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ORIGINAL PAPER

Differentially expressed genes under simulated deep-sea conditions in the psychrotolerant yeast *Cryptococcus* sp. NIOCC#PY13

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Abstract Microorganisms exhibit varying degrees of tolerance to extreme conditions of pressure and temperature. In the present study, the psychrotolerant deep-sea yeast, *Cryptococcus* sp. NIOCC#PY13, was exposed to elevated hydrostatic pressure and low temperature to explore the differentially expressed genes responsible for its survival at such extreme conditions. The suppression subtractive hybridization technique was employed for identification of expressed upregulated genes at extreme conditions of pressure and temperature. The effect of elevated pressure was found to be different than that of combined pressure and temperature exposures. Altogether, 17 and 20 upregulated genes were identified at 50 MPa and 50 MPa/5 °C, respectively. These differentially expressed genes were similar to the NCBI database ESTs (expressed sequence tags), coding for proteins such as arachidonic acid metabolism, amino acid transport and unsaturation of membrane fatty acids, which have been previously demonstrated to assist in the survival of microorganisms under stress conditions. Interestingly, about 50 % of the upregulated genes matched with hypothetical proteins at a percentage similarity of ≤96, suggesting their probability of being novel. Detailed studies of the above genes/proteins from deep-sea microorganisms are suggested for future investigations, which may shed more light on the existence and adaptation mechanisms adopted by these for their survival under such extreme conditions.

Keywords Deep sea · Differential expression · Elevated hydrostatic pressure · Psychrotolerant yeast · Stress proteins · Suppression subtractive hybridization

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Introduction

In general, elevated hydrostatic pressure in the range of several dozen MPa (megapascal) may have adverse effect on the growth of mesophilic organisms (Abe et al. 1999; Bartlett 2002; Abe 2004). The cessation of growth is accompanied by morphological changes such as formation of filaments in *Escherichia coli* (ZoBell 1970) and pseudomycelia in the marine yeast *Rhodosporidium sphaerocarpum* (Lorenz and Molitoris 1992; Lorenz 1993). Elevated hydrostatic pressure is believed to inhibit protein activities, by altering their conformation or sometimes denaturation (Molina-García 2002), and reduce membrane fluidity (Bravim et al. 2010). Similarly, low temperature has been suggested to affect the expression of genes involved in translation initiation (Broeze et al. 1978). Both these stresses result in perturbation of a variety of membrane-associated processes, including trans-membrane ion and nutrient flux, and DNA replication.

In comparison to bacteria, only a few investigations on the effect of elevated pressure and low temperature on the growth patterns of fungi have been carried out (Raghukumar and Raghukumar 1998; Damare et al. 2006). Among yeasts, *Saccharomyces cerevisiae* has been used in various

studies as a model organisms for analyzing the effects of stress conditions (Martin and Drubin 2003; Fernandes et al. 2004; Abe 2007). However, to study the genes or proteins expressed under various stresses in non-model organisms, suppression subtractive hybridization (SSH) can also be used (Straus and Ausubel 1990; Dogra and Breuil 2004). It is suppression PCR where normalization and subtraction are combined in a single procedure (Diatchenko et al. 1996). The normalization step equalizes the abundance of cDNAs within the target population and the subtraction step excludes the common sequences between the target and driver population, increasing the probability of obtaining low-abundance differentially expressed cDNA. The unique genes produced under stress conditions can be PCR amplified and sequenced to get detailed information (Akopyants et al. 1998). The technique of SSH used in conjunction with high throughput differential screening or macroarray has been reported for rapid and easy identification of differentially expressed genes in a few yeast species (Dogra and Breuil 2004; Marques et al. 2004).

The present study is an analysis of differentially expressed genes in deep-sea psychrotolerant yeast,*Cryptococcus* sp., NIOCC #PY13, subjected to elevated hydrostatic pressure of 50 MPa (equal to 500 bar, the hydrostatic pressure prevailing at 5000-m depth in the ocean) and low temperature of 5 °C using SSH. The whole cell cDNA populations of stressed and normally grown yeast were hybridized and differentially expressed genes were examined for their role in survival at such extreme conditions. To our knowledge, this is the first report on the investigation of transcriptional response of deep-sea yeast under extreme conditions of hydrostatic pressure and temperature using SSH.

Materials and methods

Yeast strain and culture conditions

The psychrotolerant yeast NIOCC#PY13 was isolated from deep-sea sediment of the Central Indian Basin (10–16.5°S and 72–77°E) on board the Russian research vessel Akademic Boris Petrov (Cruise # ABP26) during December 2006. The isolate was maintained at 5 °C on malt extract agar medium (Kohlmeyer and Kohlmeyer 1979) by repeated subculturing at an interval of 20–25 days. It was identified by amplification and sequence analysis of partial 18S and complete ITS region of SSU-rDNA (Singh et al. 2010). For inoculum preparation, it was grown into 20 ml YPD medium (0.5 % yeast extract, 1 % peptone and 1 % dextrose) and incubated overnight at 25 °C and 170 rpm on a rotary shaker incubator. One ml of this was further inoculated into 100 ml of the YPD medium and incubated on shaker until the absorbance at 600 nm reached a value of 1.0. The pre-grown culture was packed in sterilized gas-permeable polypropylene plastic bags and sealed at both the ends with sealing machine (Sevana, India) without trapping any air bubbles. The packed bags were suspended in a deep-sea culture vessel (Tsurumi & Seiki Co., Japan), filled completely with distilled water. The pressure vessels were incubated at the following temperature and pressure conditions for a period of 2 h: (a) 0.1 MPa/25 °C, (b) 50 MPa/25 °C and (c) 50 MPa/5 °C. The pressure vessels were opened after 2 h of exposure and the yeast cells were collected by centrifugation. Cells were washed with distilled water to remove the remaining growth media and the cell pellet was processed immediately for RNA isolation.

RNA isolation, mRNA purification and cDNA construction

RNA was isolated from yeast cells collected after exposure to the above-mentioned three conditions using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. Purification of polyA RNA was done using the polyA RNA purification kit (Clontech Laboratories, CA, USA). From each of the three mRNA extracted for the above three pressure and temperature conditions, 2 μg was used to generate cDNA populations by using reverse transcriptase and 1 μl of 10 μM cDNA synthesis primer, and buffer conditions as prescribed by the PCR-select subtractive library kit (Clontech Laboratories, CA, USA). A complementary strand for this cDNA was synthesized by using DNA polymerase 1 and DNA ligase (Clontech Laboratories, CA, USA).

Suppression subtractive hybridization

The PCR-select SSH kit (Clontech Laboratories, CA, USA) was used to perform SSH on cDNA populations prepared from yeast cells according to the manufacturer's instructions as follows:

1. Whole cell cDNA of the yeast subjected to 0.1 MPa/25 °C was hybridized with the cDNA of whole cells subjected to 50 MPa/25 °C for 2 h. The results are represented as genes upregulated under elevated hydrostatic pressure (P) (Table 1).
2. Whole cell cDNA of the yeast exposed to 0.1 MPa/25 °C was hybridized with the cDNA of the yeasts exposed to 50 MPa/5 °C: Results are represented as genes upregulated under elevated hydrostatic pressure and low temperature (PT) (Table 2).

In brief, cDNAs from all the three populations were subjected to blunt-ended digestion by restriction enzyme

Table 1 Details of clones representing genes upregulated at elevated hydrostatic pressure exposure of 50 MPa (P) for 2 h

Clone ID	NCBI database homolog (source), accession no.	Probable function	Identity (%)	*E* value
PH_06	Hypothetical protein SERLA73DRAFT_132214 (*Serpula lacrymans var. lacrymans* S7.3), *EGO01737.1*	Pre-mRNA splicing, telomere replication and mRNA degradation	75	2.4
PH_10	Epoxide hydrolase (*Rhodotorula mucilaginosa*), *AAV64029.1*	Arachidonic acid metabolism	58	2e−22
PH_13	Hypothetical protein RTG_00533 (*Rhodotorula glutinis* ATCC 204091), *EGU13356.1*	Amp binding enzyme	85	2e−29
PH_15	C-4 methylsterol oxidase (*Puccinia graminis f.* sp. *tritici*), XP_003325825.1	Sterol desaturase [lipid metabolism]	72	2e−24
PH_16	Beta-glucan synthesis-associated protein KRE6 (*Puccinia graminis f.* sp. *tritici*), *XP_003336377.1*	Carbohydrate transport and metabolism	55	0.021
PH_18	YALI0F05192p (*Yarrowia lipolytica*), *XP_505022.1*	Amino acid transport and metabolism	78	6e−14
PH_21	Putative chitin deacetylase (*Rhodotorula glutinis* ATCC 204091), *EGU11052.1*	Hydrolase activity, acting on carbon–nitrogen (but not peptide) bonds	65	1e−29
PH_23	CK1/CK1/CK1-G protein kinase (*Puccinia graminis f.* sp. *tritici*), *XP_003320184.1*	Serine/threonine protein kinases	85	1e−17
PH_25	Hypothetical protein RTG_00970 (*Rhodotorula glutinis* ATCC 204091), *EGU12929.1*	–	41	1e−04
PH_26	Predicted protein (*Laccaria bicolor* S238N-H82), *XP_001874954.1*	–	65	0.71
PH_27	Hypothetical protein RTG_00740 (*Rhodotorula glutinis* ATCC 204091), *EGU13027.1*	Phosphate binding	73	0.020
PH_28	Hypothetical protein MPER_03947, partial (*Moniliophthora perniciosa* FA553), *XP_002395912.1*	Amino acyl-tRNA synthetases	87	8e−10
PH_30	RING finger domain-containing protein (*Rhodotorula glutinis* ATCC 204091), *EGU11928.1*	Pre-mRNA splicing, telomere replication and mRNA degradation	57	1.6
PH_31	Hypothetical protein PGTG_01294 (*Puccinia graminis f.* sp. *tritici* CRL 75-36-700-3), *XP_003320382.1*	Nucleoside triphosphate hydrolases	58	2.5
PH_32	Methylenetetrahydrofolate reductase (*Rhodotorula glutinis* ATCC 204091), *EGU11236.1*	5,10-Methylenetetrahydrofolate is reduced to 5-methyltetrahydrofolate by methylenetetrahydrofolate reductase, a cytoplasmic, NAD(P)-dependent enzyme. The product 5-methyltetrahydrofolate is utilized by methionine synthase	71	3e−10
PH_33	C-8 sterol isomerase (*Cryptococcus neoformans var. neoformans* JEC21), *XP_567070.1*	Catalysis of the reaction which results in unsaturation at C-7 in the B ring of sterols	79	2.5
PH_35	Alpha, alpha-trehalose-phosphate synthase (*Schizosaccharomyces japonicus* yFS275), *XP_002172783.1*	Catalyzes the synthesis of alpha,alpha-1,1-trehalose-6-phosphate from glucose-6-phosphate using a UDP-glucose donor. It is a key enzyme in the trehalose synthesis pathway	90	0.006

Rsa1. In order to compare differentially expressed clones in two populations, forward and reverse subtractions were performed. In the forward subtraction, cDNA at 0.1 MPa/25 °C served as the tester and cDNA at 50 MPa/25 °C served as the driver, and in reverse subtraction, cDNA at 0.1 MPa/25 °C served as the driver and cDNA at

Table 2 Details of clones representing genes upregulated at elevated hydrostatic pressure and low temperature exposure of 50 MPa/5 °C (PT) for 2 h

Clone ID	NCBI database homolog (source), accession no.	Probable function	Identity (%)	*E* value
PTH_01	ATP-dependent RNA helicase DHX8 (*Rhodotorula glutinis* ATCC 204091), *EGU11123.1*	Polyribonucleotide nucleotidyltransferase	100	7e−05
PTH_02	Hypothetical protein, partial (*Ophiocordyceps unilateralis*), *ADI72904.1*	–	85	2e−33
PTH_03	Transcript antisense to ribosomal RNA protein, partial (*Ajellomyces capsulatus* H88), *EGC42647.1*	–	63	7e−18
PTH_07	Hypothetical protein SERLA73DRAFT_67379, partial (*Serpula lacrymans var. lacrymans* S7.3), *EGN91453.1*	–	73	2e−12
PTH_08	Helicase (*Rhodotorula glutinis* ATCC 204091), *EGU13556.1*	–	100	2e−06
PTH_12	Adenosylmethionine decarboxylase (*Rhodotorula glutinis* ATCC 204091), *EGU10839.1*	Regulatory role in the polyamine biosynthetic pathway	76	0.18
PTH_16	Hypothetical protein PICST_57317, partial (*Scheffersomyces stipitis* CBS 6054), *XP_001383614.1*	–	89	3e−36
PTH_22, PTH_23, PTH_30	Hypothetical protein SNOG_09178 (*Phaeosphaeria nodorum* SN15), *XP_001799479.1*	AMP-activated protein kinase	52	2.7
PTH_25	Family 2 glycosyltransferase (*Melampsora larici-populina* 98AG31), *EGF99634.1*	Catalyzes the incorporation of GlcNAc from substrate UDP-GlcNAc into chitin	60	5e−41
PTH_27, PTH_33	Hypothetical protein NEUTE2DRAFT_133468 (*Neurospora tetrasperma* FGSC 2509), *EGZ67539.1*	–	82	3e−32
PTH_41	Aromatic amino acid family biosynthesis-related protein (*Cryptococcus neoformans var. neoformans* JEC21), *XP_569127.1*	Dehydroquinate synthase (DHQS) catalyzes the conversion of DAHP to DHQ in shikimate pathway for aromatic compounds synthesis	48	1e−09
PTH_44	Hypothetical protein BATDEDRAFT_35978 (*Batrachochytrium dendrobatidis* JAM81), *EGF77791.1*	Protein involved in vacuole import and degradation (intracellular trafficking and secretion)	60	9e−09
PTH_46	Malate dehydrogenase (*Pyrenophora tritici-repentis* Pt-1C-BFP), *XP_001931613.1*	–	58	1.1
PTH_47, PTH_49	Hypothetical protein RTG_02039 (*Rhodotorula glutinis* ATCC 204091), *EGU11795.1*	–	64	2e−06
PTH_52	Hypothetical protein E5Q_05427 (*Mixia osmundae* IAM 14324), *GAA98739.1*	Protein kinase C-related kinase	72	2e−29
PTH_59	Ribose-5-phosphate isomerase (*Rhodotorula glutinis* ATCC 204091), *EGU12089.1*	Catalyzes the reversible conversion of ribose-5-phosphate to ribulose 5-phosphate, the first step of the non-oxidative branch of the pentose phosphate pathway	100	2e−31

50 MPa/25 °C served as the tester. The same protocol for forward and reverse subtraction was repeated for the cDNA populations at 0.1 MPa/25 °C and 50 MPa/5 °C.

In each of the forward subtraction, cDNA was split into two samples (25 ng each) to which different PCR-primer adaptor sequences were ligated (adaptor 1 for one pool of cDNA and adaptor 2R for other). A third ligation of both adaptors to the tester cDNAs (unsubtracted tester control) was performed as a negative control.

In the first hybridization, cDNA (driver) was added to each sample of testers (Tester-1 and Tester-2) and incubated at 68 °C for 8 h. Hybridization kinetics led to equalization and enrichment of differentially expressed sequences. In the second hybridization, two primary

hybridization samples were mixed together and fresh denatured cDNA (driver) was added to the mixed sample and incubated at 68 °C overnight and subsequently diluted with 200 µl of dilution buffer. The resulting cDNA molecules were then subjected to two rounds of PCR to amplify and enrich the desired differentially expressed sequences by using primers (PCR primer1, nested PCR primer1 and nested PCR primer 2R) of the PCR-select subtractive library kit, (Clontech Laboratories, CA, USA) in a PCR machine (Eppendorf, Germany). A PCR of 27 cycles was performed which included an initial step at 72 °C for 5 min to extend the adaptors and fill in overhangs, denaturing at 94 °C for 30 s, annealing at 66 °C for 30 s and extension at 72 °C for 1.5 min. To further reduce the background, the resulting PCR product was further diluted (3 µl in 27 µl) and a second round of amplification of 12 cycles was performed by using nested PCR primer1 and 2R. Denaturation was carried out at 94 °C for 30 s, annealing at 68 °C for 30 s and extension at 72 °C for 1.5 min with a final extension incubated at 72 °C for 15 min.

Subtracted cDNA library construction and screening of clones

The PCR products of forward as well as reverse subtraction were then ligated separately with pGEM-T easy vector (Promega, USA) and transformed into *E. coli* cells (Invitrogen, CA, USA), following the manufacturer's instructions. Transformed cells were grown on Luria–Bertani (LB) agar medium under X-gal and ampicillin selection. Individual white colonies that showed the presence of inserted DNA (by β-galactosidase inhibition) were randomly picked and further analyzed. Randomly picked white colonies were grown in 96-well plates overnight at 37 °C in 1 ml of Luria–Bertani broth containing 100 µg ml^{-1} of ampicillin. Overnight-grown clones were processed for plasmid isolation and purification using Millipore plasmid preparation kit (Millipore, USA). Sequencing of the plasmids was done at the National Institute of Oceanography, Goa, India, using the ABI 3130xl Genetic analyzer by chain termination method with BigDye terminator v3.1 (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis

Forward and reverse sequences were assembled using ChromasPro Version 1.34 (Technelysium Pvt. Ltd, Tewantia, Queensland, Australia). All the sequences were checked with Ribosomal Database Project (Cole et al. 2004) for the presence of chimeras. No chimeric sequences were detected. The sequences were compared with the non-redundant database using the blastx and

Table 3 GenBank accession numbers and dbEST_Id of the cDNA sequences obtained in the present study

Clones upregulated at 50 MPa	GenBank accession no.	dbEST_Id	Clones upregulated at 50 MPa/5 °C	GenBank accession no.	dbEST_Id
PH_06	JK757950	76227928	PTH_01	JK757967	76227945
PH_10	JK757951	76227929	PTH_02	JK757968	76227946
PH_13	JK757952	76227930	PTH_03	JK757969	76227947
PH_15	JK757953	76227931	PTH_07	JK757970	76227948
PH_16	JK757954	76227932	PTH_08	JK757971	76227949
PH_18	JK757955	76227933	PTH_12	JK757972	76227950
PH_21	JK757956	76227934	PTH_16	JK757973	76227951
PH_23	JK757957	76227935	PTH_22	JK757974	76227952
PH_25	JK757958	76227936	PTH_23	JK757975	76227953
PH_26	JK757959	76227937	PTH_25	JK757977	76227955
PH_27	JK757960	76227938	PTH_27	JK757978	76227956
PH_28	JK757961	76227939	PTH_30	JK757976	76227954
PH_30	JK757962	76227940	PTH_33	JK757979	76227957
PH_31	JK757963	76227941	PTH_41	JK757980	76227958
PH_32	JK757964	76227942	PTH_44	JK757981	76227959
PH_33	JK757965	76227943	PTH_46	JK757982	76227960
PH_35	JK757966	76227944	PTH_47	JK757983	76227961
			PTH_49	JK757984	76227962
			PTH_52	JK757985	76227963
			PTH_59	JK757986	76227964

blastn algorithms (Altschul et al. 1990) at the DNA analysis Web site maintained by NCBI to search the database for similar sequences. All these sequences have been submitted to EST database of NCBI GenBank. Their GenBank Accession numbers and dbEST_Ids are listed in Table 3.

Results

Culturable yeasts were isolated by incubation of the deep-sea sediment sample in nutrient media at 5 °C for a period of 1 month. One of these isolates, NIOCC#PY13, was a psychrotolerant yeast, as it exhibited growth at 5 °C as well as at 30 °C. It showed homology to *Cryptococcus vishniacii* based on 18S rRNA gene sequence (Singh et al. 2010). This yeast was selected for the analysis of differentially expressed genes in the present study due to its psychrotolerant nature. It exhibited growth at simulated deep-sea conditions, i.e., elevated hydrostatic pressure and low temperature (Singh et al. 2010). It also grew better in media prepared with seawater than with distilled water. All these characteristics indicate adaptation of this yeast to growth under deep-sea conditions, making it a suitable candidate for studying genes expressed under elevated hydrostatic pressure and low temperature.

In the present study, a total of 384 positive clones were sequenced from the forward and reverse cDNA subtracted libraries constructed for this yeast, subjected to different pressure and temperature exposures (see "Materials and methods"). After analysis, a total of 17 and 20 cDNA sequences were found to be upregulated at stress conditions of 50 MPa and 50 MPa/5 °C, respectively (Tables 1, 2). No overlapping of upregulated cDNA clones was observed between exposure of elevated hydrostatic pressure (50 MPa) alone and combined elevated pressure and low temperature (50 MPa/5 °C). Most of the cDNA sequences matched with distinct open reading frames (ORFs) of protein sequences of other fungal isolates in the NCBI database.

Out of the 17 ESTs (expressed sequence tags), upregulated under elevated hydrostatic pressure, 41.1 % matched with hypothetical proteins in the NCBI database. Among other upregulated ESTs, the genes coding for the proteins responsible for unsaturation of fatty acids, arachidonic acid metabolism, amino acid transport, carbohydrate metabolism and synthesis of trehalose were detected (Table 1). The combined effect of elevated hydrostatic pressure and low temperature resulted in differential expression of ESTs, involved in the polyamine biosynthetic pathway, glycosylation of membrane proteins and protein kinases, and synthesis of aromatic compounds and enzymes having regulatory role in the pentose phosphate pathway (PPP).

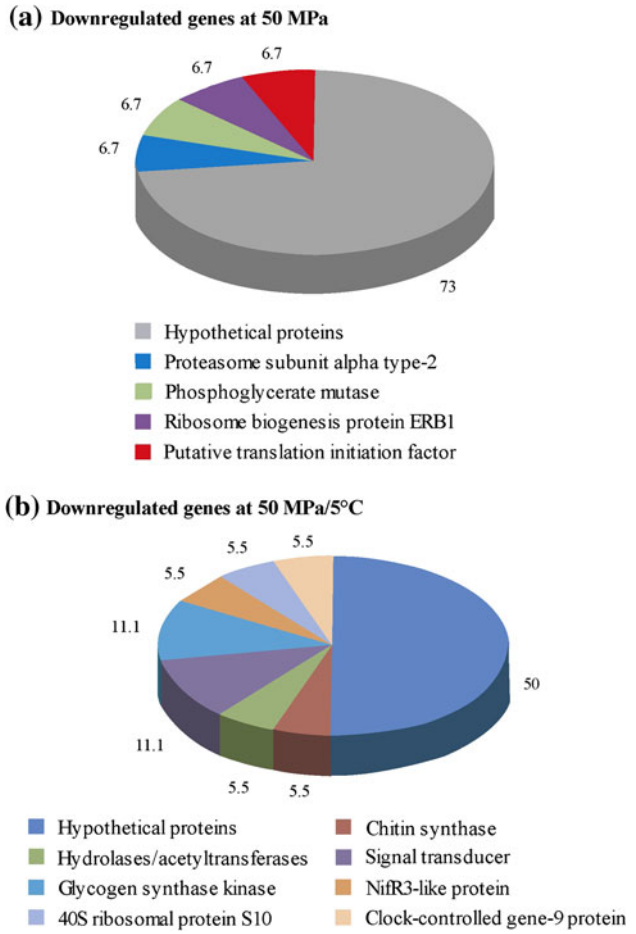

Fig. 1 Pie chart showing the percentage of downregulated genes in a deep-sea psychrotolerant yeast, NIOCC#PY13, at **a** 50 MPa and **b** 50 MPa/5 °C

Most of the upregulated genes at 50 MPa/5 °C also belonged to hypothetical proteins (60 %) having no known function (Table 2).

The ESTs appearing in the forward subtracted libraries (cDNA at 0.1 MPa/25 °C was used as tester) showed identity with genes involved in the synthesis of proteins related to ribosome assembly, transcription machinery or basic housekeeping genes (Fig. 1). However, a major proportion of these ESTs belonged to hypothetical proteins with 73 and 50 % for subtractions with cDNAs at 50 MPa and 50 MPa/5 °C, respectively (Fig. 1a, b). The ESTs detected in the forward subtracted libraries did not appear in any of the libraries where cDNAs of the pressure- or pressure/temperature-exposed cells were used as tester (Tables 1, 2). Therefore, it may be assumed that these genes were downregulated under elevated hydrostatic pressure and low temperature. Both the upregulated and downregulated cDNA clone sequences showed identity in the range of 41-100 % with existing ESTs of other fungi in the NCBI database (Tables 1, 2).

Discussion

In this study, for the first time, SSH was used to analyze the differential expression of genes at simulated conditions of deep sea, i.e., elevated hydrostatic pressure and low temperature. The SSH technique provided significant information regarding genes expressed under simulated conditions of deep sea by incorporating a hybridization step that normalizes (equalizes) sequence abundance during the course of subtraction by standard hybridization kinetics (Diatchenko et al. 1996).

A total of 17 genes including hypothetical protein-coding sequences were found to be upregulated at elevated hydrostatic pressure (50 MPa). The cDNA clones of these upregulated genes encoded for the proteins reported to be necessary for tolerance to environmental stresses (Martin and Drubin 2003; Chasse and Dohlman 2004). One of the cDNA clones, PH_10, belonged to epoxide hydrolase from *Rhodotorula mucilaginosa* in the NCBI database (Table 1). This enzyme plays a significant role in arachidonic acid metabolism (Newman et al. 2005). Arachidonic acid is a polyunsaturated omega-6 fatty acid (ω6 PUFA) which may have regulatory role toward maintaining fluidity of the yeast cell membrane under stress conditions. This has been reported to function as a precursor molecule for biosynthesis of a ω3 PUFA, eicosapentaenoic acid in fungi (Gellerman and Schlenk 1979). Eicosapentaenoic acid levels were observed to increase with decreasing temperature in an Antarctic bacterium, suggesting its role in preserving microviscosity of cell membranes at lower temperatures (Nichols et al. 1997). However, Usui et al. (2012) have emphasized the role of EPA toward maintaining proper membrane stability at extreme conditions of pressure and temperature rather than just increasing its fluidity. This suggests a more complex mechanism performed by polyunsaturated fatty acids under elevated pressure and low temperature than thought before, which needs to be explored further in detail. Other upregulated genes responsible for fatty acid desaturation mechanism involved C-4 methyl sterol oxidase and C-8 sterol isomerase (Table 1). Cellular synthesis of polyunsaturated fatty acids has also been demonstrated to enhance increased adaptation toward oxidative stress in a strain of *Saccharomyces cerevisiae* (Cipak et al., 2008).

The cDNA clone, PH_18, matched with a protein from *Yarrowia lipolytica* having a role in amino acid transport and metabolism. Since at elevated hydrostatic pressure, the protein synthesis machinery is known to be affected by inhibition of amino acid uptake and synthesis, the upregulation of proteins, assisting in amino acid transport, suggests their significant role in high pressure tolerance (Abe 2007; Abe and Minegishi 2008). Yeast cells expressing a substantial level of Tat2, i.e., tryptophan permease, were demonstrated to grow at low temperatures as well as at high hydrostatic pressures (Abe and Horikoshi 2000). Some of the upregulated genes at 50 MPa belonged to proteins responsible for mRNA degradation (Table 1). Genes involved in transcription and mRNA degradation have been reported as essential for survival at high pressure and low temperature (Abe and Minegishi 2008). Among other cDNA clones, the upregulated genes encoded for proteins required for carbohydrate metabolism, trehalose synthesis and hydrolase activities (Table 1). Trehalose is a nonreducing disaccharide known to protect proteins, membranes and other macromolecules in cells against various stresses. Iwahashi et al. (1997) reported intracellular trehalose to play a role in cell survival at extremely high pressures. All these results suggested the differential expression of genes at elevated hydrostatic pressure, enabling the yeast to survive high pressure stress by their expression (Abe 2007; Abe and Minegishi 2008).

Similarly, differentially expressed genes observed under elevated hydrostatic pressure and low temperature exposure (50 MPa/5 °C) for 2 h also belonged to proteins, mandatory for survival under stress (Table 2). These genes were identified as coding for proteins involved in polyamine biosynthetic pathway, glycosylation of membrane proteins and vacuole import and degradation (Table 2). In general, elevated hydrostatic pressure imposes adverse effect on deep-sea organisms by destabilization of protein structure and ligand binding. To counteract these adverse effects due to elevated pressure, deep-sea organisms accumulate some osmolytes inside their cells. For example, in deep-sea teleost fishes, polyamines such as trimethylamine N-oxide level has been demonstrated to increase with increasing hydrostatic pressure which help in counteracting the effect of high pressure on protein unfolding, and also maintain the affinity for substrates in certain enzymes (Yancey 2005). The upregulation of genes coding for proteins involved in the polyamine biosynthetic pathway in the present study also support this hypothesis and suggests that these polyamines play a significant role in survival under extreme conditions of deep sea.

The maintenance of appropriate membrane fluidity is thought to be one of the key factors for survival and growth under elevated pressure conditions, as suggested by the results of studies on prokaryotes (DeLong and Yayanos 1985; Abe et al. 1999). Abramova et al. (2001) speculated that cells exhibited reduced membrane fluidity under hypoxia as a possible outcome of anaerobiosis and at low temperature as a result of reduced lateral diffusion and increased microviscosity. Recycling of membranes might be crucially required for growth at high pressure and low temperature, considering the role of microautophagy (Abe and Minegishi 2008). Therefore, the upregulation of proteins involved in membrane trafficking to vacuole followed

by their degradation play an important role in survival of microorganisms under extreme conditions.

The cDNA clone, PTH_59 belonged to ribose-5-phosphate (R5P) isomerase (RpiA) (Table 2) which converts ribulose-5-phosphate (Ru5P) to R5P which is then converted by ribulose-phosphate 3-epimerase to xylulose-5-phosphate during the PPP. The end result of the reaction is essentially the conversion of the pentose phosphates to intermediates used in the glycolytic pathway. In the oxidative part of the PPP, RpiA converts Ru5P to the final product, R5P, through isomerization reaction. The oxidative branch of the pathway is a major source for NADPH, which is needed for biosynthetic reactions and protection against reactive oxygen species (Juhnke et al. 1996), proposing its role under survival during stress conditions of elevated pressure and low temperature.

The downregulated genes observed at 50 MPa and 50 MPa/5 °C, belonged mostly to the proteins required for basic cellular metabolic regulation mechanisms (Fig. 1). A few examples of these genes are 40S ribosomal protein S10, proteasome subunit alpha type-2, putative translation initiation factor, glycogen synthase kinase, chitin synthase, etc. (Fig. 1). As evident from earlier reports, the expression of basic regulatory housekeeping genes gets inhibited under different stress conditions in various microorganisms (Abe 2007). Our results are also in concordance with the above hypothesis and demonstrated inhibition of several housekeeping genes (Fig. 1) at elevated pressure and low temperature conditions. The majority of the upregulated as well as downregulated cDNA clones matched with hypothetical proteins (Fig. 1; Tables 1, 2) in the NCBI database, suggesting that they may be coding for novel proteins and need to be characterized further in detail in order to understand their role in stress survival mechanisms in yeast. The low percentage similarity with the existing database observed for the majority of the cDNA clones may also be attributed to the insufficient NCBI database for ESTs of *Cryptococcus* strain, used in the present study. Interestingly, there was no overlapping between upregulated cDNA clones at an exposure of elevated hydrostatic pressure (50 MPa) alone and combined elevated pressure and temperature (50 MPa/5 °C). However, an overlap of genes responsible for survival and growth at high pressure and low temperature has been reported during earlier studies (Abe and Minegishi 2008). The possible explanation for no overlapping genes in the present study may be unsaturation of sampling of clones from environmental libraries. A large-scale screening of forward and reverse cDNA subtracted libraries may provide greater insight into the genes responsible for elevated pressure and low temperature survival.

In conclusion, elevated hydrostatic pressure and low temperature are generally assumed to have adverse effects on biological systems, but can serve as useful parameters to enlighten dynamic structural changes associated with any reaction. Elevated pressure changes the rate of reactions in a manner separable from those due to low temperature. A combined analysis using pressure and temperature offers new insights into cellular functions such as the assembly of macromolecules, membrane trafficking or membrane protein functions. We identified several differentially expressed genes at elevated pressure and low temperature stresses by SSH in this study. This technique resulted in upregulation of various stress-related genes earlier reported as necessary for survival at extreme conditions. Further detailed analysis of the genes expressed under elevated hydrostatic pressure and low temperature may elucidate the mechanism adopted by deep-sea organisms for survival and their role in such underexplored habitats.

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