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Oxidative Stress Responses in Yeast Strains, *Saccharomyces cerevisiae*, from "Evolution Canyon", Israel

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Summary. The genome of baker's yeast, *Saccharomyces cerevisiae*, has been sequenced, many genes have been deleted for phenotypic analysis, and the tools to study gene and protein interaction and the functions of the large number of functionally unknown genes have made yeast the most advanced model for the analysis of eukaryotic organisms. The yeast research community has provided a paradigm for work in the genomics era of biology of which Professor *Ruis* has been an early protagonist. The genomics view is now contributing to initiate research in molecular evolution. We have taken strains from "Evolution Canyon", Israel, for an analysis by microarray hybridizations of the response of wild yeast accessions to environmental stress, in particular oxidative stress. Strains were selected from the "African" south-facing slope (SFS) of the canyon, characterized by xeric conditions and high irradiation, from the "European" north-facing slope (NFS), characterized by mesic conditions and low irradiation, and from the valley bottom. H_2O_2-sensitive strains included a laboratory strain (S150-2B) and most strains from the NFS. Statistically supported is a correlation between peroxide tolerance, the SFS, and micro-niche within a slope. Hierarchical clustering of regulated transcripts indicated maximum linkage of expression profiles between strains that showed the same phenotypic stress response. The analyses indicate strain-specific adaptive micro-niche evolution along the microclimatic gradient of "Evolution Canyon" that determine the response to oxidative stress.

Keywords. *Saccharomyces cerevisiae*; "Evolution Canyon"; Oxidative stress response; Microarray analysis; Adaptive evolution; Micro-climate.

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In memoriam of *H. Ruis*

Introduction

Owing to the wealth of genetic information, not the least due to the complete sequence of its genome, available for baker's yeast, *Saccharomyces cerevisiae*, this eukaryote has become the prime model for studies on functional genomics and systems biology [1–4]. The wealth of tools can be used in studies that extend our knowledge from the behavior of cells and organisms under controlled laboratory conditions to evolutionary diversity under ecological aspects and studies of populations in natural settings.

We have used strains of *Saccharomyces cerevisiae* that were collected at different locations along a microclimatic gradient at ''Evolution Canyon'' (EC), Lower Nahal Oren, Mount Carmel, Israel [5–7]. Our objective was to estimate possible microclimate-dependent adaptive genetic changes in these strains. The south- and north-facing slopes of this canyon, SFS and NFS, respectively, provide different microclimates in close physical vicinity, such that the terms ''African'' slope (SFS) and ''European'' slope (NFS) have been introduced [7]. Temperature, humidity, and irradiation differences between the slopes, separated by a few hundred meters, have recently been quantified [8, 9]. EC has already provided a model character for analyzing microclimate-dependent adaptations to extreme environments and selection mechanisms in studies with a number of organisms, including algae, fungi, plants, and animals [5, 6]. Adaptive genetic differences have been indicated for a number of species, among them fungi, vascular plants, *Drosophila melanogaster*, mammals, and cyanobacteria [10–12, 6].

Major distinguishing variables between the EC slopes are light intensity and temperature [9], which should translate into different exposure to radical oxygen stress that could determine performance and possibly survival. Stress by radical oxygen species, ROS, is known to damage DNA, oxidize lipids, and lead to protein and enzyme unfolding, denaturation, and aggregation [13]. ROS, in the form of superoxide (O_2^{-}), hydrogen peroxide (H_2O_2), and the short-lived, extremely poisonous hydroxyl radical ($OH^{-\cdot}$), which cause aging and affect life span, are controlled or detoxified by a number of systems that operate in all organisms [14–17], and the cellular mechanisms that alleviate those stresses have been described [13, 18, 19]. In budding yeast, *Saccharomyces cerevisiae*, components with a role in oxidative stress defense include thioredoxins, glutaredoxin, glutathione, superoxide dismutase, flavonoids, and catalases [20–22]. Also known are mechanisms that lead to the activation of radical defenses based on the activity of transcription factors, such as Yap1 [23] and Msn2/4 [24], and the MAPK, Hog1 [25]. These transcription factors are activated by signaling pathway, including the Sln1p-Ssk1p two-component system [26] and the phosphorylation cascade of the Hog pathway that had first been described as a salinity and osmotic signaling pathway [27]. More recently, involvement of the Hog pathway in oxidative stress signaling has been described [25].

We analyzed the ability of different strains of *S. cerevisiae*, collected at various locations and from different substrates in EC along the ''European'' and ''African'' slopes and the valley bottom. We chose oxygen radical stress as the experimental factor to challenge different strains for an investigation of genetic variability and possible divergence in the microclimatic niches that exist in close physical

proximity in this location. The results indicate a significant positive correlation between slope, location, and stress tolerance, or susceptibility.

Results and Discussion

Yeast Strains and Stress Survival

Yeast biodiversity in EC has recently been described [7] and the presence of strain of baker's yeast, *Saccharomyces cerevisiae*, has been documented. We have analyzed a collection of these strains phenotypically and by microarray hybridizations. The materials used (Table 1) included 17 strains collected from the EC south-facing slope (SFS), 19 strains from the north-facing slope (NFS), and 6

Table 1. Tolerance characteristics of *S. cerevisiae* strains from ''Evolution Canyon''; survival rates (SR) were calculated as described [29]; the list includes information on niche and strain location at EC

Strain no.	Slope (1–3)	Niche (1–7)	Survival rate (SR)/%			
			Isolate location	0.4 *M* H_2O_2	0.8 *M* H_2O_2	Phenotype H_2O_2[a]
15[b]	SFS (1)	1	Sun	88	72	5 (T)
16[b]	SFS (1)	1	Sun	73	69	4.5 (T)
17	SFS (1)	1	Shade	80	68	4.5 (T)
18	SFS (1)	1	Shade	89	79	5 (T)
20[b]	SFS (1)	1	Shade	43	5	1.5 (S)
21[b]	SFS (1)	2	Shade	72	75	5 (T)
23	SFS (1)	2	Shade	71	76	5 (T)
25	SFS (1)	2	Shade	81	89	5 (T)
26	SFS (1)	2	Shade	72	73	4.75 (T)
27	SFS (1)	2	Sun	74	51	3.75 (MT)
29	SFS (1)	2	Sun	85	87	5 (T)
30	SFS (1)	3	Shade	77	80	5 (T)
31	SFS (1)	3	Shade	84	84	5 (T)
33	SFS (1)	3	Shade	31	18	1 (S)
34	SFS (1)	3	Shade	54	10	2 (MS)
35	SFS (1)	3	Sun	49	28	1.75 (S)
36	SFS (1)	3	Sun	64	25	2.5 (MS)
37	VB (2)	4	Shade	94	94	5 (T)
39	VB (2)	4	Shade	80	76	5 (T)
40	VB (2)	4	Shade	49	27	2 (MS)
42	VB (2)	4	Sun	90	95	5 (T)
43	VB (2)	4	Sun	87	83	5 (T)
44	VB (2)	4	Sun	22	4	1 (S)
50	NFS (3)	5	Sun	49	16	1.5 (S)
51	NFS (3)	5	Sun	79	74	5 (T)
52	NFS (3)	5	Sun	51	23	1.75 (S)
53	NFS (3)	5	Shade	77	56	4 (T)

(*continued*)

Table 1 (*continued*)

Strain no.	Slope (1–3)	Niche (1–7)	Survival rate (SR)/%			
			Isolate location	0.4 *M* H_2O_2	0.8 *M* H_2O_2	Phenotype H_2O_2[a]
54	NFS (3)	5	Shade	56	14	2 (MS)
55[b]	NFS (3)	6	Sun	82	86	5 (T)
56[b]	NFS (3)	6	Sun	92	88	5 (T)
57	NFS (3)	6	Sun	61	29	2.5 (MS)
58	NFS (3)	6	Sun	64	58	3.5 (MT)
59[b]	NFS (3)	6	Shade	5	5	1 (S)
60[b]	NFS (3)	6	Shade	2	1	1 (S)
61	NFS (3)	7	Sun	83	87	5 (T)
62	NFS (3)	7	Sun	82	84	5 (T)
63	NFS (3)	7	Sun	43	1	1.5 (S)
64	NFS (3)	7	Shade	82	29	3 (MT)
65	NFS (3)	7	Shade	74	58	4 (T)
66[b]	NFS (3)	7	Shade	3	2	1 (S)
67	NFS (3)	7	Shade	71	34	2.75 (MS)
68[b]	NFS (3)	7	Shade	2	2	1 (S)

[a] Strain survival was scored in five categories (1–5), with the score 1–1.9 as sensitive (less than 40% survival), and 5 as tolerant (more than 70% survival) in H_2O_2; categories 2 (40–50%), 3 (50–60%), and 4 (60–70%) are listed as moderately sensitive or moderately tolerant; average of four numbers from each replicates of 0.4 and 0.8 *M* H_2O_2 are listed as phenotype; a phenotypic factor was calculated: tolerant (score >4; T), moderately tolerant (3–4; MT), moderately sensitive (2–3; MS), and sensitive (<2; S); SR statistical analysis was by SPSS (http://www.spssscience.com/spss11/index.cfm);
[b] assignments were confirmed by liquid culture

strains from the valley bottom. By comparison, the SFS receives 2- to 8-fold (200–800%) higher irradiation than the NFS, and is generally drier, although the distance between the slopes is 400 meters at the top, and 100 meters at the bottom of the canyon [7]. The strains are characterized by the slope, ''African'' and ''European'', respectively, and within each slope by shade or sun location of collection (Table 1). Stress conditions were chosen to test (predominantly) oxygen radical defense reactions of strains in order to monitor whether genetic variability existed which might lock different isolates into micro-climatic niches irrespective of close spatial proximity. Survival rates (SR) were determined for short time shock (30 min) by H_2O_2 (final concentrations: 0.1, 0.4, and 0.8 *M*), or the ability to grow on high concentrations of NaCl (Fig. 1, Table 1). Figure 1 exemplifies results. All strains performed well under long-term osmotic stress conditions generated by high mannitol concentration in YPD but differences existed in growth rate (not shown). Responses to NaCl and H_2O_2 seemed to be independent from each other (Fig. 1). The most sensitive strains for H_2O_2 (*e.g.*, #20, #59, and #66) showed a different response towards NaCl stress. Table 1 also indicates that relatively peroxide-sensitive strains correlated with shaded regions of the NFS, and the only peroxide-sensitive strain from the SFS had been collected from a shaded area. The data were analyzed by ANOVA and correlation analysis. Linkage-based correlation tests

showed a significant correlation between SR for hydrogen peroxide and environment. The SR for H_2O_2 showed significant negative correlation with canyon slope (Pearson correlation −0.254; significance 0.001), slope niche (Pearson correlation −0.283; significance 0.000), and light exposure (Pearson correlation −0.17; significance 0.028), indicating that strains collected from the NFS were more sensitive to H_2O_2 and exhibiting an increasing trend towards sensitivity at higher location of the SFS. Also, strains collected from shaded locations showed throughout a stronger peroxide sensitivity than those collected from sun-exposed niches. The SR for NaCl and H_2O_2 exhibited no significant relationship by ANOVA (not shown), suggesting that responses to these stresses developed independently in the strains.

The results were further tested by measuring cell survival (strains #15, 20, 56, 59, 68, and S150-2B) after shock by 0.1 *M* H_2O_2 (Figs. 1 and 2). The most tolerant (#15) resumed cell division immediately after the shock treatment, while sensitive strains (#20, 68) showed long lag times (19–23 h) before growth resumed. Intermediate was strain #59, which required less than 5 h to resume cell division. Strain #56 seemed to initiate cell divisions immediately after H_2O_2 shock, similar to strain #15, but at a much slower rate. A similar time lag of 19 h was also observed in shock treatment at 0.4 *M* H_2O_2 for strain #15, and it was correlated with survival rate (Table 1) because strain #68 did not recover at the higher concentration of 0.4 *M* H_2O_2. Cell death and/or metabolic activity, identified by staining with phloxin-B (0.4 mg/cm^3 [28]) in intervals after shock treatments, indicated that cell death played no role in the plateau phase but that cells remained alive although metabolically inactive [29].

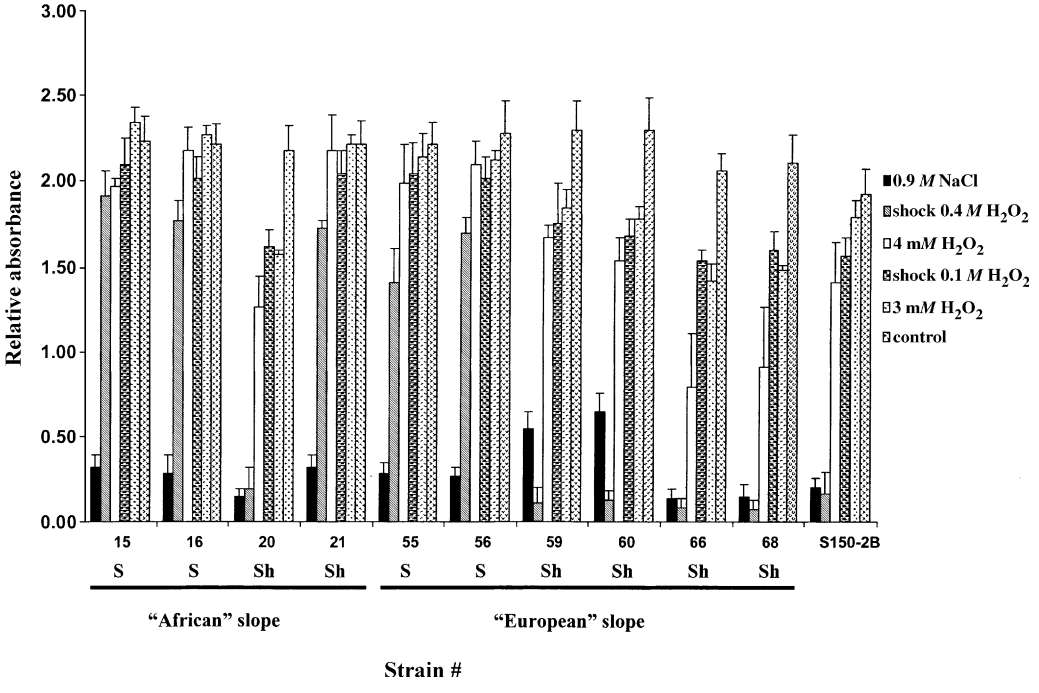

Fig. 1. Stress responses of different strains; OD600 was measured for cells (10^5 cells per sample) grown in YPD incubated at 28°C for 18 h; strains from the "African" and "European" slope, respectively, are identified by sun (S) or shade (Sh) location of collection

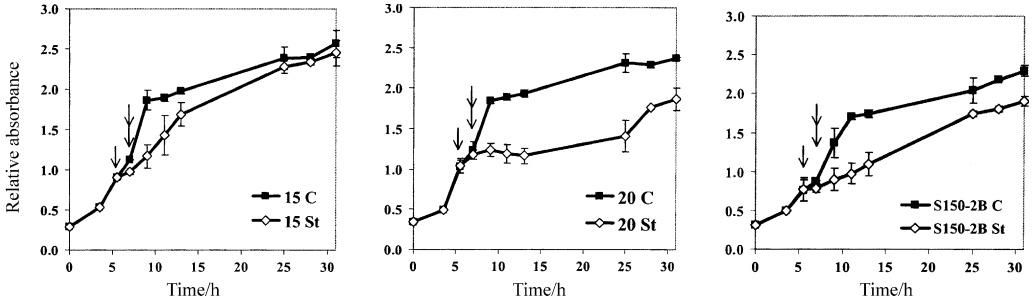

Fig. 2. Growth recovery after stress; cultures were divided equally and H_2O_2 adjusted to 0.1 *M* in one part (single arrow); after centrifugation and washing, medium without peroxide was added to the portion that had received hydrogen peroxide (double arrow); the second portion was treated by centrifugation and washing accordingly without hydrogen peroxide addition; shown are strain #15 (peroxide tolerant), #20 (sensitive), and the laboratory strain S150-2B (moderately tolerant); cell death was measured by Phloxin-B staining; in all strains, the lag phase after peroxide shock was shown to be due to metabolic arrest rather than cell death

Analysis of Expression Profiles

Microarray analyses were performed with poly(A)$^+$-*RNA* from several strains (#15, 20, 27, 50, 53, 59, 68, and S150-B) using 0.1 *M* H_2O_2 shock for 30 min. Results were normalized based on the total intensity normalization method [30–32], and log10 ratios were averaged. Data flagged due to low signal intensity (less than 100) were considered as unavailable for further analysis. In total, 3933 genes were found suitable in all hybridizations which were analyzed by hierarchical clustering with maximum linkage and maximum overlap between strains [33]. The strains with highest peroxide stress tolerance (#15, 27, 53), which had already surfaced in the growth analyses (Fig. 1), clustered most closely together, as did the previously identified sensitive strains.

Close inspection of the transcripts that are regulated and their functions indicated that the responses are fundamentally different depending on tolerance or sensitivity of the strains. The prominent characteristics that distinguish the oxidative stress response of the strains are compiled in Table 2. Focusing on coding regions that showed more than 3-fold up- or down-regulation in response to H_2O_2, three major subdivisions are recognizable. They separate the three response characters of the strains: oxidative stress tolerant, moderately tolerant, and sensitive. Highest tolerance was shown by strains #15, 27, and 53, moderately tolerant were the strains #50, 59, and the lab strain (S150-2B), and strains #20 and 68, respectively, were most sensitive.

One type of the oxidative stress response included the up-regulation in the tolerant strains of coding regions in four categories: cell wall biosynthesis, amino acid biosynthesis, ribosomal proteins and translation functions, and fatty acid/lipid biosynthesis. A number of functionally not characterized ORFs in this group include domains or similarities to genes that may also place the unknown ORFs in those four categories. To a lesser extent, moderately tolerant strains also showed up-regulation of at least some of these genes, and in no case was down-regulation observed. In the most sensitive strains, the ORFs for these genes were either not

Table 2. Analysis of regulated transcripts that distinguish yeast strains from ''Evolution Canyon''; transcripts with >3-fold changes are considered, and only those transcripts that change significantly in 3 or more strains; functional annotations are according to SGD (http://genome-www.stanford.edu/Saccharomyces/); listed are gene-IDs, functional categories, and strain numbers for up- and down-regulated transcripts

Gene	Gene ID	Category	Description
N/A	YEL071W	unknown	similar to D-lactate dehydrogenase Dld1p
GOG5	YGL225W	protein glycosylation	Golgi GDP-mannose transporter
N/A	YJL060W	unknown	unknown; similar to kynurenine aminotransferase
RPS0B	YLR048W	protein synthesis	ribosomal protein S0B
YHM2	YMR241W	unknown	unknown, suppresses HM mutant, tricarboxylate carrier activity
MET22	YOL064C	methionine biosynthesis	3′(2′)5′-bisphosphate nucleotidase
CYS3	YAL012W	methionine biosynthesis	cystathionine γ-lyase
RPS8A	YBL072C	protein synthesis	ribosomal protein S8
FEN1	YCR034W	cell wall biogenesis	β-1,3-glucan synthase subunit
HEM13	YDR044W	heme biosynthesis	coproporphyrinogen III oxidase
SNU13	YEL026W	mRNA splicing (putative)	U4/U6-U5 snRNP protein
LYS1	YIR034C	lysine biosynthesis	saccharopine dehydrogenase
ELO1	YJL196C	fatty acid metabolism	fatty acid elongation protein
ERG3	YLR056W	sterol metabolism	C-5 sterol desaturase
SUR4	YLR372W	fatty acid metabolism	conversion of 24-carbon to 26-carbon fatty acids
ASC1	YMR116C	protein synthesis (putative)	G-β like protein, WD repeat protein
ADE4	YMR300C	purine biosynthesis	amidophosphoribosyltransferase
RPS15	YOL040C	protein synthesis	ribosomal protein S15
N/A	YPL226W	unknown	unknown; similar to members of the ATP-binding cassette (AC)
FIG1	YBR040W	mating	extracellular integral membrane protein
RPS6B	YBR181C	protein synthesis	ribosomal protein S6B
RPL24B	YGR148C	protein synthesis	ribosomal protein L24B
RPS5	YJR123W	protein synthesis	ribosomal protein S5
YEF3	YLR249W	protein synthesis	translation elongation factor eEF3
RPS10B	YMR230W	protein synthesis	ribosomal protein S10B
FAA4	YMR246W	fatty acid metabolism	long-chain-fatty-acid–CoA ligase
RPL25	YOL127W	protein synthesis	ribosomal protein L25
N/A	YAL061W	unknown	unknown; similar to alcohol/sorbitol dehydrogenase
N/A	YBR147W	unknown	unknown
MAL31	YBR298C	transport	maltose permease
HXT7	YDR342C	transport	hexose permease
PDR15	YDR406W	drug resistance	putative transporter
GLC3	YEL011W	cell wall biogenesis	glycogen branching enzyme
N/A	YER067W	unknown	unknown
SPI1	YER150W	unknown	unknown; similar to Sed1p; induced in stationary phase
GSY1	YFR015C	glycogen metabolism	glycogen synthase
HXK1	YFR053C	glycolysis	hexokinase I
N/A	YGR243W	unknown	unknown
MAL12	YGR292W	maltose utilization	α-glucosidase
N/A	YHL021C	unknown	unknown
N/A	YHR087W	unknown	unknown

(*continued*)

Table 2 (*continued*)

Gene	Gene ID	Category	Description
GAL2	YLR081W	transport	glucose and galactose permease
GSY2	YLR258W	glycogen metabolism	glycogen synthase
N/A	YLR350W	unknown	unknown
HXT2	YMR011W	transport	hexose permease
PGM2	YMR105C	glycolysis	phosphoglucomutase
N/A	YNL194C	unknown	unknown; similar to Sur7p (integral to membrane)
FUN34	YNR002C	unknown	unknown
N/A	YOL157C	unknown	putative α-glucosidase
ALD4	YOR374W	ethanol utilization	mitochondrial aldehyde dehydrogenase
CSR2	YPR030W	unknown	unknown; multicopy suppressor of chs5 spa2 double mutant
NCE102	YPR149W	secretion, non-classical	unknown
N/A	YPR184W	unknown	unknown; similar to glycogen debranching enzyme
N/A	YDL204W	unknown	unknown
TRS120	YDR407C	secretion (putative)	transport protein particle (TRAPP) subunit, 120 kD
N/A	YDR516C	unknown	unknown; similar to Glk1p (glucokinase)
N/A	YER066C-A	unknown	unknown
N/A	YFL061W	unknown	unknown; similar to *E. coli* cyanamide hydratase (urea hydro-lyase)
HXT4	YHR092C	transport	hexose permease
HXT5	YHR096C	transport	hexose permease
N/A	YIL112W	unknown	unknown; similar to ankyrin and coiled-coil proteins
HXT8	YJL214W	transport	hexose permease
N/A	YLL020C	unknown	unknown
ISF1	YMR081C	RNA splicing, mitochondrial	unknown, aerobic respiration, interacts with Nam7p
N/A	YOL084W	unknown	major facilitator superfamily
N/A	YOR161C	unknown	unknown
GAC1	YOR178C	glycogen metabolism	Glc7p regulatory subunit, protein phosphatase type-1 activity
N/A	YOR228C	unknown	unknown
GPH1	YPR160W	glycogen metabolism	glycogen phosphorylase
SSA1	YAL005C	ER and mitochondrial translocation	cytosolic HSP70
HSP26	YBR072W	diauxic shift	stress-induced protein
GPD1	YDL022W	glycerol metabolism	glycerol-3-phosphate dehydrogenase
N/A	YDL222C	unknown	unknown; similar to Sur7p (integral to membrane)
N/A	YER066W	unknown	unknown; similar to Cdc4p, has one WD (WD-40) domain
CTT1	YGR088W	oxidative stress response	catalase T
BTN2	YGR142W	unknown	unknown, intracellular protein transport
SOL4	YGR248W	unknown	unknown; similar to Sol3p (pentose-phosphate shunt, oxidative branch)
HXT1	YHR094C	transport	hexose permease
SUC2	YIL162W	sucrose utilization	invertase
MHP1	YJL042W	cytoskeleton	microtubule-associated protein, cell wall organization
UGP1	YKL035W	pyrimidine metabolism	UGP1, UDP-glucose pyrophosphorylase
YJU2	YKL095W	unknown	unknown

(*continued*)

Table 2 (*continued*)

Gene	Gene ID	Category	Description
JEN1	YKL217W	transport	lactate transporter
SSA2	YLL024C	ER and mitochondrial translocation	cytosolic HSP70
N/A	YML128C	unknown	unknown, meiotic recombination
N/A	YMR250W	unknown	unknown; similar to glutamate decarboxylase
TPS3	YMR261C	trehalose utilization	α–α-trehalose-phosphate synthase
N/A	YOR225W	unknown	unknown
N/A	YAL060W	unknown	unknown; similar to alcohol/sorbitol dehydrogenase
GLK1	YCL040W	glycolysis	glucokinase
QCR10	YHR001W-A	oxidative phosphorylation	ŭbiquinol-cytochrome c oxidoreductase complex subunit
N/A	YMR145C	unknown	unknown; similar to rotenone-insensitive NADH-ubiguinone
GRE2	YOL151W	unknown	unknown; induced by osmotic stress
CYT1	YOR065W	oxidative phosphorylation	cytochrome c1
N/A	YPL004C	unknown	unknown; similar to tropomyosin

Gene	Strain number							
	#15	#27	#53	#50	#59	lab strain	#20	#68
N/A	Up	Up	Up					
GOG5	Up	Up	Up	Up				
N/A	Up	Up	Up					
RPS0B	Up	Up	Up			Up		
YHM2	Up	Up	Up		Up	Up		
MET22	Up	Up	Up		Up			
CYS3	Up	Up		Up				
RPS8A	Up		Up		Up	Up		
FEN1	Up	Up				Up		
HEM13		Up	Up	Up			Down	Down
SNU13	Up		Up	Up		Up		
LYS1		Up	Up			Up		
ELO1	Up	Up		Up			Down	
ERG3	Up	Up				Up		
SUR4	Up	Up		Up		Up		
ASC1	Up	Up				Up		
ADE4		Up	Up		Up	Up		
RPS15	Up		Up			Up		
N/A		Up	Up		Up			
FIG1			Up	Up	Up			
RPS6B			Up			Up		Down
RPL24B			Up		Up	Up		
RPS5	Up				Up	Up		
YEF3		Up				Up	Down	
RPS10B			Up		Up	Up		
FAA4		Up					Down	Down
RPL25			Up		Up	Up		
N/A	Down	Down	Down		Down	Down		

(*continued*)

Table 2 (*continued*)

Gene	Strain number							
	#15	#27	#53	#50	#59	lab strain	#20	#68
N/A	Down	Down	Down					
MAL31	Down	Down	Down	Down	Down	Down		
HXT7	Down	Down	Down	Down	Down	Down		
PDR15	Down	Down	Down	Down	Down	Down		
GLC3	Down	Down	Down	Down	Down	Down		
N/A	Down	Down	Down	Down	Down	Down		
SPI1	Down	Down	Down	Down	Down			
GSY1	Down	Down	Down	Down	Down	Down		
HXK1	Down	Down	Down	Down	Down	Down		
N/A	Down	Down	Down			Down		
MAL12	Down	Down	Down	Down				
N/A	Down	Down	Down	Down	Down	Down		
N/A	Down	Down	Down		Down	Down	Up	Up
GAL2	Down	Down	Down	Down	Down	Down	Up	
GSY2	Down	Down	Down	Down	Down	Down		
N/A	Down	Down	Down	Down	Down			
HXT2	Down	Down	Down	Down	Down	Down		
PGM2	Down	Down	Down	Down	Down	Down		
N/A	Down	Down	Down	Down	Down	Down		
FUN34	Down	Down	Down	Down	Down			
N/A	Down	Down	Down	Down				
ALD4	Down	Down	Down	Down	Down	Down		
CSR2	Down	Down	Down					
NCE102	Down	Down	Down	Down	Down			
N/A	Down	Down	Down	Down	Down	Down		
N/A	Down		Down		Down	Down		Up
TRS120	Down		Down	Down	Down	Down		
N/A	Down		Down	Down	Down	Down		
N/A	Down		Down			Down		
N/A	Down		Down	Down		Down		
HXT4		Down	Down	Down	Down	Down	Up	
HXT5		Down	Down	Down	Down	Down		
N/A	Down	Down			Down			
HXT8	Down		Down	Down	Down	Down		
N/A		Down	Down	Down	Down			
ISF1	Down		Down			Down		
N/A	Down		Down	Down	Down		Down	
N/A	Down		Down	Down	Down			
GAC1	Down		Down	Down		Down		
N/A	Down		Down	Down	Down			
GPH1	Down		Down			Down		
SSA1			Down	Down	Down			Up
HSP26	Down					Down	Up	Up
GPD1	Down			Down	Down	Down	Down	

(*continued*)

Table 2 (*continued*)

Gene	Strain number							
	#15	#27	#53	#50	#59	lab strain	#20	#68
N/A	Down				Down	Down		
N/A	Down			Down	Down			
CTT1	Down			Down	Down			
BTN2			Down	Down	Down	Up		
SOL4	Down				Down	Down		
HXT1		Down			Down		Up	
SUC2	Down			Down	Down	Down		
MHP1	Down			Down		Down		
UGP1	Down			Down	Down	Down		
YJU2	Down			Down	Down			
JEN1	Down				Down	Down		
SSA2			Down	Down	Down			
N/A	Down				Down	Down		
N/A	Down			Down	Down	Down		
TPS3	Down			Down	Down			
N/A	Down			Down	Down			
N/A					Down	Down	Up	Up
GLK1				Down	Down	Down		
QCR10						Down	Up	Up
N/A				Down	Down		Up	
GRE2						Up	Up	Up
CYT1					Down		Up	Up
N/A				Down	Down	Down		

regulated, or they were down-regulated (Table 2). A second group of regulated transcripts includes ORFs that are down-regulated in the most tolerant strains, and many of these ORFs show the same regulation in moderately tolerant strains (Table 2). If these are regulated at all in the sensitive strains, their amount is typically increased. The categories of regulated transcripts in this group show an interesting pattern. Strongly reduced are transcripts for many carbon transporters and several other transport functions, for glycogen biosynthesis, for the biosynthesis of glycerol, and, finally a few metabolite modifying enzymes that conceivably might have signaling functions. Also down-regulated are a few transcripts that are typically associated with the stress response, such as ORFs for catalase T (CTT1), for a stress-regulated (diauxic shift) protein (HSP26), two cytosolic HSP70 isoforms (SSA1, SSA2), and the regulatory GAC1 (protein phosphatase type 1 activity) or YER066w, which shows similarity to Cdc4p. A third group includes a few transcripts that are down-regulated in moderately tolerant strains, but up-regulated in sensitive strains (Table 2). Most of the ORFs in this group seem to be associated with redox and oxidative stress functions. K-means clustering [34] was used in addition to sort genes that are regulated in any of the strains for both stress treatments. In total, 1025 genes emerged from the analysis. These were clustered into

10 categories by their expression patterns of the log10 ratio between control and stress. This independent analysis (not shown) confirmed the results presented in Table 2. A further independent confirmation for transcript regulation was obtained by northern blotting and hybridizations [29]; all tested transcripts showed the same behavior in microarrays and *RNA* blots, either up- or down-regulated. The results showed very similar up- or down-regulation to those determined by microarray analysis and suggested reproducibility or at least similarity between the two techniques.

The yeast strains chosen represented a cross-section of the slopes in EC, conceivably ideal material for investigating the degree, if any, of climatic niche microadaptation. If the strains were locked into separate niches, characterized for example by a combination of light exposure, temperature, moisture, or substrate, adaptive changes in the physiological stress response and in transcription might be revealed when the strains are exposed to a defined stress. ANOVA statistical analysis showed that survival rate for H_2O_2 was indeed significantly correlated with niche, slope, and light exposure. Our analysis of responses to oxidative stress in a collection of yeast strains used shock experiments to measure survival rate (SR) generated statistically significant correlations between the tolerance character of different strains and their location in micro-niches on the two climatically divergent slopes of ''Evolution Canyon'' (Table 1). This result confirms and further supports the hypothesis of adaptive evolution and fixation of genes in niche populations that has been found in several other studies conducted in the natural laboratory that this canyon represents [6, 10, 35–37]. The additional use of transcript profiling places a number of genes and their divergent responses, depending on tolerance or susceptibility of the strain under study, in the context of oxidative stress biology. It seems that strains that show high tolerance or resistance to oxidative stress express only a subset of the common stress response that has been observed in laboratory strains [17, 38, 39]. Continued protein synthesis, and the resumption of growth (Figs. 1 and 2) that characterizes these strains can be observed not only from the nature of the up-regulated genes but also from the maintenance of transcript abundance for genes that are down-regulated in sensitive strains. Also, Table 2 lists only the most highly induced or repressed ORFs (>3-fold), while information can also be obtained from less highly regulated genes. The present results, to be elaborated elsewhere [29], display multigene genomic adaptation to stress. It will be intriguing to explore the level of parallelism or convergence of other model organisms across life in ''Evolution Canyon'' to similar and other stresses related to the natural stresses they face in the canyon of solar radiation, temperature, and drought.

Experimental

Strain Collection

Strains of *Saccharomyces cerevisiae* were identified and collected at ''Evolution Canyon'' (Lower Nahal Oren, Mount Carmel, Israel; 32°43′N; 34°58′E; [5–7]) from locations characterized by shade/sun and xeric/mesic conditions in a cross section of the canyon. Isolates were established as strains from single colonies under laboratory conditions in rich medium and numbered (Table 1). Established

strains were kept as glycerol stocks at −80°C and plated fresh for each experiment [40]. Single colonies for each strain were grown at 30°C in 2 cm^3 of liquid YPD (2% glucose, polypeptone, yeast extract, $pH = 5.5$), and one mass culture (20 cm^3) per experiment. S150-2B (*MAT-α, ura3-52, his3Δ, leu2-3; 112, trip1-289*) was used as a laboratory strain for control [41].

Stress and Culture Conditions

For hydrogen peroxide shock, cells were incubated in 0.1 *M*, 0.4 *M*, or 0.8 *M* H_2O_2 for 30 min and then plated to test for survival on plates. For transcript analysis, 0.1 *M* H_2O_2 (30 min) was used, a concentration all strains tolerated. For NaCl stress, 0.4, 0.9, or 1.4 *M* NaCl was present in the YPD plates [29]. For microarray analyses, 0.4 *M* NaCl was present for 2 h before harvesting of the cells. For liquid culture analysis, 10^5 cells per strain pre-cultured overnight were added to fresh media (YPD, YPD with 0.9 *M* NaCl, and YPD with 3 m*M* (or 4 m*M* in some experiments) H_2O_2) for continuous exposure to peroxide. Cells shocked with 0.1 *M* or 0.4 *M* H_2O_2 for 30 min were added to fresh YPD media after washing. After 18 h optical densities were measured.

Stress Response Assays and Statistical Analysis

Survival rates (SR) of strains for H_2O_2 were tested on YPD plates, and survival rates were calculated. For H_2O_2 shock, 10^6 cells were incubated for 30 min with 0, 0.1, 0.4, and 0.8 *M* H_2O_2, and 10^4 cells were plated on YPD plates. Plates were incubated at 30°C for 2 d, photographed, and analyzed by NIH Image (http://rsb.info.nih.gov/nih-image/) (not shown; [29]).

Replicates from both 0.4 *M* H_2O_2 and 0.8 *M* H_2O_2 from 42 strains (168 data points total) were used for H_2O_2 for the analysis. Factors for statistical analyses by ANOVA and linear correlation analysis included the distinction of the slopes and bottom of the canyon, SFS (1–3), NFS (5–7), VB (4), respectively, distinction of niches at different distances along the slopes, termed niches 1 to 7, and a distinction of the collection place as sunny or shady (Table 1). In addition, parameters from drop tests, growth in liquid culture, and the distinction of growth in H_2O_2 were quantified. Data from density measurements on plates were expressed by the multiplier of the means of density and drop size in relation to the multiplier of these parameters in control cells. In this way, SR was determined twice, and averaged values (%) are listed (Table 1). Strain survival was scored in five categories (1–5), with the score 1–1.9 as sensitive (less than 40% survival), and 5 as tolerant (more than 70% survival) in H_2O_2. Categories 2 (40–50%), 3 (50–60%), and 4 (60–70%) are listed as moderately sensitive or moderately tolerant. Average of four numbers from each replicates of 0.4 and 0.8 *M* H_2O_2 are listed as phenotype (Table 1). A phenotypic factor was calculated: tolerant (score >4; T), moderately tolerant (3–4; MT), moderately sensitive (2–3; MS), and sensitive (<2; S). SR statistical analysis was by SPSS (http://www.spssscience.com/spss11/index.cfm).

Phloxine-B Staining and Cell Counting

Cells were stained with Phloxin-B (0.4 mg/cm^3; [28]) for microscopic observations to monitor survival rates and cell number (n=3).

Microarray Analysis

Corning CMT™ Yeast-S228c Gene Arrays (Corning, NY), and microarray slides (Yeast Y6.4k1_v2; Microarray Centre, Ontario Cancer Institute, University Health Network (http://www.microarrays.ca/) were used. On these slides 6034 coding regions were present. For labeling, cells (OD600 = 1.0) were treated (0.1 *M* H_2O_2 for 30 min), collected, and frozen. Total *RNA* was isolated followed by poly(A)$^+$*RNA* isolation (polyATract m*RNA* isolation kit; Promega, WI), and labeling

by incorporation of cy3/cy5 dyes into 2 μg *RNA* each from control and treated cells. Microarray hybridizations were done as described [30]. Hybridization was performed in moist chambers (42°C; 20 h). The slides were washed successively in 2×SSC, 0.1% SDS, then 0.1×SSC, 0.1% SDS, 0.1×SSC for 5 min each step at 42°C. Slides were scanned and spot intensities were extracted (Genepix 4000 scanner; GenePix Pro 3.0; Axon Instruments, CA). Data from each strains #15 ($n = 5$ repeats/3 biological repeats), #20 (n=3/2), #27 (n=3/2), #50 (n=3/1), #53 (n=3/2), #59 (n=3/2), #68 (n=4/2), S150-2B (n=3/2) were averaged and analyzed using Spotfire software (http://www.spotfire.com/). Averaged data points with ratio $> \pm 3$ fold ($>$0.5 log10) were considered as up- or down-regulated, respectively.

In addition, *RNA*-blot analysis has been carried out for selected coding regions that differed in their expression according to microarray experiments (not shown; [29]).

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