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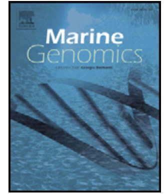

Transcriptional responses of the black-chinned tilapia *Sarotherodon melanotheron* to salinity extremes

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ABSTRACT

Sarotherodon melanotheron is one of the most euryhaline teleosts able to withstand variations in environmental salinity ranging from freshwater (FW) to 130‰ hyper-saline waters (HSW). Although significant progress has been made in exploring the cellular and molecular changes that accompany salinity adaptation in teleosts, little is known about the effects of long-term acclimation to HSW. We sought to identify in this tilapia species the genes whose transcription is induced by long-term acclimation either to HSW or FW. Two subtractive cDNA libraries were made from gills of fish acclimated for 45 days to either condition, with 320 partial cDNA sequences encoding proteins potentially involved in the response to the two salinity extremes. The ESTs comparisons with genomic databases allowed putative functions to be attributed to 197 of these genes. The suppression subtractive hybridisation (SSH) results were validated by Real-time PCR for 13 candidate genes having presumably a role in osmoregulation, supplemented by Na^+, K^+-ATPase α-subunit and carbonic anhydrase, two genes known to be implicated in this function. In fish acclimated to both salinity extremes, the functional category of cellular process was the predominant one, which may indicate high cellular turnover rates in FW and HSW-adapted fish. The acclimation to FW and HSW also appeared to trigger the expression of genes involved in transport activity, biological regulation and metabolic processes, at a higher level in fish acclimated to HSW, suggesting higher metabolic activity in this situation. These results are a first step towards the identification of key molecular processes involved in the fish acclimation to extreme salinities.

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1. Introduction

Some species of teleost fish are able to colonise aquatic habitats that undergo complex spatiotemporal variations in salinity, including some estuaries and lagoons which can range from 0‰ to more than three-fold the salinity of seawater (Sardella et al., 2004; Panfili et al., 2006; Whitfield et al., 2006). As for most aquatic organisms, such species must maintain homeostasis of internal fluids relative to environmental salinity changes, and the relative responses to freshwater (FW) and seawater (SW) are now well-established (Bartels and Potter, 2004; Evans et al., 2005). The gills are, indeed, the major active site for osmoregulation in fish and acclimation to different salinities is accompanied by important modifications in the epithelium (Marshall, 2002; Wilson and Laurent, 2002; Laiz-Carrión et al., 2005). The gill epithelium contains chloride cells, which are responsible for secreting ions in seawater (Foskett and Scheffey, 1982; Mizuno et al., 2000). In the tilapia *Oreochromis mossambicus*, morphological alterations include an enlargement of chloride cells in SW and HSW compared to FW as well as an increase in cell number (Kültz et al., 1992) and a reorganisation of cell types with a differentiation of new chloride cells (Hiroi et al., 2005b). In addition, chloride cells possess a number of ion transporters, such as the $Na^+/K^+/2Cl^-$ cotransporter (NKCC), the regulator (CFTR) channel and the Na^+,K^+-ATPase enzyme, which have been associated with the maintenance of osmotic homeostasis (Evans et al., 1999; Sakamoto et al., 2001; Evans et al., 2005; Hiroi et al., 2005a). Modifications in gill enzyme activities have also been demonstrated following a transfer to different water salinities (Evans et al., 2005).

Abbreviations: ATB, β-actin; *CA,* carbonic anhydrase; *CaM,* calmodulin; *Cyt.C,* cytochrome *C* oxydase 1; *FABP,* fatty acid binding protein; *GPX,* glutathione peroxidase; *GST,* glutathione *S* transferase; *HSP70,* heat-shock protein 70; *LDH,* lactate dehydrogenase; *MK2/3,* MAP kinase-activated protein kinase; *NADH,* NADH dehydrogenase; *NAKA,* Na^+, K^+-ATPase alpha; *SERCA,* sarco-endoplasmic reticulum (SR) Ca^{2+}-ATPase; *TF3a,* basic transcription factor 3; *VDAC,* voltage-dependent anion channel.

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New insights into some of the molecular and cellular responses of fish gills to variations in salinity have been reported in recent studies in the sea bream *Sparus sarba* (Deane and Woo, 2004), the killifish, *Fundulus heteroclitus* (Scott et al., 2004), the striped bass, *Morone saxatilis* (Tipsmark et al., 2004), the gilthead sea bream, *Sparus aurata* (Laiz-Carrión et al., 2005) and the tilapia, *Oreochromis mosambicus* (Fiol and Kultz, 2005). Most of these studies have shown that the adaptive responses to different salinities are mainly based on the modulation of gene expression for water- and ion-transporters and other enzymes such as the components of the electron transport chain, associated with osmoregulatory mechanisms in the gills. These mechanisms do not however appear to be the same amongst the species. For instance, the expression of the gene coding for the α-subunit of the Na^+, K^+-ATPase increased in the Atlantic salmon, *Salmo salar* (D'Cotta et al., 2000) and in the tilapia, *O. mosambicus* (Feng et al., 2002) upon transfer from FW to SW. In contrast, in the killifish, *F. heteroclitus*, it increased when fish were transferred from brackish water (BW) to FW (Scott et al., 2004). Likewise, carbonic anhydrase (*CA*) increased in the gills of the European sea bass, *Dicentrarchus labrax*, after a transfer from FW to SW (Boutet et al., 2006), but it also increased when the killifish *F. heteroclitus* were transferred from BW to FW (Scott et al., 2005). Furthermore, the expression of a number of genes involved in the energy metabolism, such as the electron transfer chain, fatty acid metabolism and Krebs cycle have been found to be influenced by environmental salinity, the same as their enzymatic activity (St-Pierre et al., 1998; Martínez-Álvareza et al., 2005). It is also well documented that osmotic stress is accompanied by an alteration of cell volume whose regulation involves a great diversity of intracellular signalling events (Kültz, 2001; Ollivier et al., 2006).

Most studies on fish osmoregulation involve an exposure to salinities between FW and full-strength SW (McCormick, 1996; Shepherd et al., 2005; Seale et al., 2006). The use of gene expression analysis to investigate molecular mechanisms has typically allowed comparing the acclimation to FW or SW (Feng et al., 2002; Riley et al., 2003; Boutet et al., 2006). Very few studies have considered HSW challenges, in particular with regard to underlying molecular changes (Jensen et al., 1998; Deane and Woo, 2004; Wong and Woo, 2006). Hyper-saline conditions are, in fact, increasingly observed in estuarine ecosystems as a consequence of global climate warming (Vega-Cendejas and Hernàndez de Santillana, 2004; Whitfield et al., 2006). This is especially the case for the Sahelian estuaries in West Africa, such as the Sine Saloum and Casamance in Senegal. There, the combined effects of reduced FW inputs and water evaporation have resulted in an inversion of the salinity gradient, and salinities in the upper reaches of these ecosystems can exceed SW by 3 to 4-fold.

The black-chinned tilapia, *Sarotherodon melanotheron* is an estuarine species that is extremely euryhaline and can colonise salinities ranging from 0 to 130‰ (Panfili et al., 2004; Panfili et al., 2006), but the compensatory mechanisms they use remain to be described. The present study investigated the gene transcriptional responses following acclimation of *S. melanotheron* to FW or HSW. We thus sought to identify genes implied in the long-term survivorship of fish to extreme salinity conditions. The regulation and activity of these routinely expressed genes would more likely be targets for adaptive selection pressure and this study provides the basic information for future research testing this assumption. For this reason, we focused our analyses in experimental conditions on fish that could be considered as already acclimated to extreme salinities (45 days post post-transfer). Using a transcriptomic approach, we studied differential gene expression in the gills of the black-chinned tilapia when acclimated either to FW or HSW for 45 days under controlled conditions. We first identified the genes that were up-regulated under either condition using the suppression subtractive hybridisation (SSH) method. Differential expression was then validated for 15 genes by real-time polymerase chain reaction (rt-PCR). Finally, we analysed the expression profiles of these genes in another *S. melanotheron* strain exposed to similar salinity transfers (SW to FW, SW to HSW) for only 10 days, to evaluate whether these genes responded in similar ways.

2. Materials and methods

2.1. Animals and experiments

2.1.1. Fish

Experiments were performed on juveniles of the black-chinned tilapia, *S. melanotheron* at 30 days post-fertilisation, maintained at the experimental facility of CIRAD-Gamet. Fish were obtained by natural breeding and came from 3 different couples. The initial broodstock was collected by A. Mbow (IFAN) during the rainy season in Kaolack from a wild population of the Saloum estuary in Senegal, at a water salinity of 48‰. A second acclimation experiment was performed in Dakar (Senegal) using juveniles from another wild population collected by S. Gilles (IRD) in Joal, Senegal, at a water salinity of 35‰.

2.1.2. Experimental conditions

Fish were randomly divided into 2 groups and acclimated to SW (35‰) in 30-litre aquariums for at least 2 weeks before transfer to their final test salinity. SW and HSW were prepared with a commercial sea salt (INSTANT OCEAN®) and salinity was monitored daily. The aquarium water was filtered and changed twice a week. Fish were kept under natural photoperiod and constant temperature (27 °C). They were fed *ad libitum* with commercial dry meal. Fish were fasted 24h before sampling. A second acclimation experiment was performed in Dakar (Senegal) using another black-chinned strain from Joal under experimental conditions identical to those described above.

2.1.3. Salinity transfer experiments and samplings

After the initial acclimation period in SW, one group was directly transferred to FW (0‰) whereas the other was transferred to HSW (70‰). Salinity changes were carried out by slowly emptying the SW and by replacing it either by FW or HSW. This process lasted 2h at the most. After 45 days, fish in FW and HSW were anesthetised with 2-phenoxyethanol (1.5 ml/l of water), weighed and killed by rapid decapitation. Gill tissues were collected, immediately frozen in liquid nitrogen and stored at −80 °C until library construction.

2.2. RNA extraction and construction of hypo/hyper-saline SSH libraries

Total RNA was extracted from the gills of FW or HSW acclimated fish after 45 days with TRIZOL® reagent (Gibco-BRL, USA), according to the manufacturer's instructions. The amount and quality of total RNA were checked by spectrophotometry and by agarose-electrophoresis. Two cDNA libraries, designated as FW library and HSW library, were constructed using pooled gills from four fish following the subtractive suppressive hybridisation (SSH) procedure (Clontech). Briefly, poly(A) mRNA was isolated from total RNA using Poly(A) Purist^{MT} MAG Kit (Ambion) according to the manufacturer's instructions. Equal amounts of mRNA (2 µg) from the gills of FW or HSW acclimated fish were reverse transcripted into cDNA and used as tester or driver for library construction. The cDNAs were digested with *RsaI*, ligated to different adaptors and two rounds of hybridisation and PCR amplification were performed. The secondary PCR products were directly cloned into the pCR2.1 vector (Invitrogen) and electroporated into DH10B cells. After plating, 5000 clones were randomly picked and organised in plates with 384 wells. For each library, 384 clones were randomly selected and both ends sequenced at GENOSCOPE (Evry, France).

2.3. Sequence analysis

Vector and adaptor sequences were removed and the inserted sequences were clustered using the CAP3 assembly program (http://

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