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Relationship between the occurrence of filamentous bacteria on *Bathymodiolus azoricus* shell and the physiological and toxicological status of the vent mussel

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ABSTRACT

The edifice walls of the Eiffel Tower hydrothermal vent site (Mid-Atlantic Ridge, Lucky Strike vent field) are populated with dense communities of dual symbioses harboring vent mussel *Bathymodiolus azoricus*, some of which are covered by white filamentous mats belonging to sulfur-oxidizing bacteria. Mussels were collected in both the presence and absence of the filamentous bacteria. A sample of the filamentous bacteria was collected and water measurements of temperature, CH_4 and H_2S were recorded at the collection area. The whole soft tissues were analyzed for total lipid, carbohydrate and total protein. Metallothioneins and metals (Cu, Fe and Zn) levels were determined in the major organs. The results showed no significant physiological and toxicological evidence that emphasizes the influence of associated sulfur-oxidizing filamentous bacteria on *B. azoricus* mussel shells. However, *B. azoricus* mussel seems to be well adapted to the assorted physico-chemical characteristics from the surrounding environment since it is able to manage the constant fluctuation of physico-chemical compounds.

1. Introduction

Lucky Strike is one of the largest known active vent fields (37° 18′ N, 32° 16′ W), located in the Mid-Atlantic Ridge between 1730 and 1736 m depth. The hydrothermal fluid, emitted at temperature ranging between 170 and 324 °C, presents characteristics (temperature, chlorinity, gas and metal concentration) that vary from site to site within the field (Charlou et al., 2000). The hydrothermal vent area is distributed around a lava lake, bound by the summits of three volcanic cones (Radford-Knoery et al., 1998). Both well-defined active chimneys such as Eiffel Tower belching out very hot fluids and zones where hydrothermal activity is more diffuse, can be found at Lucky Strike (Desbruyères et al., 2001). Eiffel Tower, located at 200 m from the southeastern edge of the circular lava lake, consists of chimneys combined into an edifice rising 7 m above the neighboring seafloor (Radford-Knoery et al., 1998). The structure presents numerous high temperature smokers and cracks emitting lower temperature fluids. It is colonized nearly uniformly by the vent mussel *Bathymodiolus azoricus*, several patches of mussel been covered by dense white filamentous microbial mats (Sarradin et al., 1999; Desbruyères et al., 2001) while other are not at all. The filamentous microbial mats are mainly composed of a species of *Beggiatoa* (Mattison et al., 1998). This species is a colonizer, very abundant in environments characterized by the presence of hydrogen sulfide (Wörner and Zimmermann-Timm, 2000). These chemoautotrophic bacteria gain energy by the oxidation of reduced sulfur compounds (Nelson et al., 1989; Hagen and Nelson, 1997; Erbacher and Nelskamp, 2006). *B. azoricus* hosts "dual symbioses", involving the stable coexistence of chemoautotrophic (also referred to as thiotrophic) and methanotrophic bacteria harbored within the gill (Fiala-Médioni et al., 2002; Duperron et al., 2005). Dual symbioses provide obvious advantages to host individuals recruiting to environments where the availability of substrates is unpredictable or fluctuating (Cavanaugh et al., 1992; Fiala-Médioni et al., 2002). This dual symbioses has also been described in other mussel species that live in reducing environments with high sulfide and methane concentrations, such as hydrothermal vents (Duperron et al., 2005; Stewart et al., 2005) and cold seeps (Fisher et al., 1993). The physiology and biochemistry of the hydrothermal vent mussel *B. azoricus* must be adapted to the rapid fluctuating composition of its environment composed of a mixture of seawater and hydrothermal fluid (Childress and Fisher, 1992). The presence and absence of sulfur-oxidizing filamentous bacteria on the hydrothermal mussel beds could be an indicator of a fluctuating environment, since these chemolithoautotrophic bacteria proliferate in environments with reduced sulfur compounds (Brinkhoff and Muyzer, 1997) and grow in habitats with oxic–anoxic interfaces (Moyer et al., 1995). These chemical conditions may alter the chemistry of mussel habitats (Le Bris et al., 2006) and consequently their physiological and toxicological condition. The aim of our investigation was to study the physiological condition and metal accumulation of

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B. azoricus collected from a mussel bed where the presence and absence of filamentous bacteria were observed.

2. Materials and methods

2.1. Sampling

Samples were collected during the EXOMAR cruise (with the R/V "Atalante") in July 2005, at 1690 m depth on the Eiffel Tower hydrothermal site. The mussels were sampled by the manipulator arm and brought to the surface using the Remotely Operated Vehicle (ROV) "Victor 6000". Samples were collected in two neighboring areas, where mussel shells were covered by filamentous bacteria (designated hereafter as "mussels + mats") or not covered (designated hereafter as "mussels"). From each area 25 individuals were collected and measured. Individuals from the group "mussels + mats" presented a mean total length of 4.3 cm (±0.5 SD) and individuals from the group "mussels" presented a mean total length of 3.0 cm (±0.3 SD). For each group, the whole soft tissues of 15 mussels were separated from the shells and kept frozen (−80 °C) until lyophilization and analysis. Left 10 mussels were dissected into gill, mantle, foot, digestive gland and remaining soft tissues and kept frozen (−80 °C) until lyophilization and analysis. A sample of microbial mats, that covered the mussels, was collected using the water pumping device of the ROV "VICTOR 6000". Water samples (5 l) were filtered on board and filters were preserved at −80 °C for further analysis.

2.2. Lipid analysis

Because of small soft tissue weights, 5 mussels were pooled together for single measurement of total lipids levels at each group. The lipid content was determined according to the modified method of Bligh and Dyer (1959) by extracting lipids from a dry powdered in a water–dichloromethane–methanol mixture and by weighing after evaporation to dryness, the organic layer. Level was expressed as mg g^{-1} of dry weight.

2.3. Carbohydrate analysis

The whole soft tissue of 5 individuals from each group of mussels was used for carbohydrate analysis. The carbohydrate content was determined colorimetrically in a NaCl extract, in the presence of 5% phenol and concentrated H_2SO_4, as described by Dubois et al. (1956). The concentration was determined in glucose equivalents from a glucose calibration curve using glucose as a standard. The levels of carbohydrate were expressed as mg g^{-1} of dry weight.

2.4. Samples preparation for total protein, metallothionein and metal analyses

The tissues of the 10 dissected mussels from each group, were lyophilized, weighed and homogenized in 6 ml of ice-cold 100 mM Tris buffer, pH 8.1, containing 10 mM ß-mercaptoethanol. The homogenates were centrifuged for 30 min at 25,000 g, at 4 °C to separate the supernatants (S_1) from the insoluble fraction (pellets), used for the study of intracellular metal distribution. Aliquots (1 ml) of the S_1 were used for metallothioneins determination. Pellets and remaining supernatants were digested in an Ethos Plus microwave oven with 5 ml of HNO_3 (69% v/v) for metal analysis.

2.4.1. Total protein analysis

Total protein levels were determined in supernatants S_1 from whole soft tissues of 5 individuals from each group of mussels, following the BioRad protein assay kit for the Bradford method (Bradford, 1976). BSA (Bovine Serum Albumin) was used as reference standard. Results were expressed as mg g^{-1} of dry weight.

2.4.2. Metallothionein and metal analysis

The aliquot of the supernatant S_1, was heat-denatured (90 °C, 15 min) and centrifuged (13,000 g, 10 min, at 4 °C) in order to separate the thermostable metallothioneins (MTs) from thermolabile proteins. The heat stable fractions (S_2) were used for quantification of MTs by Differential Pulse Polarography (DPP) according to Olafson and Sim (1979) improved by Thompson and Cosson (1984). A standard addition calibration curve was obtained using rabbit liver MT-I as reference. Results were expressed as mg g^{-1} of dry weight.

After pellets and remaining supernatant S_1 digestion, solutions were dried at 60 °C and diluted by adding 2 ml 0.5 N HNO_3. Metal levels (Cu, Fe, Zn) were measured by flame atomic absorption spectrophotometry (AAS) with deuterium background correction. The accuracy and precision of the method used were established by regular analysis of certified reference materials of mussel tissue CE278 (European Reference Materials of Belgium) and lobster hepatopancreas TORT-2 (National Research Council of Canada) (Table 1). Certified reference materials and blanks were taken through the procedure in the same way as the samples. Metal levels were calculated and expressed as mg g^{-1} of dry weight.

2.5. Microbial mat molecular diversity

A preliminary study of the microbial mat diversity was performed by using 16S rRNA gene sequencing. DNA was extracted from frozen mat sample pellets as described in Alain et al. (2002). Archaeal DNA was amplified using the primer A24F (5′-TTC CGG TTG ATC CTG CCG GA-3′) and the reverse primer 1407R (5′-GAC GGG CGG TGW GTR CAA-3′). Bacterial DNA was amplified using the primer E8F (5′-AGA GTT TGA TCA TGG CTC AG-3′) and the reverse primer U1492R (5′-GTT ACC TTG TTA CGA CTT-3′). PCR reactions were performed on a Robocycler Gradient 96 (Stratagene) (Wery et al., 2002; Nercessian et al., 2003). PCR products were then checked on a 0.8% (w/v) agarose gel and directly cloned using the TOPO TA Cloning® kit (pCR2.1 vector), according to the manufacturer's instructions (Invitrogen). Sequences were analyzed as previously described by Postec et al. (2005).

2.6. In situ temperature measurements, CH_4 and H_2S estimated values

Eight autonomous temperature probes (thermistor, Vemco Minilog 12 TR 64K probes) were deployed on each sampling point for two days. The temperature data were corrected against the bottom seawater temperature (4.4 °C). The sampling period was 30 s. CH_4 concentrations were estimated using the significant temperature/CH_4 linear relationship obtained during the ATOS cruise on 16 samples from the Eiffel Tower edifice (Sarradin et al., 2003). Total sulfide (ΣS) concentrations were estimated using the significant temperature/ΣS linear relationship obtained in 2006 during the MoMARETO cruise with the CHEMINI in situ chemical analyzer (Vuillemin et al., 2009). Results were expressed as µmol l^{-1}.

2.7. Statistical analysis

All the results are given as mean level by individual/tissues dry weight. The statistical calculations were performed with STATISTICA

Table 1
Levels of Cu, Fe and Zn found in certified reference material, mussel tissue CE278 (ERM-Belgium) and lobster hepatopancreas TORT-2 (NRCC-Canada).

Certified reference material		Cu (mg g^{-1}) $n=6$	Fe (mg g^{-1}) $n=8$	Zn (mg g^{-1}) $n=11$
CE278	Certified	–	–	0.08 ± 0.002
	Observed	–	–	0.10 ± 0.001
TORT-2	Certified	0.11 ± 0.01	0.11 ± 0.01	–
	Observed	0.11 ± 0.002	0.11 ± 0.01	–

Results as mean ± SD, in mg g^{-1} dry weight.