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2-D difference gel electrophoresis approach to assess protein expression profiles in *Bathymodiolus azoricus* from Mid-Atlantic Ridge hydrothermal vents

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ABSTRACT

Hydrothermal vent mussels *Bathymodiolus azoricus* are naturally exposed to toxic chemical species originated directly from vent chimneys. The amount of toxic elements varies significantly among vent sites along the Mid-Atlantic Ridge and *B. azoricus* must be able to adapt to changes in hydrothermal fluid composition, temperature and pressure. The aim of this work was to study changes in the proteome in the "gill-bacteria complex" of mussels *B. azoricus* from three hydrothermal vent sites with distinct environmental characteristics using 2-D Fluorescence Difference Gel Electrophoresis (2-D DIGE). Results showed that 31 proteins had different expression profiles among vent sites and both cluster and principal component analysis confirm a clear separation of mussels between sites. This suggests the existence of specific parameters grouping individuals from the same hydrothermal site. Protein spots of the more abundant differentially expressed proteins were excised, digested with trypsin and identified by mass spectrometry. All identified proteins (actin, ubiquinone, S-adenosylhomocysteine hydrolase, cysteine peptidases, chaperonin and catalase) have been related previously with oxidative stress conditions and are known to be affected by ROS inducing stressors, including metals. Results point out to specific adaptations at the proteome level of *B. azoricus* depending on the level of toxicants present in their environment.

1. Introduction

Deep-sea mussels of the genus Bathymodiolus are vent endemic bivalve species that are among the dominant macro-organisms in these communities. *B. azoricus* is a well-described specie present in the North Atlantic vent fields, south-west of the Azores Triple Junction, Mid-Atlantic Ridge (MAR): Menez Gwen (37°51′N, 31°31′W, 850 m), Lucky Strike (37°17′N, 32°16′W, 1700 m) and Rainbow (36°13′N, 33°54′W, 2300 m) [1]. Genetic population studies shows that *B. azoricus* mussels are confined to these northern MAR Ridge vent fields and are genetically different from other Bathymodiolus species, including those found in the southern MAR vents (*B. puteoserpentis*) [2]. A remarkable feature of *B. azoricus* is the presence of dual endosymbiosis with both

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thioautotropic and methanotrophic bacteria located in specialised cells (bacteriocytes) of the gill epithelial tissue as the main source of energy [3–5], although they retained the capability of suspension-feeding and can digest organic matter particles [6,7]. This mussel forms extensive communities surrounding the hydrothermal active area, covering the base and walls of vent chimneys forming copious animal clusters around active chimneys [8–10] and have to cope with different conditions of hydrostatic pressure, temperature and hydrothermal fluid composition in terms of dissolved oxygen, methane and sulphide concentrations as well as high levels of metals. The fluid composition in these three hydrothermal vents is mainly determined by the permeability structure of the ocean crust and the location of the heat source that influence how the hydrothermal fluids circulate. Therefore, pressure, temperature, water–rock ratio, rock composition and reaction time are the parameters that determine the fluid composition [11–14]. The main characteristics of hydrothermal fluids in the three vent sites are in Table 1. Nevertheless, *B. azoricus* live in the hydrothermal "mixing zone", i.e., the interface between the hot anoxic upflow zone and cold, oxidized seawater [15]. This mixing zone is characterized by a non-steady state equilibrium with large spatial and temporal variations of environmental conditions, and its chemistry is complex and still poorly understood. In the last few decades several studies aimed to understand how hydrothermal vent organisms can survive in such extreme conditions. Due to the wide distribution and abundance of the *Bathymodiolus* genus in hydrothermal vent sites worldwide, this mussel has been elected as a model organism to understand the mechanisms of exposure to metals in terms of accumulation and depuration processes [16–18] accumulation of petroleum hydrocarbons [19], and the biological responses or biomarkers to these potentially toxic chemical compounds, such as DNA damage [20], heat shock proteins [21] and more specific detoxification mechanisms like metallothioneins [22–24] and antioxidant enzymes [25–27]. These studies contributed enormously for the knowledge of biological, physiological and biochemical adaptations of the mussel *B. azoricus* to these extreme environments. Nevertheless, the study of single biomarkers is limited and there is a need to have a holistic approach to understand the responses to different conditions, especially in extreme toxic environments. Unlike the single biomarker responses, proteomics examines how multiple expression changes are associated to the presence of contaminants or a harmful environment. Consequently, proteins involved in toxicological responses that have not been described previously may be revealed. Thus, proteomics-based approaches represent an effective method to identify qualitative and quantitative differences between complex protein samples and to provide protein expression signature (PES) that could be robust and unbiased [28]. In the last years, proteomic studies have been used in several coastal bivalve species in response to contaminants, including in the clams *Ruditapes decussatus* [29,30], *Tapes semidecussatus* [31], and mussels *Mytilus galloprovincialis* [32], and *Mytilus edulis* [33–38]. Relatively few proteomic studies were conducted in hydrothermal vent organisms, namely in the deep-sea tube worm *Riftia pachyptila* and their bacterial endosymbionts [39,40], the proteomic characterization of a thermophilic *Geobacillus* bacteriophage isolated from deep-sea vents [41], and recently the effects of oxygen stress in the polychaete *Alvinella pompejana* was study by proteomic approach [42]. To the best of our knowledge this is the first time 2D-DIGE was used to study the proteome of hydrothermal vent organisms. 2D-DIGE shows advantages over traditional 2D-PAGE in several aspects, including multiple pre-labelling of samples, introduction of a pooled internal standard, co-detection and wider dynamic range. Hence, the purpose of this work was to use two-dimensional difference gel electrophoresis (2D-DIGE) to investigate differentially expressed proteins in the gills of *B. azoricus* from three distinct hydrothermal vent fields. Identification of these proteins may provide information about the overall adaptation of this mussel to these extreme environments.

Table 1 – Temperature, pH and concentration of toxic chemical species in the hydrothermal vent sites Menez-Gwen, Lucky Strike and Rainbow compared to average seawater (adapted from Douville et al., 2002).

Site	Menez-Gwen	Lucky Strike	Rainbow	Seawater
T (°C)	271–284	185–324	365	–
pH	4.5	3.4–5.0	2.8	7.8
H_2S (mM)	1.5	0.6–3.4	1.0	~0
CO_2 (mM)	17–20	8.9–28	<16	–
CH_4 (mM)	1.35–2.63	0.5–0.97	2.2–2.5	~0
Ag (nM)	4.3–17	4.7–25	47	0.023
Cd (nM)	<9–12	18–79	130	0.7
Cl (mM)	380–400	413–554	750	546
Co (μM)	<2	<2	13	<2
Cu (μM)	<2	<2–30	140	0.0033
Fe (μM)	<2–18	70–920	24000	0.0045
Mn (μM)	59–68	77–450	2250	0.0013
Ni (μM)	<2	<2	3	<2
Si (mM)	8.2–11.2	8.2–16	6.9	<0.2
Zn (μM)	<2	<2–40	160	0.028

2. Materials and methods

2.1. Animal collection

Deep sea hydrothermal mussels *B. azoricus* were collected from three hydrothermal vent sites located in the northern Mid-Atlantic Ridge Menez-Gwen (37° 51′N and 31° 31.2′ W, 840 m), Lucky Strike (37° 17′ 30″N and 32° 165.3′ W, 1700 m) and Rainbow (36° 13′N and 33° 54.1′W, 2400 m) using a remote operated vehicle during the EU-funded *VENTOX* Project [43]. Mussels were brought to the surface in an isolated compartment of the ROV module to a cold room (10 °C) in the research vessel Atalante (IFREMER). Organisms used for proteomic studies were immediately dissected on ice and the gills frozen in liquid nitrogen and stored at −80 °C until further analysis at the laboratory of proteomic (ProteoRed) at the University of Valencia (Spain).

2.2. Sample preparation and Cy-dye labelling

Symbiotic bacteria were not isolated from the fresh tissues, therefore the gill samples reflect the "gill-bacteria complex" functional unit with contributions from both the host tissue (*B. azoricus*) and thioautotropic and methanotrophic bacteria. The cellular ultrastructure of bacteriocytes in the gills of fresh collected *B. azoricus* is well described by [44]. These specialized