Fish and Shellfish Immunology 86 (2019) 246–252

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

# Gill symbionts of the cold-seep mussel *Bathymodiolus platifrons*: Composition, environmental dependency and immune control

Jiajia Yu[a,c], Minxiao Wang[a,b], Baozhong Liu[a,b], Xin Yue[a,*], Chaolun Li[a,b,**]

[a] CAS Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Center for Ocean Mega-Science, Chinese Academy of Sciences, 7 Nanhai Road, Qingdao, 266071, China

[b] Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, 1 Wenhai Road, Qingdao, 266000, China

[c] University of Chinese Academy of Sciences, Beijing, 100049, China

ARTICLE INFO

*Keywords:*
*Bathymodiolus platifrons*
Methanotrophs
FISH
qRT-PCR
Lysosomal system

ABSTRACT

Deep-sea *Bathymodiolus* mussels depend on the organic carbon supplied by symbionts inside their gills. In this study, optimized methods of quantitative real-time PCR and fluorescence in situ hybridization targeted to both mRNA and 16S rRNA were used to investigate the gill symbionts of the cold-seep mussel *Bathymodiolus platifrons*, including species composition, environmental dependency and immune control by the host. Our results showed that methanotrophs were the major symbiotic bacteria in the gills of *B. platifrons*, while thiotrophs were scarce. In the mussels freshly collected from the deep sea, methanotrophs were housed in bacteriocytes in a unique circular pattern, and a lysosome-related gene (*VAMP*) encoding a vesicle-associated membrane protein was expressed at a high level and presented exactly where the methanotrophs occurred. After the mussels were reared for three months in aquaria without methane supply, the abundance of methanotrophs decreased significantly and their circle-shaped distribution pattern disappeared; in addition, the expression of *VAMP* decreased significantly. These results suggest that the symbiosis between *B. platifrons* and methanotrophs is influenced by the environment and that the lysosomal system plays an important immune role in controlling the abundance of endosymbionts in host. This study provides a reliable method for investigating symbionts in deep-sea mussels and enriches the knowledge about symbionts in *B. platifrons*.

## 1. Introduction

Mussels of the genus *Bathymodiolus* are one of the dominant species in two kinds of unique deep-sea habitats, cold seeps and hydrothermal vents, which are characterized by abundant reduced compounds (i.e., $CH_4$, $H_2S$). These mussels exhibit a very high biomass in the harsh deep-sea environment [1], owning to their symbiosis with chemosynthetic bacteria which can provide energy. Methanotrophs and thiotrophs have been reported to be the most typical symbiotic bacteria in *Bathymodiolus* mussels. Some mussel species, e.g., *B. childressi*, *B. japonicas* and *B. thermophilus*, harbor only one kind of symbiotic bacteria [2–4]. Some species of *Bathymodiolus*, e.g., *B. azoricus*, *B. sp.* and *B. brooksi*, live in dual symbioses with two types of bacteria [5–8]. *B. platifrons*, first described by Hashimoto and Okutani in 1994, is a deep-sea mussel common in both cold seeps and hydrothermal vents in the Western Pacific Ocean [9]. High densities of symbionts, which appeared to be type I methanotrophs that use methane as an electron donor and energy source, have been observed in the gills of *B. platifrons* [10,11]. However, thiotrophs, another type of typical symbiotic bacteria in *Bathymodiolus* mussels, have not been investigated in *B. platifrons*. A study investigating the composition of symbiotic bacteria in *B. platifrons* would provide more information about this mussel and its deep-sea adaptations.

A large number of symbionts are able to present inside *B. platifrons* and be controlled without unregulated proliferation. Therefore, there must be a control or immunity mechanism in the host mussel responsible for maintaining the balance of endosymbionts. The lysosomal system plays an important role in the immune response to bacteria by a host [12]. Previous studies in pea aphids showed that the lysosomal system could regulate the *Buchnera* endosymbiont population and maintain homeostasis by degrading the symbionts [13,14]. In a study comparing deep-sea mussels to the shallow-water mussel *Modiolus*

* Corresponding author.
** Corresponding author. CAS Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Center for Ocean Mega-Science, Chinese Academy of Sciences, 7 Nanhai Road, Qingdao, 266071, China.
*E-mail addresses:* yuexin@qdio.ac.cn (X. Yue), lcl@qdio.ac.cn (C. Li).

https://doi.org/10.1016/j.fsi.2018.11.041
Received 8 June 2018; Received in revised form 16 October 2018; Accepted 16 November 2018
Available online 17 November 2018

*kurilensis*, Zheng et al. [15] found that the expression of most genes involved in the lysosomal system were upregulated in the deep-sea mussels. The concurrence of an upregulated lysosomal gene expression and a high endosymbiosis biomass suggests a key role of the lysosomal system in controlling the abundance of symbionts. The vesicle-associated membrane protein (*VAMP*) is a key component of the lysosomal system and is required for the formation of specific membrane compartments, in which symbionts are hosted in a controlled manner by the host [16]. In this study, the expression of *VAMP* was analyzed to identify the role of the lysosomal system in controlling the symbionts in the deep-sea mussel *B. platifrons*.

The abundance of chemosynthetic symbionts in *Bathymodiolus* mussels is reportedly environmentally dependent. In *B. azoricus*, the relative abundances of methanotrophs and thiotrophs differed between mussels from two hydrothermal vent sites [17]. In addition, Bettencourt et al. [18] transferred *B. azoricus* from a vent to aquaria at atmospheric pressure and found that after two weeks, the number of symbionts decreased significantly. Sun et al. [19] investigated the change in the abundance of methanotrophs in *B. platifrons* after a 34-day rearing and found that the abundance of symbionts declined significantly. Nonetheless, limited information is available regarding the response of symbionts in deep-sea mussels after a longer laboratory-rearing period, e.g., more than two months.

Several different detection methods have been used to investigate the change in symbionts abundance in *Bathymodiolus* mussels, including fluorescence in situ hybridization (FISH) [17,20,21] and quantitative real-time PCR (qRT-PCR) [19,22]. For FISH, two kinds of probes were used, including the 16S rRNA probe and mRNA probe targeted to a specific functional gene of the symbiotic bacteria. For qRT-PCR, it also commonly targets to the 16S rDNA or a specific functional gene of the symbiotic bacteria. FISH technique helped to visually study the distribution pattern of symbionts, while qRT-PCR was used for quantifying the abundance of symbionts. The combination of FISH and qRT-PCR provides comprehensive information on both the distribution and quantity of symbionts.

In this study, FISH and qRT-PCR methods were applied to analyze symbiont composition in the gills of *B. platifrons* and to detect both the change in symbionts abundance and the expression variance of *VAMP* in *B. platifrons* under different environments (i.e., cold seep in the deep sea or laboratory-reared in methane-absent seawater for three months). We focused on whether two kinds of popular symbiotic bacteria (i.e., methanotrophs and thiotrophs) occurred in *B. platifrons* and the environmental dependency of these bacteria; furthermore, the role of the lysosomal system in the control of symbionts by the host was investigated.

## 2. Materials and methods

### 2.1. Sample collection and preparation

*B. platifrons* were collected from a cold seep at a depth of 1100 m in the South China Sea (119°17′6.580"E, 22°06′57.144"N) in 2016. Mussels were obtained using the remotely operated vehicle *Faxian*, which was operated from the RV *Kexue*. The gills of nine mussels (control group) were dissected immediately on board after being sampled from the deep sea and then preserved in liquid nitrogen for DNA and RNA extraction or in cold 4% paraformaldehyde for paraffin section preparation. Other mussels were transferred immediately to a tank with fresh cooled seawater during shipment and were then reared in a laboratory tank at atmospheric pressure without methane. The water temperature was regulated to 4 °C, similar to the temperature where the mussels were collected. After a 3-month rearing period, the gills of three mussels (treatment group) were sampled and preserved in liquid nitrogen or cold 4% paraformaldehyde.

**Table 1**
Primers used in this study.

| Primer | Sequence(5'-3') | Tm |
|---|---|---|
| 16S rDNA-1 | F:AAGCGTTCGTAGGCGGTTATT<br>R:CTTTCGTTCCTCAGCGTCAGT | 60 °C |
| 16S rDNA-2 | F:GAGTAACGCGTAGGAATCTGC<br>R:CGAAGGTCCTCCACTTTACTCCATAGAG | 58 °C |
| *VAMP*-RT-1 | F: CACAGAAGTCACAGACCAGA<br>R: TGCTCGAACACCATACTGA | 60 °C |
| 28S rDNA | F:CCATCTAAGGCTAAATACCGACAC<br>R:GCGGGTCTTTACAGATCCCTATC | 60 °C |
| *VAMP*-1 | F: GAACAACTGTCTTAGCCAAAT<br>R: CACCACAAGCTGCTGATAT | 58 °C |

### 2.2. PCR amplification of 16S rDNAs/specific genes for methanotrophs and thiotrophs

To validate the existence of methanotrophs and thiotrophs in *B. platifrons*, PCR amplifications of the 16S rDNAs and specific genes of methanotrophs and thiotrophs were performed. The primer pairs specific to the 16S rDNA of methanotrophs (16S rDNA-1, Table 1) and thiotrophs (16S rDNA-2, Table 1) were designed by Primer Premier 5 based on the 16S rDNA sequences of methanotrophs or thiotrophs in deep-sea mussels provided by the NCBI database. The primer pairs used to amplify *pmoA*, a gene encoding subunit A of the particulate methane monooxygenase (A189gc and Mb661), and *aprA*, a gene encoding the alpha-subunit of adenosine-5'-phosphosulfate reductase (AprA-1-FW and AprA-5-RV), correspond to sequences from previous studies [21,23,24]. Total genomic DNA was extracted from the gills of each sampled mussel using a commercial DNA extraction kit especially for marine animals (Tiangen, China). The DNA mixture of all freshly collected individuals from deep sea was used as the PCR template. The PCR reactions were performed using a LA PCR kit (TaKaRa, Japan). The PCR parameters were as follows: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, nºC (n = Tm of each primer pair, Table 1) for 30 s and 72 °C for 1min, followed by the final extension at 72 °C for 10 min. The PCR products were purified and then cloned into the pGEM-T-Easy vector (Promega, USA). The recombinant plasmids were transformed into competent *Escherichia coli* cells, which were cultured on the LB plates at 37 °C overnight. Positive clones with the recombinant plasmid were further confirmed by PCR and then were cultured and regrown in LB broth. A large number of the recombinant plasmids were extracted from the bacteria culture and then sequenced by the Sanger method. The sequences were analyzed with BLAST against the NCBI database.

### 2.3. qRT-PCR

To compare the methanotroph abundance in the gills between mussels *in-situ* and those acclimated in the laboratory for three months, qRT-PCR method was used. The qRT-PCR, targeting to the methanotrophs 16S rDNA sequence, was performed using the specific primer pair 16S rDNA-1 (Table 1). The total genomic DNA extracted from the gills of each sampled mussel was used as the qRT-PCR template. The DNA concentration was quantified by Nanodrop machine (Thermo, USA).

The relative mRNA expression level of *VAMP* in the gills of mussels under different environments (i.e., cold seep in the deep sea or laboratory-reared in methane-absent seawater for three months) was also compared by qRT-PCR using the specific primer pair *VAMP*-RT-1 (Table 1). Total RNA was isolated from the gills of each sampled mussel using a Total RNA Kit (Omega, USA) according to the manufacturer's instructions. The concentration of the total RNA isolated from each sample was quantified by Nanodrop machine (Thermo). Then cDNA was synthesized from the same amount of the RNA, respectively, and was used as the qRT-PCR template.

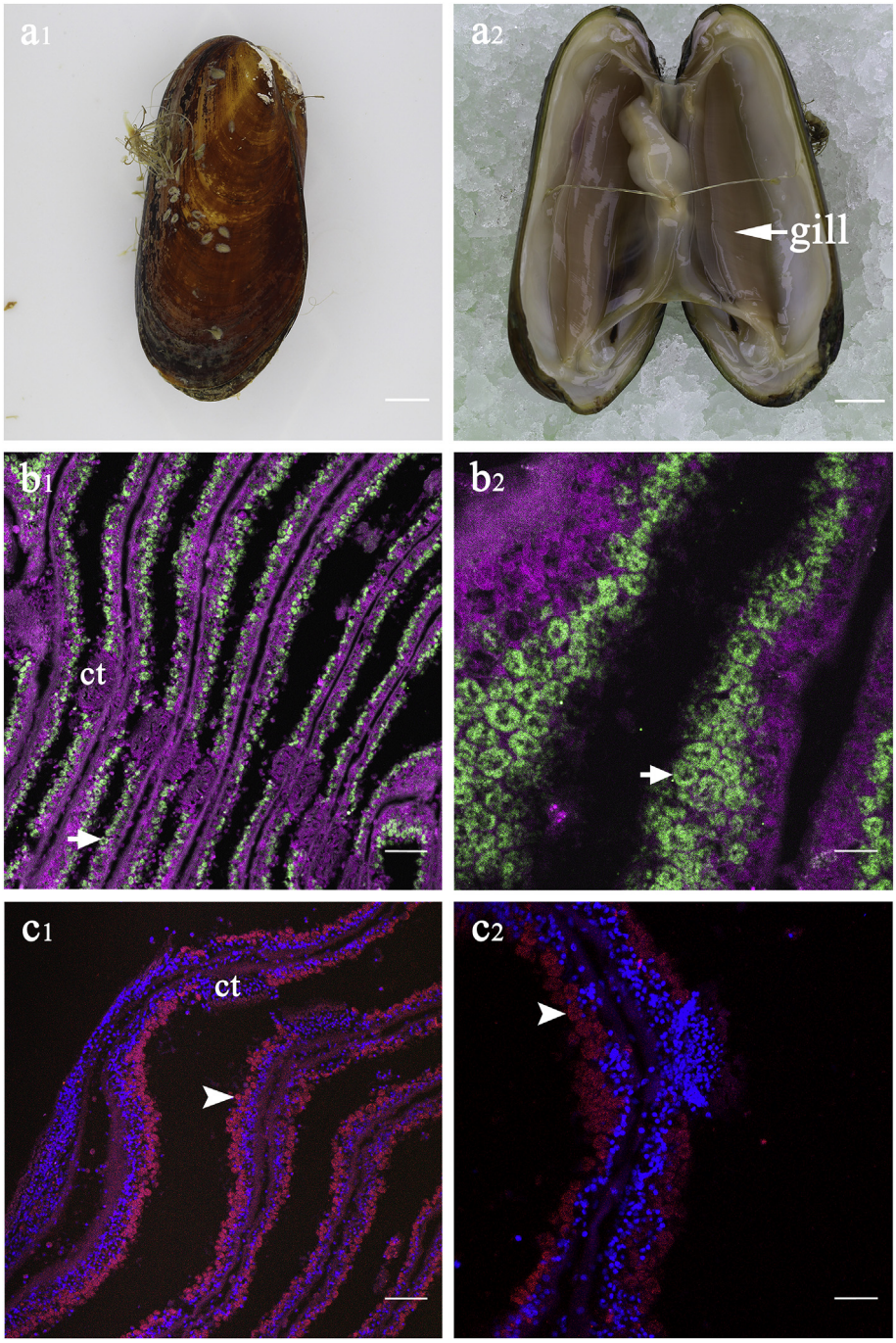


**Fig. 1. a1 & a2.** The deep-sea mussel *B. platifrons* collected from the cold seep. **b1 & b2.** FISH target to *pmoA* of methanotrophs (green fluorescence, arrow) in the gills (magenta fluorescence) of *B. platifrons*. **c1 & c2.** FISH target to 16S rRNA of methanotrophs (red fluorescence, arrowheads) in the gills of *B. platifrons*. Nuclei are revealed by blue fluorescence. ct, ciliary tuft. Scale bar: (a1) 1 cm; (a2) 1 cm; (b1) 100 μm; (b2) 20 μm; (c1) 100 μm; (c2) 50 μm.

qRT-PCR was performed using a QuantStudio 6 Flex machine (ABI, USA). Each reaction was performed in triplicate and in a 10-μl volume containing 20 ng of template (cDNA or DNA), 0.3 μM of each primer and 5 μl of SYBR premix (TaKaRa, Japan). The qRT-PCR conditions were as follows: 95 °C for 30 s and 40 cycles of 95 °C for 5 s, 60 °C for 34 s, followed by the melting curve determination. Each reaction was performed in triplicate. 28S rDNA, which was amplified using the 28S rDNA primer pair (Table 1), was used as the internal reference to normalize the relative methanotroph abundance or the relative expression levels of *VAMP* between samples. The $2^{-\Delta\Delta CT}$ method was used to analyze the relative methanotroph abundance and the relative mRNA expression level of *VAMP* [25]. The data were analyzed by *t*-test, and when $P < 0.05$, the difference was considered significant.

### 2.4. FISH

Routine paraffin section preparation was applied to gills of *B. platifrons* fixed in 4% paraformaldehyde. Sections with a 6 μm thickness were cut and then transferred onto poly-L-lysine-coated slides (Boster, China). FISH targeted to 16S rRNA or *pmoA* mRNA was used to investigate the abundance of methanotrophs, while FISH targeted to *VAMP* mRNA was used to investigate the expression of *VAMP* mRNA in these gill sections.

For the FISH targeted to *pmoA*/*VAMP* mRNA, the DNA fragment of

*pmoA* (470 nt) was amplified using the specific primers A189gc and Mb661 [23], and the cDNA fragment of *VAMP* (600 nt) was amplified using the specific *VAMP*-1 primer pair designed by Primer Premier 5 (Table 1). Then, a digoxigenin-labeled RNA probe with the antisense sequence or sense sequence of *pmoA/VAMP* was synthesized, following the general protocol of the DIG RNA labeling kit (Roche, Germany) [26]. The hybridization method was modified based on the method described in Pernthaler & Amann [27] and Yue et al. [28] using a DIG DNA Detection Kit (Roche). Briefly, dewaxed sections of the slides were prehybridized in the hybridization buffer without the probe for 2 h at 58 °C and then hybridized in the hybridization buffer with 1 ng $\mu l^{-1}$ of antisense probe or sense probe (negative control) for 12–18 h at 58 °C. For stringent washing, slides were placed into 50% (v/v) formamide-2 × SSCT for 20 min at 58 °C, followed by two washes in 2 × SSCT at 58 °C for 20 min each and a subsequent 20 min washing in 0.2 × SSCT at 58 °C. Then, slides were blocked in the blocking buffer for 30 min at 37 °C and incubated with anti-digoxigenin-fluorescein Fab fragments in the blocking buffer for 2 h at 37 °C. Evans blue dye (Sigma, USA) was used as a background stain (magenta fluorescence) for gill tissues. Unbound antibodies were removed by washing slides five times in 1 × PBST for 5 min each. Finally, slides were mounted with Prolong Diamond Antifade Mountant (Invitrogen, USA), covered with a coverslip and photographed with a LSM710 confocal laser scanning microscope (Zeiss, Germany). The positive signals of *pmoA/VAMP* mRNA were shown as the green fluorescence.

The procedure of FISH targeted to 16S rRNA was modified based on that described in Duperron et al. [6]. Briefly, sections of the slides were dewaxed and rehydrated in decreasing ethanol series. After incubating in proteinase K (10 μg $ml^{-1}$, pH 8) and lysozyme solution (5 μg $ml^{-1}$, pH 8), slides were dehydrated in 70% ethanol and dried in absolute ethanol. Then, slides were hybridized for 3 h at 46 °C in the hybridization buffer (0.9 M NaCl, 0.02 M Tris-HCl, 0.01% SDS and 30% formamide) with 10 ng $\mu l^{-1}$ of probe. After hybridization, slides were washed in the washing buffer (0.1 M NaCl, 0.02 M Tris-HCl, 0.001% SDS, 5 mM EDTA) for 15 min at 48 °C and then mounted with ProLong Diamond Antifade Mountant with DAPI (Invitrogen, USA) and a coverslip. The 16S rRNA-based specific probe Mγ993 (5′ ACAGATTCTCT GGATGTC 3′) labeled with Cy3 (methanotrophs, red fluorescence) [29] and a 16S rRNA-based specific probe (5′ AACAAACCACCTACGCACGCT 3') labeled with FITC (thiotrophs, green fluorescence) were used in FISH. Hybridized slides were photographed under a LSM710 confocal laser scanning microscope (Zeiss, Germany).

## 3. Results and discussion

The mussel *B. platifrons* has been widely found in the harsh deep-sea environment, including cold seeps and hydrothermal vents [9]. The establishment of endosymbiosis in *B. platifrons* gills is undoubtedly an important step in deep-sea adaptation [30]. In this study, several *B. platifrons* individuals were collected from a cold seep at a depth of 1100 m in the South China Sea (Fig. 1a1 & 1a2). We applied FISH and qRT-PCR methods to analyze the composition of symbionts in the mussel gills. In addition, we compared the symbiont abundance and expression of *VAMP* between mussels from the deep sea and mussels reared in a methane-absent laboratory environment for three months.

### 3.1. Detection for methanotrophs and thiotrophs in the gills of B. platifrons

We planned to detect two kinds of symbiotic bacteria in *B. platifrons*, namely, methanotrophs and thiotrophs, which are common in other *Bathymodiolus* mussels. Normal PCR for 16S rDNAs and specific genes (*pmoA* and *aprA*) were performed to detect methanotrophs and thiotrophs in the mussels.

Based on the peak map of Sanger sequencing for the PCR products, the PCR products amplified by the primers 16S rDNA-1 were homogenous. The BLAST results against the NCBI database showed that the sequence of the fragment amplified using 16S rDNA-1 (Fig. S1) had the highest similarity to the methanotrophs 16S rDNA. In addition, *pmoA*, a specific gene of methanotrophs, was able to be amplified. These results confirm the existence of methanotrophs in the gills of *B. platifrons*, which was consistent with a previous study [3].

Based on the peak map of Sanger sequencing for the PCR products, the PCR products amplified by 16S rDNA-2 were heterogeneous. The BLAST results against the NCBI database showed that sequence of the amplified fragment carried by one *E. coli* clone (Fig. S1) had a high similarity to the thiotroph 16S rDNA, while sequence of the fragment carried by another *E. coli* clone had a high similarity to the uncultured bacterium clone B78-54 16S rDNA. This may be due to the non-specificity of 16S rDNA-2. However, *aprA*, an indicator of thiotrophs, was not amplified. Similar results were found in previous studies. For the thiotrophic bacteria *Pelagibaca bermudensis*, *aprA* was not amplified [24]. Similarly, in the deep-sea mussel *Bathymodiolus puteoserpentis*, although the thiotrophic symbionts were present, no *aprA* signal was detected by FISH [21]. Therefore, it is possible that *aprA* can not be detected in thiotrophs. One possible reason is that the primer we used failed to amplify the target sequences, another possible reason is that the thiotrophs are so scarce that the expression level of *aprA* is out of the limit of detection. Although our results suggest that thiotrophs present in the gills of *B. platifrons*, the quantity of thiotrophs might be very low. Therefore, it is reasonable to conclude that methanotrophs are the dominant symbiotic bacteria in the *B. platifrons* collected from a cold seep in the South China Sea.

### 3.2. Distribution pattern of methanotrophs in the gills of B. platifrons

To display the distribution pattern of methanotrophs in the gills of mussels, FISH targeted to both *pmoA* and 16S rRNA was performed. The result of FISH targeted to *pmoA* showed that methanotrophs were abundant in the gill epithelium, while almost no methanotrophs were observed in the ciliary tufts (ct) of the gill (Fig. 1b1), which is similar to the distribution pattern of methanotrophs in other *Bathymodiolus* mussel species [11,31]. The high magnification of gill filaments revealed that the green fluorescence signal indicating methanotrophs was circular in shape (Fig. 1b2). The results of FISH targeted to 16S rRNA exhibited the same distribution pattern and circle-shaped signals (Fig. 1c1 & 1c2). Fisher et al. [32] observed by transmission electron microscope (TEM) that symbiotic bacteria were housed in gill bacteriocytes in *B. thermophilus*. Moreover, by TEM, Frenkiel et al. [33] found that thiotrophs inhabited gill bacteriocytes with a diameter of 10–15 μm in *Lucina pectinate*. In this study, the signal indicating methanotrophs was circle-shaped and had a similar diameter as that of a bacteriocyte, suggesting that each circular signal may be a bacteriocyte. Since *aprA* was not amplified by normal PCR, FISH targeted to thiotroph 16S rRNA was performed. As Fig. S2 shows, the green fluorescence indicating thiotrophs was on the surface of the ct of the gill, and no signal was detected in the putative bacteriocyte-covered area. The scarce thiotrophs we detected in the gills were probably environmental bacteria in deep-sea water, not endosymbionts. Based on our FISH results, methanotrophs are abundant in the gills of *B. platifrons* and concentrated in bacteriocytes. In addition, the unique circle-shaped FISH signal in *B. platifrons* has not been detected in other species of *Bathymodiolus* mussel. The results of FISH targeted to mRNA were consistent with those of FISH targeted to 16S rRNA, providing precise spatial information for methanotrophs.

### 3.3. Change in abundance of methanotrophs after a 3-month rearing period

To understand the relationship between symbionts and the surrounding environment, we compared the relative abundance of methanotrophs in the gills of *B. platifrons* freshly collected from the deep sea and those maintained in a methane-free aquaria for three months. The qRT-PCR results indicate that the methanotrophs in the mussels

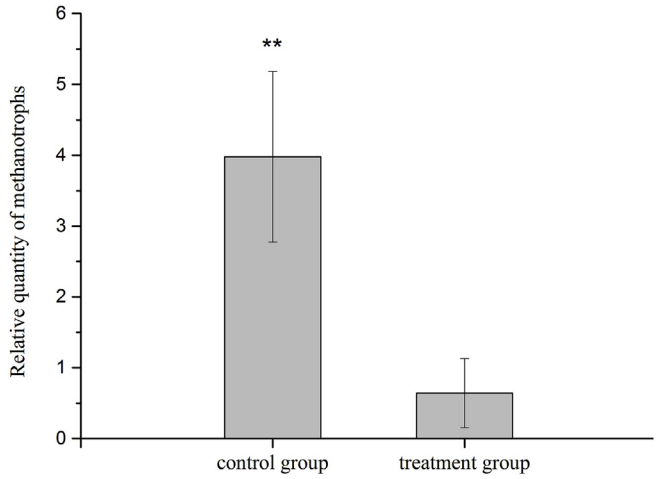


**Fig. 2.** Relative quantity of methanotrophs in the gills of *B. platifrons* under different environment, including the control group (i.e., mussels freshly collected from deep-sea) and treatment group (i.e., mussels reared in the laboratory for three months). ** indicates that there is an extremely significant difference ($P < 0.01$). Error bars indicate the standard deviation (SD).

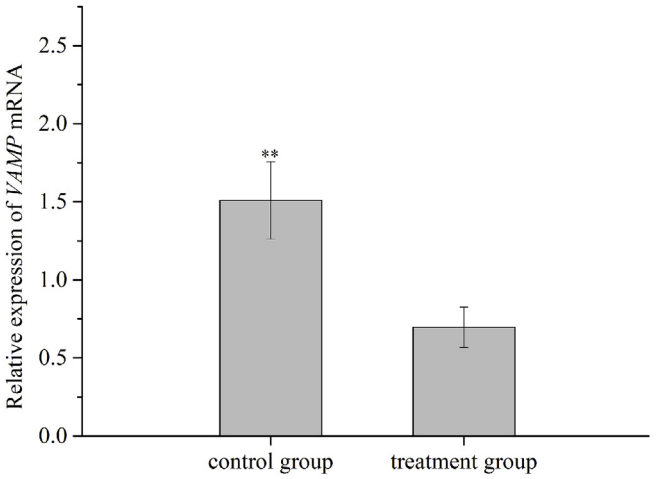


**Fig. 4.** Relative quantity of *VAMP* in the gills of *B. platifrons* under different environment, including the control group (i.e., mussels freshly collected from deep-sea) and treatment group (i.e., mussels reared in the laboratory for three months). ** indicates that there is an extremely significant difference ($P < 0.01$). Error bars indicate the SD.

decreased significantly after a 3-month rearing without methane supply ($P < 0.01$) but did not completely disappear (Fig. 2). In addition, FISH targeted to both *pmoA* and 16S rRNA was performed to visually understand the change in abundance of methanotrophs. The FISH results also showed that the number of methanotrophs in the gills decreased significantly after a three-month laboratory rearing. The decrease of methanotrophs was homogeneous in the gill and not related to the locations (Fig. 3). Notably, after the laboratory rearing, the circle-shaped fluorescence signal in the gill disappeared and changed into spots, indicating that the methanotrophs in a bacteriocyte decreased after the laboratory rearing (Fig. 3c and d). Sun et al. [19] also found that methanotrophs in bacteriocytes decreased but still grouped together in bacteriocytes after rearing for one month. Similarly, Kádár et al. [34] reported that methanotrophs and thiotrophs in *B. azoricus* gradually decreased over a one-month acclimatization period under laboratory conditions without sulfur or methane supply. These reports suggest that

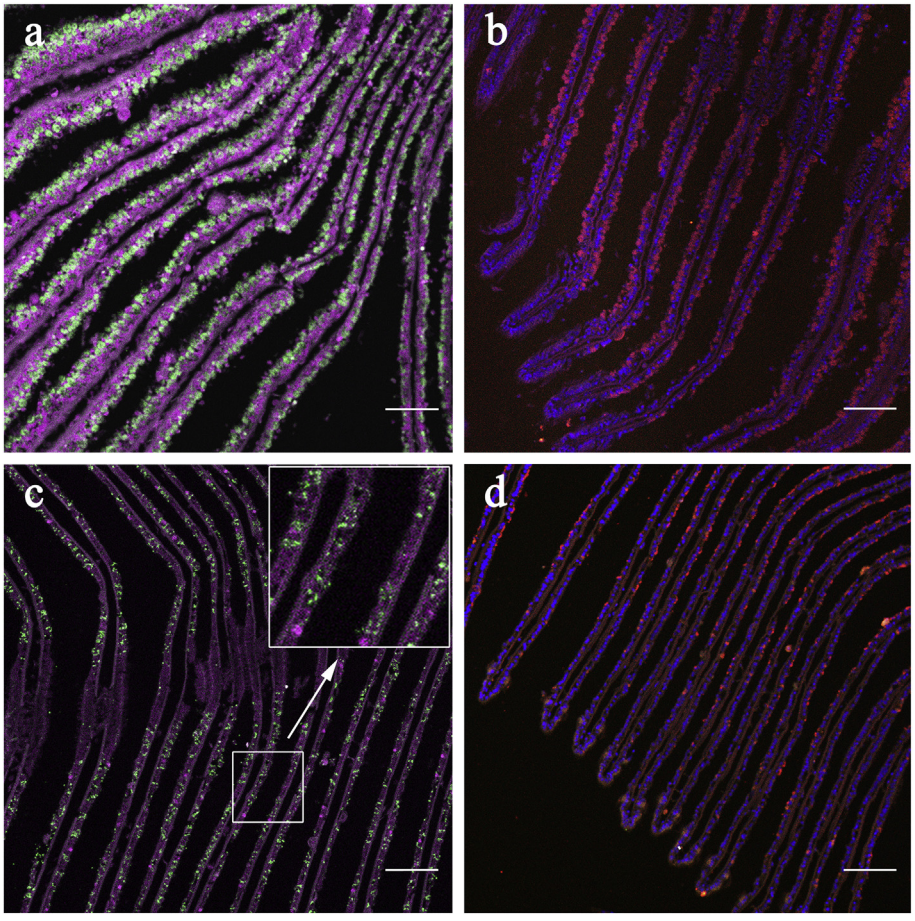


**Fig. 3.** FISH of methanotrophs in the gills of *B. platifrons* under different environment. FISH target to *pmoA* (green fluorescence) (**a**) and 16S rRNA (red fluorescence) (**b**) in the gills of mussels from the control group (i.e., mussels freshly collected from deep-sea). FISH target to *pmoA* (green fluorescence) (**c**) and 16S rRNA (red fluorescence) (**d**) in the gills of mussels from the treatment group (i.e., mussels reared in the laboratory for three months). Contrasting background of the gill tissues were revealed by magenta fluorescence (**a, c**) or were revealed by blue fluorescence indicating nuclei (**b, d**). Scale bar: 100 μm.

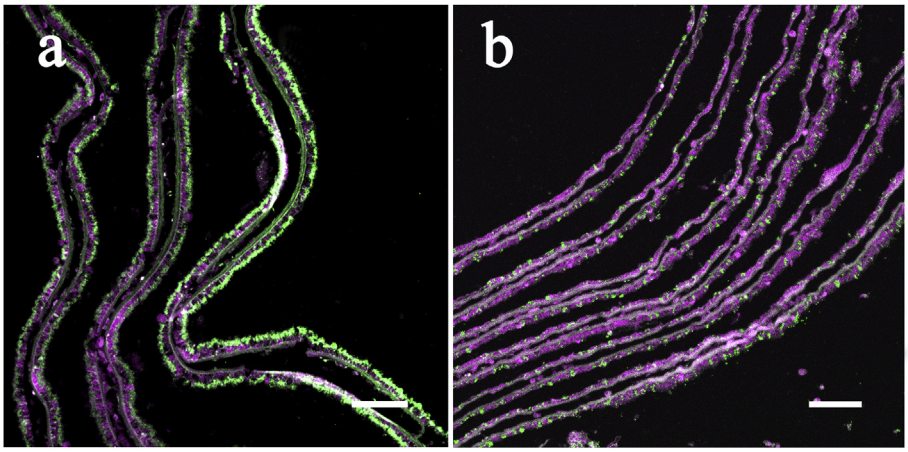


**Fig. 5.** FISH target to *VAMP* mRNA (green fluorescence) in the gills (magenta fluorescence) of *B. platifrons* under different environment, including mussels from the control group (i.e., mussels freshly collected from deep-sea) (**a**) and those from treatment group (i.e., mussels reared in the laboratory for three months) (**b**). Scale bar: 100 μm.

the necessary chemical environment plays an important role in symbiont maintenance in deep-sea shellfish. Our study also supports that methane is critical for the maintenance of methanotrophs in *B. platifrons*. However, the methanotrophs did not disappear completely in the gills of *B. platifrons* after a 3-month rearing without methane supply, indicating that the symbiosis between *B. platifrons* and methanotrophs is not as delicate as we previously believed. A different result was deserved in the deep-sea clam *Calyptogena okutanii*, in which the symbionts disappeared completely after acclimation to laboratory conditions for 91 days [35]. It is therefore implied there are differences in symbiont maintenance among different species, which may be related to the different adaptation abilities of species to the unstable chemical environment of the deep sea.

### 3.4. Expression variation in a lysosome-related VAMP gene after a 3-month rearing period

To identify the role of the lysosomal system in controlling the symbionts in *B. platifrons*, the variations in the expression of *VAMP*, a lysosome-related gene, were analyzed in the gills of freshly collected mussels and those reared for three months in the laboratory. The qRT-PCR results showed that freshly collected mussels had a higher mRNA expression level of *VAMP* than those reared in the laboratory for three months (Fig. 4). Similar to the qRT-PCR results, the results of FISH targeted to *VAMP* mRNA also showed that *VAMP* had a higher expression in the gills of freshly collected mussels than in the gills of mussels reared for three months (Fig. 5a and b). Combined with the observed change in abundance of methanotrophs, these results indicate that the expression of *VAMP* is positively related to the abundance of methanotrophs in the gills of *B. platifrons*. The FISH signals of *VAMP* mRNA in the freshly collected mussels were also circular in shape and present exactly where methanotrophs occurred (Figs. 3a and 5a); in addition, similar to the FISH signals of methanotrophs, the expression of *VAMP* also decreased in the gills of mussels reared for three months (Figs. 3c and 5b). These results further suggest that there is a relationship between *VAMP* and methanotrophs. *VAMP* is reportedly required for the formation of a specific membrane compartment, in which the symbionts are hosted in a controlled manner by the host [16]. The high expression of *VAMP* in gills with a high abundance of symbionts is likely necessary for the host mussel to control symbionts. When symbionts were reduced or disappeared, the host mussel did not need to consume energy to control symbionts; thus, the expression of *VAMP* decreased, and the circle-shaped distribution pattern of methanotrophs disappeared. Our results suggest that the lysosomal system plays an important immunity role in controlling the abundance of endosymbionts in *B. platifrons*.

## 4. Conclusion

In this study, we investigated the gill symbionts of the cold-seep mussel *B. platifrons*, including species composition, environmental dependency and immune control. Modified methods of FISH targeted to both mRNA and 16S rRNA were used to detect symbionts. The *pmoA*-based RNA probe was used to detect methanotrophs in *B. platifrons* for the first time. Similarly, the 16S rRNA-based probe used in this study has not been applied to *Bathymodiolus* mussels before. In addition, a new primer pair specific to the 16S rDNA of methanotrophs (16S rDNA-1) was produced and can be applied to the future PCR detection and quantification of methanotrophs in *B. platifrons*. Our results showed that methanotrophs were the major symbiotic bacteria in the gills of *B. platifrons*, while thiotrophs were absent or scarce, which is consistent with the results of previous studies. In the mussels freshly collected from the deep sea, methanotrophs were housed in bacteriocytes in a unique circle-shaped pattern, and a similar expression pattern was also found for a lysosome-related *VAMP* gene. After a 3-month rearing period in aquaria without methane supply, the abundance of methanotrophs decreased significantly, and their circle-shaped distribution pattern disappeared; meanwhile, the expression of *VAMP* decreased significantly. These results suggest that the symbiosis between *B. platifrons* and methanotrophs is influenced by the environment, and the lysosomal system plays an important immune role in controlling the abundance of endosymbionts in host.

## Acknowledgements

This work was funded by Strategic Priority Research Program of the Chinese Academy of Sciences (XDA11030202), the International Partnership for Innovative Team Program (20140491526) from the Chinese Academy of Sciences, the Youth Foundation Project (YQ2018NO07) and the Scientific and Technological Innovation Project (2015ASKJ02) from Qingdao National Laboratory for Marine Science and Technology.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.11.041.

## References

[1] E.M. Spiridonova, B.B. Kuznetsov, N.V. Pimenov, T.P. Tourova, Phylogenetic characterization of endosymbionts of the hydrothermal vent mussel *Bathymodiolus azoricus* by analysis of the 16S rRNA, *cbb*L, and *pmo*A genes, Microbiology 75 (2006) 694–701.

[2] D.L. Distel, C.M. Cavanaugh, Independent phylogenetic origins of methanotrophic and chemoautotrophic bacterial endosymbioses in marine bivalves, J. Bacteriol.

176 (1994) 1932–1938.
[3] Y. Fujiwara, K. Takai, K. Uematsu, S. Tsuchida, J.C. Hunt, J. Hashimoto, Phylogenetic characterization of endosymbionts in three hydrothermal vent mussels: influence on host distributions, Mar. Ecol. Prog. Ser. 208 (2000) 147–155.
[4] D.L. Distel, D.J. Lane, G.J. Olsen, S.J. Giovannoni, B. Pace, N.R. Pace, D.A. Stahl, H. Felbeck, Sulfur-oxidizing bacterial endosymbionts: analysis of phylogeny and specificity by 16S rRNA sequences, J. Bacteriol. 170 (1988) 2506–2510.
[5] A. Fiala-Médioni, Z.P. McKiness, P. Dando, J. Boulegue, A. Mariotti, A.M. Alayse-Danet, J.J. Robinson, C.M. Cavanaugh, Ultrastructural, biochemical, and immunological characterization of two populations of the mytilid mussel *Bathymodiolus azoricus* from the Mid-Atlantic Ridge: evidence for a dual symbiosis, Mar. Biol. 141 (2002) 1035–1043.
[6] S. Duperron, T. Nadalig, J.-C. Caprais, M. Sibuet, A. Fiala-Medioni, R. Amann, N. Dubilier, Dual symbiosis in a *Bathymodiolus* sp. mussel from a methane seep on the Gabon continental margin (Southeast Atlantic): 16S rRNA phylogeny and distribution of the symbionts in gills, Appl. Environ. Microbiol. 71 (2005) 1694–1700.
[7] S. Duperron, M. Sibuet, B.J. MacGregor, M.M.M. Kuypers, C.R. Fisher, N. Dubilier, Diversity, relative abundance and metabolic potential of bacterial endosymbionts in three *Bathymodiolus* mussel species from cold seeps in the Gulf of Mexico, Environ. Microbiol. 9 (2007) 1423–1438.
[8] S. Duperron, The diversity of deep-sea mussels and their bacterial symbioses, in: S. Kiel (Ed.), The Vent and Seep Biota, Springer, Netherlands, 2010, pp. 137–167.
[9] A. Kyuno, M. Shintaku, Y. Fujita, H. Matsumoto, M. Utsumi, H. Watanabe, Y. Fujiwara, J.-I. Miyazaki, Dispersal and differentiation of deep-sea mussels of the genus *Bathymodiolus* (Mytilidae, Bathymodiolinae), J. Mar. Biol. 2009 (2009) 295–305.
[10] J.P. Barry, K.R. Buck, R.K. Kochevar, D.C. Nelson, Y. Fujiwara, S.K. Goffredi, J. Hashimoto, Methane-based symbiosis in a mussel, Bathymodiolus platifrons, from cold seeps in Sagami Bay, Japan. Invertebr. Biol. 121 (2002) 47–54.
[11] M. Fujinoki, T. Koito, Y. Fujiwara, M. Kawato, Y. Tada, K. Hamasaki, M. Jimbo, K. Inoue, Whole-mount fluorescence in situ hybridization to visualize symbiotic bacteria in the gills of deep-sea mussels, Aquat. Biol. 14 (2012) 135–140.
[12] F. Renoz, C. Noel, A. Errachid, V. Foray, T. Hance, Infection dynamic of symbiotic bacteria in the pea aphid *Acyrthosiphon pisum* gut and host immune response at the early steps in the infection process, PloS One 10 (2015) e0122099.
[13] P. Simonet, K. Gaget, S. Balmand, M. Ribeiro Lopes, N. Parisot, K. Buhler, G. Duporta, V. Vulstekeb, G. Febvaya, A. Heddia, H. Charlesa, P. Callaertsb, F. Calevro, Bacteriocyte cell death in the pea aphid/*Buchnera* symbiotic system, Proc. Natl. Acad. Sci. U.S.A. 115 (2018) E1819–E1828.
[14] K. Nishikori, K. Morioka, T. Kubo, M. Morioka, Age- and morph-dependent activation of the lysosomal system and *Buchnera* degradation in aphid endosymbiosis, J. Insect Physiol. 55 (2009) 351–357.
[15] P. Zheng, M. Wang, C. Li, X. Sun, X. Wang, Y. Sun, S. Sun, Insights into deep-sea adaptations and host-symbiont interactions: a comparative transcriptome study on *Bathymodiolus* mussels and their coastal relatives, Mol. Ecol. 26 (2017) 5133–5148.
[16] S. Ivanov, E.E. Fedorova, E. Limpens, S.D. Mita, A. Genre, P. Bonfante, T. Bisseling, *Rhizobium*-legume symbiosis shares an exocytotic pathway required for arbuscule formation, Proc. Natl. Acad. Sci. U.S.A. 109 (2012) 8316.
[17] S. Duperron, C. Bergin, F. Zielinski, A. Blazejak, A. Pernthaler, Z.P. McKiness, E. EdChaine, C.M. Cavanaugh, N. Dubilier, A dual symbiosis shared by two mussel species, *Bathymodiolus azoricus* and *Bathymodiolus puteoserpentis* (Bivalvia: Mytilidae), from hydrothermal vents along the northern Mid-Atlantic Ridge, Environ. Microbiol. 8 (2006) 1441–1447.
[18] R. Bettencourt, P. Dando, D. Rosa, V. Riou, A. Colaco, J. Sarrazin, P.-M. Sarradin, R.S. Santos, Changes of gill and hemocyte-related bio-indicators during long term maintenance of the vent mussel *Bathymodiolus azoricus* held in aquaria at atmospheric pressure, Comp. Biochem. Physiol. A 150 (2008) 1–7.
[19] Y. Sun, M. Wang, L. Li, L. Zhou, X. Wang, P. Zheng, H. Yu, C. Li, S. Sun, Molecular identification of methane monooxygenase and quantitative analysis of methanotrophic endosymbionts under laboratory maintenance in *Bathymodiolus platifrons* from the South China Sea, PeerJ 5 (2017) e3565.
[20] S. Halary, V. Riou, F. Gaill, T. Boudier, S. Duperron, 3D FISH for the quantification of methane-and sulphur-oxidizing endosymbionts in bacteriocytes of the hydrothermal vent mussel *Bathymodiolus azoricus*, ISME J. 2 (2008) 284.
[21] A. Wendeberg, F.U. Zielinski, C. Borowski, N. Dubilier, Expression patterns of mRNAs for methanotrophy and thiotrophy in symbionts of the hydrothermal vent mussel *Bathymodiolus puteoserpentis*, ISME J. 6 (2012) 104.
[22] H. Guezi, I. Boutet, A.C. Andersen, F.H. Lallier, A. Tanguy, Comparative analysis of symbiont ratios and gene expression in natural populations of two *Bathymodiolus* mussel species, Symbiosis 63 (2014) 19–29.
[23] A.M. Costello, M.E. Lidstrom, Molecular characterization of functional and phylogenetic genes from natural populations of methanotrophs in lake sediments, Appl. Environ. Microbiol. 65 (1999) 5066–5074.
[24] B. Meyer, J. Kuever, Molecular analysis of the diversity of sulfate-reducing and sulfur-oxidizing prokaryotes in the environment, using *aprA* as functional marker gene, Appl. Environ. Microbiol. 73 (2007) 7664–7679.
[25] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method, Methods 25 (2001) 402–408.
[26] X. Wang, B. Liu, G. Wang, B. Tang, J. Xiang, Molecular cloning and functional analysis of cathepsin B in nutrient metabolism during larval development in *Meretrix meretrix*, Aquaculture 282 (2008) 41–46.
[27] A. Pernthaler, R. Amann, Simultaneous fluorescence in situ hybridization of mRNA and rRNA in environmental bacteria, Appl. Environ. Microbiol. 70 (2004) 5426–5433.
[28] X. Yue, P. Huan, G. Xiao, B. Liu, Expression patterns of an i-type lysozyme in the clam *Meretrix meretrix* along with larval development, Dev. Comp. Immunol. 41 (2013) 27–32.
[29] G. Eller, S. Stubner, P. Frenzel, Group-specific 16S rRNA targeted probes for the detection of type I and type II methanotrophs by fluorescence in situ hybridisation, FEMS Microbiol. Lett. 198 (2001) 91–97.
[30] M.Y. Kellermann, F. Schubotz, M. Elvert, J.S. Lipp, D. Birgel, X. Prieto-Mollar, N. Dubilier, K.-U. Hinrichs, Symbiont–host relationships in chemosynthetic mussels: a comprehensive lipid biomarker study, Org. Geochem. 43 (2012) 112–124.
[31] C. Wentrup, A. Wendeberg, M. Schimak, C. Borowski, N. Dubilier, Forever competent: deep-sea bivalves are colonized by their chemosynthetic symbionts throughout their lifetime, Environ. Microbiol. 16 (2014) 3699–3713.
[32] C.R. Fisher, J.J. Childress, R.S. Oremland, R.R. Bidigare, The importance of methane and thiosulfate in the metabolism of the bacterial symbionts of two deep-sea mussels, Mar. Biol. 96 (1987) 59–71.
[33] L. Frenkiel, O. Gros, M. Mouëza, Gill structure in *Lucina pectinata* (Bivalvia: Lucinidae) with reference to hemoglobin in bivalves with symbiotic sulphur-oxidizing bacteria, Mar. Biol. 125 (1996) 511–524.
[34] E. Kádár, R. Bettencourt, V. Costa, R.S. Santos, A. Lobo-da-Cunha, P. Dando, Experimentally induced endosymbiont loss and re-acquirement in the hydrothermal vent bivalve *Bathymodiolus azoricus*, J. Exp. Mar. Biol. Ecol. 318 (2005) 99–110.
[35] K. Ohishi, M. Yamamoto, A. Tame, C. Kusaka, Y. Nagai, M. Sugimura, K. Inoue, K. Uematsu, T. Yoshida, T. Ikuta, T. Toyofuku, T. Maruyama, Long-term cultivation of the deep-sea clam *Calyptogena okutanii*: changes in the abundance of chemoautotrophic symbiont, elemental sulfur, and mucus, Biol. Bull-US 230 (2016) 257.