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Differential expression of genes involved in immunity and biomineralization during Brown Ring Disease development and shell repair in the Manila clam, *Ruditapes philippinarum*

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ABSTRACT

Severe drop in Manila clams production in French aquacultured fields since the end of the 1980's is associated to Brown Ring Disease (BRD). This disease, caused by the bacteria *Vibrio tapetis*, is characterized by specific symptoms on the inner face of the shell. Diseased animals develop conchiolin deposit to enrobe bacteria and form new calcified layers on the shell. Suppression subtractive hybridization was performed to identify genes differentially expressed during the early interaction of *V. tapetis* and *Ruditapes philippinarum*. Results revealed 301 unique genes differentially expressed during *V. tapetis* challenge. Several candidates involved in immune and biomineralization processes were selected from libraries. Transcriptional expression of selected candidates was determined in hemolymph and mantle tissues and revealed spatial and temporal variations. At 56 days after infection, when clams were in phase of shell repair, transcripts of galectin and ferritin in hemocytes showed higher expression. Ca-like and serpin transcripts were specifically expressed in mantle and could contribute to defense against BRD.

1. Introduction

Microbial diseases such as vibriosis have been well-studied in aquacultured animals (Paillard et al., 1994; Lacoste et al., 2001; Travers et al., 2008; Goarant and Merien, 2006). Studies were performed to better understand cellular and humoral mechanisms of defense responses in bivalves (Ford et al., 1993; Allam and Ford, 2006; Mateo et al., 2009). Additionally, several molecular approaches such as SSH (suppression subtractive hybridization) (Diatchenko et al., 1996) were used to identify differentially expressed genes during bacterial or protistan infections (Tanguy et al., 2004; De Lorgeril et al., 2008; Perrigault et al., 2009; Morga et al., 2011). Brown Ring Disease (BRD) affects populations of Manila clams *Ruditapes philippinarum* along the North East coast of Europe (Paillard, 2004). This disease is caused by Gram-negative bacterium, *Vibrio tapetis* (Paillard and Maes 1990). A previous molecular study using SSH was performed on hemocytes from *R. philippinarum* incubated *in vitro* with *V. tapetis* (Brulle et al., 2012). This study led to the identification of genes involved in immunity such as B-cell translocation gene and early growth protein factor. One characteristic of the BRD is the formation of a brown deposit of conchiolin on the inner shell at the edge of the mantle, the main organ involved in the shell formation. Resistance to BRD development is characterized by shell repair processes with brown deposits formation encapsulating bacteria (Trinkler et al., 2011). Mantle tissue is therefore essential in the defense of *R. philippinarum* against *V. tapetis*. The first step of *V. tapetis* colonization is observed 72 h post injection and occurs on the periostracal lamina secreted by the mantle (Paillard and Maes, 1995a). Increases in total hemocyte counts and in lysozyme activity were described 24 h post injection. High total hemocyte counts were also observed between days 7 and 15 post injection (hemocytosis) and were followed by an immunodepression after 1 month (Oubella et al., 1993; Allam et al., 2000). One flaw of our previous molecular study was characterized by the *in vitro* approach used to identify differentially expressed genes of *R. philippinarum* in response to *V. tapetis*. To complete this molecular approach of *R. philippinarum* defense response to *V. tapetis* and overcome *in vitro* limitations, a second SSH was performed on mantle tissue during an *in vivo* infection, to identify and characterize defense mechanisms involved in the early development of BRD in clams 1 day after *V. tapetis* challenge. Candidate genes were thereafter selected from SSH libraries and their transcriptional variations were analyzed in mantle tissue and hemolymph cells over 56 days.

2. Materials and methods

2.1. Biological materials

Three hundred adult Manila clams (36–40 mm) were collected from the Lanveur Bay, Brittany, in France. Clams were acclimated

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in tanks with circulating seawater and held at 14 °C for 1 week before experiment.

V. tapetis CECT4600 strain was grown on Zobell medium during 24 h at 18 °C. The bacterial suspension was rinsed with sterile seawater and the concentration determined by spectrophotometry at 490 nm absorbance.

2.2. Infection experiment

Experimental infection was carried out by injecting 100 µl of a *V. tapetis* suspension (5×10^8 CFU ml^{-1}) per individual in the pallial cavity with a sterile syringe according to Paillard and Maes (1990). One hundred infected clams were maintained in three 50-L tanks with aerated water for 56 days. Forty animals sampled after 1 day (20 controls and 20 injected) were used for the generation of subtractive libraries.

One hundred control clams were inoculated with 100 µl of sterile seawater in the pallial cavity and maintained in three separate 50-L tanks. Before the challenge (time T0), hemolymph and mantle samples from 20 animals were collected. Twenty animals were collected at each sampling subsequent time (Day 1, Day 14 and Day 56).

2.3. Tissue sampling

Hemolymph was withdrawn by inserting a needle through the hinge into the adductor muscle of clams. Hemocytes were isolated by centrifugation (1 min, 800*g*). Mantle samples were dissected preferentially in the area where the syringe was inserted for the challenge, a region usually associated to the appearance of the first BRD symptoms. All clam tissues were removed and shells were kept for BRD diagnostics. Mantle and hemocytes were individually homogenized with Tri-reagent and stored at −80 °C until RNA extraction.

2.4. Brown Ring Disease diagnosis

At each incubation time, BRD development or CDS (Conchiolin Deposit Stage) was determined from animal shells according to the classification developed by Paillard and Maes (1994). Briefly, CDS 0 was associated to asymptomatic clams and seven gradual stages (CDS 1 to CDS 7) represented the different disease development stages. Signs of repair were also determined by different SRS (Shell Repair Stage) stages: from SRS 0 (no repair) to SRS 3 (total repair) according to Paillard and Maes (1994). An additional stage, SRS 2.5, was added corresponding to a near complete recovery from BRD, with only few small persisting black points (Paillard, 2004).

2.5. RNA extraction

For each sample time, RNA was extracted from mantle tissue of three animals per condition (bacteria-challenged and control) using Tri reagent (Invitrogen, Cergy-Pontoise, France) according to manufacturer's instruction. Samples at day 1 were used for mantle SSH librairies. Then, polyadenylated RNA was isolated from the total RNA using the PolyAtract mRNA isolation System (Promega, Madison, WI, USA) according to manufacturer's instruction. For each time sample, hemocyte RNA from three individual clams was pooled to obtain sufficiently amount of RNA, Quality and quantity of total RNA and mRNA samples were determined with a NanoDrop spectrophometer and Agilent Bioanalyser (Agilent Technologies, Waldbronn, Germany).

2.6. Suppression subtractive hybridization

Two micrograms of mRNA were used as templates for the SSH following the PCR-select cDNA subtraction kit procedure (Clontech) (Diatchenko et al., 1996). Forward subtraction was carried out using mRNA from mantle tissue of 1 day post-challenged clams as the tester and mRNA from mantle tissue of control clams collected at the same time as the driver. Reverse subtraction was performed as the opposite of the forward subtraction. Differentially expressed genes were cloned into pGEM-T vector (Promega) and transformed in XL1-Blue competent cells (Agilent Technology). For each library, 528 white colonies were grown on LB/ampicillin/IPTG/XGal plates and then cultured onto agar plates supplied by AGTC Biotech Company. A total of 1056 clones were sequenced by AGTC Biotech Company using the M13-FP primer (5′-TGTAAAACGACGGCCAGT-3′). Sequences were analyzed using the Seqman II™ software (DNASTAR Inc., Madison, WI, USA). Unique sequences (>100 pb) (from contigs and singletons) were subjected to BlastN and BlastX analysis (http://www.ncbi.nlm.nih.gov) against the non-redundant nucleotide and protein databases of NCBI. Homologies with *E*-value $<1e^{-4}$ were considered as significant. Gene function was inferred from the AmiGO classification (http://www.geneontology.org).

2.7. Real time PCR analysis

Specific primers were designed using the Primer3 software (Rozen and Skaletsky, 2000). Total RNA was extracted from mantle and hemocytes as previously described (Sections 2 and 3). Total RNA was treated with RQ1 DNAse (1 U/µg total RNA, Promega) to remove genomic DNA contamination.

First strand cDNA was synthesized from 4 µg of DNAse-treated total RNA using the $Oligo(dT)_{18}$ primer and the RevertAid™ HMinusFirst Strand cDNA Synthesis Kit (Fermentas) according to manufacturer's protocol.

Real time PCR was performed in triplicate with 3 µl cDNA (1:50 dilution) in a total volume of 20 µl using a 7300 Real-Time PCR System (Applied Biosystems) in 96-microwell plates. Each well contained 1X Absolute™ Blue QPCR SYBR® Green ROX Mix (ABgene) and 75 nM of each primer. Oligonucleotide primer sequences used to amplify specific gene products are listed in Table 1. Cycling conditions were defined by: 15 min activation at 95 °C followed by 40 cycles (denaturation 15 s at 95 °C, elongation 20 s at 55 °C and extension 30 s at 72 °C). Melting curves were also performed (95–60 °C) by decreasing the temperature 0.5 °C every 10 s. Negative controls (cDNA free) were included to rule out DNA contamination. For each primer pair, PCR efficiency was determined by the slope of standard curves obtained from serial dilutions analysis of cDNA (1:5–1:20000). Amplification efficiencies were calculated according to the following equation $E = 10^{(-1/\text{slope})}$ (Pfaffl, 2001). Expression of candidate genes was normalized to the reference gene Elongation Factor 1 alpha (EF1a), which had a low variation

Table 1
General characteristics of SSH libraries and ESTs obtained from mantle of *R. philippinarum*. Forward library: enriched for genes expressed by infected clams. Reverse library: enriched for genes expressed by control clams.

	Total	Forward	Reverse
Total number of clones sequenced	1030	510	520
Total number of EST sequences analyzed	1020	505	515
Unique gene object	301	64	237
Contigs	74	23	51
Singletons	227	41	186
Total number of EST with no match to database	112	15	80
Redundancy (%)	71	87	54