Cellular responses to environmental salinity in the halophilic black yeast *Hortaea werneckii*

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Summary

The development of crop plants with increased salt tolerance necessitates the study of naturally salt-tolerant eukaryotic species. We studied the biosynthesis of glycerol as a compatible solute in the halophilic eukaryotic microorganism, black yeast *Hortaea werneckii*. A restriction fragment–differential display technique was used to investigate the transcriptome of the organism. Eight differentially expressed genes were identified in response to growth at different salinities. Although the putative functions of their products, P-type ATPase, ubiquinone reductase, aconitase, RNA helicase, Asn-tRNA ligase, isoamyl alcohol oxidase, and phosphatidylinositol-3-kinase, are not intimately related within the cellular machinery, the results presented here are sufficient to propose a model which describes how *H. werneckii* adapts to extremely high salinities. Some of these mechanisms of adaptation to raised environmental salinity are similar to those in other salt-sensitive species, e.g. glycerol accumulation, there also appear to be novel mechanisms present such as the use of different energy production mechanisms and post-transcriptional regulation of gene expression. Our results have also provided new data on two genes from two other fungal species, the *Neurosperia crassa* B1D1.130 gene and the *Aspergillus ustus* amdS-A gene.

Introduction

Progressive salinization of irrigated land is one of the main problems for agriculture world-wide. This trend necessitates research into the development of genetically engineered crop plants with greater salt tolerance. An important step in this direction is to understand the mechanisms of salt tolerance in those eukaryotic organisms which are salt-tolerant by nature. Studies on salt tolerance in fungal species have been to this date largely performed with salt-sensitive model species, such as *Saccharomyces cerevisiae* (Varela and Mager, 1996; Blomberg, 2000), *Aspergillus nidulans* (Redkar *et al*., 1996) and others. While these species are more convenient from the experimental point of view, their cellular machinery is not adapted to highly saline environments and their mechanisms of adaptation are expected to differ at least partially from those in naturally salt-tolerant species. For a deeper understanding of salt tolerance in eukaryotes, studies on halotolerant and halophilic species are therefore required.

High saline concentration environments prevent growth of the majority of organisms. There are some specially adapted organisms, mostly prokaryotic, that can grow at extremely high salt concentrations. Little is known about eukaryotic halophilic organisms, let alone about their adaptation to such conditions at the molecular level. One notable exception is the marine yeast *Debaryomyces hansenii* which has been quite extensively studied for its halophilic character (Adler et al., 1985; Prista et al., 1997; Neves et al., 1997; Ramos, 1999; Almagro et al., 2000). However, the precise and complete picture of how this species can grow with up to 20% NaCl in its environment is still not available (Ramos, 1999).

*Hortaea werneckii* (Dothideales, Ascomycota) is a melanized yeast-like fungal species, member of an arbitrary group of the so-called black yeasts. Previous studies have shown that it is an extremely halotolerant or perhaps even halophilic species (de Hoog and Gerrits van den Ende, 1992; Petrovič et al., 1999; Gunde-Cimerman et al., 2000). The natural habitat of *H. werneckii* is hypersaline waters, such as those in solar salterns where it was found to be the dominant fungal species (Gunde-Cimerman et al., 2000). The apparent ability to cope with substantial changes in environmental salinity is demonstrable also under laboratory conditions, since it can grow in media without added salt and up to 30% (5.2 M) NaCl concentrations (U. Petrovič, unpublished). *H. werneckii* is thus one of the most salt-tolerant species among all microorganisms and as such a highly appropriate model organism in which to study salt tolerance in eukaryotes. Although it has been studied because it causes *tinea nigra*, a non-pathologic dermal change on human palms...
(de Hoog and Gerrits van den Ende, 1992), its metabolism and genetics have not previously been investigated on the molecular level.

In this study we have investigated glycerol accumulation, previously described as an important mechanism in salt tolerance in salt-sensitive species. However, our attention was focused primarily on potentially unique mechanisms of *H. werneckii*, which we attempted to find by using a global experimental approach, namely restriction fragment–differential display. By constructing a model based on the data, we have revealed some possible mechanisms of adaptation in this halophilic black yeast to different environmental salinities. The findings presented here could open a whole new world in understanding the metabolic adaptation of halophilic eukaryotes to extremely saline environments.

**Results**

**Glycerol as a compatible solute**

One of the main features of the majority of microorganisms, when transferred to an environment with increased osmolarity, is the production of compatible solutes. In eukaryotic species, exemplified by the yeast *S. cerevisiae* and the green alga *Dunaliella salina*, glycerol is the main compound that increases the intracellular osmotic potential and simultaneously protects cellular structures from adverse conditions (Blomberg and Adler, 1992; Oren, 1999). We determined intra- and extracellular glycerol concentrations in *H. werneckii* cells grown at different salinities.

A steady increase in intracellular glycerol concentration was observed with environmental salinities from 0% to 10% NaCl (Fig. 1). At higher salinities, the concentration remained virtually unchanged. Extracellular glycerol concentrations were low and independent of salt concentration between 0% and 17% NaCl. One of the possible interpretations for this result is that synthesized glycerol is efficiently kept inside the cells, although we cannot exclude the possibility that glycerol crosses the plasma membrane and is then actively taken up into the cells, which is in accordance with previous reports from other organisms (Nevoigt and Stahl, 1997; Blomberg, 2000). At salinities higher than 17%, the extracellular concentration of glycerol started to increase. This effect correlates with the diminished growth of *H. werneckii* at salinities above 17% NaCl. Growth rate of *H. werneckii* depends on the salinity of the media. The generation times were 8 h in the saltless medium, down to 6 h at salinities between 3% and 7.5% NaCl, increased up to 15 h at salinities between 10% and 17% NaCl, and as high as 80 h at 25% NaCl.

The expression of the gene for a putative glycerol-3-phosphate dehydrogenase was measured. This is a key enzyme for glycerol production in *S. cerevisiae* (Albertyn et al., 1994) that catalyses production of glycerol-3-phosphate from dihydroxyacetone phosphate and thus turns the metabolic flux from glycolysis into glycerol production. A part of the *H. werneckii* glpD gene, coding for a putative glycerol-3-phosphate dehydrogenase, was cloned and sequenced (accession number: AJ420183). This sequence was used for Northern blot analysis and for quantitative RT-PCR analysis which revealed a several-fold increase in gene expression level when cells grown in a medium supplemented with 17% and 25% NaCl were compared with those grown in the absence of NaCl (Fig. 1). These findings are in accordance with the hypothesis that, as in *S. cerevisiae*, glycerol biosynthesis in *H. werneckii* is regulated mainly through transcriptional regulation of glycerol-3-phosphate dehydrogenase, and that glycerol is an important compatible solute in *H. werneckii* when exposed to elevated environmental salinities. Unchanged intracellular glycerol concentration between 10% and 25% NaCl points to the presence of (an)other compatible solute(s) in the cells of *H. werneckii* grown at salinities above 10% NaCl.

**Restriction fragment – differential display (RF–DD)**

In order to measure changes in the transcriptome in response to different environmental salinities, *H. werneckii* was grown at three different salinities: 0%, 17% and 25% NaCl. Both the growth characteristics and our previous data (Petrovič et al., 1999 and unpublished data) indicated that *H. werneckii* thrives best at moderate salinities up to 17% NaCl, and that it experiences hyposaline...
Sequences were compared against GenBank or EMBL databases with the use of different BLAST and FASTA algorithms. Nine sequences were unambiguously identified as homologues of known genes and were named SOL1-SOL9 (Table 1). Eight of these ESTs were selected from differential display (DD) as differentially expressed genes, while one (SOL3) was chosen as a gene whose expression does not change with the salinity. Quantitative RT-PCR was used to confirm the expression profiles of these nine genes and to calculate their relative expression levels (Fig. 2). In the case of SOL5 the primer set used produced an alternative band from cDNA isolated from cells grown at 25% NaCl (data not presented) and the expression profile of SOL5 was therefore measured by Northern blot analysis (Fig. 2). In general, three clusters of genes with similar expression profiles emerged. The first cluster consists of the genes whose expression is highest at 17% NaCl in the medium (SOL1, SOL2, SOL6 and SOL7), the second of genes whose expression is highest at 25% NaCl (SOL4 and SOL8), and the third from genes SOL3 and SOL9, whose expression remains virtually unchanged when cells are exposed to different salinities.

While differential display enables one to compare transcriptomes of cells grown under different conditions in a single experiment, addition of restriction of cDNA and ligation of specific adapters prior to DD allows studies on completely unknown transcriptomes with reduced numbers of false positive results (Ivanova and Belyavsky, 1995). The high number of false positive results that often occur with DD (Debouck, 1995) was shown to be partially alleviated with the addition of restriction/ligation prior to DD (Ivanova and Belyavsky, 1995). This was also the case in our experiments since out of the nine identified genes whose expression profiles we confirmed with RT-PCR (Fig. 2), transcription of only one (i.e. SOL9) was found to be virtually unaffected by differences in environmental salinity, although it was selected as a differentially expressed gene. Its expression profile was also confirmed by Northern blot analysis (U. Petrović, unpublished). Therefore, in our hands the RF–DD technique gave only 11% false positive results, compared to more than 70% sometimes reported for classical DD (Sompayrac et al., 1995). The biggest caveat of the technique in our opinion was the fact that only less than half (i.e. 9 out of 19) cloned ESTs could be identified with database homology searches. One reason for this might be the fact that only ESTs shorter than 500 base pairs could be successfully

<table>
<thead>
<tr>
<th>H. werneckii gene/accession number</th>
<th>Closest homologue in the database</th>
<th>Algorithm</th>
<th>E-value</th>
<th>FASTA</th>
<th>FASTAX</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOL1/AJ420787</td>
<td><em>Emericella nidulans</em> plasma membrane H⁺-ATPase</td>
<td>BLASTN</td>
<td>2e-07</td>
<td>✓</td>
<td>NA</td>
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<tr>
<td>SOL2/AJ420788</td>
<td><em>Neurospora crassa</em> ferredoxin-like iron-sulphur subunit of mitochondrial complex I</td>
<td>BLASTN</td>
<td>1e-20</td>
<td>✓</td>
<td>NA</td>
</tr>
<tr>
<td>SOL3/AJ420789</td>
<td>Schizosaccharomyces pombe hypothetical protein with unknown function</td>
<td>BLASTX</td>
<td>0.052</td>
<td>–</td>
<td>✓</td>
</tr>
<tr>
<td>SOL4/AJ420790</td>
<td><em>Neurospora crassa</em> conserved hypothetical protein B1D1.130 (Na⁺/X symporter)³</td>
<td>BLASTX</td>
<td>2e-10</td>
<td>✓</td>
<td>NA</td>
</tr>
<tr>
<td>SOL5/AJ420791</td>
<td>Aspergillus terreus aconitase</td>
<td>BLASTN</td>
<td>3e-14</td>
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<tr>
<td>SOL6/AJ420792</td>
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<td>2e-21</td>
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<td>NA</td>
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<tr>
<td>SOL7/AJ420793</td>
<td>Schizosaccharomyces pombe probable nuclear RNA helicase (DEAD family)</td>
<td>BLASTX</td>
<td>3e-37</td>
<td>✓</td>
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</tr>
<tr>
<td>SOL8/AJ420794</td>
<td><em>Aspergillus oryzae</em> mreA gene for isoamyl alcohol oxidase</td>
<td>TBLASTX</td>
<td>8e-03</td>
<td>–</td>
<td>✓</td>
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<tr>
<td>SOL9/AJ420795</td>
<td>Schizosaccharomyces pombe probable phosphatidylinositol 3-kinase</td>
<td>BLASTX</td>
<td>7e-05</td>
<td>–</td>
<td>✓</td>
</tr>
</tbody>
</table>

a. The function of the *Neurospora crassa* B1D1.130 gene was proposed in this study. The X represents an unknown molecule (see text for details).  

b. The alternative open reading frame in the *Aspergillus ustus* amdS-A gene was identified in this study (see text for details).  

For each gene, the accession number, the closest homologue according to the E-value found in the GenBank database, algorithm by which the homologue was found and the respective E-value, are shown. Also shown are the results of the verification of the homology between the two sequences by two different FASTA algorithms against the database of all fungal sequences in the EMBL database, FASTA and FASTAX. ✓, identification confirmed; -, identification denied; NA, not analysed.


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New insights into old genes from other fungal species

When looking for homologues of SOL8, two genes with different functions were found as possible homologues, *Aspergillus oryzae mreA* gene, coding for isoamyl alcohol oxidase (Yamashita *et al.*, 2000), and *Aspergillus ustus amdS-A* gene which codes for acetamidase (Table 1). However, further analysis showed that the similarities found by TBLASTX algorithm corresponded to two similar predicted amino acid sequences, which could also be functionally related. It became evident that the *amdS-A* gene could code for isoamyl alcohol oxidase in an alternative reading frame, or that a pseudogene might be present there. Therefore we concluded that we may have identified as differentially expressed and cloned with the use of our protocols. In addition, the lack of related fungal sequences in the databases prevents identification of genes in a non-characterized species such as *H. werneckii*.

The closest homologue of SOL4 gene was found to be the *Neurospora crassa B1D1.130* gene. Since the expression of SOL4 increased with the salinity, with a profile that is very similar to that of *glpD* and also very interesting in the present context, we analysed the sequence of *B1D1.130*, whose function was unknown, to find any possible links to its function. The PSI-BLAST algorithm revealed that the putative amino-acid sequence derived from *B1D1.130* contains an SNF motif, shared by mammalian sodium:neurotransmitter symporters (SNF protein family, Pfam 00209), and two hypothetical Na⁺-dependent transporters from *Haemophilus influenzae* (http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF00209). Since it is also known for the canine transporters from this family that their mRNA expression responds to a hyperosmolar environment (Takenaka *et al.*, 1995), it is possible that *N. crassa B1D1.130* and *H. werneckii SOL4* are new members of this family of conserved putative Na⁺-dependent transporters.

**Cellular responses of H. werneckii to changes in environmental salinity**

Data on differentially expressed genes (Fig. 2) combined with both our previously published data on *H. werneckii* (Petrović *et al.*, 1999; M. Turk, L., Méjanéllé, M. Šentjurc, J. O. Grimalt, N. Gunde-Cimerman and A. Plemenitaš, submitted) and with biochemical data presented here (Fig. 1) led us to propose a mechanism, described below, of how *H. werneckii* adapts to varying environmental salinity.

*H. werneckii* is an obligate aerobic species so that, when grown on glucose as the sole carbon and energy source, glycolysis and Krebs cycle constitute its main energy metabolism. This metabolism appears to be perturbed with increase in environmental salinity to 17% or 25% NaCl, when both *glpD* expression level and glycerol concentration increase. Since glycerol presumably becomes one of the most abundant molecules produced by the cells, relatively less metabolic flux remains for the late stages of glycolysis and presumably also for the Krebs cycle. The latter assumption is supported by our finding that CO₂ production per dry weight is significantly higher in cells grown without salt than in those grown at 17% or 25% NaCl (Fig. 3A). Increased expression of SOL5, a putative aconitase gene, at 17% NaCl and 25% NaCl (Fig. 2), could also be related to this phenomenon. When Krebs cycle becomes less important, a cell could gain enough ATP through alternative electron donors, such as the glycerophosphate shuttle (Larsson *et al.*, 1998) and the isovaleryl-CoAdehydrogenase/electron-transferring-flavoprotein:ubiquinone oxidoreductase system (Ikeda

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Fig. 2. Expression profiles of newly identified genes (see Table 1) at three salinities: no NaCl, 17% NaCl, and 25% NaCl. The expression profiles of the genes were measured by quantitative RT-PCR, with the exception of SOL5 whose expression was measured by Northern blot analysis (see Experimental procedures).
profiles at the three chosen salinities were found for genes SOL1, a putative P-type H\textsuperscript{+}-ATPase, and SOL2, a putative subunit of the mitochondrial electron chain complex I (NADH-ubiquinone reductase). These two enzymes do not have obviously related functions, unless we speculate that H. werneckii NADH-ubiquinone reductase behaves like the NADH-ubiquinone reductase of moderately halophilic bacteria Salinivibrio costicola, which functions as a Na\textsuperscript{+} exporter (Udagawa et al., 1986; Ventosa et al., 1998). The subcellular localization of SOL2p is currently unknown, but it could be present in the plasma membrane and, together with SOL1p, function as a system for Na\textsuperscript{+} efflux and electrochemical potential across the membrane, presumably together with an as yet unidentified Na\textsuperscript{+}/H\textsuperscript{+} antiporter. However, the two genes could also be indirectly involved in generating energy for the cell under conditions where Krebs cycle is deprived, as discussed above. Also SOL4p is likely to be connected to Na\textsuperscript{+} transport. Its closest homologue, the Neurospora crassa B1D1.130 gene, was identified as a member of the SNF protein family (see above). The mammalian members of the SNF protein family co-transport Na\textsuperscript{+} and neurotransmitters or other molecules, such as betain (Takenaka et al., 1995). Very similar molecules as betain, on the other hand, function as compatible solutes in the prokaryotic microorganisms that live in extremely high saline environments (Oren, 1999). According to these data and to the expression profile of SOL4, which is very similar to that of glpD (Figs 1 and 2), we speculate that SOL4p could be a co-transporter of Na\textsuperscript{+} and a putative compatible solute into the cells.

Next, co-ordinated expression of genes hmgR (U. Petrović, unpublished), coding for hydroxymethylglutaryl CoA reductase (HMGR), and SOL8, coding for isoamyl alcohol oxidase, was observed. Their expression increases dramatically at 25% NaCl (Fig. 2), indicating that these two genes could be functionally related in the response to extremely high salinities. HMGR is a key regulatory enzyme of the mevalonate pathway whose main products in eukaryotes are sterols (Brown and Goldstein, 1990). However, although the increase also in the activity of HMGR was measured at 25% NaCl in H. werneckii (Petrović et al., 1999), the level of sterols does not increase under such conditions (Turk et al., 2002). Thus, an alternative pathway downstream of HMGR and mevalonate could be important at 25% NaCl in H. werneckii cells. This pathway could include production of isopentenoid isoamyl alcohol, followed by its oxidation, an enzyme presumably coded by SOL8. The product of isoamyl alcohol oxidase catalysed reaction, isovaleraldehyde, is known to be an attractant in oomycetes (Cameron and Carlile, 1981). However, if we assume the subsequent transformation of isovaleraldehyde to isovalerate and further to isovalerate-CoA, this path-

Fig. 3. CO\textsubscript{2} production of H. werneckii cells grown at different salinities.

A. Production of CO\textsubscript{2} by H. werneckii cells grown at different NaCl concentrations in the media, calculated per dry weight. Values were calculated relatively to the highest value, measured after 5 h by gas chromatography for the cells from 0% NaCl medium, which was assigned a value 1.

B. Calculated amounts of CO\textsubscript{2} produced for duplication of biomass at three different salinities. Values were calculated as measured data points after 5 h (Fig. 3A), multiplied by the generation times at the respective salinities, and plotted in relation to the highest value.

and Tanaka, 1983), or it could make use of Na\textsuperscript{+} and/or H\textsuperscript{+} electrochemical potentials across the plasma membrane – genes SOL1, SOL2 and SOL8 could be part of this scenario (see also below). To estimate the energy consumption of H. werneckii cells at different salinities we calculated the amount of CO\textsubscript{2} production required for doubling the biomass at different salinities. The results in Fig. 3B show that the highest production was required when cells were grown at 25% NaCl, which is in accordance with the inhibited growth observed under this hypersaline condition, but that the lowest production was observed at 17% NaCl, lower than at 0% NaCl. This could either mean that 17% NaCl is in terms of energy more amenable for H. werneckii than an environment without salt, or more likely that different energy-producing systems are at work under these conditions, such as those mentioned above.

The other parts of the model are based on the concerted changes in expression of some identified genes, which often indicates functional relationship (DeRisi et al., 1997; Ideker et al., 2001). Almost identical expression

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way could also help generating ATP by providing electrons from electron transport through the isovaleryl-CoA dehydrogenase/electron-transferring-flavoprotein: ubiquinone oxidoreductase system in a fashion described above.

Lastly, but not least, identical expression profiles of SOL6, a putative Asn-tRNA ligase, and SOL7, a putative RNA helicase (Fig. 2), together with a putative salt-sensing RNA helicase, ssrH (U. Petrović, unpublished), indicate that post-transcriptional regulation could be important in the response of H. werneckii to elevated salinities.

To summarize, we are proposing a model by which the glycerol accumulation, the use of alternative energy production mechanisms, such as the isovaleryl-CoA dehydrogenase/electron-transferring-flavoprotein: ubiquinone oxidoreductase system, or the Na$^+$ and/or the H$^+$ electrochemical potentials, and the post-transcriptional regulation enable H. werneckii to thrive at NaCl concentrations unparalleled by other eukaryotic species. This model should provide a good starting point for further studies on this and other highly salt-tolerant eukaryotic species.

Experimental procedures

Strain and growth conditions

Hortaea werneckii MZKI B-736 strain was used in all experiments. Cells were grown, as described (Petrović et al., 1999), in a defined minimal medium with glucose as the sole energy and carbon source and with different NaCl concentrations. In all experiments, cells from the mid-exponential growth phase were used.

Glycerol concentration determination

Total and extracellular concentrations of glycerol were determined as described (Larsson et al., 1990), using an enzymatic test kit (Glycerol Test Combination, Roche Molecular Biochemicals). Intracellular concentrations were calculated by subtracting extracellular concentration from the total.

CO$_2$ concentrations

A Becker gas chromatograph (Packard Model 417) was used to measure CO$_2$ concentrations in the atmosphere inside the hermetically closed bottles containing the identical volumes of mid-exponential cultures of cells grown in the presence of different NaCl concentrations. Samples for GC analysis were taken every 30 min for 5 h, and the concentrations increased linearly with time. All the results were calculated relative to the highest data values which were assigned a value 1.

DNA extraction and glpD partial sequence

For genomic DNA isolation, a protocol described by Rozman and Komel (1994) was used. The partial gene sequence of a putative glycerol-3-phosphate dehydrogenase gene was obtained by PCR at an annealing temperature of 58°C, using oligonucleotide primers GPD-p1: TCCATCGACCAT ACTTCCA and GPD-p2: CTTTGAGCGATTGACCACT. The fragment obtained was cloned (TOPO-TA Cloning kit, Invitrogen) and sequenced.

RNA extraction and quantitative RT-PCR

Total cellular RNA was isolated from the cells in the mid-exponential growth phase by the guanidine thiocyanate method (Breekvær et al., 1991). cDNA was obtained by adding 1.5 μl of 12.5 μM 5'-T$^3$-V'-3' oligo to 10 μg of total RNA (dissolved in 10 μl). Samples were heated for 10 min at 70°C, then put on ice for 10 min. To each sample 100 units of RT (Thermo-RT, Display Systems), dNTP mixture (final concentration 1 mM each) and appropriate amount of RT buffer were added and the mixture incubated at 42°C for 2 h. RNA was then degraded by adding 8 units of RnaseH. Twelve units of DNA polymerase 1 and 7.5 μl dNTP mix (5 mM each) were added to each sample, and the volume was adjusted to 70 μl with nuclease free dH2O. Samples were incubated at 16°C for 2 h and then DNA was extracted using phenol and then ethanol extractions. Equality of the amounts of cDNA obtained was checked by agarose gel electrophoresis. 50 ng of cDNA per reaction was used for the PCR reaction. The total mixture volume was 40 μl and it consisted of: 1 μl cDNA (50 ng μl$^{-1}$), 15 μl each oligo primer (10 μM), 3.2 μl dNTP mix (2.5 mM each), 4 μl 10 × Taq poly buffer and 0.4 μl Taq polymerase (5 U μl$^{-1}$, Perkin Elmer). The PCR reaction was programmed for up to 40 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 45 s. Aliquots (10 μl) of each sample were taken after various cycle numbers and analysed on agarose gel electrophoresis. The oligo primers used were as following: glpD, GPD-p1: TCCATCGACCATACTTCCA; GPD-p2: CTTTGAGCGATTGACCACT; SOL1, SOL1-p1: GGCTACTCGATTTGA; SOL1-p2: CGAAGCGCTATCCAGCT; SOL2, SOL2-p1: CTCCCACCTGACCCATTTTTG; SOL2-p2: GCATCTACGTGGCTTCTGC; SOL3, SOL3-p1: AGCGGA TGGTGACAATGAT; SOL3-p2: GCACAA GAACGATATGTA; SOL4, SOL4-p1: ATGCAACAATCGATTTCC; SOL4-p2: TGCTTACACTCGACCAAGC; SOL6, SOL6-p1: CTACGGCGAGAAGACATC; SOL6-p2: GCAGGTCGTCGAAAGTGAT; SOL7, SOL7-p1: CTGGAGTGTGGTTCCAGAT; SOL7-p2: CACCTCTTCTCAAGCACAA; SOL8, SOL8-p1: GACAA CTGCGAAGCCTTCC; SOL8-p2: ATCGCTTGAACCGGAGA; SOL9, SOL9-p1: TTCTGCGTGTGAG AGCACCTC; SOL9-p2: CGGGGAGGTAACAGATT.

Northern blot

The total RNA and the glycerol-3-phosphate dehydrogenase specific probe (i.e. glpD partial sequence) were obtained as described above. 20 μg of each RNA species (from 0%, 17% and 25% NaCl) were run on a formaldehyde-agarose gel and blotted onto a neutral nylon membrane (Amersham) and dried for 2 h at 80°C. The probe was labelled with [32P]dCTP (Prime-It Random Primer Labelling kit, Stratagene). The membrane was first prehybridized for 2 h at 65°C in a
prehybridization solution (Ausubel and Frederick, 1998). Hybridization was then performed for 20 h at 65°C using a hybridization solution and the H. werneckii specific glycerol-3-phosphate dehydrogenase probe. The membrane was then washed for 2 h at 65°C with 2×SSC/0.3% SDS solution, and exposed to photographic film (X-OMAT, Kodak) for 48–72 h at –70°C.

Restriction fragment – differential display (RF–DD)

A commercially available kit, displayPROFILE kit (Display Systems Biotech), was used for RF–DD and the technique was performed according to the manufacturer’s instructions. 1 μg of total RNA, obtained as described above, was used for each of the three conditions tested (no NaCl, 17% NaCl and 25% NaCl in the growth media). Analysis of the amplified ESTs was performed by using ABI Prism 310 Genetic Analyzer (PE Applied Biosystems) and also by using pre-cast polyacrylamide gel electrophoresis (GeneGel Clean, Amersham Pharmacia Biotech) and silver staining. Bands corresponding to differentially expressed genes (>5-fold difference between at least two different growth conditions was selected as threshold) were excised from the gel, PCR reamplified, cloned (TOPO-TA Cloning kit, Invitrogen) and sequenced. Identification of the genes was done by using different BLAST and FASTA algorithms against GenBank and other nucleotide databases.

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