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Response of larval barnacle proteome to CO_2-driven seawater acidification

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ABSTRACT

The majority of benthic marine invertebrates have a complex life cycle, during which the pelagic larvae select a suitable substrate, attach to it, and then metamorphose into benthic adults. Anthropogenic ocean acidification (OA) is postulated to affect larval metamorphic success through an altered protein expression pattern (proteome structure) and post-translational modifications. To test this hypothesis, larvae of an economically and ecologically important barnacle species *Balanus amphitrite*, were cultured from nauplius to the cyprid stage in the present (control) and in the projected elevated concentrations of CO_2 for the year 2100 (the OA treatment). Cyprid response to OA was analyzed at the total proteome level as well as two protein post-translational modification (phosphorylation and glycosylation) levels using a 2-DE based proteomic approach. The cyprid proteome showed OA-driven changes. Proteins that were differentially up or down regulated by OA come from three major groups, namely those related to energy-metabolism, respiration, and molecular chaperones, illustrating a potential strategy that the barnacle larvae may employ to tolerate OA stress. The differentially expressed proteins were tentatively identified as OA-responsive, effectively creating unique protein expression signatures for OA scenario of 2100. This study showed the promise of using a sentinel and non-model species to examine the impact of OA at the proteome level.

1. Introduction

As a result of the dissolving of anthropogenic atmospheric CO_2 into the surface oceans over the past two centuries (Sabine et al., 2004), the carbonate chemistry equilibrium has shifted toward a higher hydrogen ion concentration and a lower carbonate (CO_3^{2-}) ion concentration, in a process commonly known as ocean acidification (OA) (Feely et al., 2004). The current global average surface seawater pH is around 8.1 units, a drop of 0.1 units compared to pre-industrial levels (Raven et al., 2005), and it is projected to drop by 0.3 to 0.5 pH units within this century (Caldeira and Wickett, 2003; Solomon, 2007). Such a drastic decrease in pH is unprecedented to contemporary marine organisms, which have evolved in a relatively chemically-static marine environment over the past 20 to 30 Ma (Pearson and Palmer, 2000).

Barnacles are representative organisms in studies of the ecology of intertidal shores and of the larval development of intertidal benthic invertebrates (Gosselin and Qian, 1996; Holm et al., 2000; Leslie et al., 2005). A growing amount of empirical evidence suggested that they are generally robust to OA, albeit discrepancy in sensitivity has been observed between certain populations and species. A pilot companion experiment showed that larval attachment and metamorphic success was compromised with increasing pCO_2 (under pH 7.9, 7.6 and 7.3 regimes) in the larval barnacle *Balanus amphitrite* (Lane et al., unpublished data). Another study on the same species by McDonald et al. (2009) showed that their larval development until the cyprid stage as well as the percentage of nauplius larvae survived to metamorphosis were not affected by rearing the larvae under the level of OA condition down to a pH of 7.4 units. In addition, Thomsen et al. (2010) showed that settlement of barnacles, such as *Balanus improvisus*, was abundant between summer and autumn in a natural CO_2-rich coastal habitat with a pH value that can be lower than pH 7.5 (but see Findlay et al., 2009a,b, 2010a,b,c for opposite responses such as compromised adult survival, delayed embryonic development as well as compromised post-larval growth and calcification in a few other barnacle species). These observations suggested that CO_2 responses and thus sensitivity may vary between barnacles depends on taxonomic group, life history and habitat patterns, as well as degree of the OA stress (Widdicombe and Spicer, 2008; Dupont and Thorndyke, 2009; Lannig et al., 2010).

To cope with OA stress, barnacles could adjust the expression patterns of proteins as short-term adaptation. This is a common strategy adopted by organisms to tolerate abiotic stressors (known as 'plastic proteome responses') (Lopez et al., 2001; González-Riopedre et al., 2007). For example, a marine snail has two ecotypes that result from proteome variation elicited by different habitats (Martínez-Fernández et al., 2008). Recently, it has been observed that plastic proteome stress responses include the post-translational modification (PTM) of proteins following their synthesis (Mann and Jensen, 2003). Altogether, dynamics in protein expression and the state of PTM provide parallel, overlapping, or complementary levels of regulation in cellular functions (Scroggins and Neckers, 2007; Tomanek, 2010).

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One type of PTM that is better studied in marine invertebrate larvae is protein phosphorylation, which is postulated to play a central role in the transduction of cellular signals to modulate and coordinate a spectrum of biological processes, including larval metamorphosis onset (Thiyagarajan et al., 2009; Wong et al., 2010; Zhang et al., 2010a) and responses to environmental shifts (e.g. Burlando et al., 2006; Ulrich and Marsh, 2009). On the other hand, glycosylation of specific proteins appears to regulate stress response in higher organisms (Hart, 1992; Henle et al., 1993). However, a large scale analysis of proteome and protein post-translational modification response to environmental stressors is rare in marine invertebrates.

The field of proteomics, specifically two dimensional gel electrophoresis (2-DE)-based proteomics, has been increasingly applied to study proteome responses in many non-model species (those without a completely sequenced genome) to various environmental stresses (Apraiz et al., 2006; Chora et al., 2008). Despite the lack of genomic data, the wealth of molecular information contained in these organisms can be characterized using the concept of protein expression signatures (PES) (Shepard and Bradley, 2000). The PES of an organism consists of the set of proteins expressed at any given time, some of which may represent a particular stress response or be associated with a transient developmental stage (Bradley et al., 2002). By establishing the PES for animals exposed to various stressors, proteomics can potentially be used to study the stress response of a non-model species at the molecular level. In this respect, proteomics enables testing hypothesis surrounding the molecular basis for stress responses in these organisms (Knigge et al., 2004).

Most marine invertebrates are non-model species and have a biphasic life cycle, where the fitness of the pelagic larval stage directly impacts the success of the benthic adult stage (reviewed in Pechenik, 2006). During development, larvae are confronted with various stressors through which they must overcome in addition to finding an appropriate substrate, attaching, and then finally metamorphosing into their adult form (reviewed in Thiyagarajan, 2010). Larvae of many species are capable of physiological and behavioral adaptation to various environmental stressors, including OA (reviewed in Dupont and Thorndyke, 2009). Therefore, larval forms, especially those responsible for the settlement (attachment and metamorphosis) process, may show more profound and more readily detectable proteome responses to environmental stressors compared to the adult form due to their rapid development, greater susceptibility and the complex developmental reprogramming in the proteome during such a larval–juvenile transition (Thiyagarajan and Qian, 2008; Thiyagarajan et al., 2009; Thiyagarajan, 2010). Clearly, an examination of protein expression patterns would help better understand the molecular basis of OA stress responses in larvae during attachment and metamorphosis.

It has been suggested that the precisely orchestrated expression of larval proteins is crucial to environmental cue recognition, settlement signal conveying, as well as preliminary preparation of the larvae for subsequent juvenile tissue production (Thiyagarajan and Qian, 2008; Thiyagarajan et al., 2009; Zhang et al., 2010b). The expression of proteins that provide essential functions related to larval attachment and metamorphosis may change in response to an environmental stressor. In this respect, exposure to stressful environmental conditions such as OA may elicit changes in the regulation of protein expression, corresponding to specific PES, indicative of the molecular preparations for attachment and/or biochemical responses to OA stress. In this study, larvae of the barnacle *B. amphitrite* were used as model to examine the above hypothesis at the total protein level as well as two PTM levels (phosphoprotein level and glycoprotein level) using 2-DE based proteomic approach. This species is used because it has been a representative organism in the study of ecology (Rittschof et al., 1992) and biofouling research (Hung et al., 2007). In addition, its larval development, survival and metamorphic success in response to OA have been recently examined in detail (McDonald et al., 2009; Lane et al., unpublished data).

2. Materials and methods

2.1. Study organism

Adults of the barnacle *B. amphitrite* (Cirripedia, Balanidae) (=*Amphibalanus amphitrite*) were scrapped off from the concrete posts at a pier of Pak Sha Wan, Kowloon, Hong Kong (22°21′45″ N, 114°15′35″E) on 29 March 2010. Adults (about 100 to 200 individuals) were induced to release their larvae by the aerial exposure method (Thiyagarajan et al., 2003). The newly hatched stage I nauplii larvae developed into stage II within 30 min and were used in the following experiment.

2.2. Experimental design

The swimming stage II nauplii larvae were reared to the competent larval stage (i.e. the stage exploring hard substrate before attaching and metamorphosing onto it), called cyprids (Supplementary Fig. S1), in the control and the OA treatment aquaria using optimized culturing techniques (Thiyagarajan and Qian, 2008). The control and the treatment represented the current (pH 8.1) and the projected levels of OA for 2100 (pH 7.6), respectively (Caldeira and Wickett, 2003). The nominal value of pH 7.6 was selected because it is also within the environmentally relevant range that can be encountered by the barnacle *B. amphitrite*. Surface coastal waters close to Pak Sha Wan (the adult barnacle collection site) had a pH range from 7.3 to 8.6 units from 2005 to 2009 (Environmental Protection Department, Hong Kong — Marine water quality data, Port Shelter, sample station PM6, at http://epic.epd.gov.hk/ca/uid/marinehistorical/p/1). In addition, it is also suggested that intertidal organisms encounter frequent pH fluctuation lower than 7.5 units (Marchant et al., 2006). Hence, pH 7.6 should be an appropriate level to examine the fundamental mechanisms that the barnacle larvae employ to tolerate environmental pH variation in the natural intertidal settings, as well as to unravel the potential strategy they adopt to tolerate OA stress, at the proteome level.

In this study, the pH values were used as a proxy for the changes in seawater carbonate system (CO_2, HCO_3^-, CO_3^{2-}, pH) in response to the dissolving CO_2 (Dickson et al., 2007). There were 3 replicate culture tanks for control and treatment. The carbonate system in each treatment tank was adjusted by bubbling CO_2-enriched air, while each control tank was bubbled with ambient air. During the entire larval culture period, temperature, salinity and pH were measured 2 to 3 times per day. The pH was measured using a pH probe (Mettler-Toledo SG2). TA levels of each tank at a time point were calculated using the Gran Plot method (Gran, 1952; Brewer et al., 1986), and validated against certified seawater reference materials (Batch 98, A. G. Dickson, Scripps Institution of Oceanography). Carbon chemistry of each tank was calculated using the program CO2SYS developed by Lewis and Wallace, 1998 by inputting pH, TA, temperature and salinity of the culturing seawater immediately before and approximately a few hours after seawater change. The following parameters were used in the CO2SYS program: seawater pH was in total scale, carbonate dissociation constant from Roy et al. (1993), KSO_4 constant from Dickson (1990), and concentrations of silicate and phosphate for FSW were both set as default (i.e. 0.0 μmol kg SW^{-1}) at the salinity level corresponding to each of the culture tank.

Roughly 10,000 nauplii larvae were introduced into each of the 6 culture tanks (<2 larva mL^{-1}). More than 90% nauplii larvae developed into the cyprid stage, irrespective of pH treatments, in 4 days. At the end of the culture period, cyprids were sieved out using a 240 μm mesh and immediately fixed for 2-DE work.

2.3. Sample preparation for 2-dimensional gel electrophoresis (2-DE)

Cyprids were washed with Milli-Q water and then lyzed in 2-DE buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT, and