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The identification of genes from the oyster Crassostrea gigas that are differentially expressed in progeny exhibiting opposed susceptibility to summer mortality

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Abstract: Summer mortality associated with juveniles of the oyster Crassostrea gigas is probably the result of a complex interaction between the host, pathogens and environmental factors. Genetic variability in the host appears to be a major determinant in its sensitivity to summer mortality. Previously, divergent selection criteria based on summer survival have been applied to produce oyster families with resistant and susceptible progeny. In this paper, we describe the use of suppression subtractive hybridization to generate 150 C. gigas clones that were differentially regulated between resistant and susceptible F2 progeny. The nucleotide sequence of these clones was determined. In 28%, the inferred amino sequence was found to match the products of known genes, 14% matched hypothetical proteins and a further 14% appeared to contain open reading frames (ORFs) whose product had no obvious homologue in the nucleotide databases. It has been hypothesized that differences exist in the level of energy generation and immune function between resistant and susceptible progeny. In light of this, clones encoding homologues of cavortin, cyclophilin, isocitrate dehydrogenase, sodium glucose cotransporter, fatty acid binding protein, ATPase H+ transporting lysosomal protein, precerebellin, and scavenger receptor were analyzed by real-time PCR. These transcripts were induced in resistant progeny when compared to their susceptible counterparts. A bacterial challenge of oysters resulted in the suppression of six of these transcripts in only those that were resistant to summer mortality. This study has identified potential candidates for further investigation into the functional basis of resistance and susceptibility to summer mortality.

Keywords: Bacterial challenge; Bivalves; Differentially regulated genes; Oysters; Suppression subtractive hybridization

1. Introduction

Under intensive or extensive aquaculture conditions, marine species are exposed to various stressors that can lead to an overall reduction in performance (growth, reproduction) and increased susceptibility to disease (Pickering, 1992). Significant mortality has been reported in the Pacific cupped oyster *Crassostrea gigas* for many years (Cheney et al., 2000) and is a major concern of oyster farmers (Goulletquer et al., 1998). Summer mortality is especially problematic in juveniles and is associated mainly with high temperatures and the oyster reproductive period. Though two oyster pathogens, Herpes like virus (Renault et al., 1994) and *Vibrio spp* (Lacoste et al., 2001; Le Roux et al., 2002), have been reported previously, neither was associated systematically with summer mortality. Indeed, summer mortality may well be the result of complex interactions between the host, one or more pathogens and numerous environmental factors.

Increasing the tolerance of animals of economic importance to stress and diseases by selective breeding has long been considered as feasible (Satterlee and Johnson, 1988). For oyster, genetic variability is suspected to be a major determinant in sensitivity to summer mortality (Hershberger et al., 1984) and in bivalve defence mechanisms. Within the recently established French national multidisciplinary program “Morest”, which was set-up to study the causes of summer mortality in *C. gigas* juveniles, divergent selection criteria were applied (Dégremont et al., 2003). A strong genetic basis for survival was observed and F2 oyster progeny were separated into two groups; resistant (R) and susceptible (S), depending on their summer survival rates (Dégremont et al., 2003). In the present study, an analysis of the molecular events underpinning the physiological differences between the R and S progeny was undertaken using suppression subtractive hybridization (SSH), a PCR-based technique that allows the identification of genes that are differentially expressed in response to stimuli. This technique combines normalization and subtraction, allowing the suppression of abundant

transcripts while rare transcripts are enriched to the same order of magnitude (Diatchenko et al., 1996). This approach is currently used to identify genes implicated in molecular mechanisms involved in cancer, immunity and development (Bayne et al., 2001; Hofsaess and Kapfhammer, 2003). Until now, only a few transcripts encoded by genes that may be involved in oyster stress or immune responses have been reported (Jenny et al., 2002; Gueguen et al., 2003; Boutet et al., 2004).

This project was designed to compare the differential expression of *C. gigas* genes between R and S progeny during a summer mortality event that had affected only the S progeny. One hundred and fifty clones were partially sequenced and those putatively implicated in immunity or energy metabolism, systems suspected to be implicated in summer mortality, identified. The nucleotide sequence of these clones was extended and their differential expression in R and S samples analysed. The expression of these transcripts was also measured in two-year-old R and S progeny before and after a bacterial challenge to asses their value in determining susceptibility to summer mortality.

2. Material and Methods

2.1 Biological material

F2 oyster progeny were bred during March 2002 at the Ifremer hatchery in La Tremblade (France) according to divergent selection criteria and based on results of *in situ* survival of F1 bi-parental families (Dégremont et al., 2003). These oysters were then cultured at the Ifremer station in Bouin (France). For SSH experiments, progeny from 3 R and 3 S families were placed in the same experimental raceway (April 2002) in seawater filtered to 20 µm and fed 10^9 *Skeletonema costatum* day^{-1} oyster^{-1}. When the first oysters died in the raceway (July 2002), 10 individuals were collected from each family and their mantle-gonad immediately dissected and stored in liquid nitrogen. No oysters were collected that were clearly dying.

Frozen samples were crushed to a fine powder with a Dangoumau grinder for total RNA extraction. The remaining oysters were reared in the same experimental conditions. Dead oysters were counted daily and removed from the experimental raceway.

2.2 Bacterial challenge

A bacterial challenge was carried out at 19°C (November 2003) as described in Montagnani et al. (2002). *Vibrio splendidus* was grown overnight at 24°C in marine broth. Bacterial cells were collected by centrifugation (3500 rpm, 15 min), washed and resuspended in 10ml sterile seawater. Two-year-old oysters from the same R and S progeny described above were challenged by injecting either 100µl of sterile seawater (isw) or 100 µl *V. splendidus* into the adductor muscle. Sixty oysters were injected with *V. splendidus* (iv), 60 with sterile seawater (isw) and a further 60 were untreated (ni). The oysters were then returned to seawater raceways for 10 h after which time the mantle-gonad was dissected from 25 oysters per group and total RNA extracted. The remaining 35 oysters in each group continued to be reared under the same experimental conditions. Dead oysters were counted daily and removed from the experimental raceways.

2.3 RNA extraction

Total RNA was isolated using Trizol reagent (Gibco BRL) at a concentration of 1 ml/50 mg of tissue. Samples were then treated with DNAse I (Sigma) (1 U/µg total RNA). For SSH experiments, polyadenylated RNA was isolated using the Quickprep micro mRNA purification kit (Amersham). RNA concentrations were measured at 260 nm using the conversion factor 1 OD = 40 µg/ml RNA, and RNA quality was checked by electrophoresis through a denatured agarose gel.

2.4 Suppression subtractive hybridization

mRNA extracted from the mantle-gonad tissue of R or S oysters was pooled and 2 µg of each pool used as the template for SSH following the PCR-select cDNA subtraction kit procedure (Clontech). Hybridization and subtraction steps were carried out in both directions, i.e. for forward subtraction the R sample (tester) was subtracted with the S sample (driver) and *vica versa* for reverse subtraction. The PCR products from the forward subtraction were cloned into pCR 2.1® TOPO plasmid using TOP10 One Shot® competent cells for transformation (Invitrogen).

2.5 Screening of the subtracted clones

To eliminate cDNA clones common to both the R (tester) and S (driver) samples in the subtracted tester, a PCR-select method (Diatchenko et al., 1999) was employed on subtracted clones following the recommendations of the PCR-select differential screening kit (Clontech). Inserts cloned into pCR 2.1 were amplified by PCR using adaptor specific primers. Each PCR product was blotted onto Hybond-N+ nylon membranes (Amersham) in duplicate after denaturation by adding one volume of 0.6N NaOH. Oyster *actin* was cloned (**AF026063**) and blotted in duplicate onto each nylon membrane after PCR amplification. DNA was cross-linked to the membrane at 70°C for 2 h. In total, 7 different membranes were prepared in quadruplicate for hybridization with 4 different cDNA probes corresponding to the forward-subtracted probe, the unsubtracted tester probe, the reverse-subtracted probe and the unsubtracted driver probe. After RsaI digestion, probes were prepared using the Ready-to-go DNA labelling beads [-dCTP] kit (Amersham) and 50 µCi α-P^{32} CTP. The labelled probes were purified from unincorporated dNTPs using AutoSeq G-50 columns (Amersham). The specific activity of each probe was measured using a scintillation counter (Packard Instruments, France) after TCA precipitation.

Membranes were prehybridized for 1 h at 42°C in 50% deionised formamide, 5x SSC, 5x Denhardt's, 0.5% SDS and 100 µg/ml denatured herring sperm DNA. Hybridization was performed overnight at 42°C in prehybridization buffer containing the radio-labelled probe. After hybridization, membranes were washed for 20 min at 68°C in prewarmed low-stringency solution (2x SSC/0.5% SDS) and then twice for 20 min at 68°C in prewarmed high-stringency solution (0.2x SSC/0.5% SDS). Membranes were then exposed to autoradiographic film (Kodak Biomax MS). The signal intensity was quantified using Multi-analyst software (Biorad, CA) with the background signal removed. The value obtained is the spot intensity expressed as mean count per pixel and multiplied by the spot surface area. Clones were sequenced using an ABI Prism Big Dye Terminator Cycle Sequencing kit (PE Applied Biosystems). Database searches were carried out using the BlastX program (http://www.ncbi.nlm.nih.gov/BLAST/). BlastN analyses were carried out using the specific oyster database "GigasBase" (Gueguen et al., 2003). Only E-values less than 10^{-2} were considered significant. Contigs were built using the CAP3 assembly program (Huang and Madan, 1999).

2.6 Full length cDNA

A cDNA library constructed in λ–ZAP II from *C. gigas* mantle-edge mRNA was screened as described by Lelong et al. (2000). Specific primers, and where required, nested primers, were designed for selected cDNAs (Table 1). Amplified fragments were subcloned into pCR 2.1® TOPO plasmid and sequenced as described above.

2.7 Real-time PCR analysis of gene expression

The expression level of 8 mRNA transcripts were investigated by real time PCR using an Icycler (Biorad). One microgram of total RNA isolated from oysters inoculated with either *V.*

splendidus or seawater or from unchallenged oysters were reverse-transcribed as described by Huvet et al. (2003) and amplified by real time PCR using specific primers (Table 1).

Table 1. Primers used for the screening of the cDNA library and real-time PCR assay.

cDNA	Primers	Oligonucleotide sequences (5'- 3')
cavortin	Cav_qf	CTT CAT gCC Agg CAA CCT
	Cav_qr	TgA CgT TgA ATC Cgg TCA
	Cav_1f	gAg Agg TgA Atg CTA CCA ggA CTT TC
	Cav_1r	ACA gAC AgA AgC TCA TTT CCA AAg
fatty acid binding protein	Fab_qf	CAC gAA ggg ACC CAA AgA
	Fab_qr	CAT gTg ACC Agg gCC TTC
	Fab_1f	AAT ACT gAT gTC TgA ggg ACT TTg T
	Fab_1r	CTg gCA TTg TCC CAT ATA TCA AC
sodium glucose cotransporter	Glt_qf	Cgg AAg gCT gTg TgT CCT
	Glt_qr	gAg gTg ATg gCC Tgg ATg
	Glt_1f	ACg Tgg gAC TTC TTT CTT TAg ATg
	Glt_1r	Tgg gCT gAg AAT TAA gTA AgT TgC
	Glt_4r	ACT ACC gCA CTC TCT CTC ACA AAT A
cyclophilin	Cyc_qf	CgC Cgg TAg gAT TgT CAT
	Cyc_qr	AgC CAA AgC CTT TCT CTC CT
	Cyc_1f	CTTCAgCTggAAgTTCTCATCAg
isocitrate dehydrogenase	Idh_qf	CCg ACg gAA AgA CTg TCg
	Idh_qr	CTg gCT ACC ggg TTT gTg
	Idh_1f	gCAggATACAAAACCgTgTgAC
	Idh_1r	AgCTATTAgTTCACACCCgAGTTC
atpase H+ transporting lysosomal protein	Atp_qf	ggC gAC ATg gAg AgC AAg
	Atp_qr	TCT CTT gAg TgC CAC CTC CT
CK172358	Lin_qf	CAA gAg CTT ggA CTT Tgg gTA
(putative *precerebellin*)	Lin_qr	CAA AgA gCT ATg ACC gAg Tgg
	Lin_2f	gAT TTC AAA gAg CTA TgA CCg AgT
	Lin_1r	CTg TgT CAA TAg ATg Agg CAT TTC
	Lin_2r	ACT TAg TAg CCT CCT TgT gAC ACC
CK172401	Sca_qf	ATg TgC Agg TCA gCA TTg TAA
(putative *scavenger-receptor*)	Sca_qr	TCT CCC TCC TCC TTT gAT TCT
	Sca_1f	AAT ATC AAT CTC CCT CCT CCT TTg
	Sca_1r	ACT ggg Agg AAT TgA TCT TAC TTg
	Sca_2r	CTg TAC AAC TTC CAT TCC AAC AAg

r: reverse primer; f: forward primer; q: indicated primers used for the real time PCR analysis. Other primers were used for the screening of the cDNA library.

Amplification of *actin* and *elongation factor I* cDNAs were performed in order to confirm the steady-state level of expression of a housekeeping gene to provide an internal control for gene expression. *Actin* and *elongation factor I* primers were those used by Huvet et al. (2003) and Fabioux et al. (2004) respectively.

The real-time PCR assay was performed in triplicate with 5 µL cDNA (1/10 dilution) in a total volume of 15 µL. The concentrations of the reaction components were as follows: 0.33 µM each primer, 1.5 µL fluorescein and 1X Quantitect SYBR Green PCR kit (Qiagen). This reaction was performed using Taq Polymerase as follows: activation at 95°C for 15 min followed by 45 cycles of 30 sec at 95°C, 1 min at 60°C, and a melting curve program from 95°C to 70°C by decreasing the temperature 0.5°C every 10 seconds. Each run included a positive cDNA control (one S sample of the present experiment analyzed in each amplification plate), negative controls (each total RNA sample with DNAse I treatment) and blank controls (water) for each primer pair. PCR efficiency (E) was determined by drawing standard curves from a serial dilutions analysis of cDNA from R and S samples to ensure that E ranged from 99 to 100% for each primer pair.

The calculation of relative mRNA levels was based on the comparative Ct method (Livak and Schmittgen, 2001). No significant differences between Ct values were observed for the two house keeping genes (*actin, elongation factor I*), between R and S samples or between injected and non injected oysters (t-test after Bonferroni adjustment: $P = 0.151$ and 0.085; coefficient of variation = 3.4 and 3.5% for *actin* and *elongation factor I*, respectively). Therefore, the relative quantification value of the sample was normalized to the *actin* gene (because of its lower coefficient of variation) and relative to the positive control, and was expressed as $2^{-\Delta\Delta Ct}$, where ΔCt = [Ct(cDNA sample) – Ct(positive cDNA control)] and $\Delta\Delta Ct$ = ΔCt of cDNA - ΔCt of actin. Comparison of the level of mRNA (relative to *actin* mRNA) between R and S progeny was performed by Student's *t*-test using SYSTAT 9.0 by SPCC.

Multiple comparisons of the level of mRNA (relative to *actin* mRNA) between injected and non-injected groups were performed within sibling progeny by analysis of variance using the least significant difference (LSD) pairwise multiple comparisons test using the same software.

3. Results

3.1 Experimental conditioning

In April 2002, F2 progeny from 6 oyster families selected as either resistant (R) or susceptible (S) to summer mortality were placed in the same experimental raceway and maintained under the same conditions. The first oysters died on July 4th 2002 and one week later the cumulative mortality was estimated at 74.5 ± 11.0% and 4.1 ± 3.2% for the S and R progeny respectively. Bacterial analysis identified *Vibrio splendidus* as making up 50 to 80% of the total bacterial population of the dying S oysters. In live R and S oysters, however, a greater diversity in the bacterial population was observed (data not shown).

3.2 Suppression Subtractive Hybridization

Tester mRNA obtained by forward subtraction was used to construct a subtracted cDNA library. In total, 376 clones were isolated (Table 2). To eliminate cDNA clones common to both the R (tester) and S (driver) samples, all the subtracted clones were arrayed after PCR amplification onto nylon membranes and hybridized with 4 different probes (forward-subtracted probe, unsubtracted tester probe, reverse-subtracted probe and unsubtracted driver probe). Mean spot intensity were attributed to each clone for each probe allowing their classification into 2 categories:

(1) clones that hybridized only to the forward-subtracted probe; clones that hybridized to the forward-subtracted probe and unsubtracted tester probe but not to the reverse-subtracted probe or unsubtracted driver probe and clones that hybridized to both subtracted probes

when the difference of signal intensity was higher than 3. The 150 clones (40%) classified into this category were confirmed by PCR-select to be differentially expressed, their expression being induced in R compared to S progeny.

(2) clones that hybridized to both subtracted probes when the difference of signal intensity was equal to or lower than 3; clones that hybridized equally to both subtracted probes and to both unsubtracted probes. In this category, 226 clones (60%) were considered as false positive or non-differentially expressed clones. The *actin* clone was classified into this second category with similar values of mean spot intensity in the R and S samples (mean value = 0.37 and 0.33, respectively).

The nucleotide sequence of the 150 clones from category 1 was established: 28% matched with products of known genes (42 sequences); 14% matched hypothetical proteins (21 sequences); 14% displayed ORFs of significant length but whose product was unknown (21 sequences), 35% appeared to be non coding sequences (53 sequences) and 9% were unreadable sequences (13 sequences which were then excluded from the analysis). Genbank accession numbers of the 137 analysable sequences are **CK172301-CK172437**. These sequences have a mean size of 452 bp and coalesced into 74 singletons and 22 contigs indicating a redundancy of 30% among the 137 clones sequenced (Table 2).

Table 2. General characteristics of the subtracted library and cDNA sequences from C. gigas mantle-gonad.

Total number of subtracted clones	376
Total number of differentially expressed clones (confirmed with blots)	150
Total number of cDNA sequences deposed in Genbank	137
Average sequence size (range)	452 bp (156 – 847)
ORF	84
Contigs	22
Singletons	74
Redundancy	30%

Among the 42 sequences that matched with the products of known genes (Table 3), 25 were unique and clustered into 6 categories (Figure 1): 24% were active in general metabolism, 12% in energy metabolism, 20% in cell signalling, cell cycle and cell structure, 16% in putative immune functions, 16% in ribosomal proteins and 12% in replication, repair and transcription of DNA. Among the 22 identified contigs, 9 matched with the products of known genes. These were isocitrate dehydrogenase, sarcoplasmic calcium-binding protein, cytochrome C oxidase, tRNA splicing phosphotransferase, DNA topoisomerase, DNA replication factor, bone morphogenic protein, KIAA1007 protein and 28S mitochondrial ribosomal protein. Three contigs encoded proteins with no known function and 10 displayed ORFs of significant length but whose hypothetical product did not match any sequence in Genbank.

Figure 1. Functional classification of the SSH sequences which matched with known genes (42 sequences corresponding to 25 unique genes). They were clustered into 6 categories according to their putative biological function.

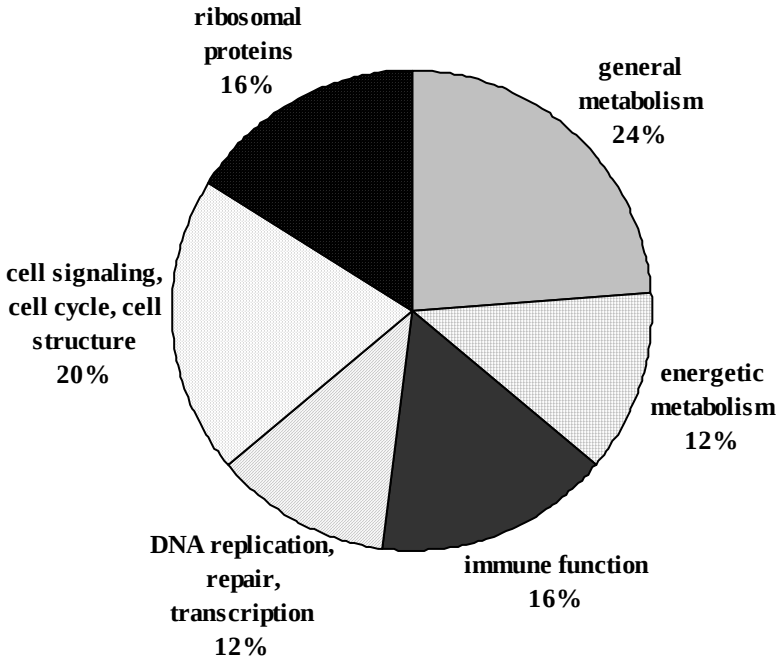


Table 3. Identified cDNAs clones in oyster subtracted library.

Putative match	Species match	E value	Length	Accession number
Cavortin	*Crassostrea gigas*	6e-28 1e-89 *	353 194 aa*	**CK172315** **AY551094***
Peptidyl-prolyl cis-trans isomerase (Cyclophilin)	*Mus musculus* *Blattella germanica **	6e-52 1e-69 *	841 164 aa *	**CK172388** **AY551095***
Isocitrate dehydrogenase	*Homo sapiens*	7e-12	735, 735, 739	**CK172330, CK172366, CK172386**
	*Danio rerio **	1e-174*	470 aa*	**AY551096***
Fatty acid binding protein	*Myotis lucifugus*	4e-4 5e-16 *	386 141 aa *	**CK172312** **AY551097***
Sodium glucose cotransporter	*Homo sapiens*	2e-2 1e-162*	523 653 aa *	**CK172416** **AY551098***
Atpase H+ transporting lysosomal protein	*Caenorhabditis elegans*	4e-7 4e-7 *	412 61 *	**CK172372** **AY551099***
Scavenger-receptor	*Sus scrofa*	1.5e-1	296	**CK172401**
	BQ426240 (*C. gigas*)	1e-144		
Precerebellin	*Oncorhynchus mykiss*	4.8	756	**CK172358**
	BQ426725 (*C. gigas*)	7e-12		
PP2A inhibitor	*Tetraodon fluviatilis*	3e-18	600	**CK172303**
DNA replication licensing factor mcm5 (cdc46 homolog)	*Xenopus laevis*	5e-69	498	**CK172304**, **CK172410**
Putative Cutinase	*Phytophthora capsici*	6e-11	243	**CK172308**
Sarcoplasmic calcium-binding protein	*Mizuhopecten yessoensis* *Meretrix lusoria*	3e-4 6e-4 1e-44	357 322 517	**CK172313,** **CK172314** **CK172346**
NADH Dehydrogenase 6	*Crassostrea gigas*	2e-29	495	**CK172316**
tRNA splicing 2' phosphotranferase	*Homo sapiens*	4e-39 7e-39	722 723	**CK172320** **CK172355**
Cytochrome C Oxidase	*Crassostrea gigas*	6e-68	804	**CK172365**
DNA Topoisomerase I	*Homo sapiens*	7e-12 1e-3 6e-11 3e-12 2e-14 2e-16 2e-10	363 371 369 363 422 363 365	**CK172310** **CK172354** **CK172360** **CK172369** **CK172383** **CK172406** **CK172430**
Bone Morphogenic Protein	*Dugesia japonica*	8e-7 8e-7	703 702	**CK172353** **CK172362**
Gamma-kafirin preprotein precursor	*Sorghum bicolor*	3e-2	648	**CK172377**
Glycoprotein 120	*Crassostrea gigas* (**BQ427259**)	8e-7	366	**CK172407**
Kiaa1007 protein	*Homo sapiens*	5e-34 5e-38	392 391	**CK172341** **CK172418**

Poly(a)-specific ribonuclease	*Rattus norvegicus*	4e-41	845	**CK172399**
S8 ribosomal protein	*Drosophila melanogaster*	6e-42	470	**CK172301**
28S mitochondrial ribosomal protein S18c	*Drosophila melanogaster*	1e-19	514	**CK172352, CK172379**
		8e-11	522	**CK172391**
		1e-19	514	**CK172415**
40S ribosomal protein s15a	*Drosophila melanogaster*	1e-58	470	**CK172414**
Ribosomal protein L34	*Branchiostoma belcheri tsingtaunese*	3e-21	171	**CK172385**

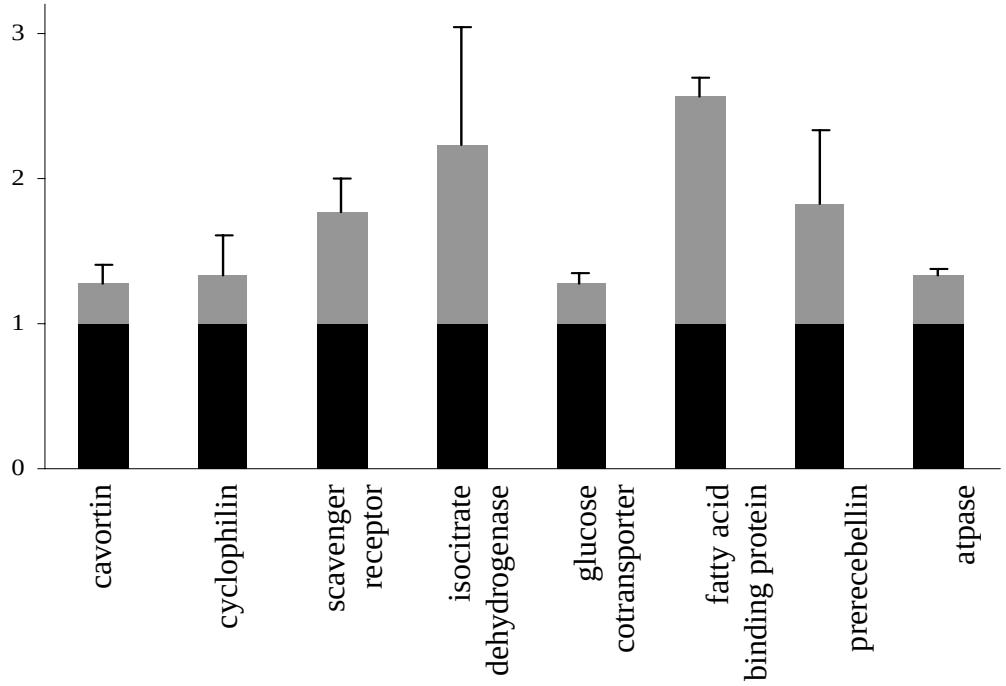
cDNAs appearing in bold were extended (*v-atpase* cDNA is not complete), and their full length (in aa), E-value and Genbank accession number are reported with *.

3.3 Sequence and expression analysis of ESTs implicated in energy generation or immune function

The transcripts clustered into categories corresponding to energy generation or immune function were fully sequenced and found to encode homologues of the following proteins: cavortin (**AY551094**), cyclophilin (PPIase, **AY551095**), isocitrate dehydrogenase (IDH, **AY551096**), sodium glucose cotransporter (SGLT, **AY551098**), fatty acid binding protein (FABP, **AY551097**) and ATPase H+ transporting lysosomal protein (V-ATPase; **AY551099**). Clones showing doubtful homology with scavenger receptor (SR, **CK172401**) and precerebellin (**CK172358**), two proteins reported to have a role in immune function, were also extended. After the extension of these two clones, no significant homology of sequence was found in databases (E-value >0).

For these 8 transcripts, the mRNA level of the samples used for the SSH was estimated by real time PCR and was significantly higher (relative to the *actin* transcript) in the R compared to the S progeny at the 5% level for *cavortin*, *ppiase*, *idh*, putative-*precerebellin*, and at the 1% level for putative-*sr*, *sglt*, *fabp*, *v-atpase*. The mean additional expression observed in the R progeny was 1.7 ± 0.4 ranging from 1.28 to 2.57 for *cavortin* and *fabp* respectively (Figure 2).

Figure 2. The amount of gene transcript relative to *actin* transcript in susceptible (S, black bar) and resistant (R, black plus grey bars) progeny. The additional amount of relative gene expression in R families compared to S families is represented by the grey bars and is significant at the 5% level for all the measured genes (Student's *t*-test). The error bars represent one standard deviation of that additional amount.



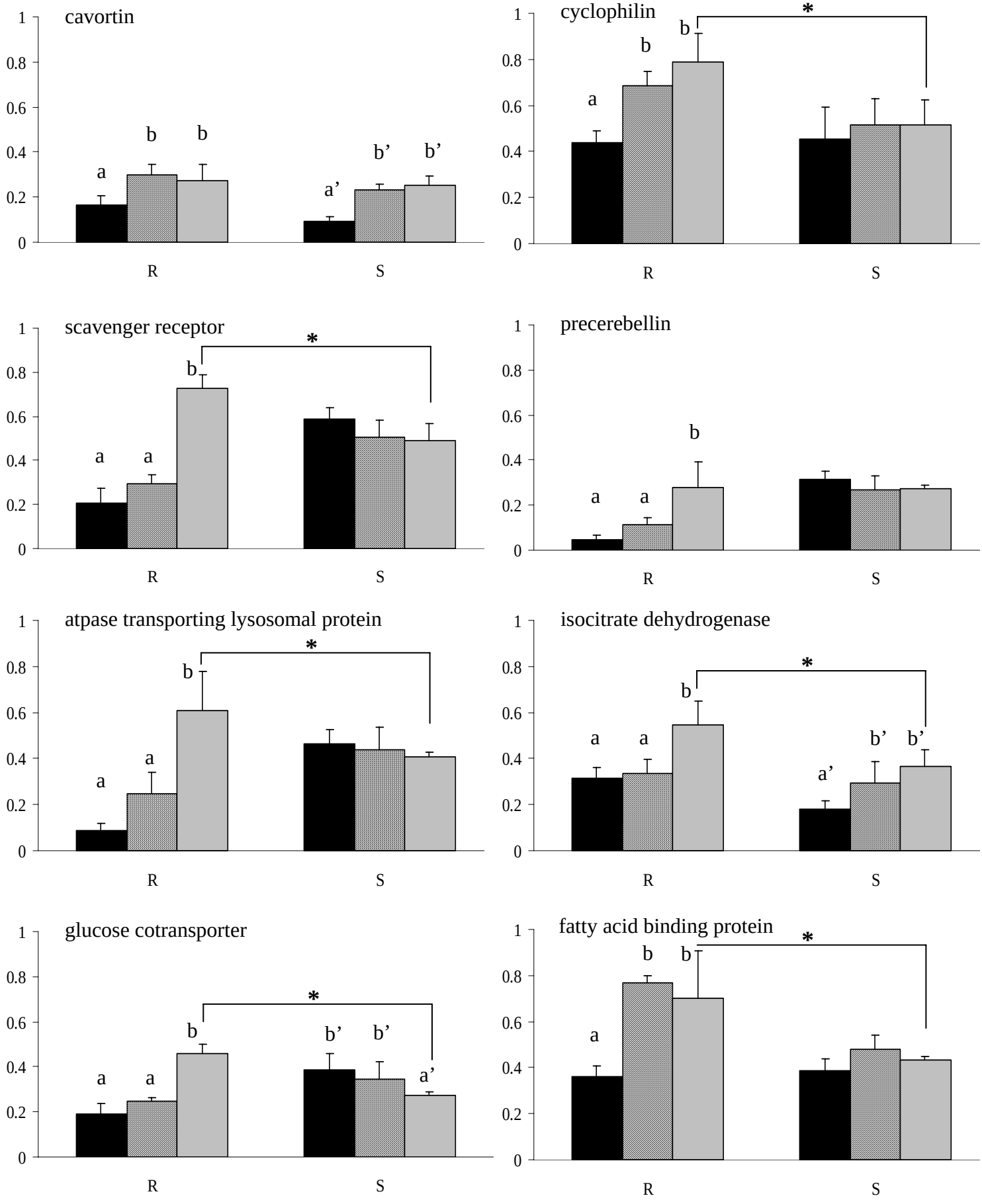
3.4 Bacterial challenge

Gene expression analysis of the 8 selected transcripts showed the level of 6 (*ppiase*, *idh*, *sglt*, *fabp*, *v-atpase*, putative-*sr*, Figure 3) of the transcripts to be significantly higher in the non injected-R (ni-R) group compared to the non injected-S (ni-S) group at the 5% level. The mean additional expression observed in the ni-R group was 1.6 ± 0.1 ranging from 1.5 to 1.7 for *idh* and *sglt*, respectively. mRNA levels of *cavortin* and putative-*precerebellin* relative to *actin* transcript, were not significantly different between the ni-R and ni-S groups ($P > 0.05$). To try to reproduce the particular summer mortality event we observed in the first experiment, a bacterial challenge was carried out with *V. splendidus* isolated during this event. Ten hours after challenge, significant changes in the mRNA level (relative to *actin* mRNA) were reported between ni-S and challenged S groups for *sglt*, *cavortin* and *idh* only. The level of *cavortin* and *idh* transcripts decreased in injected-*vibrio*-S (iv-S) compared to ni-S whereas a

slight significant increase of *sglt* transcript (1.4-fold higher) was observed in iv-S compared to ni-S ($P = 0.018$). In contrary, in R progeny significant reductions in mRNA levels (relative to *actin* mRNA) were reported in injected-*Vibrio*-R (iv-R) compared to ni-R for all 8 genes. These reductions ranged from 1.7 to 7.2-fold for *cavortin* and *v-atpase* respectively (mean decrease = 3.3 ± 1.9) (Figure 3). Furthermore, significant reductions were also observed in injected-seawater-R (isw-R) compared to ni-R for 5 different transcripts (*idh*, *sglt*, *v-atpase*, putative-*sr*, putative-*precerebellin*). These reductions were less intense (mean decrease = 1.7 ± 0.7) compared to those measured between iv-R and ni-R but no significant differences were observed between iv and isw mRNA levels.

Four days after injection, cumulative mortality was 79.5 ± 12.0% and 72 ± 9.0% for iv-R and iv-S progeny respectively. No mortality was observed in uninjected oysters or oysters injected with sterile seawater.

Figure 3. The amount of gene transcript relative to *actin* transcript in resistant (R) and susceptible (S) progeny 10 hours after challenge. Oysters were injected with either *Vibrio splendidus* (black bars), sterile seawater (hatched bars) or not injected (grey bars). The error bars represent one standard deviation. Comparison of the level of mRNA (relative to *actin* mRNA) between non-injected R and S groups was performed by Student's *t*-test; (*) significant at the 5% level. Homogenous groups were estimated within progeny between injected and non injected groups using the least significant difference (LSD) pairwise multiple comparisons test.



4. Discussion

4.1 Suppression subtractive hybridization

SSH was conducted in the present study using *C. gigas* R and S F2 progeny obtained following divergent selection criteria (Dégremont et al., 2003). Three hundred and seventy six clones were obtained in the subtracted library and the differential expression of these clones was confirmed for 150 using a PCR-select method (Diatchenko et al., 1999). The remaining 60% represent non-differentially expressed 'background' transcripts and were therefore eliminated from the analysis. An enhanced level of mRNA expression in the R compared to the S progeny was then confirmed for 8 selected transcripts by real time PCR. For example there was a 1.3 to 2.6-fold enhancement of expression in the R progeny for *cavortin* and *fabp* respectively.

4.2 Induced genes in R compared to S progeny

BlastX analysis of the 150 partially sequenced differentially regulated clones resulted in 25 unique homologues being identified (Table 3) of which 16 have never been reported previously in any marine bivalve species. These genes appeared to be divided into 6 functional categories, including general metabolism, cell signaling, cell cycle and cell structure, and DNA replication, repair and transcription. Based on published data related to summer mortality in mussels (Tremblay et al., 1998) and on initial data from the 'Morest' program (non shown), it has been hypothesized that *C. gigas* summer mortality is the result of an energetic and/or immunological dysfunction. Twelve and 16% of the characterized cDNA clones respectively were in these categories. The nucleotide sequence of 8 cDNAs which were classified as being involved in either energy metabolism or putative immune function were extended. Transcripts encoded homologues of the following proteins: Cavortin, PPIase, IDH, SR, Precerebellin, V-ATPase, SGLT and FABP. For Cavortin, PPIase, IDH, V-ATPase,

SGLT and FABP, extending their cDNA sequences to full length size (cDNA sequence of V-ATPase is not complete) confirmed their identity. However, extending the two other cDNA sequences changed the doubtful identity of cDNAs encoding homologues of SR and Precerebellin to having no significant homology (Table 3).

Based on the function of some genes identified in the subtracted library, energy and especially its mobilization would appear to be a key difference between the R and S progeny. Reported SSH sequences encoded SGLT and FABP; SGLT mediates glucose uptake into cells driven by a Na^+ gradient (Wright, 2001) and is of major importance in the sustenance of a cell's energetic requirement. FABP is a cytosolic protein involved in the uptake, transport and compartmentalization of fatty acids (Dhar et al., 2003) and would be stimulated under a variety of circumstances for mobilization of lipid to provide the metabolic fuel required.

In our initial experiment in which the cumulative mortality of S and R progeny reared in summer conditions with a plentiful food supply was determined, we found *V. splendidus* to comprise up to 50 to 80% of the total bacterial population of the dying S oysters. In contrast, R progeny displayed a high survival rate under the same conditions. This result is additional evidence for the strong genetic basis of the survival observed in the divergent selection criteria applied (Dégremont et al., 2003). Immune function might therefore be another difference between R and S progeny. Some subtracted transcripts encoded proteins implicated in immune pathways such as Cyclophilin, a peptidylprolyl isomerase known in mammals to accelerate the folding of proteins and to mediate signalling events leading to T-cell activation (Shida et al., 2003). In the case of bacterial infection, a cellular immune response is engaged in the host initially when SR and/or Toll-like receptors on haemocyte membranes recognize surface constituents of bacteria and activate NFκB pathways and phagocytosis. The NFκB pathway can also be stimulated by inflammatory cytokines such as bone morphogenic protein (BMP)-4 (Sorescu et al., 2003). A member of the BMP family was characterised in our

subtracted oyster library and clone **CK172329** matched with Toll-8 but without significant blast value. When pathogens are phagocytosed, haemocytes produce reductive oxygen intermediates (ROIs) that together with lysosomal cytotoxic components kill pathogens. Lysosomal processes require acidification of the intracellular compartment (Forgac, 1999). This is the function of V-ATPase, a multisubunit enzyme characterised in the subtracted library. ROIs produce oxidative damage to biological macromolecules such as DNA, RNA, protein and lipids. A greater capacity to rapidly detoxify the ROIs could perhaps explain differences in oyster survival rather than the capacity of haemocytes to synthetize ROIs. Some subtracted cDNAs encoding Cytochrome C oxidase, IDH and Cavortin, may contribute to cellular host protection against ROIs: IDH is able to supply NADPH which is needed for GSH production (Ciriolo et al., 1997; Kim and Park, 2003) and Cytochrome C oxidase converts molecular oxygen into water in the mitochondrial respiratory chain (Tian et al., 1998). Furthermore, Cavortin, recently characterized as a self-aggregating haemolymph glycoprotein in mussel (called Pernin), has a superoxide dismutase (SOD) domain (Scotti et al., 2001). SOD activity converts $O_2^{\cdot-}$ to the less dangerous H_2O_2 and is needed to prevent host cellular oxidative damage. To date, no SOD activity has been found directly associated with cavortin but this is still under investigation (Scotti P., Pers. Comm.).

The present study identified candidate genes implicated in the underlying physiological differences between R and S progeny. To begin to address their potential role in summer mortality and consequently identify pathway(s) that may significant to the higher rate of mortality of S progeny, we analyzed the expression of these genes in an *in vitro* experimental summer mortality event.

4.3 Bacterial challenge

The relative levels of gene expression in unchallenged oysters showed that, irregardless of age, season or physiological status (i.e. resting period *versus* reproductive period), most of the measured transcripts (*ppiase, idh, sglt, fabp, v-atpase,* putative-*sr*) were induced in the R compared to the S progeny. This suggests fundamental functional differences between the two groups of progeny for those genes. This is the case for both cDNAs involved in energy mobilization (*sglt*, *fabp*) and for those such as *ppiase*, *idh* and *v-atpase* that are probably implicated in immune or cellular host protection against ROIs.

The high energetic cost associated with the reproductive process, when combined with high summer temperatures, are thought to weaken oysters and make them more susceptible to opportunistic pathogens possibly leading to death. This was bourn out by our preliminary conditioning experiment when *V. splendidus* comprised 50 to 80% of the total bacterial population of the dying S oysters. The identification of such relatively high levels *V. splendidus* in S oysters allowed us to carry out a bacterial challenge with this isolate with the aim of mimicking the observed experimental summer mortality event. Some transcripts (*cavortin*, *idh*), which are suggested to act in cellular host protection against ROIs, displayed a similar reduction in mRNA levels in R and S progeny in response to *Vibrio* inoculation. However, other transcripts (*v-atpase, sglt,* putative-*sr,* and putative-*precerebellin*) showed a significant decrease in gene expression solely in R progeny in response to *Vibrio* and seawater injections, probably corresponding to a general response to stress, and for which no down-regulation in S progeny was observed. For two other transcripts, one probably involved in immune function (*ppiase*) and the second in energetic mobilization (*fabp*), the decrease of mRNA levels appeared to be specifically in response to inoculated bacteria and solely in R progeny. Such a decrease of mRNA levels was reported for *penaeidin* in shrimp during the first 12 hours following a bacterial challenge (Munoz et al., 2002). The authors suggested that

this strong decrease was the result of the migration of haemocytes towards injured tissues and visualized by a massive accumulation of *penaeidin*-producing haemocytes around the site of injection. Here, the haemocytes constituted the main cellular mediators of the defence system but are also involved in the transport of nutrients in marine invertebrates (Cheng, 1996). If we extrapolate this observation to the present study we might hypothesize that the observed decrease of mRNA level in mantle-gonad tissue of challenged R oysters corresponded to mobilization of haemocytes towards the muscle where we injected *Vibrio*. Thus, a greater capability to rapidly recruit phagocytes towards infected tissues, kill pathogens and/or provide the metabolic fuel required for tissue regeneration would be expected in R oysters compared to S oysters. However, no difference was observed between R and S progeny in response to bacterial injection though both suffered a high mortality rate. Injecting 10^8 *V. splendidus* is generally fatal for oysters. Effects of lower doses, alternative method of bacterial challenge (close to natural infection), and temporal responses of oysters will be among our future experiments.

In conclusion, this work constitutes the first step towards elucidating the physiological and genetic basis of summer survival of R and S selected progeny. From the genes and pathways suggested to operate differentially between F2 S and R progeny, our data suggest *fabp* (lipid mobilization) and *ppiase* (immune mediation) expression during an infection episode as possible starting points for further research. Finally, a strong capacity to down-regulate some metabolic pathways was solely observed in R progeny after injection of pure sea water into the adductor muscle. This may be a reaction to the general stress of the injection and requires further investigation.

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