CHAPTER 6

Stress Response of *Rhodotorula mucilaginosa*

Introduction

Rhodotorula mucilaginosa is a highly pigmented red yeast that has the ability to grow across a wide range of conditions. Found in water, soil, food products as well as mammals, this yeast inhabits many niches and tolerates a wide range of temperatures. *R. mucilaginosa* has a long history, the type strain, CBS 316, was originally deposited in the National Collection of Type Cultures in London in 1912. It has a long list of synonyms, well over fifty in fact, with *R. rubra* and *Torulopsis mucilaginosa* the more common (Fell and Statzell-Tallman, 1998b). This yeast has been isolated from a wide range of substrates including beer, capsicums, marine and fresh water habitats as well as humans. Previously isolated from Antarctic soil samples around Lake Vanda by Goto *et al.* (1969) it was reported as *R. rubra*. More recently, this yeast has been isolated from Shirmacher Oasis, Antarctica in 1989 (Ray *et al.*, 1989). In the present study, *R. mucilaginosa* was isolated from soil samples collected in the summer of 1994/1995, from the Vestfold Hills region of Antarctica.

Extreme conditions in Antarctica such as low temperatures, strong winds, soil salinity, oxidative stress and prolonged exposure to UV-irradiation impose severe limitations on all life forms. For most species of yeasts, environmental or external temperature ranges for viability are relatively narrow 4 - 40°C, indeed for many Antarctic yeasts the maximum temperature for growth is between 20 and 25°C. The ability of microorganisms to circumvent such extreme conditions indicate that these species have developed mechanisms to deal with environmental stresses that their mesophilic counterparts do not encounter.

Life under stress

Heat shock proteins & thermotolerance

The heat shock proteins (hsps) are a large family of proteins found in both prokaryotic and eukaryotic cells and are identified by their molecular weights as determined by 1D-SDS-PAGE. They play a critical functional role maintaining cellular homeostasis and therefore have been highly conserved throughout evolution. Many of the hsps are constitutively expressed, performing essential housekeeping functions in the cell, including preventing proteins from unfolding, refolding partially denatured proteins and eliminating proteins that are irreversibly damaged (Morimoto *et al.*, 1990). These properties have led to the description of some hsps as molecular chaperones. Increased expression of hsps has been shown to protect cells against subsequent lethal stress induced by heat, oxygen, UV and osmotic stressors, exposure to heavy metals and cytotoxic drugs, hyperthermia as well as shifts in intracellular redox balance (Macario and Conway de Macario, 2000; Calabrese *et al.*, 2003). Figure 6.1 summarizes the physiological signals that activate hsp expression in cells.

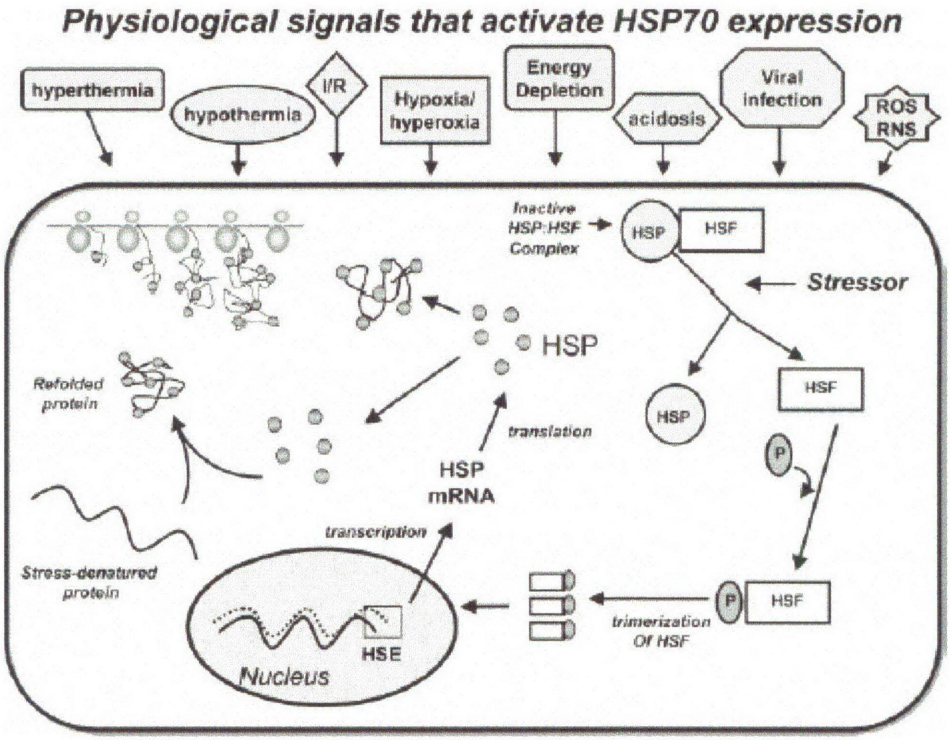

Figure 6.1. A schematic view of physiological factors that activate hsp70 within a cell. A variety of stressors can activate heat shock factors (HSFs) resulting in a cascade of events beginning with phosphorylation of the HSFs and ending with newly synthesized HSPs (Kregel, 2002).

Table 6.1: Location and function of reprentative yeast heat shock proteins

HSP Family	Cellular Location	Function
Hsp26	**Cytoplasm**	**Microfilament stabilization, antiapoptotic**
Hsp40 family		
Ydj1	**Cytosol**	**Facilitates protein translocation across membranes, cochaperone**
Sis1	**Nucleus**	**Initiates translocation, cochaperone**
Hsp60	**Mitochondria**	**Refolds proteins and prevents aggregation of denatured proteins**
Hsp70 family		
Ssb1	**Cytosol, nucleus**	**Protein folding, cytoprotection**
Ssb2	**Cytosol, nucleus**	**Molecular chaperones**
Ssc1	**Mitochondria**	**Molecular chaperones**
Bip/KAR2	**ER**	**Cytoprotection, molecular chaperones**
Hsp90	**Cytosol, ER, nucleus**	**Regulation of steroid hormone receptors, protein translocation**
Hsp104	**Cytosol**	**Protein folding**

(adapted from (Gething, 1997) Hsp – heat shock protein; ER – endoplasmic reticulum

Acquired thermotolerance is a characteristic feature of the heat shock response whereby cells exposed to a prior mild non-lethal heat shock acquire resistance to a normally lethal heat stress. This phenomenon, known as the heat shock response, and which results in a transient resistance to a subsequent heat stress has been reported in almost all organisms examined to date and include mammalian cells (Kampinga, 1993), *Drosophila* (Ritossa, 1962), plants (Baniwal *et al.*, 2004), yeasts (Watson, 1990), parasites (Martinez *et al.*, 2002) and bacteria (Stewart and Young, 2004). However, the precise mechanism by which acquired thermotolerance occurs is yet to be elucidated particularly in mammalian cells where increased expression of hsp70 may be part of a complex series of reactions to stress (Kregel, 2002).

Lahav *et al.* (2003) recently reported increased expression of hsp70 following osmotic stress in a strain of *R. mucilaginosa* isolated from chemical waste. Initial protein synthesis was reduced in cells when shifted to a high salt medium followed by a transient adaptation period. Detection of two members of the hsp70 family was carried out using SDS-PAGE and western immunoblot analysis indicating down regulation of Ssb1/2p, while expression of Kar2p was

significantly increased. This response was not seen in *S. cerevisiae* suggesting that this response may be related to the ability of *R. mucilaginosa* to withstand hypersaline environments in contrast to *S. cerevisiae* which is salt sensitive (Lahav *et al.*, 2003).

Deegenaars and Watson (Deegenaars and Watson, 1998b) produced protein profiles following heat shock at various temperatures, for the psychrophilic Antarctic yeasts *Mrakia frigida, M. gelida, M. stokesii* and *Leucosporidium antarcticum* and two psychrotolerant Antarctic yeasts, *L. fellii* and *L. scottii*. They were able to characterize the heat shock response in these organisms and show that similarities exist among psychrophilic yeasts from Antarctica.

In the present study, an Antarctic strain of *R. mucilaginosa* was initially observed to be highly UV resistant. This observation prompted further investigation to examine whether hsps synthesis might be related to the ability of this yeast to survive high UV levels associated with the Antarctic environment.

The high concentration of carotenoids in *Rhodotorula* yeasts has been postulated to be protective against oxidative stress (Moore *et al.*, 1989). *Rhodotorula* yeasts characteristically form pink to bright red colonies due to the production of a variety of carotenoids, including the industrially important astaxanthin (Barnett *et al.*, 1990). The so-called industrial red yeasts (*R. glutinis*, *Sporidiobolus salmonicolor* and *Phaffia rhodozyma)* are rich sources of astaxanthins. Carotenoid production is increased in these strains under various exogenous stress conditions, such as osmotic stress, oxidative stress, heavy metals, UV-irradiation and ethanol stress, either alone or in a variety of combinations (Drabkova *et al.*, 2003; Koci *et al.*, 2003a; Koci *et al.*, 2003b; Pokorna *et al.*, 2003). Given the protective properties that carotenoids exhibit, it not unreasonable to hypothesis that they may serve as a mechanism of survival for yeasts found in extreme habitats such as Antarctica. Carotenoid synthesis is known to be induced by exposure to UVB radiation (Cockell and Knowland, 1999) and carotenoid synthesis in an Antarctic moss has recently been shown to be induced by UVB (Newsham, 2003). The paper by Newsham (2003) presents a summary of recent evidence of the influence of UVB radiation on pigment formation in a number of diverse species, including plants.

Prolonged exposure to UV-irradiation, particularly UVA, induces oxidative stress, leading to the generation of reactive oxygen species and free radicals (Parsons, 1997). Cellular

damage caused by reactive oxygen species (ROS), including hydrogen peroxide and reactive free radicals, occurs due to normal cellular processes. Autoxidation of the electron transport carriers in mitochondria are a known source of superoxide anion generation and as such elicit damage to proteins, lipids and DNA (Davidson and Schiestl, 2001). In preliminary studies from this laboratory, it was shown that a number of Antarctic yeasts were resistant to UVA and UVB-radiation (Tsimako *et al.*, 2002) indicating a survival response not observed in other yeast species. A number of studies on animal model systems, and a limited number of human studies, have indicated that antioxidants may help protect cells from free radical-related damage (Jones *et al.*, 1999). Interestingly, UVA but not UVB stress has also been shown to up-regulate hsp70 synthesis in a human fibrosarcoma cell line with maximal expression occurring at 8 – 12 hours after exposure (Trautinger *et al.*, 1999).

Ubiquinone

Ubiquinone (coenzyme Q, CoQ) is an essential lipid-soluble redox component of the respiratory chain located in the inner mitochondrial membrane of eukaryotes. As well as its role in electron transport, ubiquinone in the reduced form (ubiquinol, $CoQH_2$) [Fig 6.2] functions as an antioxidant preventing lipid peroxidation and is found in such structures as the Golgi apparatus, lysosomes, endoplasmic reticulum, peroxisomes and the plasma membrane. Importantly, the ratio of ubiquinol to ubiquinone ($CoQH_2/CoQH_2 + CoQ$), has been used as a marker of oxidative stress in bacteria (Søballe and Poole, 1999; 2000; Dunlap *et al.*, 2002) and in human plasma (Beyer *et al.*, 1996; Yamamoto and Yamashita, 1997). Moreover, reduced levels of ubiquinol have been linked to various human disorders such as Parkinson's disease, hepatitis, liver cirrhosis, hyperlipidemia and cardiovascular disease (Kontush *et al.*, 1997; Soja and Mortensen, 1997; Tomasetti *et al.*, 1999; Yamamoto and Yamashita, 1999; Gotz *et al.*, 2000).

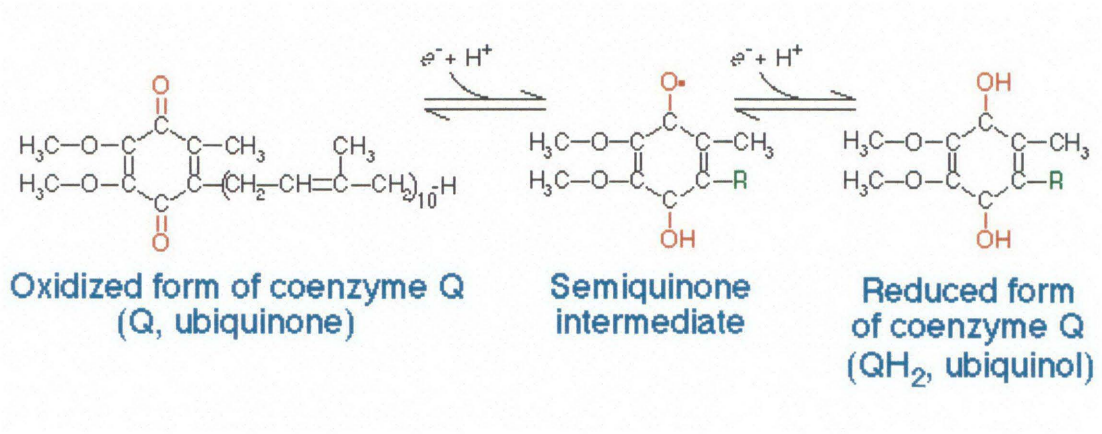

Figure 6.2. Chemical reaction resulting in the reduced form of coenzyme Q

Ubiquinone has been shown to have antioxidant properties by increasing resistance to oxidation through *in vitro* as well as *in vivo* studies in various systems such as lymphocytes, plasma membranes and lipoproteins (Kontush *et al.*, 1997; Tomasetti *et al.*, 1999; Kaikkonen *et al.*, 2002) by inhibiting lipid peroxidation and scavenging free radicals. In the present studies, identification of the coenzyme Q system was used as a taxonomic tool at the generic level as the length of the isoprene chain is usually consistent within a monophyletic group (Kuraishi *et al.*, 2000). The identification of the principal coenzyme Q homolog in the Antarctic yeasts correlated in some but not in all cases with results provided by molecular techniques. Although conclusions based on the nature of the ubiquinone system alone are contentious, closely related species may be separated by this identification (Yamada *et al.*, 1989).

Fatty acid composition

It has long been established that the fatty acid composition of yeast membranes are important for the ability of cells to function at low temperatures (Watson, 1987). In particular the unsaturated fatty acid index (defined as 1(% monoenes) + 2(% dienes) + 3(% trienes)/100) was an important parameter of membrane fluidity (Watson, 1980). Early studies (Watson and Arthur, 1976) investigated the fatty acid composition of three psychrophilic Antarctic yeasts (*Leucosporidium frigidum, L. gelium, L. nivalis*) and found a predominance for C_{18} unsaturated fatty acids, particularly the polyunsaturated linoleic ($C_{18:2}$) and linolenic ($C_{18:3}$) fatty acids. Although cellular fatty acid composition has been used extensively in bacterial taxonomy, it has only received recognition in fungal taxonomy quite recently (Veys *et al.*, 1989; Augustyn

et al., 1990; Brondz and Olsen, 1990; Amano *et al.*, 1992). In the present study, fatty acid composition was established for *R. mucilaginosa* as a marker to establish the psychrophilic nature of the Antarctic yeasts.

Preliminary data for *R. mucilaginosa* (Tsimako *et al.*, 2002) prompted a further study in responses to various stress conditions such as UVA, UVB and heat. Responses examined included hsp expression following heat shock and UVA radiation, acquired thermotolerance and coenzyme Q redox balance in response to UVA.

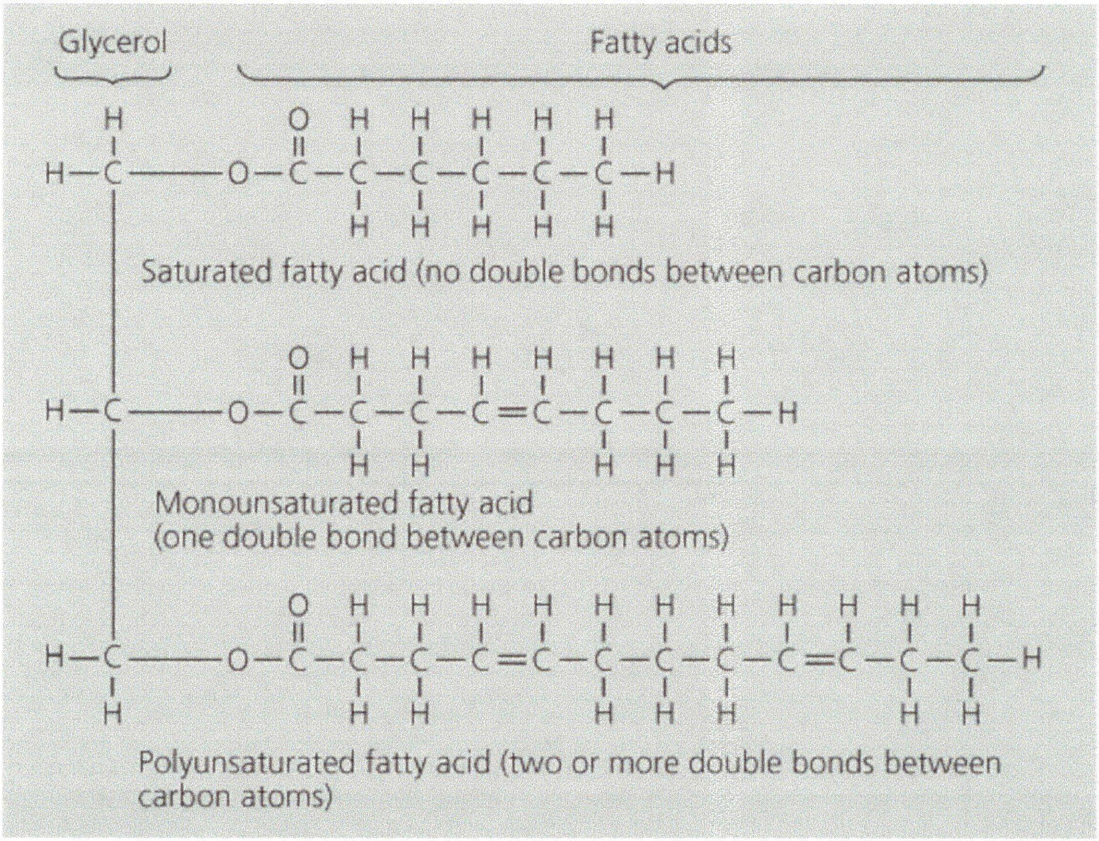

Figure 6.3. Structure of saturated, monounsaturated and polyunsaturated fatty acids

Results

Molecular & physiological analyses

Sequence analyses of the D1/D2 region of the 26S ribosomal DNA and the ITS regions identified isolate UNE1130a (in-house reference) as *Rhodotorula mucilaginosa* and molecular analyses placed it into the phylogenetic tree of related Basidiomycetous yeasts [Fig 6.4]. Comprising of four major clades, species of *Rhodotorula* are represented in the *Microbotryum, Sporidiobolus* and *Erythrobasidium* clades, but are not found in the *Agaricostilbum* clade. Physiological analyses of *R. mucilaginosa* confirmed the phylogenetic results and included the inability to assimilate inositol, positive reactions to urease and Diazonium Blue B. Starch formation was negative. No sexual state was observed from cultures plated for 3-6 months at 6°C or 15°C on YEP agar, cornmeal agar, malt agar or nitrogen base agar. In *R. mucilaginosa* a high percentage of total fatty acid was unsaturated, particularly the monounsaturated oleic acid ($C_{18:1;}$ 42%) with smaller but significant amounts of the polyunsaturated linoleic acid ($C_{18:2}$; 9%) and much smaller amounts of linolenic acid ($C_{18:3}$, 1%) [Fig 6.5]. Optimal growth temperature studies indicated growth across a wide temperature spectrum, with maximum growth at between 15 and 20°C, but good growth also up to 37°C and as low as 5°C.

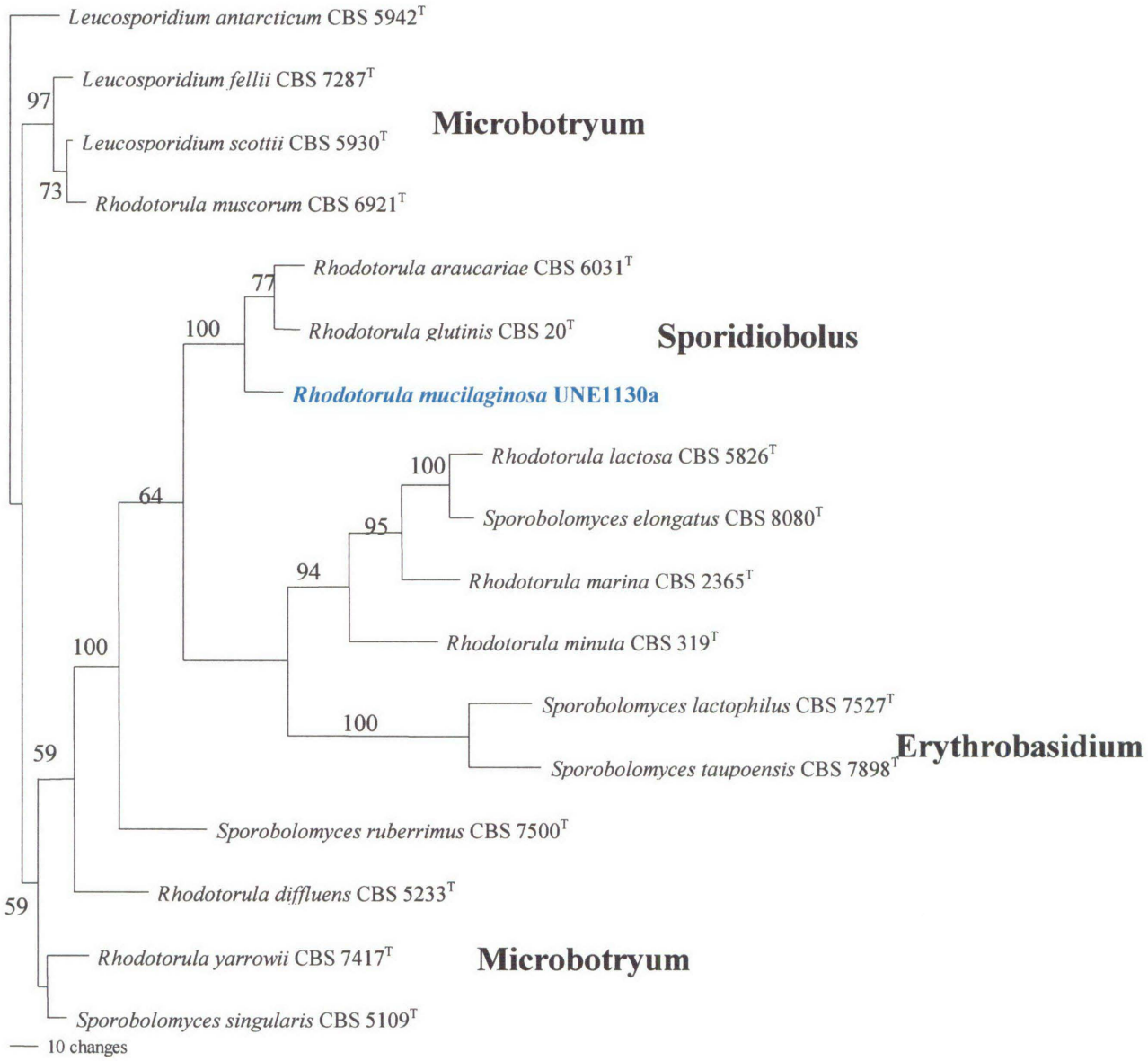

Figure 6.4. Phylogenetic tree showing placement of *Rhodotorula mucilaginosa* (UNE1130a) among related basidiomycetous yeasts derived from maximum parsimony analysis of the 26S rDNA domain D1/D2. Bootstrap replications from 100 full heuristic replications using the neighbour joining method are displayed. Labelled clades have representative species only.

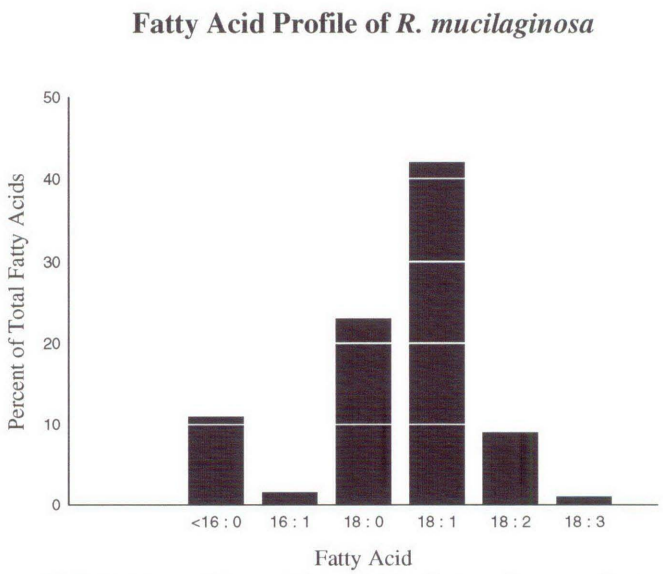

Figure 6.5. Fatty acid profile of *Rhodotorula mucilaginosa (UNE1130a)*

Stress tolerance

Cells grown at 25°C and subjected to a mild heat shock (37°C/1 hr) exhibited heat shock-induced thermotolerance to a normally lethal heat stress (52°C/2-4 hr) as illustrated in Figure 6.6. Viability decreased dramatically to < 15% in cells not previously heat shocked and exposed to a 52°C heat stress for 90 min. By contrast, cells previously heat shocked at 37°C retained > 60% viability at the same time point (90 min at 52°C). In these cells, viability (> 60%) was maintained up to at least 150 min at 52°C after which there was a marked decrease in viability.

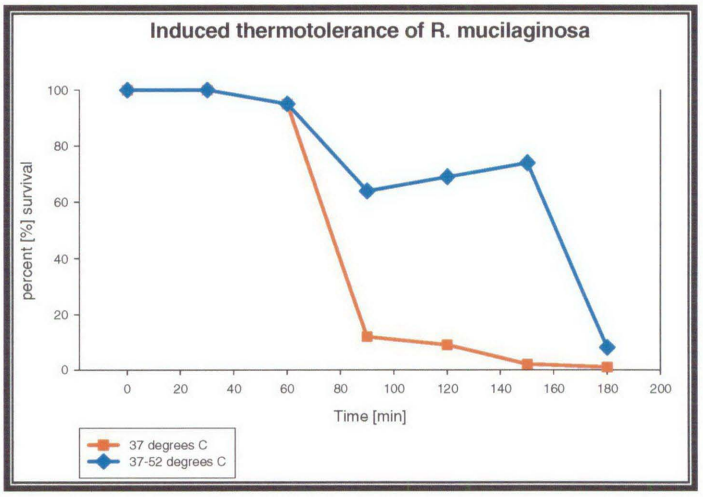

Figure 6.6. Induced thermotolerance of *Rhodotorula mucilaginosa (UNE1130a)* grown at 25°C

Heat shock induced proteins were identified by ^{35}S-methionine labeling on SDS-PAGE. Labeling was performed either by addition of ^{35}S-methionine just prior to the heat shock at 37°C or added immediately after the heat shock. Autoradiographs of ^{35}S-methionine labeled proteins in control and heat shocked cells are shown in Figure 6.12. Cells of *S. cerevisiae* K7 were used as a control as this strain has been used as a model system for the heat shock response in yeast over many years in this laboratory (Gross and Watson, 1998). In the case of K7, heat shock inducible proteins hsp70 and hsp90 were observed following a mild heat shock at 37°C [Fig 6.12]. By contrast, in *R. mucilaginosa* the only obvious heat shock inducible protein was a band at around 30 kDa. This band was more prominent in the sample in which ^{35}S-methionine was added post heat shock [Fig 6.12].

Response to UVA radiation was measured over a time course (2, 3 and 4 hr) and cell viability estimated by serial dilution plate count. Essentially 100% cell survival up to 4 hr [Fig 6.8a] was observed in the case of the Antarctic yeast *R. mucilaginosa*, as compared with very few survivors at 4 hr for the mesophilic yeast *S. cerevisiae* K7 [Fig 6.8b]. The latter produced small colonies following exposure to UVA (4 hr) [Fig 6.8b], UVB (85-380 sec) or UVC (30-60 sec) radiation. The small colonies of *S. cerevisiae* K7 were found by cytochrome difference spectra to be deficient in cytochrome a + a_3 and cytochrome b but with relatively high levels of cytochrome c [Fig 6.10, courtesy of Shanchita Khan, 2004]. The Antarctic yeast *R. mucilaginosa* also produced small colonies following UVA, UVB or UVC irradiation [Fig 6.7, courtesy of Shanchita Khan, 2004]. However, unlike *S. cerevisiae* K7, none of these colonies had an impaired respiratory chain as demonstrated by cytochrome difference spectra and oxygen uptake [Fig 6.9, courtesy of Shanchita Khan, 2004].

UVA induced proteins were identified by ^{35}S-methionine labeling on SDS-PAGE [Fig 6.11]. Autoradiographs of protein profiles of *Cr. watticus* and UNE116c, both of which were resistant to UVA radiation (Shanchita Khan, personal communication), showed essentially no differences between control and UVA-treated cells [Fig 6.11]. In the case of *R. mucilaginosa* there appeared to be increased synthesis of a protein at approximately 30 kDa, following the 12 hr post-UVA recovery period. On the other hand, protein bands corresponding to hsp90 and hsp70 were enhanced in *S. cerevisiae* K7 following the 12 hr post-UVA recovery period.

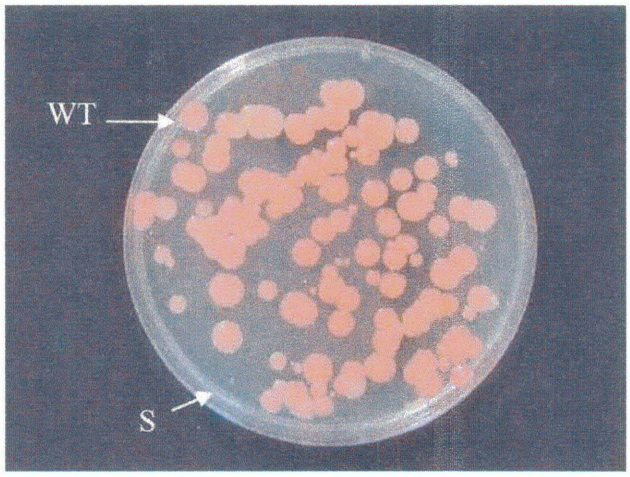

Figure 6.7. *R. mucilaginosa* on YEP agar showing wild-type (WT) and small (S) colonies following UVC irradiation

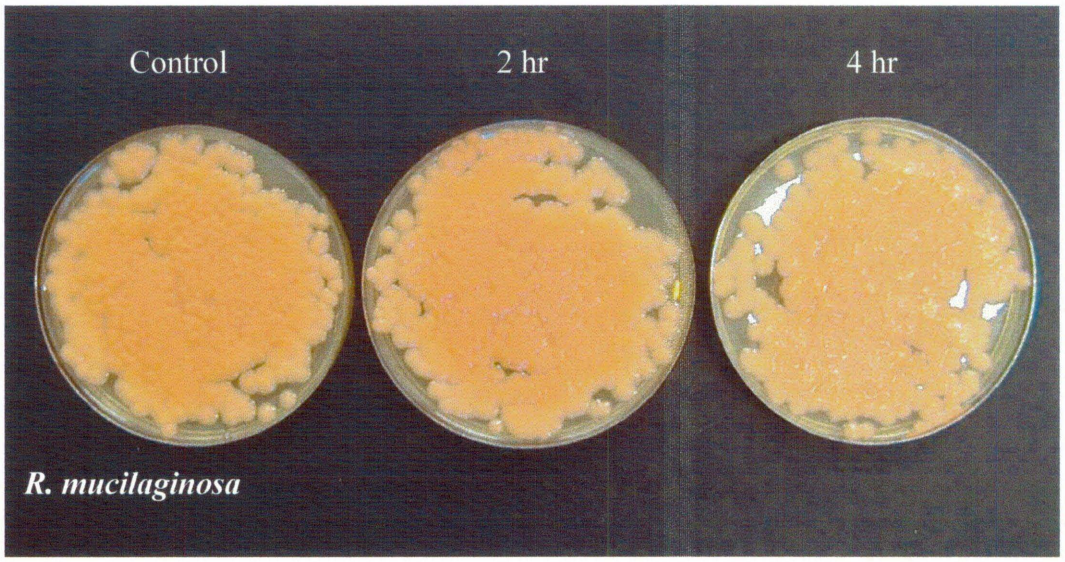

Figure 6.8a. Agar plates of colonies of *Rhodotorula mucilaginosa* following UVA-irradiation at 2 hr and 4 hr

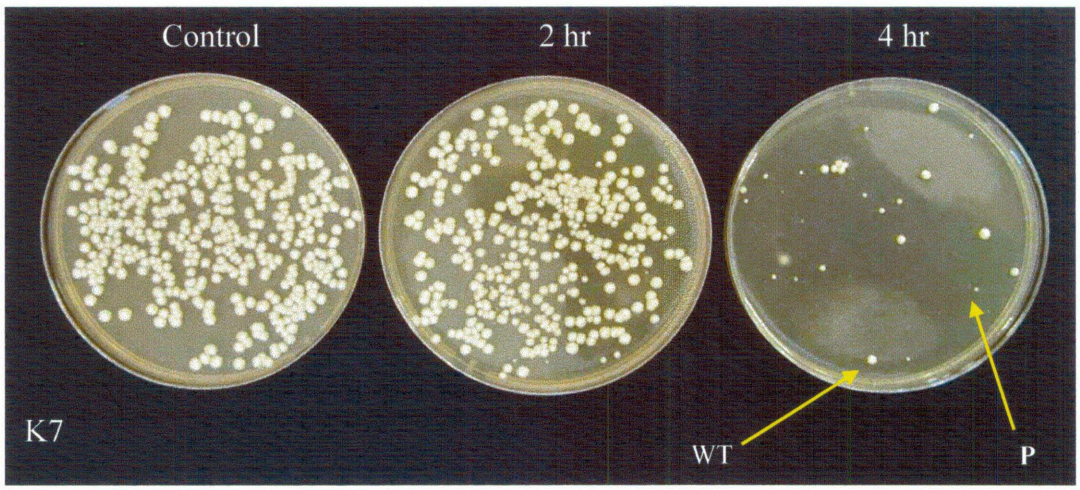

Figure 6.8b. Agar plates of colonies of *Saccharomyces cerevisiae* K7 following UVA-irradiation showing wild-type [WT] and petite colonies [P]

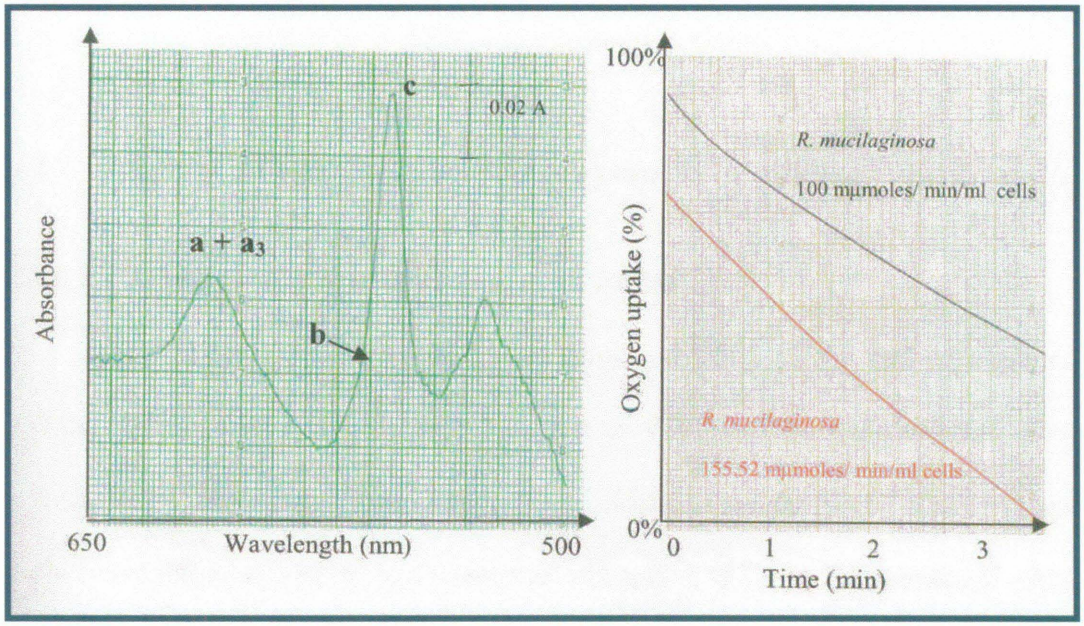

Figure 6.9. (a) Cytochrome difference spectra of small colonies from UV-irradiated *R. mucilaginosa* (b) Oxygen uptake of the same cells as measured by an oxygen electrode, black line oxygen uptake for small colonies, red line oxygen uptake for normal wild type colonies.

$a + a_3$, cytochrome $a + a_3$
b, cytochrome b
c, cytochrome c

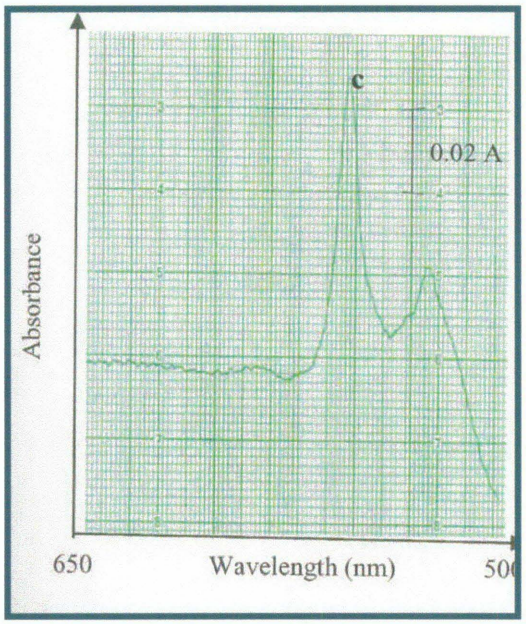

Figure 6.10. Cytochrome difference spectra of small colonies from UV-irradiated *S. cerevisiae*.

c, cytochrome c

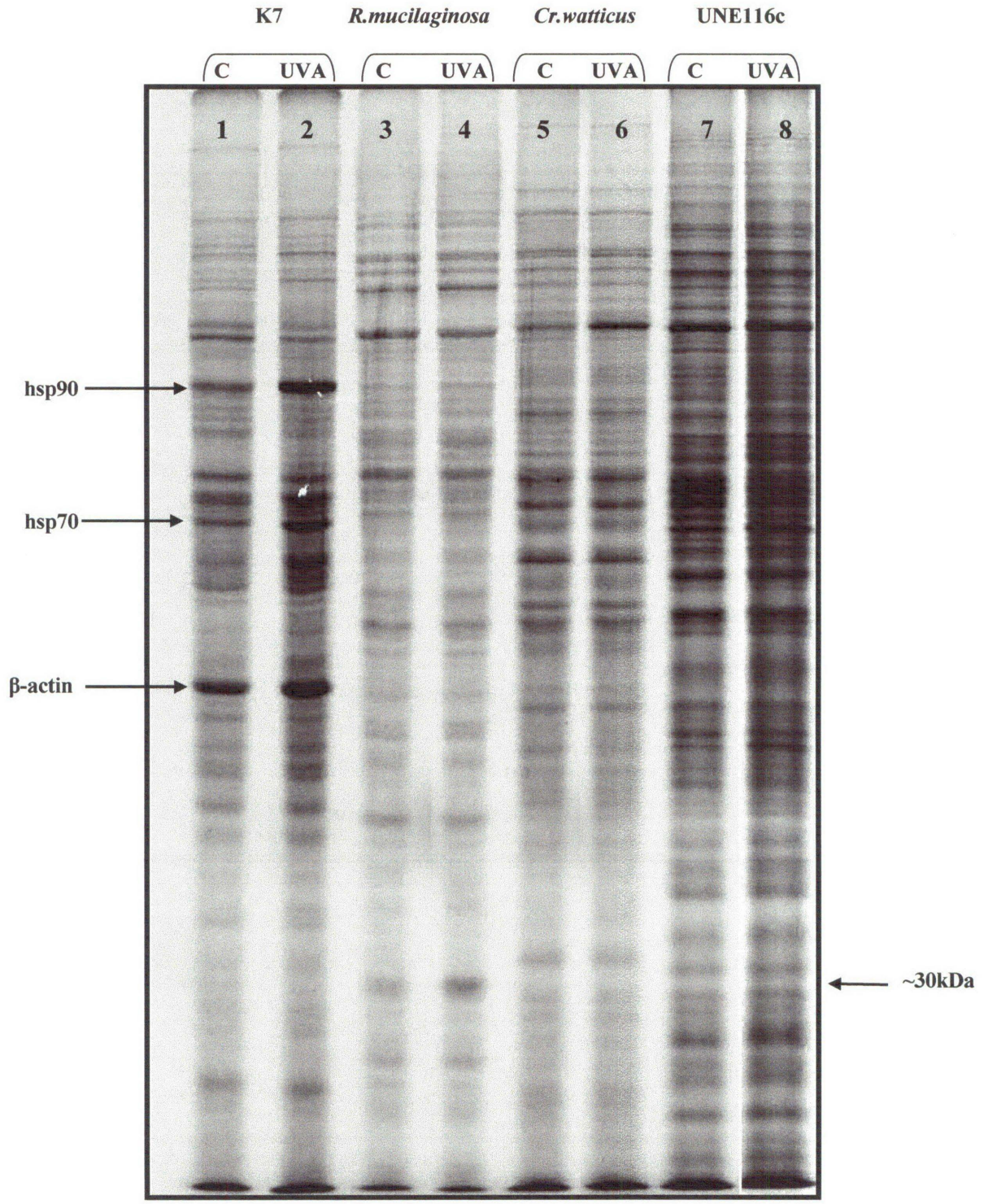

Figure 6.11. Autoradiograph of ^{35}S-methionine labeled proteins following UVA exposure
Lane 1 - *S. cerevisiae* K7 control **Lane 2** - *S. cerevisiae* K7 12 hr post UVA
Lane 3 - *R. mucilaginosa* control **Lane 4** - *R. mucilaginosa* 12 hr post UVA
Lane 5 - *Cr. watticus* control **Lane 6** - *Cr. watticus* 12 hr post UVA
Lane 7 - UNE116c control **Lane 8** - UNE116c 12 hr post UVA
Lanes 2, 4, 6 & 8 - ^{35}S-methionine added post UVA exposure

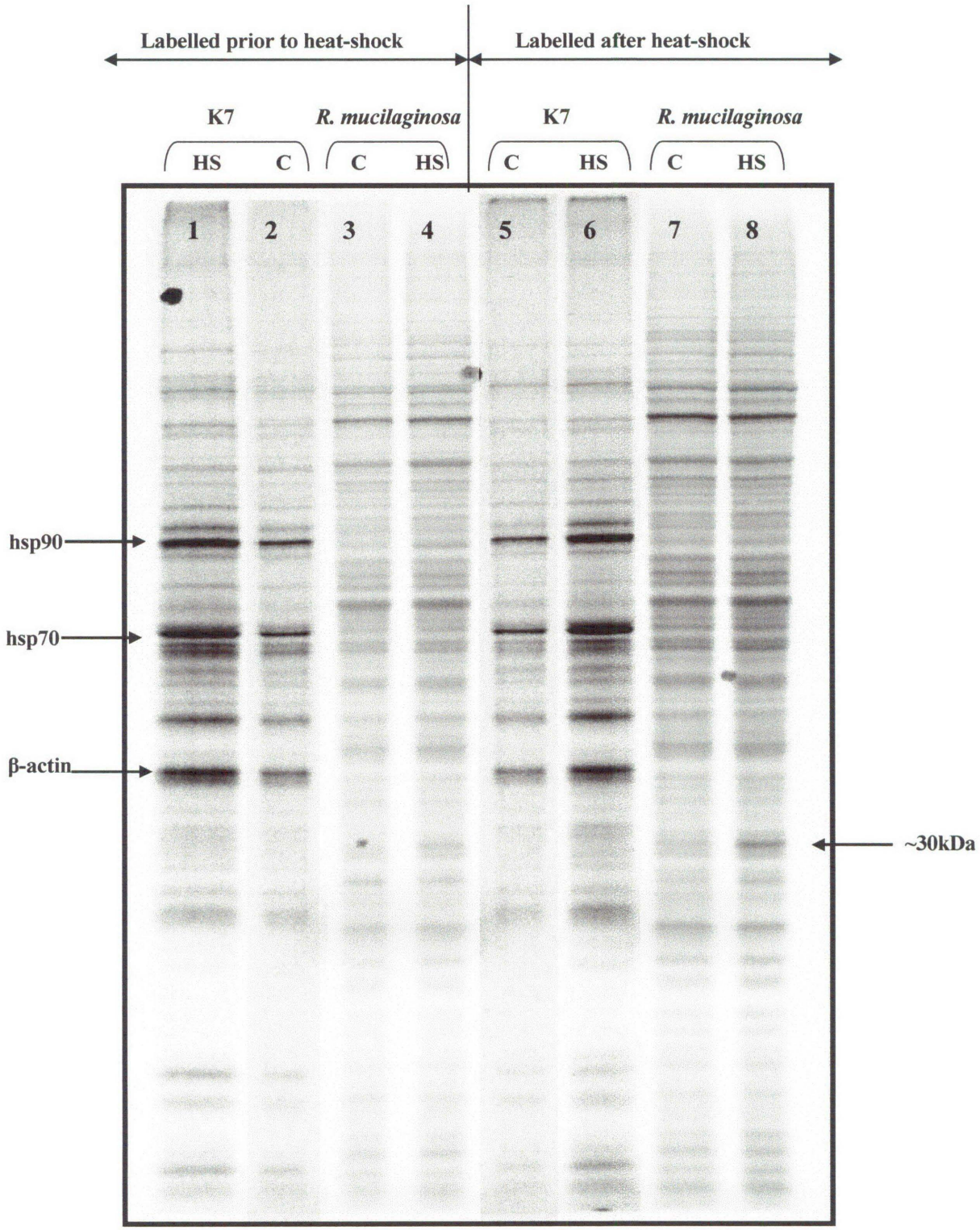

Figure 6.12. Autoradiograph of ^{35}S-methionine labeled proteins extracted from *S. cerevisiae* [K7] and *R. mucilaginosa*

Lanes 1 & 6 - K7 heat shock
Lanes 2 & 5 - K7 control
Lanes 3 & 7 - *R. mucilaginosa* control
Lanes 4 & 8 - *R. mucilaginosa* heat shock
Lanes 1 to 4 - ^{35}S-methionine added prior heat shock
Lanes 5 to 8 - ^{35}S-methionine added post heat shock

The coenzyme Q content was examined in numerous species of yeasts recently isolated from Antarctica as well as *Mrakia gelida*, type strain CBS 5272. Of the isolates studied, CoQ_{10} was the predominant component found, with only five isolates containing CoQ_9 and four with CoQ_8, the latter including *R. mucilaginosa* (Table 6.2). Relative CoQ and $CoQH_2$ concentrations were determined by HPLC analysis. It was noteworthy that the reduced form of coenzyme Q ($CoQH_2$) was the predominant species present (61 – 98%) in the yeasts isolated as shown in Table 6.2. In three of the yeasts, *Cr. gastricus, Cr. gilvescens* and *Cr. nyarrowii* (UNE172a), the oxidised form of ubiquinone (CoQ) was the predominant component (>50%). In two others, namely *R. mucilaginosa* and *Cr. nyarrowii* (UNE116b), the $CoQH_2$ component was just over 50%.

Representative HPLC analyses of Antarctic yeasts with CoQ_8 (*M. gelida*), CoQ_9 (*Cr. nyarrowii* UNE180e) and CoQ_{10} (*Cr. nyarrowii* UNE64a) as main homologs are illustrated in Figure 6.14, Figure 6.15 and Figure 6.16 respectively. The Antarctic yeast UNE116c was unusual in that although CoQ_9H_2 was the predominant species (63%) it also contained significant amounts of CoQ_8H_2 and lesser amounts of $CoQ_{10}H_2$ (Fig 6.17). *R. mucilaginosa* is highly UV resistant, therefore examination of the CoQ redox response in this organism was warranted as it has been recently reported that UV-resistant tropical marine bacteria demonstrate a reductive shift in CoQ redox balance upon UVA exposure (Dunlap *et al.*, 2002). The coenzyme Q ratio, a sensitive measure of cellular redox potential and oxidative stress, was measured in *R. mucilaginosa* cells by HPLC over a 4 hr period of exposure to UVA, followed by a further 2 hr period in the absence of UVA [Fig 6.13]. The CoQ redox balance was expressed as the percentage ratio, $\%CoQH_2 = 100 \times CoQH_2/(CoQ + CoQH_2)$. The reduced CoQ ratio, initially at a baseline of 54%, gradually increased to reach a peak of UVA exposure of 90% after 4 hr. During the 2 hr recovery period (without UVA) ubiquinol remained steady at about 90% [Fig 6.18].

NAD(P)H:quinone oxidoreductase (NQR, DT-Diaphorase) is a two-electron quinone reductase that maintains coenzyme Q in the reduced state. The activity of this enzyme in *R. mucilaginosa* was followed every 30 min for 4 hr during exposure to UVA radiation. Results of using this assay to measure quinone reductase activity in UVA-irradiated cells indicated an increased rate of enzyme activity over a 4 hr period [Fig 6.19]. Irradiated cells did not show an

initial response (baseline reading of 0.41μmol/min) during the first hour of exposure, but then gradually increased reaching a peak of 5.87 μmol/min at 4 hr as shown in Figure 6.19.

Table 6.2. CoQ ubiquinol/ubiquinone redox status in Antarctic yeasts

Species	UNE Strain No.	Major CoQ Component	%$CoQH_2$
Candida norvegica	1140a	8	90
Candida parapsilosis	1217a	9	95
Cryptococcus gastricus	25b	10	25
Cryptococcus gilvescens	124g	10	42
	189e	10	72
	189d	10	71
	1026b	10	92
Cryptococcus nyarrowii	64a	10	88
	73a	10	83
	116b	10	54
	125a	10	88
	157a	10	98
	172a	10	47
	180e	9	88
Cryptococcus watticus	22c	10	90
	41b	10	76
	180c	10	89
Cryptococcus victoriae	76c	10	86
	98a	10	61
	151b	10	69
Debaryomyces hansenii	176a	9	94
	1151a	9	81
Rhodotorula laryngis	1266b	10	62
Rhodotorula minuta	17b	10	86
Rhodotorula mucilaginosa	1130a	8	54
Unknown Ascomycete	98e	8	74
	116c	9	63
Mrakia gelida	CBS 5272^T	8	75

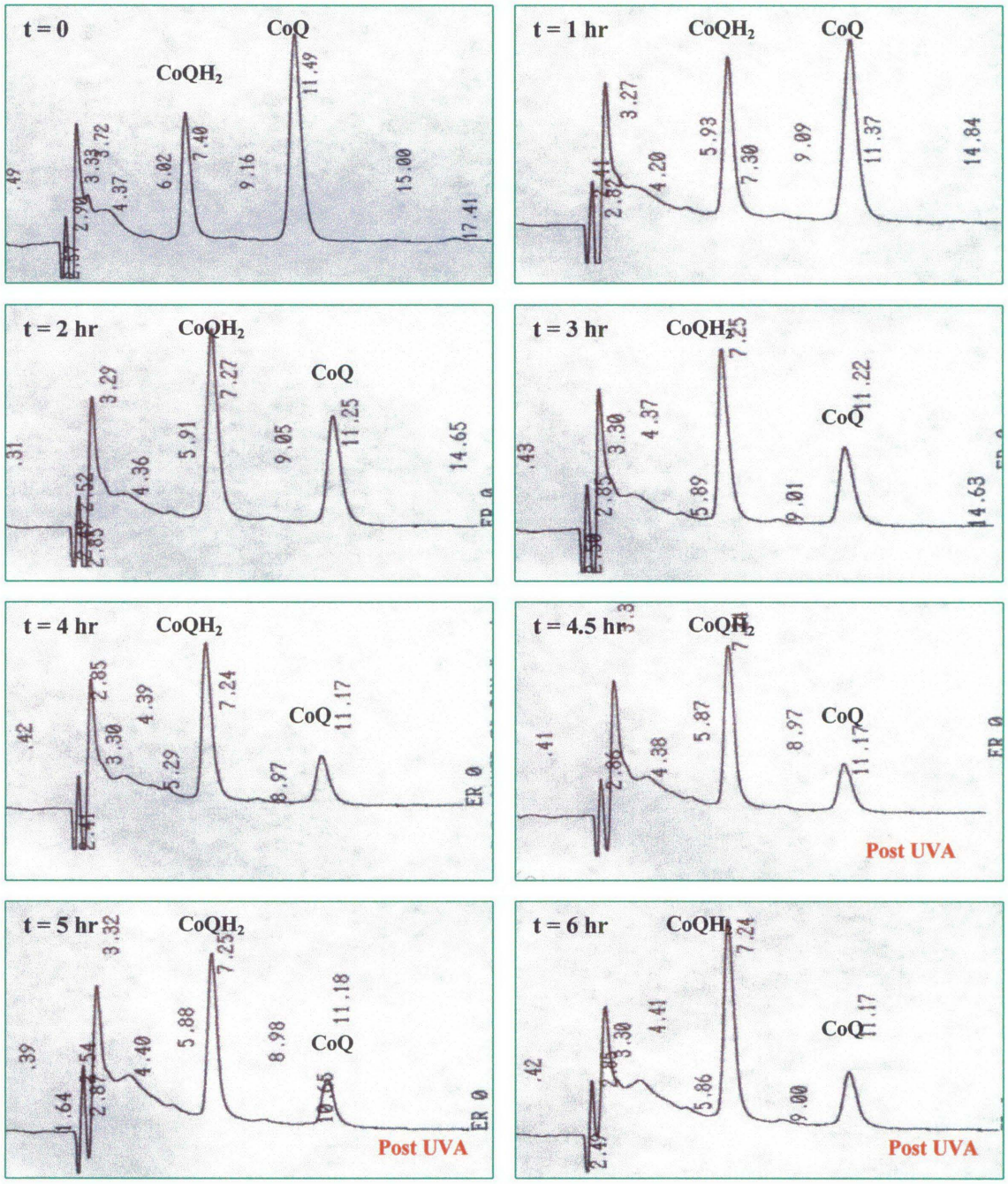

Figure 6.13. Chromatographic analysis of CoQ redox balance on UVA exposure of *R. mucilaginosa* UNE1130a. Retention times (min) are shown on the chromatographs.

t = 1, 2, 3 and 4 hr: time of exposure to UVA
t = 4.5, 5 and 6 hr: time post-UVA exposure

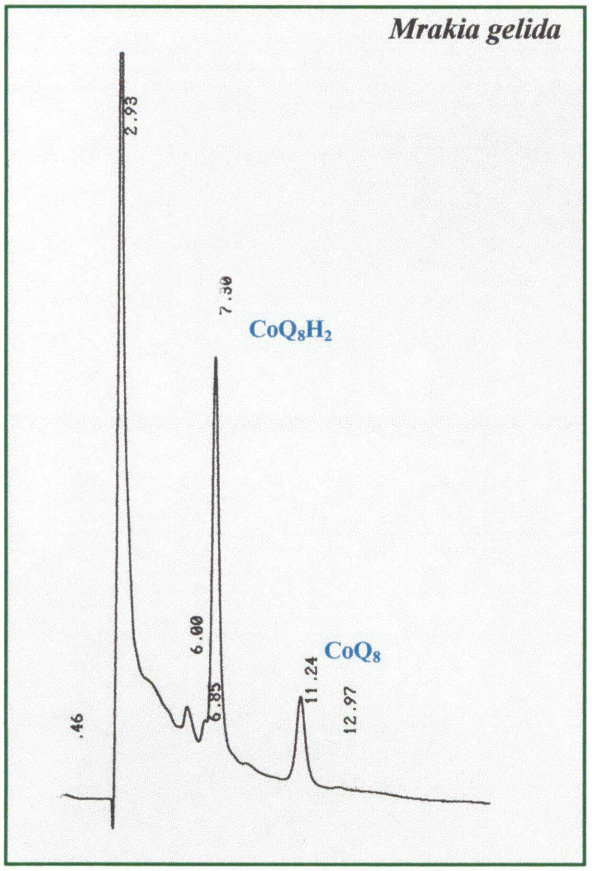

Figure 6.14. HPLC analysis of CoQ redox balance of *Mrakia gelida*. Major CoQ component is CoQ_8. Retention times (min) are shown on the chromatographs.

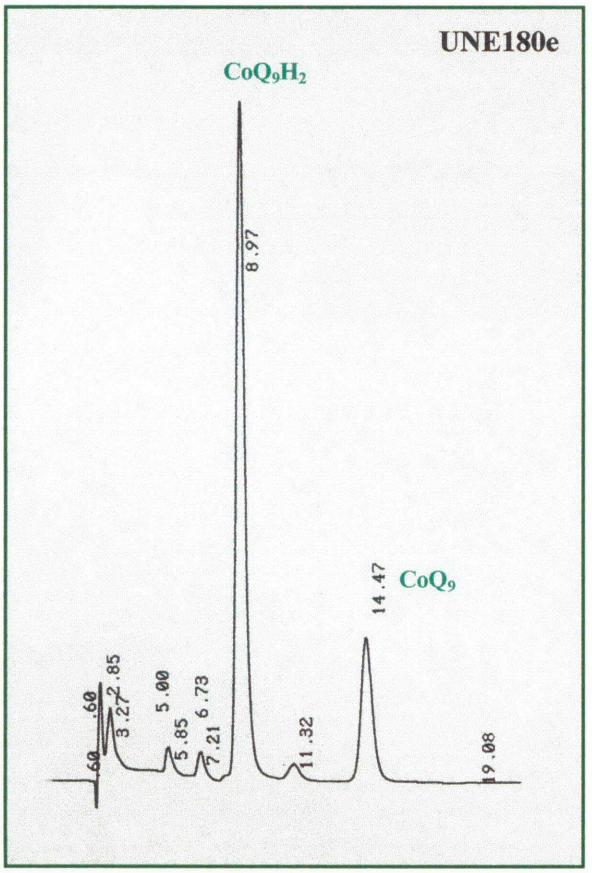

Figure 6.15. HPLC analysis of CoQ redox balance of *Cr. nyarrowii* (UNE180e). Major CoQ component is CoQ_9. Retention times (min) are shown on the chromatographs.

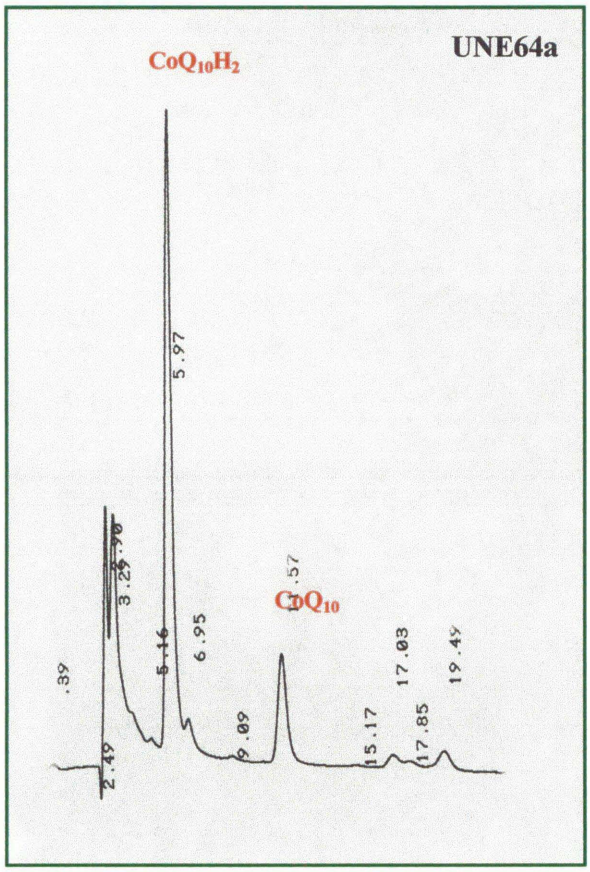

Figure 6.16. HPLC analysis of CoQ redox balance of *Cr. nyarrowii* (UNE64a). Major CoQ component is CoQ_{10}. Retention times (min) are shown on the chromatographs.

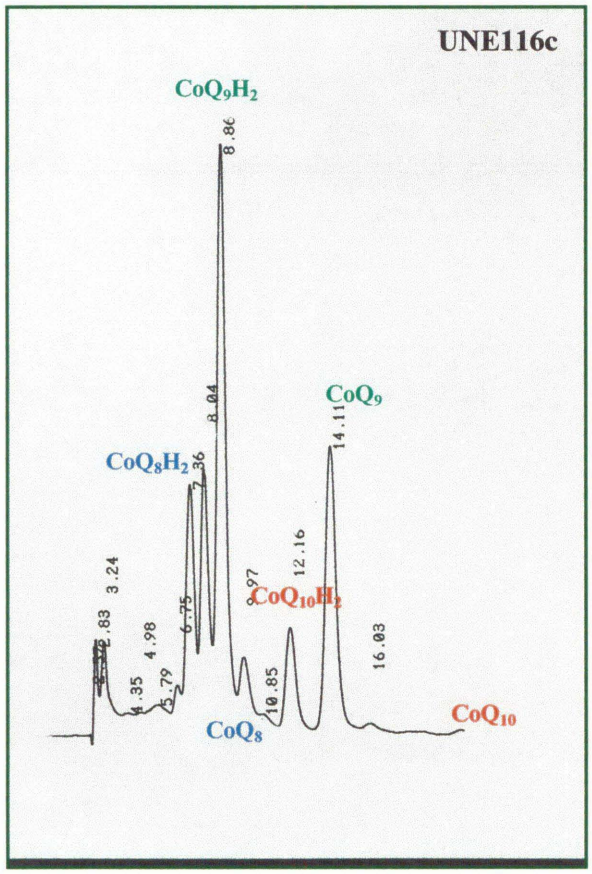

Figure 6.17. HPLC analysis of CoQ redox balance of UNE116c. Major CoQ component is CoQ_9 with smaller amounts of CoQ_8 and CoQ_{10}. Retention times (min) are shown on the chromatographs.

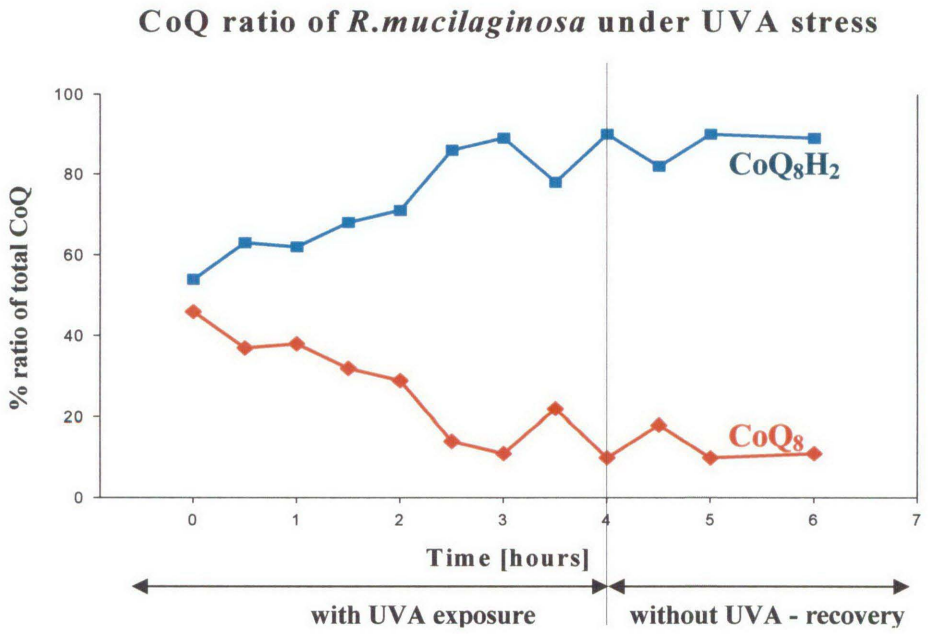

Figure 6.18. Change in CoQ_8 redox balance of *R. mucilaginosa* UNE1130a during UVA exposure

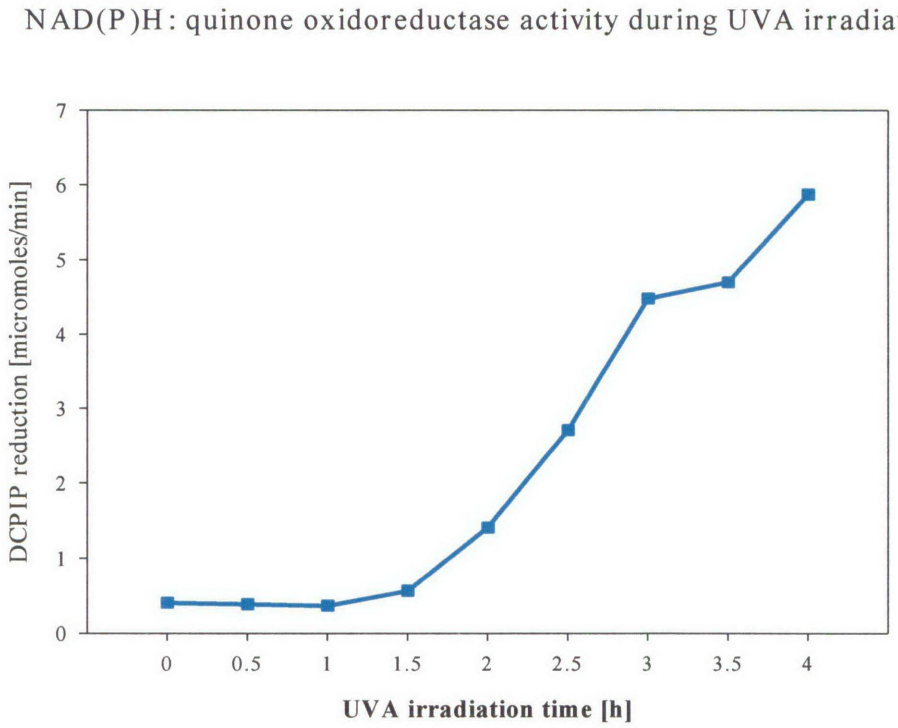

Figure 6.19. Change in rate of NADPH-DCPIP reduction during UVA exposure of *R. mucilaginosa* UNE1130a

Discussion

Phylogenetic & physiological analysis

Sequence analyses of the D1/D2 region of the 26S ribosomal DNA and the ITS regions identified isolate UNE1130a (in-house reference) as *Rhodotorula mucilaginosa* and molecular analyses placed it into the phylogenetic tree of related Basidiomycetous yeasts [Fig 6.4]. *Rhodotorula spp.* are commonly characterised by pink/red colonies and, along with *Sporobolomyces* and *Tilletiopsis* are anamorphic genera assigned to the Urediniomycetes (Fell *et al.*, 2000). Comprising four major clades, species of *Rhodotorula* are represented in the *Microbotryum, Sporidiobolus* and *Erythrobasidium* clades, but are not conventionally found in the *Agaricostilbum* clade. Although carotenoid pigments are not used for taxonomic purposes, the *Sporidiobolus* clade can be differentiated based on this feature (Fell *et al.*, 2000). Physiological analyses of *R. mucilaginosa* confirming the phylogenetic results included the inability to assimilate inositol, positive reactions to urease and Diazonium Blue B and negative for starch formation. No sexual state was observed from cultures plated for 3-6 months at 6°C or 15°C on YEP agar, cornmeal agar, malt agar or nitrogen base agar.

Lipid analysis

R. mucilaginosa (UNE1130a) had a relatively high percentage of total fatty acids as unsaturated, predominantly $C_{18:1}$ with smaller amounts of $C_{18:2}$ and $C_{18:3}$ [Fig 6.5]. Zlatanov *et al.* (2001) reported a similar profile in a *R. mucilaginosa* strain isolated from Livingston Island, Antarctica, using the same growth temperature as this study. This result confirmed earlier reports by Watson (1980) and Watson & Arthur (1976) in which it was reported that psychrophilic yeasts had a predominance for C_{18} unsaturated fatty acids associated with the need to maintain functional membrane fluidity at low temperatures. A study of the fatty acid composition of thirteen *Rhodotorula* strains, including *R. mucilaginosa*, supported these results with the major fatty acid in all strains being $C_{18:1}$ followed by $C_{18:2}$ (Perrier *et al.*, 1995). These authors also reported up to 100 μg per (g dry weight)$^{-1}$ of total carotenoid content in these strains with *β*-carotene comprising approximately 70%. Increased carotenoid production has long been established in the so-called industrial red yeasts *Rhodotorula glutinis*, *Sporidiobolus salmonicolor* and *Phaffia rhodozyma*, under various exogenous stress

conditions including UV radiation (Pokorna *et al.*, 2003). In view of the potential protective properties that carotenoids demonstrate against UV stress, it is quite possible that they may serve as a survival mechanism for yeasts found in extreme habitats such as Antarctica. Optimal growth temperature studies indicated that *R. mucilaginosa* could grow across a wide temperature spectrum. Although maximum growth occurred between 15 and 20°C, adequate growth occurred from 4°C up to 37°C. The production of substantial amounts of carotenoid pigments as well as significant levels of unsaturated fatty acids coupled with fast growth rates and undemanding growth requirements, makes this yeast a prime target for commercial applications. These issues are further explored in Chapter 8.

Expression of hsps following heat and UVA stress

Cells grown at 25°C and subjected to a mild heat shock (37°C/1 hr) exhibited heat shock-induced thermotolerance to a normally lethal heat stress (52°C/2-4 hr) [Fig 6.7]. Previous studies in this laboratory have used *S. cerevisiae* K7 as a control for studies on the heat shock response (Deegenaars and Watson, 1998a). In these studies, heat shock inducible proteins have been described for Antarctic yeasts of the genera *Mrakia* and *Leucosporidium*. Hsps 110, 90, 70 as well as a number of smaller hsps were shown to be heat shock inducible under various heat shock conditions (Deegenaars and Watson, 1998a). The 110 kDa hsp was heat shock inducible in all of the psychrophilic strains studied with the exception of *L. antarcticum* and it was postulated that this hsp substitutes the equivalent role that hsp104 plays in thermotolerance in the mesophilic yeast *S. cerevisiae*. Hsp90 was heat shock inducible in the *Mrakia* strains but not the *Leucosporidium* strains, suggesting that the presence (or absence) of a particular hsp is not enough to distinguish between psychrophilic and psychrotolerant species, but may be a species-specific characteristic (Deegenaars and Watson, 1998a).

By contrast, in the present studies, 1D-SDS-PAGE showed no increased expression of hsp70 or hsp90 following a heat shock of 37°C for 1 hr but did show an increased expression of a smaller hsp at ~ 30 kDa following UVA exposure [Fig 6.11]. The nature of the protein at ~30 kDa was not further explored in the present studies. This result indicated that under the conditions used in this study, expression of hsp110, hsp90 and hsp70 is not induced upon exposure to a heat shock. Whilst this Antarctic yeast exhibited the classic heat shock response

of acquired thermotolerance, increased expression of the heat shock proteins normally associated with thermotolerance (e.g. hsp70) was not observed.

In response to UVA exposure for a 4 hr period, *R. mucilaginosa* did not show any induction of hsp expression as detected by 1D-SDS-PAGE [Fig 6.12]. In contrast, *S. cerevisiae* had increased expression of both hsp70 and hsp90, which was more pronounced during the 12 hr recovery period following UVA exposure. Importantly, two other Antarctic yeasts studied, *Cr. watticus* and as yet unidentified Ascomycetous yeast (UNE116c), produced similar results as *R. mucilaginosa* showing no induction of hsps following UVA exposure. Although these observations indicated a lack of the classic heat shock response following both a heat and UV stress in these Antarctic yeasts, it could just be that these yeasts are not *stressed* under UV conditions normally considered stressful to mesophilic yeasts.

UVR response

Response to UVA radiation was measured over a time course (2, 3 and 4 hr) and cell viability estimated by serial dilution plate count. Essentially 100% cell survival was observed in the case of the Antarctic yeast *R. mucilaginosa*, as compared with 0% survival for the mesophilic yeast *S. cerevisiae*. The latter produced small colonies following exposure to UVA (4 hr), UVB (85-380 sec) or UVC (30-60 sec) radiation. The small colonies of *S. cerevisiae* K7 were found to be deficient in cytochrome a + a_3 and cytochrome b indicative of an impaired respiratory chain, however, normal levels of cytochrome c were observed. These properties of small respiration-deficient colonies were characteristic of the well established petite mutation in *S. cerevisiae* which can be induced by exposure of cells to UVC irradiation (Mayer, 1970; Moustacchi and Enteric, 1970). The Antarctic yeast *R. mucilaginosa* produced small colonies following UVA, UVB and UVC irradiation. However, unlike *S. cerevisiae* K7, none of these colonies had an impaired respiratory chain as demonstrated by cytochrome difference spectra and oxygen uptake and were thus not respiratory-deficient mutants.

The small colonies of *R. mucilaginosa* exhibited essentially normal cytochrome difference spectra with the full complement of respiratory chain cytochromes and high oxygen uptake. In the case of *R. mucilaginosa*, the cytochrome ratio for the wild-type was 1: 0.5: 1.6 and for the small colonies (from UVC exposure) it was 1: 0.6: 2.8, again indicating a marked increase in cytochrome c content. The respiratory rate for the small colonies were typically

around 100 to 150 mμmoles oxygen/min/ml cells, with the higher the UVC exposure, the lower the rate.

Coenzyme Q redox balance following UVA

The cellular coenzyme Q status is a balance of the rate of metabolic ubiquinol oxidation and the conversion rate of ubiquinone to ubiquinol (from the inactive to the active state). This conversion is due to the enzyme NAD(P)H: quinone oxidoreductase (NQR). In many cases, including human plasma, coenzyme Q is constitutively expressed at high levels in the reduced state with metabolic stress indicated by an oxidative shift in the cellular coenzyme Q ratio (Lagendijk *et al.*, 1996; Yamamoto and Yamashita, 1997, 1999; Tang *et al.*, 2001). Studies of tropical marine bacteria have indicated a wide range of constitutive levels of CoQ in the reduced state, with a number of bacterial isolates having much lower levels of the reduced form. It has been demonstrated that some of these bacterial strains with lower basal levels of $CoQH_2$ (>50%) showed a reductive response to UVA radiation. Those isolates that showed such a response were also UV-tolerant (Dunlap *et al.*, 2002). Corresponding with the increased levels of $CoQH_2$, was a significant increase in NQR activity under the same UVA stress conditions, thus indicating that NQR is indeed responsible for the conversion of ubiquinone to ubiquinol in marine bacteria (Dunlap *et al.*, 2005).

During initial studies of determining CoQ components (for taxonomic purposes), of a number of newly isolated Antarctic yeasts, it was noticed that a number of these yeasts also had low to intermediate basal levels of reduced CoQ. One of these isolates, *R. mucilaginosa*, was found to be highly UV-resistant prompting further investigation to examine whether this isolate was capable of increasing cellular $\%CoQH_2$ under UVA stress in a similar fashion to the tropical marine bacteria. Preliminary results indicated a reductive response in cellular CoQ balance, increasing the $CoQH_2$/total CoQ ratio on exposure to UVA radiation [Figs 6.13 & 6.18]. Two other Antarctic yeasts (*Cr. watticus* and UNE116c) were also examined under the same conditions but no viable cells were detected after 1 hr of UVA radiation. Not all yeasts with a low to intermediate basal level of reduced CoQ were examined for their response to UVA due to time limitations, however, it is not unreasonable to expect that possibly some of these yeasts may also show a reductive response to UV exposure.

This finding is significant as it is the only report to date of a reductive CoQ redox response in a eukaryotic organism. The discovery of this response in tropical marine bacteria has prompted research into the plausibility of using this bacterial model to examine the regulation of NQR activity in mammalian mitochondria (Dunlap *et al.*, 2005). Whilst it has been shown that impaired capacity to maintain adequate reduced levels of ubiquinol have been linked to various human disorders such as cardiovascular disease (Stocker *et al.*, 1991; Soja and Mortensen, 1997; Singh *et al.*, 1998; Sarter, 2002) and various cancers (Walker and Doolittle, 1983; Marin *et al.*, 1997; Marks-Hull *et al.*, 1997; Clairmont *et al.*, 1999; Larson *et al.*, 1999; Lin *et al.*, 1999; Steiner *et al.*, 1999; Wiemels *et al.*, 1999; Asher *et al.*, 2001; Lewis *et al.*, 2001; Smith *et al.*, 2001; Yin *et al.*, 2001; Anwar *et al.*, 2002; Asher *et al.*, 2002a), it is yet to be shown whether these transduction pathways mediating CoQ balance is sufficiently conserved between prokaryotes and eukaryotes, and in particular mammalian mitochondria, given the vast distance in evolutionary divergence. However, the discovery of this pathway in a yeast provides a more appropriate model for examining the mammalian system. Although results thus far are promising, the question remains, is the enzyme NQR directly activated by a UVA-signal or is it a redox response to the production of ROS induced by UVA radiation. A number of degenerative diseases in humans have been linked to a diminished capacity of ubiquine reduction, further study utilizing *R. mucilaginosa* as a model organism may provide insightful knowledge as to the regulation of coenzyme Q redox balance in eukaryotes and it's future possible role in areas such as human ageing (see also Chapter 7).

The results of this study indicate that while this strain of *R. mucilaginosa* is highly resistant to UVR, the classic heat shock response, that is, the increased expression of hsp70 is not responsible for this protection. Increased synthesis of hsps was not induced under either heat shock conditions, which did elicit a heat shock response from *S. cerevisiae,* nor in response to UVA radiation. Nonetheless, heat shock acquired thermotolerance was observed in this strain, which is normally indicative of a heat shock response. Given these results, it can be postulated that the unique CoQ redox response of this yeast to UV-photooxidative stress is a cellular adaptation to the high levels of UVR found in Antarctica.
