiguous positive signals with the three probes used in this study. Bacterial symbionts were located in the apical half of most bacteriocytes located in the lateral zone of all gill filaments examined. The methanotroph-specific probe ImedM hybridized with large bacteria, while the thiotrophic-specific probe hybridized with small bacteria that appeared more tightly packed together (Figure 1). Methanotrophs and thiotrophs were mixed, and did not display any peculiar distribution. No bacteria were detected that hybridized only with Eub338, indicating that bacteriocytes only contained methanotrophs and thiotrophs. In four mussel specimens out of 13, a fraction of bacteriocytes displayed only one or the other bacterial symbiont (see Table 1).

Total volume occupied by bacteria in bacteriocyte sections

Density distributions of the total volume occupied by symbiotic bacteria in 10-µm-thick sections of bacteriocytes followed a normal distribution in all groups investigated (LS1, LS2, MG, MG6h and MG7d). Mean values and s.d. are displayed in Table 1. Variances were neither homogeneous between LS1, LS2 and MG ($P = 1.28 \times 10^{-14}$) nor among MG, MG6h and MG7d ($P = 7.23 \times 10^{-8}$).

ANOVA and pairwise *t*-tests indicated significant difference in volume occupied by bacteria among bacteriocyte sections from the LS1, LS2 and MG groups, representing bacteriocytes from specimens fixed directly after recovery (Table 2). Volume was significantly higher in the MG group ($444 \pm 33 \mu\text{m}^3$) than that in the LS1 ($344 \pm 15 \mu\text{m}^3$) and LS2 groups, the latter displaying the smallest volume ($159 \pm 9 \mu\text{m}^3$) (Table 1).

Figure 1 Fluorescence *in situ* hybridization of symbiotic bacteria on transverse sections of gill filaments from *Bathymodiolus azoricus*. Sulphur oxidizers appear in purple, methane oxidizers in orange. (a) Overview of a filament. Scale bar = 10 µm. (b) Three-dimensional reconstruction of a 10-µm-thick bacteriocyte section.

In the sulphide pulse/starvation experiment, the total volume occupied by bacteria per bacteriocyte section was not significantly different between MG and MG6h groups ($P=0.78$). The volume of bacteria in bacteriocytes from MG7d specimens starved during 7 days after the sulphide pulse was significantly lower (by 20.8%) than that in the MG and MG6h groups ($P=0.02$).

Relative abundances and volume of bacterial symbionts in bacteriocyte sections

Density distributions of the fraction of total bacterial volume occupied by sulphur oxidizers fitted a normal distribution in bacteriocytes from LS2, MG and MG6h mussels (see Figure 2a). These fractions displayed non-normal distributions in bacteriocytes from LS1 and MG7d mussels (see Figure 2b). In these two groups, approximately 10% of the bacteriocytes displayed an abnormally high abundance (>90%) of either type of symbiont, mostly methanotrophs in LS1 and sulphur oxidizers in MG7d

Table 1 Data acquired after 3D reconstruction of bacteriocyte sections and image analysis

Number of specimens	Number of bacteriocytes	Number of 'outlayers'	Total volume of bacterial fraction	Volume of SOX	Volume of MOX	Volume proportion of SOX (%)	Volume of a single MOX	Number of MOX
LS1	3	59	344.42 ± 14.52	216.88 ± 9.66	157.03 ± 4.88	56.23 ± 6.97	1.92 ± 0.36	81.78
LS2	3	122	158.5 ± 8.69	113.82 ± 6.06	44.67 ± 3.12	68.52 ± 9.71	1.65 ± 0.36	27.07
MG	3	52	444.06 ± 33.04	242.05 ± 20.28	202.01 ± 14.8	53.05 ± 10.29	1.16 ± 0.21	174.14
MG6h	2	63	454.40 ± 28.26	301.71 ± 20.47	152.69 ± 9.33	64.98 ± 8.55	1.67 ± 0.45	91.43
MG7d	2	121	359.89 ± 13.35	215.66 ± 8.87	144.23 ± 6.25	59.44 ± 12.16	1.46 ± 0.33	98.78

Abbreviations: LS1, specimens collected at Lucky Strike at a depth of 1642 m; LS2, specimens collected at Menez Gwen; MG, specimens collected at Menez Gwen; MG6h, specimens collected at Menez Gwen 6 h after the end of the pulse; MG7d, specimens collected at Menez Gwen 7 days after the end of the pulse; MOX, methane-oxidizing symbionts; SOX, sulphur-oxidizing symbionts; 3D, three dimensional. Volumes (expressed in µm³ ± s.d.) and numbers of methanotrophs are given per bacteriocyte section (10 µm thickness).

Table 2 Pairwise *t*-tests with risk correction comparing the measured parameters between bacteriocytes from the different groups (LS1, LS2, MG, MG6h and MG7d)

		<i>Mean homogeneity</i>	
		<i>Pairwise t-test with risk correction</i>	
Total volume of bacteria	LS2	LS1	LS2
	MG	*	**
	MG7d	**	—
MG	0.78	*	—
Volume occupied by sulphur oxidizers	LS2	LS1	LS2
	MG	**	—
	MG7d	0.39	**
MG	*	**	—
Volume occupied by methanotrophs	LS2	LS1	LS2
	MG	*	**
	MG7d	0.30	—
MG	*	*	—
Percentage of volume occupied by sulphur oxidizers	LS2	LS1	LS2
	MG	**	—
	MG7d	0.10	**
MG	**	**	—
Volume of a single methanotroph	LS2	LS1	LS2
	MG	**	—
	MG7d	**	**
MG	**	**	—

Abbreviations: LS1, specimens collected at Lucky Strike at depth of 1642 m; LS2, specimens collected at Lucky Strike at depth of 1692 m; MG, specimens collected at Menez Gwen; MG6h, specimens collected at Menez Gwen 6 h after the end of the pulse; MG7d, specimens collected at Menez Gwen 7 days after the end of the pulse

*Indicates a *P*-value <0.05, **indicate a *P*-value <0.001, *P*-values >0.05 are displayed.

All parameters followed normal distributions in all groups, variances were unequal.

(Table 1). FISH images indicated that these bacteriocytes displayed normal size and morphology, but contained bacteria that were much more tightly packed together than what was observed in typical bacteriocytes. Because of their unusual structure, their occurrence in only 4 out of 13 specimens and because distributions would otherwise fit normal curves, such as observed in LS2, MG and MG6h, we suggest that 'outlayer bacteriocytes' are not representative of the typical mussel bacteriocyte, and discarded them from further analysis. Mean values and s.d. are given in Table 1. Variances were not homogeneous among the different groups.

Fractions of bacterial volume occupied by sulphur oxidizers were not significantly different between

Figure 2 Density distribution of the proportion of bacterial volume occupied by sulphur-oxidizing symbionts in the two groups of bacteriocytes. x axis represents percentages of volume occupied by sulphur oxidizers. (a) Normal distribution in bacteriocytes from mussels collected at LS2. (b) Non-normal distribution in bacteriocytes from mussels collected 7 days after the end of the sulphur pulse (MG7d), the distribution shows high densities of bacteriocytes displaying only one type of symbiont (<10 or >90% sulphur oxidizers, hatched).

bacteriocytes from mussels collected at LS1 and MG ($P=0.1$). For these two mussel groups, sulphur-oxidizing bacteria represented 56.2% and 53.1%, respectively, of the total bacterial volume per bacteriocyte section. Sulphur oxidizers represented a significantly higher fraction of the total bacterial volume in bacteriocytes from LS2 mussels, occupying 68.5% of the total bacterial volume ($p_1 = 2.5 \times 10^{-13}$ and $p_2 = 3.1 \times 10^{-10}$).

In the sulphide pulse/starvation experiment, a significant increase, from 53.05% to 64.98% of the volume fraction occupied by sulphur oxidizers, was observed between bacteriocytes from specimens fixed directly after recovery (MG) and those fixed 6 h after the end of the pulse (MG6h) ($P = 5.7 \times 10^{-8}$). This corresponded to a 24.6% increase in the absolute volume of sulphur oxidizers, from $242.1 \mu\text{m}^3$ to $301.7 \mu\text{m}^3$ (Table 1). Bacteriocytes from specimens recovered 7 days after the pulse (MG7d) displayed a lower proportion of sulphur oxidizers (59.4%, $P = 4.10 \times 10^{-8}$), corresponding to a 28.5% decrease in their absolute volume ($215.7 \mu\text{m}^3$). Volume occupied by methanotrophs in bacteriocyte sections decreased all along the experiment, by 24.4% (from 202.0 to $152.7 \mu\text{m}^3$) between MG and MG6h mussels, and further decreased by 5.60% to $144.2 \mu\text{m}^3$ between MG6h and MG7d mussels. Results are summarized in Table 1 and Figure 3.

Estimation of the volume and amount of methanotrophic bacteria

For each condition, the mean volume of a single methanotrophic bacterium and the respective

Figure 3 Graph displaying the variation of volume, in cubic micrometre, occupied by sulphur (squares) and methane-oxidizing symbionts (circles) within 10- μm -thick sections of bacteriocytes from MG mussels exposed to an artificial sulphur pulse followed by starvation in sterile sea water. The x axis represents time after mussel collection; injection of sulphide is indicated by the black arrow, each interval represents 6 h. Mussels were collected before the pulse at $t=0$ (MG) and 6 h (MG6h) and 7 days (MG7d) after the end of the pulse.

Figure 4 Density distribution of the volume of individual methanotrophs and sulphur-oxidizers in bacteriocyte sections from mussel specimens collected 6 h after the end of the sulphur pulse (MG6h). Distribution of volumes of methanotrophs (left) fits a normal curve, while the distribution of sulphur-oxidizers (right) does not.

abundance were estimated (Table 1). Distributions of both parameters followed normal distributions (see Figure 4 left). Methanotrophs from LS1 and LS2 mussels had slightly different volumes ($V_{LS1} = 1.9 \mu\text{m}^3$ and $V_{LS2} = 1.6 \mu\text{m}^3$), and more methanotrophs were present in bacteriocyte sections from LS1 ($N_{LS1} = 81.8$ and $N_{LS2} = 27.1$). In mussels collected at Menez-Gwen, the volume of a single methanotroph was smaller ($V_{MG} = 1.2 \mu\text{m}^3$) and their number was higher ($N_{MG} = 174.1$).

In the pulse/starvation experiment, the abundance of methanotrophs decreased from 174.1 to 91.4 bacterial cells per bacteriocyte section 6 h after the pulse and 98.8 cells per section 7 days after the pulse.

Regarding sulphur-oxidizing symbionts, volumes measured for the isolated cells were highly variable,

ranging from $0.3 \mu\text{m}^3$ to $14 \mu\text{m}^3$. Their distribution indicated a high abundance of small bacteria ($< 1 \mu\text{m}^3$), but few very large bacteria were also seen ($5\text{--}14 \mu\text{m}^3$) (Figure 4 right). Because distributions do not follow normal curves, estimates based on mean volumes would be unreliable, and thus no estimation of bacterial numbers was performed.

Discussion

Comments on the methodology

Investigating the interaction among hosts, multiple bacterial symbionts and their environment is a key issue to understand the factors controlling the distribution and functioning of symbiont-bearing invertebrates at hydrothermal vents, cold seeps and more generally chemosynthesis-based ecosystems. An accurate quantification of bacterial symbionts and their relative abundances is necessary. Until now, most studies used direct counts of bacteria within bacteriocytes observed on transmission electron microscopy micrographs to assess relative bacterial abundances, benefiting from the morphological difference between small thiotrophs and large membrane-bearing methanotrophs (Cavanaugh *et al.*, 1987; Fiala-Médioni *et al.*, 2002). However, the method is limited because only two-dimensional images are treated, cell count is not automated and transmission electron microscopy does not allow to distinguish among different bacteria that do not display peculiar morphological features, such as the multiple symbionts of *Idas* sp. (Duperron *et al.*, 2008). More recently, quantification of 16S rRNA was used to assess symbiont relative abundances in *Bathymodiolus* species from the Gulf of Mexico (Duperron *et al.*, 2007). This approach is limited because the amount of ribosome per bacterium depends on their type and activity and is thus not always correlated with cell numbers (Stahl *et al.*, 1988; Amann *et al.*, 1995).

The choice to focus on volumes occupied by bacterial symbionts seems a more reasonable option for several reasons. The volume occupied by different symbionts appears as a better indicator than cell counts of the potential contribution of symbionts to the host nutrition or to detoxication processes. Indeed, the volume of a single methanotrophic bacterium represents approximately four times that of a sulphur oxidizer. Furthermore, the volume occupied by bacteria can be measured precisely from stacks of FISH images using computation tools, while bacterial numbers can only be estimated with potential error (Daims *et al.*, 2001).

For this study, we focused on bacteriocytes and not on the whole gill tissue because a bacteriocyte is finite, while it is very difficult to quantify the number of bacteriocytes present in a gill. Because of tissue autofluorescence and fluorochrome bleaching during long acquisitions, we investigated only 10- μm sections of bacteriocytes to get good quality

images for automated processing. However, we are confident that the trends observed in symbiont absolute and relative abundances reflect a general trend at the scale of a bacteriocyte and a gill because all distributions fit normal curves. This indicates that all or at least most of the bacteriocytes within a gill display similar behaviours, and that the volumes occupied by bacteria as well as the proportion of each symbiont type are not stochastic. In most cases, variances were not homogeneous, but the ANOVAs we employed are not very sensitive to unequal variances and most *P*-values were well below 0.05 (Campbell, 1967).

Variability of symbiont abundances in mussels from different sites

The volume occupied by bacteria within bacteriocytes varied significantly among mussel specimens from different sites (LS2, LS1 and MG). Because we did not quantify the total number of bacteriocytes per gill, we cannot address whether this implies that different total amounts of bacteria were present in the gills. Nevertheless, this indicates that the volume of bacteria present in a bacteriocyte is variable between *B. azoricus* specimens from different sites.

The fact that bacteriocytes from LS2 mussels display a markedly higher proportion of sulphur oxidizers compared to those from LS1 and MG mussels could be a consequence of their local chemical environment. Indeed, previous studies pointed out the link between fluid composition and symbiont-relative abundances and contribution to the host in MAR mussels. It has been demonstrated that symbiont-relative abundances in mussel gills varied among different MAR sites (Fiala-Médioni *et al.*, 2002; Duperron *et al.*, 2007). Hydrothermal fluids at LS and MG have different chemical compositions, suggesting different nutritional regimens for the mussels (Trask and Van Dover, 1999; Colaco *et al.*, 2002; Fiala-Médioni *et al.*, 2002; Duperron *et al.*, 2007). At the scale of a single site, Trask and Van Dover (1999) observed that specimens of *B. azoricus* displayed distinct symbiont-relative abundances between two locations at LS based on transmission electron microscopy counts. By providing quantitative data, we confirm that differences are statistically significant, reflecting different responses to environmental parameters. The differences observed in this study between the two LS patches also emphasize the need to properly investigate local characteristics of the environment at the relevant scale for organisms.

Variability of symbiont abundances following a sulphur pulse and starvation

Pulses of sulphur-rich fluids are known to occur in the vicinity of mussels at hydrothermal vents (Le Bris *et al.*, 2006). Such pulses represent a

potential threat, as H_2S is toxic to all metazoans (Vismann, 1991). The increase in total volume and proportion of sulphur-oxidizing symbionts measured 6 h after the end of the pulse clearly indicates that the presence of sulphur compounds promotes the rapid growth of sulphur oxidizers. Hypothesis of rapid growth of symbionts is supported by the presence of dense aggregates of sulphur-oxidizers on the micrographs of bacteriocytes from the MG6h group. Recent work on sulphur-oxidizing symbionts associated with lucinid clams provided evidence that symbionts contain multiple copies of their genome, which could allow them to divide very quickly following a sulphur pulse (Caro *et al.*, 2007). The decrease in the volume of sulphur oxidizers observed after 7 days of starvation indicates that the effect of sulphur compounds is temporary. The total volume and number of methanotrophs decreased rapidly between the beginning of the experiment and 6 h after the end of the pulse. During the next 7 days after the pulse, methanotrophs only slightly decreased. This could indicate that absence of methane is less deleterious to populations of methanotrophs than abundance of sulphide, which the other symbiont is benefiting. Survival of some methanotrophs in the absence of methane could be due, for example, to the presence of a functional Calvin–Benson cycle for carbon uptake (Elsaied *et al.*, 2006). Complete loss of methanotrophs was previously reported after a 24 h sulphur pulse following a 30 day starvation period (Kadar *et al.*, 2005).

Although the rapid loss of bacterial symbionts in the absence of their electron or carbon sources was reported in *B. azoricus*, with bacteria barely detectable after 30 days, this is to our knowledge the first quantification of the effect of a sulphur pulse on symbiont abundances (Kadar *et al.*, 2005). The rapid growth of sulphur-oxidizing symbionts following a sulphur pulse could be beneficial to the host by both absorbing the excess H_2S , preventing intoxication of host's respiratory chains and by producing organic matter available to the host. It could also indicate that the host does not regulate symbiont abundances at a short timescale. These results confirm that the symbiotic association is highly dynamic and is influenced by local environmental parameters at a short timescale. However, caution is needed as experiments were performed *ex situ* and at atmospheric pressure. A parallel study investigating the effects of a methane pulse is needed.

The protocol used in this study allows to rapidly and automatically quantify symbionts in a great number of bacteriocyte sections. Most parameters measured displayed normal distributions, indicating that bacteriocytes from a specimen tend to display similar behaviours. The consequence for future studies is that for a given effort, it is probably relevant to investigate little less bacteriocytes per specimen but more specimens. Variability of symbiont-absolute and -relative abundances were

evidenced both in mussels recovered from vent sites and in mussels exposed to an artificial sulphur pulse. 3D FISH and image analysis allow to perform large quantitative studies of symbiotic populations and dynamics, and to point out significant differences that would not be detected with other techniques.

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