Proteomic response to physiological fermentation stresses in a wild-type wine strain of *Saccharomyces cerevisiae*

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We report a study on the adaptive response of a wild-type wine *Saccharomyces cerevisiae* strain, isolated from natural spontaneous grape must, to mild and progressive physiological stresses due to fermentation. We observed by two-dimensional electrophoresis how the yeast proteome changes during glucose exhaustion, before the cell enters its complete stationary phase. On the basis of their identification, the proteins representing the *S. cerevisiae* proteomic response to fermentation stresses were divided into three classes: repressed proteins, induced proteins and autoprolysed proteins. In an overall view, the proteome adaptation of *S. cerevisiae* at the time of glucose exhaustion

seems to be directed mainly against the effects of ethanol, causing both hyperosmolarity and oxidative responses. Stress-induced autoprolysis is directed mainly towards specific isoforms of glycolytic enzymes. Through the use of a wild-type *S. cerevisiae* strain and PMSF, a specific inhibitor of vacuolar proteinase B, we could also distinguish the specific contributions of the vacuole and the proteasome to the autoprolytic process.

Key words: ethanol, glucose, oenology, two-dimensional electrophoresis, yeast.

INTRODUCTION

Upon exposure to a specific environmental stress, cells mount a protective response. The evolutionary conservation between organisms and the universal distribution of general stress responses make the budding yeast *Saccharomyces cerevisiae* an ideal model system to investigate the ways in which cells adjust to changes in their environment. In fact, yeast has the same defence mechanisms as higher eukaryotes, and offers the possibility of genome/proteome studies, since *S. cerevisiae* was the first eukaryotic organism whose genome was completely sequenced in 1996 [1]. Moreover, the number of each expressed gene has been determined [2], and protein–protein interactions have been investigated [3].

This makes *S. cerevisiae* the optimal eukaryotic model in which to study cellular control under stress conditions. Boucherie [4] pioneered proteomic investigation by two-dimensional (2D) PAGE of budding yeast strains, demonstrating the occurrence of new proteins as a result of glucose exhaustion. This was followed by other studies using similar approaches to investigate oxidative [5], hyperosmolarity [6] and cadmium stress responses [7]. Proteomic studies can help to relate the genome and proteome to cell functions, and they are complementary to genomic and transcriptomic studies, since mRNA abundance is not perfectly correlated quantitatively with gene expression [8].

In addition to being a model system for mammalian cells, yeast is also utilized widely as a biotechnological tool in the food industry. Baker's *S. cerevisiae* strains have been widely studied by both genome sequencing and other types of characterization, whereas the proteomes of brewing or wine yeasts are still largely unknown. However, better knowledge of specific strains for biotechnological applications is desirable in order to optimize industrial processes.

Alcoholic fermentation is the main step in wine production using strains of *S. cerevisiae*. Therefore optimization of biotechnological processes in wine-making involves mainly the selection of natural yeast populations from fermenting musts and the characterization of *S. cerevisiae* strains able to overcome stuck fermentation due to nutrient consumption or other types of shock. Knowledge of the effects of stress on wine *S. cerevisiae* is important for at least two reasons. First, all wine strains are subjected to several stresses, since wine fermentation media are characterized by a high initial concentration of sugar followed by glucose deprivation, as well as a high ethanol concentration, a low content of assimilable nitrogen, oxygen shortage and low pH. Secondly, the peculiarity of these parameters in natural winery musts cause stresses that must be controlled and possibly overcome by *Saccharomyces* strains with specific features.

Cell stress studies are usually carried out with baker's yeast strains, often genetically manipulated, under extreme environmental conditions that are very unlike physiological ones. For example, nutrient starvation stress has been studied by analysing the cell response to the sudden removal of a nutrient from the growth medium. Alternatively, stressful agents (e.g. ethanol) are added at single concentrations, and their effects are observed at single time points. In contrast, our present study is the first to observe how a wild-type wine *S. cerevisiae* strain adapts physiologically to the progressive depletion of an essential nutrient, glucose, during batch culture, and how its proteome changes up to and beyond the point of glucose exhaustion. In fact, the nutrient 'starvation' response is considered to be different from the nutrient 'limitation' response [9]. The culture conditions under which the present study was carried out differ from those used in previous studies, since we used a wild-type wine *S. cerevisiae* strain grown under semi-aerobiosis, and with an initial glucose concentration of 100 g/l in order to mimic as far

Abbreviations used: 2D, two-dimensional; Hsp26 (etc.), heat-shock protein 26 (etc.); YPD, yeast peptone dextrose.

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as possible yeast growth conditions in grape must. We monitored the natural spontaneous fermentation of *S. cerevisiae* and observed its responses to mild physiological stresses, due mainly to glucose shortage.

We found that the proteomic response to physiological fermentation stress involves both the induction and the repression of specific proteins which, in several cases, differ from those reported for baker's yeast. We also found that the yeast response induces intracellular proteolysis that seems to be directed to defined classes of proteins.

Moreover, we wanted to distinguish the specific contribution of the vacuole to the response of *S. cerevisiae* to alcoholic fermentation stress. To prevent vacuole activity, we utilized PMSF, a specific inhibitor of vacuolar proteinase B that does not affect proteasome functionality [10], in parallel with the occurrence of stressful environmental conditions causing auto-proteolysis. Proteomic evaluation of the stress response in the presence and absence of the vacuole inhibitor allowed us to identify which proteins were specifically degraded by this organelle.

EXPERIMENTAL

General materials

All high-purity reagents were from Sigma-Aldrich (Milano, Italy), Bio-Rad Laboratories (Segrate, Italy), Merck Eurolab (Milano, Italy), Carlo Erba (Rodano, Italy), Serva (Heidelberg, Germany), Amersham Pharmacia Biotech Italia (Cologno Monzese, Italy) or Difco Laboratories (Detroit, MI, U.S.A.). All water used was Milli-Q quality (Millipore, Bedford, MA, U.S.A.).

Yeast strain and culture conditions

The *S. cerevisiae* K310 strain was isolated from spontaneous wine fermentation. K310 was grown in YPD (yeast peptone dextrose) medium at 25 °C with rotary shaking up to saturation. An aliquot of the saturated culture was inoculated in YPD, adjusted to a final pH of 4.5 by adding 0.2 M citrate/phosphate buffer containing 100 g/l glucose, to obtain an initial cell concentration of 1×10^4 cells/ml. The cell suspension was incubated at 25 °C without shaking, to allow semi-anaerobic growth, for 89 h. Samples were collected at time 0 and after 12, 16, 19, 22, 27, 36, 44, 50 and 62 h. At each sampling, the pH of the cell suspension was checked and growth was monitored by measuring the absorbance of the culture at 660 nm.

PMSF (1 mM final concentration, in absolute ethanol) was added to cell suspensions, cultured in parallel, at 36 and 40 h. An equal volume of ethanol was added to parallel control cultures.

Determination of glucose, ethanol, trehalose and glycogen

The levels of glucose and ethanol were determined in culture supernatants using the Sigma glucose and ethanol assays (kit codes 510-A2 and 332-C respectively). The levels of trehalose and glycogen were determined in whole yeast cells using the protocols of Parrou and Francois [11].

Preparation of yeast cell extracts and 2D electrophoresis

A *S. cerevisiae* K310 cell suspension was centrifuged for 5 min at room temperature at 3000 *g* in a Beckman model J2-21 centrifuge equipped with a JA10 rotor. The supernatant was discarded and the pellet was washed with distilled water and then with yeast medium buffer at pH 4.5 (50 mM Mes, 10 mM EDTA, 10 mM

MgCl₂). The pellet was then added to 2 vol. of a solution consisting of yeast medium buffer containing a protease inhibitor cocktail (50 µg/ml leupeptin and 0.1 M PMSF) and an equal volume of glass beads (425–600 µm). Disruption of the cells was achieved by vortexing the sample for 8 min, alternating 30 s of vortexing with 30 s of rest in an ice bath. Cells were then centrifuged for 5 min at room temperature at 300 *g* in a Beckman model J2-21 centrifuge, using a JA20 rotor. The supernatant was collected and its protein concentration was determined.

The sample was denatured in the buffer for the first dimension of 2D PAGE, which comprised 8 M urea, 4% (w/v) CHAPS, 40 mM Tris, 65 mM dithioerythritol and a trace of Bromophenol Blue. The diluted yeast sample was then loaded on to an Immobiline (immobilized pH gradient; Amersham Pharmacia Biotech) gel strip.

2D electrophoresis was carried out according to procedures detailed elsewhere [12]. Briefly, 45 µg (analytical) or 1 mg (preparative) of protein sample was applied to an Immobiline strip consisting of a non-linear gradient, pH range 3.5–10, rehydrated previously according to the manufacturer's protocol. Isoelectric focusing was carried out on a horizontal electrophoresis system, Multiphor II (Amersham Pharmacia Biotech). The voltage was increased linearly from 300 to 3500 V during the first 3 h and then stabilized at 5000 V for 22 h (total 110 kV·h). The immobilized pH gradient strip was then equilibrated in 6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS, 0.05 M Tris/HCl, pH 6.8, and 2% (w/v) dithioerythritol, and later also with 2.5% (w/v) iodoacetamide. Electrophoresis in the second dimension was carried out on a 9–16% (w/v) polyacrylamide non-linear gradient gel (18 cm × 20 cm × 1.5 mm) at a constant current of 40 mA until the dye front reached the bottom of the gel. The analytical gel was stained with ammoniacal silver nitrate as described previously [13]. The digitalized image was obtained by scanning the gel with a Laser Densitometer (4000 × 5000 pixels; 12 bits/pixel) from Molecular Dynamics (Sunnyvale, CA, U.S.A.), and analysed qualitatively and quantitatively using Melanie II 2D PAGE software (Bio-Rad). Spot intensities were obtained in pixel units and normalized to the total absorbance of the gel. The increasing/decreasing index (fold change) was calculated as the ratio of spot intensities (relative volumes) between the 44 h and 16 h gel maps.

N-terminal microsequence analysis and database searching

Following preparative 2D PAGE runs, the protein spots were electrotransferred [12] on to a PVDF membrane (Bio-Rad; 20 cm × 20 cm, 0.2 µm pore size) using a semi-dry blotting apparatus (Novablot II; Amersham Pharmacia Biotech). Blots were stained with 0.1% (w/v) Coomassie Brilliant Blue R250 in 50% (v/v) methanol for 2 min, and then destained in 40% (v/v) methanol/10% (v/v) acetic acid. The PVDF membrane spots were excised and transferred to a protein sequencer (Model 241; Hewlett Packard, Palo Alto, CA, U.S.A.). Protein sequence tags were used as probes for searches in the SWISS-PROT and TrEMBL annotated protein sequence databases using both BLAST and Blitz software and the sequence search tools at the Yeast Proteome Database [14]. Additional information was obtained from the Saccharomyces Genome Database [15].

MS analysis

Spots from 2D PAGE were excised from the gel, triturated and washed with water and acetonitrile. Proteins were in-gel reduced, S-alkylated and digested with trypsin, as reported previously [16]. Protein digests were analysed with a Voyager DE-PRO

MALDI-TOF (matrix-assisted laser-desorption ionization-time-of-flight) spectrometer (Applied Biosystems) as reported previously [16]. ProteinProspector and PROWL software packages were used to identify spots from *S. cerevisiae* sequence databases without any limitation in M_r or pI. Candidates with the highest scores were evaluated further by comparison of their calculated M_r and pI values with the experimental values obtained from 2D PAGE. The occurrence of a degraded protein species was assessed on the basis of the simultaneous occurrence of the following events: definite protein identification (ProteinProspector's MOWSE score > 100000 and/or PROWL's Est'd Z score > 2), an evident discrepancy with respect to the expected M_r value of the intact protein ($\Delta M_r > 30\%$) and a non-uniform distribution of the MS-detected peptides on the primary structure (i.e. occurrence at specific polypeptide regions). This criterion was verified as always being correct for all samples for which a parallel microsequence analysis was also performed.

RESULTS

K310 is a wild-type *S. cerevisiae* strain isolated from the natural must of spontaneous grape fermentation, and is often selected as a potential starter for the guided fermentation of high-quality wines. This is due to the ability of the K310 strain to overcome environmental stresses and thus complete the fermentation process even under adverse conditions. Individuality, along with the enormous amount of general information provided by genomics, is important in biotechnological applications, since it is strain specificity that makes one yeast preferable to another. For example, differential regulation of fermentative capacity and glycolytic enzyme levels between industrial and laboratory yeast strains has been demonstrated [17], clearly indicating that physiological and biochemical studies cannot necessarily be extrapolated from one *S. cerevisiae* strain to another.

In the present study, yeast cells were cultivated in a synthetic medium, to allow experimental reproducibility, which was modified in order to mimic more closely the conditions of natural wine fermentation. For the same reason, we also adopted semi-aerobic growth. We then observed how the proteome of the K310 strain changed following the natural, physiological exhaustion of glucose (together with citrate, the only carbon source in the culture medium), without any external intervention. Our attention was focused on glucose withdrawal, since this is the main cause of unwanted stuck fermentation during vinification. We allowed glucose limitation to increase naturally and progressively, and analysed the protein repertoire when cells were completely deprived of this nutrient, attempting systematic identification of the gene products of the stress response.

Cell growth

Growth of the K310 yeast strain in modified YPD containing 10% (w/v) glucose was monitored by measuring turbidity and cell density. Under the conditions adopted, K310 cells exhibited changes in growth (Figure 1A). The exponential phase ended after 44 h of fermentation, when cells began to reach the stationary phase, in which cell proliferation ceased. Determination of the glucose concentration in the medium showed that entry of the cells into the stationary phase was concomitant with the disappearance of glucose from the medium. The ethanol concentration in the culture medium reached a maximum at the same time, and did not change noticeably during the initial period of the stationary phase (Figure 1A). The external pH (4.5) never changed during cell growth (results not shown).

Figure 1 Growth of the K310 yeast strain in modified YPD medium containing 10% (w/v) glucose, and variations in the levels of some metabolites during growth

The yeast was grown as described in the Experimental section. Growth (▲) was followed turbidimetrically (A). Glucose (●) and ethanol (■) in the culture (A) and intracellular glycogen (▲) and trehalose (□) (B) were measured as described in the Experimental section. Glycogen and trehalose concentrations are expressed in mg/g cells (dry weight). The results shown are the means of three experiments performed in duplicate.

In parallel, glycogen began to accumulate in yeast cells in the late exponential phase, reached a maximum at the beginning of the stationary phase, and then decreased. Intracellular trehalose did not begin to accumulate until the glucose concentration in the medium had dropped to approx. 4 g/l; it increased rapidly during the late exponential phase and remained stable during the next 18 h (Figure 1B).

Monitoring of growth and of the glucose concentration, as well as the concentrations of glycogen and trehalose (used as hallmarks of changing nutritional conditions), indicated that the stages of the cell life that we analysed by proteome comparison corresponded to the exponential phase (16 h) and the very early stationary phase (44 h). The typical presence of Ssb1, Ura7 and Asc1 in the 16 h 2D map (see below) particularly underlines that peculiar moment of cell life, since these proteins are induced in

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Table 1 Identified proteins present at 16 h of culture time but repressed following glucose exhaustion

Data for spot volumes are relative to the total volume of all spots in the gel.

Gene	Relative volume of spot (%)			Type of analysis	Cellular function	Repressed under stress conditions
	16 h gel	44 h gel	Decrease at 44 h (fold)			
<i>SSE1</i>	0.359	0.143	2.5	Gel matching; MS	Protein folding; heat shock	
<i>STI1</i>	0.095	0.035	2.7	Gel matching; MS	Protein folding; heat shock	
<i>SSB1</i>	0.573	0.189	3.0	Gel matching; MS	Protein folding; heat shock	
<i>HSP82</i>	0.081	0.028	2.9	Gel matching; MS	Protein folding; heat shock	Cold shock
<i>KAR2</i>	0.092	0.043	2.0	Gel matching; MS	Protein folding; heat shock	Cold shock
<i>ALD6</i>	0.119	0.048	2.5	MS	Carbohydrate metabolism	
<i>GPP1</i>	0.448	0.296	1.5	Gel matching; MS	Carbohydrate metabolism	
<i>PFK1</i>	0.086	0.038	2.3	Gel matching; MS	Carbohydrate metabolism	
<i>PFK2</i>	0.117	0.067	2.4	Gel matching; MS	Carbohydrate metabolism	
<i>ENO1</i>	0.057	0	Present	MS	Carbohydrate metabolism	
<i>URA7</i>	0.104	0.040	2.6	MS	Protein synthesis	Heat shock
<i>EFT1</i>	0.429	0.197	2.2	Gel matching; MS	Protein synthesis	Oxidative stress
<i>ASC1</i>	0.825	0.514	1.5	Gel matching	Protein synthesis	Heat shock; hyperosmotic shock, cadmium stress
<i>YEF3</i>	0.070	0.030	2.3	MS	Protein synthesis	
<i>RPL22A</i>	0.134	0.064	2.0	MS	Protein synthesis	
<i>FRS1</i>	0.086	0.036	2.4	MS	Protein synthesis	
<i>RPL5</i>	0.108	0.057	1.9	MS	Protein synthesis	
<i>PAB1</i>	0.206	0.090	2.3	MS	Protein synthesis	
<i>GRS1</i>	0.121	0.051	2.4	MS	Protein synthesis	
<i>RRP6</i>	0.190	0.060	3.2	MS	RNA turnover	
<i>GUA1</i>	0.105	0.058	1.8	MS	Nucleotide biosynthesis	
<i>SPE3</i>	0.091	0.059	1.6	MS	Cell growth	
<i>SAM1</i>	0.068	0.026	2.6	Gel matching; MS	Cell growth	
<i>YBR025C</i>	0.309	0.096	3.2	MS	Unknown	
<i>YIR035C</i>	0.062	0.024	2.6	MS	Unknown	
<i>YIR036C</i>	0.080	0.040	2.0	MS	Unknown	
<i>STR2</i>	0.090	0.012	7.5	MS	Amino acid metabolism	

the early exponential phase and are repressed in the stationary phase and the diauxic shift. On the other hand, hyperexpression of Hsp26 (heat-shock protein 26), Tdh1 and Ubi4 in the 44 h gel (see below) is an indication of a very early diauxic shift, while many typical proteins of the stationary phase are missing from our 2D map [18].

Proteome changes

To evaluate the proteome changes that occur during fermentation, we compared the 2D PAGE maps of the K310 strain grown under different nutritional conditions (culture medium glucose concentration) and extracted at different times during the fermentation. 2D gels were produced with protein extracts collected at 16 h (100 g/l glucose in the culture medium), 22 h (30 g/l) and 44 h (0 g/l) of culture. Only gels at 16 h and 44 h are reported (Figure 2), since the 22 h gels did not differ substantially from those collected at 16 h. Undesirable *in vitro* proteolysis phenomena were evaluated carefully and excluded totally by blocking yeast proteases with high concentrations of inhibitors during cell lysis. Reproducibility was assessed by performing the culture experiments three times, and sets of 12 gels were produced for each collection time; all the gels from the same collection time were completely superimposable. Moreover, additional experiments were carried out with an initial glucose concentration of 200 g/l. The corresponding protein maps were substantially similar to those with 100 g/l glucose (results not shown). Determination of the pI and M_r scales on the gels was performed by gel matching with the calibrated yeast reference gel contained in the Swiss-2D database (<http://www.expasy.ch/ch2d/>). The cali-

brated 16 and 44 h 2D maps were then compared qualitatively and quantitatively.

Out of a total of around 2500 spots, 54 spots were typically present only in the 16 h gel, while 138 appeared only in the 44 h gel. Most of the latter were contained in the lower part of the map, thus corresponding to M_r values lower than 30000. Interestingly, due to the different specificities of dyes, these low- M_r polypeptides were much better visualized by Coomassie Blue staining on the PVDF replicas adopted for microsequence analysis (not shown).

Changes in the intensity of the spots could be recognized by simple visual inspection. For most of the spots, we observed a complete absence from the map at 16 h and, conversely, a presence at 44 h, or vice versa. For only a few spots, we found an increasing or decreasing density in the maps. In this case, a quantitative and comparative evaluation was carried out by laser densitometry, and the relative abundance of individual polypeptides was calculated. Differences by a factor greater than 1.5 between the maps at 16 and 44 h were considered to be significant, taking into account the mild stress conditions under which cultures were carried out; this evaluation was carried out for different culture batches and gel sets.

Identification of the spots was carried out by microsequence analysis, MS and gel matching (a few cases). The results are summarized in Tables 1–3. Gel matching was carried out by comparing our gels with the reference yeast gel of the Swiss-2D database. Both microsequencing and MS analyses sometimes revealed the presence of multiple proteins under the same 2D spot; in these cases, additional letters were used in Table 2 to indicate different protein species.

Table 2 Identified proteins found to be induced at 44 h of culture time

Data for spot volumes are relative to the total volume of all spots in the gel.

Gene	Relative volume of spot (%)			Type of analysis	Amino acid sequence	Cellular function	Induced under stress conditions
	16 h gel	44 h gel	Increase cf. 16 h (fold)				
<i>SOD1</i>	0.013	0.046	3.6	Gel matching		Cell stress	
<i>TSA1</i>	0.022	0.158	7.1	MS		Cell stress	Oxidative stress
<i>CTT1</i>	0.031	0.68	2.2	Gel matching		Cell stress	Osmotic, oxidative stress; heat shock
<i>ZWF1</i>	0.049	0.075	1.5	Gel matching		Pentose phosphate pathway; cell stress	Oxidative stress
<i>HSP26</i>	0.049	0.501	7.0	Microsequencing; MS	Blocked (acetylation)	Protein folding; cell stress	Glucose starvation; heat, ethanol shock
<i>SBA1</i>	0.018	0.045	2.1	Gel matching		Protein folding	Amino acid starvation; oxidative stress
<i>HSP12</i>	0.057	0.118	2.5	Gel matching; MS		Protein folding; cell stress	Heat shock; starvation, oxidative, cadmium stress
<i>CYP5</i>	0.003	0.055	18.3	Gel matching		Protein folding; vesicular transport	Heat shock
<i>UBI4</i>	0.090	0.140	15.5	Gel matching		Protein degradation; cell stress	Heat shock; starvation, oxidative, cadmium stress
<i>VMA4</i>	0.066	0.200	2.1	Gel matching		Vacuole transport	Alkaline shock
<i>PUP3</i>	0.025	0.076	3.0	MS		Protein degradation	
<i>TFS1</i>	0.067	0.231	3.5	MS; microsequencing	Blocked (acetylation)	Protein degradation	Oxidative stress
<i>FBA1</i>	0.026	0.116	4.4	MS		Glycolysis	
<i>GPM1</i>	0	1.532	Present	Microsequencing	P ¹ KLVLVRHG	Glycolysis	
<i>TDH1</i>	0	0.039	Present	Microsequencing; MS	M ¹ IRIAI	Glycolysis; cell stress	Glucose starvation; heat shock; osmotic stress
<i>PDC5</i>	0.012	0.119	9.9	MS		Carbohydrate metabolism	
<i>ENO1</i>	0.344	0.557	1.5	Gel matching		Carbohydrate metabolism	Osmotic and oxidative stress
<i>GPP2</i>	0.086	0.079	0.7*	MS; gel matching		Carbohydrate metabolism	Osmotic and oxidative stress
<i>GUP1</i>	0.022	0.150	3.8	Gel matching; MS		Glycerol transport	
<i>RPL15B</i>	0.095	0.233	2.5	Microsequencing; MS	Blocked (acetylation)	Protein synthesis	
<i>RPS12</i>	0.050	0.093	1.9	MS		Protein synthesis	
<i>HYP2</i>	0.212	0.630	3.0	Gel matching		Protein synthesis	
<i>RPS0A</i>	0.002	0.031	15.5	Gel matching; MS		Protein synthesis	
<i>EFB1</i>	0.037	0.103	2.8	Gel matching		Protein synthesis	
<i>RPS0B</i>	0.007	0.056	8.6	Gel matching; MS		Protein synthesis	Cold shock
<i>IPP1</i>	0.285	0.322	1.5	Gel matching		Phosphate metabolism	
<i>IDI1</i>	0	0.154	Present	MS; gel matching		Lipid, fatty acid and sterol metabolism	
<i>PST2</i>	0.084	0.207	2.5	Microsequencing	P ¹ RVAII	Unknown	Oxidative stress
<i>YKL056C</i>	0.034	0.095	2.7	Gel matching		Unknown	
<i>BMH1</i>	0.074	0.156	2.3	Gel matching; MS		Unknown	Rapamycin stress
<i>BMH2</i>	0.117	0.270	2.3	Microsequencing; MS	Blocked (acetylation)	Unknown	Rapamycin, cadmium stress
<i>YLR301W</i>	0.006	0.038	6.3	MS		Unknown	
<i>SHE1</i>	0	0.046	present	Microsequencing; MS	Blocked (predicted acetylation)	Unknown	

* The change in Gpp2 is shown, although it was lower than 1.5-fold, to allow a comparison with the Gpp1 value in Table 1.

Table 3 Vacuole-attributed (A) and PMSF-insensitive (B) protein fragments

Listed are (A) protein fragments present in the K310 *S. cerevisiae* proteomic map at 44 h of growth (see Figures 2B and 3B), but absent at 16 h or at 44 h following treatment with PMSF, and (B) protein fragments whose presence in the proteomic map at 44 h of fermentation was not affected by treatment with PMSF (see Figures 2B and 3C). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GEF, guanine-nucleotide exchange factor; CDC, cell division control; PSS, phosphoribosylamidoimidazolesuccinocarboxamide synthase.

Spot no.	Analysis for identification	Amino acid sequence found	Protein fragment derived from:	Gene name	Cellular role	Cellular localization of whole protein
(A)						
86	MS		GAPDH 1	<i>TDH1</i>	Glycolysis	Cytoplasm and nucleus
56	MS		GAPDH 1	<i>TDH1</i>	Glycolysis	Cytoplasm and nucleus
88	MS		GAPDH 1	<i>TDH1</i>	Glycolysis	Cytoplasm and nucleus
122a	Microsequencing	T ⁷⁴ YQERDPANL	GAPDH 1	<i>TDH1</i>	Glycolysis	Cytoplasm and nucleus
38	Microsequencing	T ¹⁷⁹ ATQKT	GAPDH 2/3	<i>TDH2/ TDH3</i>	Glycolysis	Cytoplasm and nucleus
9	Microsequencing	T ²⁴² VKLNK	GAPDH 3	<i>TDH3</i>	Glycolysis	Cytoplasm and nucleus
33	Microsequencing	M ⁴² FKYDS	GAPDH 2/3	<i>TDH2/ TDH3</i>	Glycolysis	Cytoplasm and nucleus
34	Microsequencing	M ⁴² FKYDS	GAPDH 2/3	<i>TDH2/ TDH3</i>	Glycolysis	Cytoplasm and nucleus
106	Microsequencing	N ³¹ DPFIT	GAPDH 3	<i>TDH3</i>	Glycolysis	Cytoplasm and nucleus
11	Microsequencing	N ⁶⁴ IALEK	Phosphoglycerate mutase 1	<i>GPM1</i>	Glycolysis	Cytoplasm
122b	Microsequencing	N ⁶⁶ IALEKADRLW	Phosphoglycerate mutase 1	<i>GPM1</i>	Glycolysis	Cytoplasm
99	MS		Phosphoglycerate mutase 1	<i>GPM1</i>	Glycolysis	Cytoplasm
120	MS		Phosphoglycerate mutase 1	<i>GPM1</i>	Glycolysis	Cytoplasm
6	MS		Phosphoglycerate kinase 1	<i>PGK1</i>	Glycolysis	Cytoplasm
24	MS		Phosphoglycerate kinase 1	<i>PGK1</i>	Glycolysis	Cytoplasm
39	Microsequencing	A ⁵⁹ SHLGR	Phosphoglycerate kinase 1	<i>PGK1</i>	Glycolysis	Cytoplasm
98	MS		Phosphoglycerate kinase 1	<i>PGK1</i>	Glycolysis	Cytoplasm
97	MS		Phosphoglycerate kinase 1	<i>PGK1</i>	Glycolysis	Cytoplasm
60	Microsequencing	V ³²⁵ AKAKT	Phosphoglycerate kinase 1	<i>PGK1</i>	Glycolysis	Cytoplasm
41	Microsequencing	D ²⁸⁷ AFSAD	Phosphoglycerate kinase 1	<i>PGK1</i>	Glycolysis	Cytoplasm
16	Microsequencing	A ²²¹ FGNCHGL	Fructose bisphosphate aldolase 1	<i>FBA1</i>	Glycolysis	Periplasm
83	MS		Fructose bisphosphate aldolase 1	<i>FBA1</i>	Glycolysis	Periplasm
89	MS		Enolase 1	<i>ENO1</i>	Glycolysis	Cytoplasm
93	Microsequencing	G ³¹⁴ IQIVADDIT	Enolase 2	<i>ENO2</i>	Glycolysis	Cytoplasm
42	Microsequencing	A ¹ VSKVY	Enolase 2	<i>ENO2</i>	Glycolysis	Cytoplasm
34S	Microsequencing	A ¹¹⁶ ARAAA	Enolase 2	<i>ENO2</i>	Glycolysis	Cytoplasm
81	Microsequencing	G ³¹⁴ IQIVADDIT	Enolase 2	<i>ENO2</i>	Glycolysis	Cytoplasm
81a	Microsequencing; MS	G ³¹⁴ IQIVADDIT	Enolase 2	<i>ENO2</i>	Glycolysis	Cytoplasm
17	Microsequencing	G ³⁴⁹ TLSESIK	Enolase 2	<i>ENO2</i>	Glycolysis	Cytoplasm
57	Microsequencing; MS	A ¹ VSKVY	Enolase 2	<i>ENO2</i>	Glycolysis	Cytoplasm
44S	MS		Enolase 2	<i>ENO2</i>	Glycolysis	Cytoplasm
44	Microsequencing	A ¹ VSKVY	Enolase 2	<i>ENO2</i>	Glycolysis	Cytoplasm
58	Microsequencing; MS	A ¹ VSKVY	Enolase 2	<i>ENO2</i>	Glycolysis	Cytoplasm
45	MS		Glucose-6-phosphate isomerase	<i>PGI</i>	Glycolysis and gluconeogenesis	Cytoplasm
46	MS		Glucose-6-phosphate isomerase	<i>PGI</i>	Glycolysis and gluconeogenesis	Cytoplasm
81b	Microsequencing; MS	Blocked (acetylation)	Pyruvate decarboxylase isoenzyme 1	<i>PDC1</i>	Carbohydrate metabolism	Cytoplasm
121	Microsequencing; MS	Blocked (acetylation)	GEF for Ras1p and Ras2p	<i>CDC25</i>	Carbohydrate metabolism	Plasma membrane
37	Microsequencing; MS	P ¹ SHFDT	Met17 protein	<i>MET17</i>	Amino acid metabolism	Cytoplasm
24a	MS		Met17 protein	<i>MET17</i>	Amino acid metabolism	Cytoplasm
106	MS		Met17 protein	<i>MET17</i>	Amino acid metabolism	Cytoplasm
59	MS; microsequencing	Blocked (unknown modification)	Elongation factor 1 α	<i>TEF1</i>	Protein biosynthesis	Cytoplasm
53	MS		Heat-shock protein Ssa1	<i>SSA1</i>	Protein folding; heat and oxidative stress response	Cytoplasm and nucleus
87	MS		Heat-shock protein Ssa1	<i>SSA1</i>	Protein folding; heat and oxidative stress response	Cytoplasm and nucleus
(B)						
21	MS		D,L-Glycerol-3-phosphatase	<i>GPP1</i>	Glycogen metabolism	Cytoplasm
36a	Microsequencing; MS	Blocked (possible acetylation)	PSS	<i>ADE1</i>	Nucleotide metabolism	Unknown
85	MS		Glycolytic gene transcriptional activator Gcr1	<i>GCR1</i>	Glycolytic enzyme gene expression regulation	Nucleus (probable)
13	Microsequencing	D ⁸⁵⁹ VTGQP	CDC protein 15	<i>CDC15</i>	Mitosis	Nucleus
36b	Microsequencing	E ⁴³⁴ FDTGN	Putative Cox1/Oxi3 intron 2 protein	<i>A12</i>	Mitochondrial membrane system	Mitochondria

On the basis of their identification, the protein spots representing the K310 proteomic response to fermentation stresses were divided into three classes: repressed proteins, induced proteins and autoprolysed proteins. For each class, a further functional classification is reported in Tables 1–3.

In *S. cerevisiae*, the breakdown of autophagic bodies in the vacuole is dependent on vacuolar proteinase B. Therefore the addition of the proteinase B inhibitor PMSF to starving wild-

type cells leads to the accumulation of autophagic bodies and impairment of vacuole activity [10]. We carried out parallel cultures in which PMSF was added at 36 and 40 h of cell growth in order to prevent *in vivo* proteolysis due to vacuole activity, and the corresponding proteomes were analysed. When PMSF was added at 36 h, significant inhibition of autodigestion was observed (Figure 3C), while addition at 40 h did not have such an effect (results not shown), probably because this time is too late

Figure 3 Specific contribution of the vacuole to the protein autoproteolytic process in yeast K310 strain

Low- M_r details of silver-stained 2D PAGE proteomic maps of K310 at 16 h (A) and 44 h (B) of growth, and at 44 h after the addition of PMSF (C). The digitalized images were obtained by laser densitometry (4000 × 5000 pixels; 12 bits/pixel) and analysed using Melanie II 2D PAGE software on a Sun workstation. Circles in (B) indicate protein fragments that are part of the autoproteolytic process. Protein spots still present after treatment with PMSF are circled in (C).

with respect to the beginning of the vacuole and does not affect proteasome activity. Reproducibility was again assessed by repeating culture experiments three times, and sets of 12 gels were produced for each collection time. Since PMSF is a specific inhibitor of vacuole functionality, we hypothesized that the polypeptide fragments still present in the 44 h gel following PMSF addition were derived from proteins digested by the

proteasome (Figure 3C). On the other hand, polypeptides that disappeared from the 44 h map after treatment with PMSF can be assumed to be proteolysed by the vacuole. The identification of polypeptides, through microsequencing and/or MS, allowed their classification into PMSF-sensitive (vacuole-attributed; Table 3A) and PMSF-insensitive (proteasome-attributed; Table 3B) protein fragments. Protein spots reported in Table 3 were not

detectable either at 16 h or in the presence of PMSF, whereas they were quantitatively appreciable in the 44 h gel.

DISCUSSION

We report here the first proteomic characterization of a wine *S. cerevisiae* strain during its response to stress conditions that were presumed to be similar to those occurring during winery fermentation. The monitoring of several parameters during yeast growth confirmed that the observed response was due to glucose exhaustion and to a mild ethanol shock. Since the cultures were carried out at a constant 25 °C temperature and under semi-aerobiosis, we can exclude the occurrence of heat/cold and oxidative and anaerobic stresses respectively. The pH value was measured throughout growth, and no variation from the value of 4.5 was observed, indicating that no change in ion composition that might cause shock occurred.

The comparison of proteomes of the K310 strain in the exponential growth phase and the very early stationary phase revealed that its adaptive response to glucose exhaustion involves three mechanisms: underexpression, overexpression and auto-digestion of cell proteins.

Proteins repressed at the time of glucose exhaustion

Of the 54 proteins whose levels decreased more than 1.5-fold between 16 and 44 h of culture, 27 were identified unambiguously. They belong mainly to three functional classes: (i) protein folding and cell stress, (ii) protein synthesis and RNA turnover, and (iii) carbohydrate metabolism. The greatest number of these proteins are involved in protein synthesis, cell growth and RNA metabolism. Their down-regulation is thought to reflect growth arrest that occurs during stress; this phenomenon is necessary to save energy and to allow the cell to adapt to new conditions. Three proteins, Ybr025C, Yir035C and Yir036C, have unknown functions. None of these proteins has been found previously to be underexpressed under nutrient withdrawal conditions.

Four of the identified proteins, Eft1, Asc1, Hsp82 and Kar2, all of which are involved in protein synthesis/folding, have been reported to be repressed under heat/cold, oxidative or hyper-osmotic stress [5,19–22], but none of them has been associated previously with glucose starvation. Sti1 and Sse1 can also be associated with this group, since they function as co-chaperonins [23] together with Hsp82. This finding supports the partial cross-responsiveness of eukaryotes to different types of stress [24,25]. The markedly decreased protein abundance of the protein synthesis/folding and cell growth gene products is also related to the control exerted by the nutritional status of the cell, particularly glucose starvation, on the yeast's biosynthetic apparatus [18]. Moreover, Ssb1 and Asc1 are known to be induced by the early exponential phase and repressed by the diauxic shift [26]. For Sse1, Sti1, Eft1, Asc1 and Pfk1, a decreased abundance was reported when growth was shifted from a glucose medium to an ethanol one [20], similar to our present observations. Although we adopted a rich medium, analogous conditions probably occurred after 44 h of K310 culture, following fermentation progress, when the glucose concentration was zero and that of ethanol was 33.6 g/l.

The carbohydrate metabolism-related proteins Gpp1, Pfk1 and Pfk2 were found to be underexpressed in the present study. Gpp1 is one of two highly conserved D,L-glycerol-3-phosphatase isoenzymes that control osmoregulation in *S. cerevisiae* through glycerol production, and different roles of these proteins in normal and stressed cells have been proposed [27,28]. We found repression of Gpp1 and a slight induction of Gpp2 during K310

growth under semi-aerobic conditions, thus confirming differential expression of this phosphatase depending on cell status. Moreover, in addition to other types of gene transcription regulation [28], Gpp1 expression may be controlled by cellular protein degradation, since a fragment of this isoenzyme was observed at the time of glucose exhaustion, when ethanol production during fermentation was maximal (Figure 3 and Table 3B).

Pfk1 and Pfk2 are both required for phosphofructokinase activity, being key regulators of glycolytic flux. Unlike what we observed in the K310 wine strain, it has been reported previously that phosphofructokinase is induced under aerobic conditions in budding yeast and regulates some periodic metabolic oscillations, which may be correlated with oscillations in cellular stress resistance [29]. The repression of the synthesis of both isoenzymes reported in our present study may be related to the glucose shortage, and it could be a peculiarity of the wine strain's response to gradual glucose starvation.

Proteins induced at the time of glucose exhaustion

We identified 33 proteins whose synthesis was induced during fermentation in culture medium. A classification by cellular function allowed us to group them into the following functional categories: (i) protein folding and cell stress, (ii) carbohydrate metabolism, (iii) protein synthesis and (iv) protein degradation. Six proteins, i.e. Pst2, Ykl056C, Bmh1, Bmh2, She1 and Gup1, have unknown cellular functions.

Although related to other stress responses, Ctt1, Zwfl, Pst2, Tfs1, Sod1, Rps0b, Vma4, Sba1, Bmh1, Bmh2, Ubi4, Cyp5, Eno1 and Gpp2 have never been reported previously to be induced by glucose shortage, and they can be attributed to the specificity of the K310 strain, or at least of wine *S. cerevisiae*. The growth conditions and monitoring of parameters adopted in the present work seem to exclude the possibility that the observed response was due directly to stresses other than glucose exhaustion and mild ethanol shock [4% (v/v) at 44 h of fermentation]. A significant cross-responsiveness is a remarkable feature of the cell reaction to stress, and resistance to one type of shock is known to induce a certain tolerance to other kinds of stresses. This has important implications when yeast strains are used, as in our case, for vinification of high quality wines.

Many of the proteins that were induced at 44 h are part of a response against oxidative stress. Under semi-aerobiosis, Cct1 (catalase T) can protect cells from the oxidizing effects of ethanol [30], exerting this action in synergy with the co-expression of Sod1 (superoxide dismutase), as has been observed for resistance to paraquat. Hsp26, Pst2, Sba1, Eno1, Tfs1, Ubi4, Rps0b and Tsa1 [5] are also overexpressed under conditions of H₂O₂ oxidative stress. Zwfl overproduction is also protective against oxidation, since it is required for NADPH production by the oxidative part of the pentose phosphate pathway [6]. Nevertheless, we found increases in Hyp2, Ykl056c, Efb1 and Asc1 levels, which instead have been reported to be repressed or unaffected by H₂O₂ treatment. Moreover, Gpp2 has not previously been reported to be induced by oxidative stress, while Ssa1, which should be overexpressed in the presence of H₂O₂, was found to be repressed in our study. This implies that the oxidative stress that we observed differs from drastic hydrogen peroxide shock, thus indicating a differential reaction under semi-anaerobic conditions, in which the K310 response is probably directed mainly towards the oxidizing effects of ethanol.

In the second group, the induced proteins Ctt1, Tdh1, Rps0b, Eno1 and Gpp2 form part of a response to hyperosmotic stress.

This is consistent with repression of Asc1. Again we suggest that this adaptation is related to a progressive increase in osmolarity throughout ethanol fermentation [31]. Hyperexpression of Hyp2, Rps0a and Rps0b, whose abundance is, in contrast, markedly decreased upon heat shock [19], shows that the ethanol/heat shock cross-response is not total, at least for the K310 wine strain.

Regarding proteins involved in carbohydrate metabolism, we observed a consistent synthesis of the two glycolytic enzymes enolase and glyceraldehyde-3-phosphate dehydrogenase, in agreement with Boucherie [4]. There was also an increase in Zwfl, which indicates the use of the pentose phosphate pathway as an alternative sugar metabolism pathway to glycolysis.

Overall, the proteomic adaptation of K310 to glucose exhaustion seems to be directed mainly against the effects of ethanol, including both hyperosmolarity and oxidative responses. Ethanol stress has been associated with oxidative lesions through the increased synthesis of oxygen free radicals. Many of the cytotoxic effects of ethanol seem to be due to acetaldehyde production. In fact, Sod1 is one of the most important enzymic defences against this type of stress [32]. Moreover (and consistent with our results), ethanol induces both heat-shock proteins and trehalose to stabilize membranes and proteins and to suppress protein aggregation respectively [33]. High intracellular levels of trehalose, which increase rapidly during exponential growth of yeast cells, stabilize dry membranes in anaerobic organisms, providing resistance to dehydration and tolerance to ethanol stress [32]. Both the ethanol and glucose starvation responses observed in K310 only partially reflect what has been reported in previous studies with baker's yeast.

Autodigested proteins

Some of the proteins that were induced at 44 h of fermentation are involved in intracellular proteolysis. Bmh1, Bmh2, Eno1 and Gpm1 are proteasome-interacting proteins, and thus can be considered as potential substrates or regulators of protein autodigestion [34]. Ubi4 and Tfs1 play a role in protein degradation, and Sod1 is involved in vacuole biogenesis [35]. Pup3 is a component of the 20 S proteasome. Moreover, Vma4 is a vacuolar ATPase involved in maintaining the acid environment that is necessary for vacuole functionality [36].

Most of the protein spots appearing in the 2D map when glucose was completely exhausted represent protein fragments derived from intracellular proteolysis. Autodigestion processes are known to occur following several types of cell stress, mainly to enrich the cytoplasmic amino acid pool or to contribute to turnover by providing protein building blocks, and both the vacuole and the proteasome contribute to this stress response through ubiquitination [37]. Nevertheless, the peptide composition of these protein fragments has not been examined previously. We determined their nature by microsequencing and MS (Tables 3A and 3B).

It is noteworthy that many protein fragments are parts of single isoforms of glycolytic enzymes, which suggests a possible role of intracellular proteolysis in regulating the abundance of specific isoenzymes. For example, most enolase peptides were derived from Eno2, which is known to be down-regulated by ethanol. Similarly, Gpm1 and Gpp1 were degraded, whereas Gpm2 and Gpp2 were induced.

Through the use of a wild-type *S. cerevisiae* strain and PMSF, a specific inhibitor of vacuolar proteinase B [10,38], we could also distinguish the specific contributions of the vacuole and proteasome to the autoproteolytic process.

Vacuole-proteolysed proteins

A general observation is that the vacuole seems to have a quantitatively greater role than the proteasome in the adaptive response of *S. cerevisiae* to physiological stresses. This observation is consistent with other reports [39] that, under starvation conditions, 85% of overall protein degradation depends on the activity of the vacuolar proteinases.

When a functional classification is carried out, a striking feature of the vacuole-attributed protein fragments is the predominance of enzymes involved in carbohydrate metabolism, particularly glucose metabolism; this indicates that autodigestion is directed mainly towards those enzymes whose primary substrate is lacking. Although our analysis is not exhaustive but only indicative, the observation that almost all the polypeptides analysed were derived from proteins of the glycolytic pathway conflicts partially with the overall aspecificity of vacuole bulk autophagocytosis, since a greater variety of fragmented proteins would have been expected. The abundance of protein types [8] only partially agrees with what was observed, since the digested proteins are very abundant in the cytoplasm in some cases (Eno2, Fba1), but to a lesser extent in other cases (Ssa1, Met17, Pdc1). Moreover, no peptide was found to be derived from other very abundant cytosolic proteins with roles other than in the glycolytic pathway.

A detailed analysis of the functional nature of single processed proteins seems to suggest that their proteolysis may not be random, and that a metabolism-related explanation can be tentatively suggested. The *MET17* gene encodes the bifunctional *O*-acetylserine/*O*-acetylhomoserine sulphydrylase; this is the only enzymic activity responsible for the incorporation of reduced sulphide in *S. cerevisiae*, since the trans-sulphuration pathway is the only one by which cysteine is synthesized in this yeast [40]. Met17 levels can vary greatly from strain to strain [41]. It has recently been reported that Met17 overactivity is related not to increased cysteine production, but to H₂S production and release via a pathway whose regulation is highly stress-specific [42]. H₂S is a by-product of yeast alcoholic fermentation and can give the final industrial products an undesirable smell. Therefore yeasts used for wine production should not produce high concentrations of H₂S, and this can be an important selection criterion for strains with biotechnological applications. The K310 strain was selected on the basis of the organoleptic features of the wine it produces; thus it produces only trace amounts of H₂S. Nevertheless, several environmental factors have been implicated in the appearance of H₂S, e.g. nutrient limitation, particularly nitrogen withdrawal, which in turn can be a consequence of ethanol stress, similar to what we observed. On the basis of what was reported previously [42], we can hypothesize that fermentation stress can lower Met17 activity in the K310 strain due to substrate shortage and, as for the glycolytic enzymes, regulation of its cytoplasmic level can be achieved through proteolysis.

The abundance of Tef1 (elongation factor 1 α) was reported to decrease by 10% when the cell passes from glucose to ethanol as a carbon source [43]. Interestingly, the synthesis of Tef1 is induced by Gcr1 (glycolytic genes transcriptional activator), and both were degraded under the fermentation stress in the present study; this suggests a parallel down-regulation of protein synthesis under critical conditions.

Ssa1 is a cytoplasmic chaperone and heat-shock protein of the Hsp70 family that is involved in protein folding and translocation; together with Ssa2, it is required for cytoplasm-to-vacuole targeting, but not for the autophagocytic pathway. The abundance of Ssa1 decreases following the diauxic shift and glucose starvation [16].

When a classification on the basis of cellular location is considered, almost all of the proteins degraded by the vacuole appear to be cytosolic proteins. The exception is Cdc25. Cdc25, a guanine-nucleotide exchange factor for Ras1p and Ras2p, is an unstable, short-lived plasma membrane protein of low abundance. Its proteolysis by vacuolar proteinase B provides new information, since this vacuolar activity was considered to be directed towards long-lived cytoplasmic proteins [39]. Degradation by the vacuole of plasma membrane proteins, particularly stress-stimulated permeases, is mediated by ubiquitination [38] and regulates the cytoplasmic levels of proteins no longer necessary during starvation. This provides an example of vacuole specificity. Five lysine residues accessible for ubiquitin conjugation within the first or last 31 amino acids at the N- or C-terminus are necessary for such plasma membrane proteins to be degraded by the vacuole [38]. Interestingly, Cdc25 has seven lysine residues in its C-terminal sequence. Similarly, Tef1, although cytoplasmic, has eight lysine residues in its C-terminus.

Proteasome-proteolysed proteins

Although the proteasome is the predominant site of protein breakdown in mammalian cells, it appears to play a more restricted role in yeast [38]. The present work also indicates that the proteasome has a limited role in stress-induced autoprotoleolysis in *S. cerevisiae*. We found only five protein fragments derived from proteasomal proteolysis whose production was unaffected by PMSF treatment. This may also be due to the fact that the proteasome does not produce intermediary polypeptides that are visible on 2D gels, but cleaves proteins into very short peptides or single amino acids, thanks to trypsin-like activity [44,45].

A shared feature of these proteins is that they are all low-abundance molecules. Another common feature is that they are mainly short-lived proteins, since PMSF does not inhibit the proteasome or the breakdown of long-lived proteins [38]. Unlike the vacuole-proteolysed proteins, the proteasome-attributed protein fragments belong to molecules also located in the nucleus and mitochondria, re-inforcing the idea of the diverse activities of these organelles.

Role of proteolysis

Autophagy does not exclude the existence of other, more specific, mechanisms for the uptake of certain cytoplasmic proteins into the vacuole, as described for the enhanced transport of some proteins to the lysosome in cells of higher eukaryotes under starvation stress [46].

We cannot exclude the possibility that we observed intermediary products that also occur under standard conditions but are usually degraded rapidly to amino acids and consequently are not visible in 2D gels. The high rate of alcoholic fermentation of oenological strains may reduce or inhibit this degradation, allowing the accumulation and observation of partially proteolysed proteins.

Greater knowledge of stress-induced protein processing in *S. cerevisiae* can influence its biotechnological applications. Cell changes of *S. cerevisiae* during fermentation (autoprotoleolysis) and aging (autolysis) give wine its specific organoleptic properties [47]. The amount of nitrogen in autolysates and the concentrations of free amino acids differ significantly according to strain, which can markedly affect the composition and quality of the final product [48].

A role for proteolytic enzymes has been suggested in the release of nitrogen compounds before and during the yeast

autolysis process under wine manufacturing conditions. In fact, in addition to amino acids, polypeptides of M_r 10 000–16 000 and peptides with M_r s lower than 10 000 are also released. These are transformed further into smaller peptides by enzymes released by the yeast, which in turn modify the taste of the wine [30]. Moreover, under conditions of nutrient limitation and fermentation, wine *S. cerevisiae* can release amino acids into the medium that are derived from protein processing or from *de novo* synthesis from ethanol. It has been proposed that the yeast cell is able to use amino acids not only as nitrogen sources but also as redox agents to balance the oxidation–reduction potential under critical environmental conditions [49].

Conclusions

In conclusion, we have evaluated the overall adaptive response of a wine *S. cerevisiae* strain to a global set of stresses that occur physiologically during progressive fermentation. The mild nature of these shocks induced a mild but complex response. The yeast cell progressively encountered stresses derived mainly from a high glucose concentration, glucose exhaustion and a high ethanol concentration, leading to mild hyperosmolarity and oxidative shock. A good wine yeast strain must have the ability to overcome such stresses. The present study was conducted on a single wine *S. cerevisiae* strain, and the research should be extended to other fermentation starters. Indeed, it should be underlined that the stress response is always strain-specific [6,41,43]. K310 is a naturally selected wine strain of *S. cerevisiae* that has been adapted to grow under winery fermentation stress conditions; thus it provides a natural model in which to analyse gene expression or proteome changes.

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