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Stress responsive proteins of a flor yeast strain during the early stages of biofilm formation

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ABSTRACT

Flor yeasts metabolism configures organoleptic properties in Sherry type wines during biological aging. Along this process, yeasts form a biofilm known as *flor* velum, being the presence of certain stress proteins essential for survival and making them excellent organisms for stress response investigations. In this study, an OFFGEL fractionator coupled to LTQ Orbitrap XL MS equipment was used to identify in a flor yeast strain, the maximum possible number of stress proteins under biofilm formation conditions (BFC) and under non-biofilm formation conditions (NBFC), used as reference. A total of 85 stress response proteins have been detected in a higher content than in the reference condition of which 18 are involved in the response to the lack of fermentable carbon source, 39 in the response to the ethanol stress, 3 in the acetaldehyde resistance and 46 in the oxidative stress response. Three proteins of these 85 were found to respond to three different stresses (Por1p and Tps2p in lack of fermentable carbon source, resistance to ethanol and resistance to oxidative stress; and Tkl1p in the resistance to ethanol, resistance to acetaldehyde and resistance to oxidative stress). Results of this study might lead to the genetic improvement of flor yeast strains.

1. Introduction

In some wine-producing regions around the world: California (USA), Sardinia (Italy), Jura (France), Jerez and Montilla-Moriles (Spain), among others, biological aging method is used for the elaboration of some special white types of wine known as Sherry wines. During this process, the final organoleptic properties of the oenological product are modified principally due to the metabolism performed by peculiar yeast strains, so-called "flor yeasts" [1,2].

Flor velum formation takes place after fermentation when flor yeasts become predominant [3]. In this moment, the medium is characterized by very low sugar and high ethanol content that result from a previous fermentative metabolism and an oxidative stress coming from a non-fermentable carbon respiratory metabolism developed by the yeasts when fermentation is finished. In addition, high acetaldehyde levels are achieved during biological aging, being well known to be more toxic than ethanol for yeasts [4]. In order to survive in the mentioned harsh environmental conditions, flor yeasts have the capacity to develop biological systems where proteome plays an important role [1,5,6]. For instance, formation of the biofilm appears to be an adaptive mechanism because it ensures access to oxygen and therefore permits continued growth on non-fermentable carbon sources [7]. Proteins like Flo11p, by increasing surface hydrophobicity [8], Hsp12p found to be essential to the biofilm formation [9] and Ccw7p which exerts molecular reorganization of the cell wall during stress adaptation [10]; all contribute to the biofilm development. On the other hand, high ethanol and acetaldehyde content together with the oxidative stress also provokes a proteomic stress response. Alcohol dehydrogenase I and II isoenzymes have a role in the redox balance maintenance during hostile biofilm formation conditions [11] and Hsp12p both respond to ethanol and acetaldehyde high concentration [12] meanwhile superoxide dismutases (Sod1p and Sod2p) are known to be implicated in the response to ethanol and oxidative stresses [13].

Nowadays, genes that codify stress response proteins have been used as targets for the genetic improvement of wine yeast strains to enhance the fermentation performance [14]. Also, in the biological aging process, a higher quality in velum and a higher cell viability have been attained by the overexpression of *FLO11*, *SOD1*, *SOD2* and *HSP12* genes [15–17]. Certainly, another proteins involved in

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Fig. 1. Formation of thick flor velum biofilms by *Saccharomyces cerevisiae* G1 at 29th day from the inoculation. It can be observed a very thin layer of yeast cells settled in the bottom of the Erlenmeyer flasks [11].

the response to one or various stresses at the time, will constitute attractive targets for overexpression in order to improve winemaking, ethanol production and other fermentative processes.

In the present study, proteomic response of a flor yeast strain to the stresses present during the first stage of biofilm formation have been characterized. This knowledge alongside that provided by experiments aimed to definitely confirm the necessity of these proteins synthesis in the biological aging, may serve for the selection of target genes in order to construct flor yeast strains by genetic engineering and hence prolongs in time the flor yeast viability and as consequence flor endurance in the biological aging process for Sherry wine production.

2. Materials and methods

2.1. Microorganism and cultivation conditions

Saccharomyces cerevisiae G1 (ATCC: MYA-2451), a wild type of industrial wine flor yeast from the Department of Microbiology (University of Cordoba, Spain) collection was used in this work. *S. cerevisiae* G1 produces a thick flor velum about 30 days after inoculation with a cellular viability higher than 90% and a negligible proportion of sediment cells in the bottom of flasks (Fig. 1, [11]).

Cultivation conditions: Biofilm formation condition (BFC) was performed in a biofilm formation medium (0.67% w/v YNB without amino acids, 10 mM glutamic acid, 1% w/v glycerol and 10% v/v ethanol) incubated at 21 °C w/o shaking for 29 days. Non-biofilm formation condition (NBFC) was developed in a non-biofilm formation medium (0.67% w/v YNB w/o amino acids, 10 mM glutamic acid, and 17% w/v glucose) incubated at 21 °C under gentle shaking for 12 h, used as a reference. Experiment was carried out using eleven 250 mL flasks with 250 mL BFC medium and three 500 mL flasks with 250 mL NBFC medium. All flasks were sterilized and stoppered with hydrophobic cotton. A concentration of 1×10^6 cells/mL was inoculated to each flask. Cell viability was carried out on plating in YPD agar (1% yeast extract, 2% peptone, 2% glucose and 2% agar).

2.2. Proteome analysis

Sampling time was chosen, at 29 days from BFC and 12 h from NBFC, when yeast cells were in exponential phase of growth, cellular viability higher than 90% and the number of expressed proteins was high [11,18–20]. At day 29th yeast cells are in the initial phase of velum formation (Ph I) and the biofilm is completely formed under the air–liquid surface (BFC) [21].

All cells from the NBFC cultures were harvested by centrifugation (4500 × *g* during 10 min, Rotina-38 apparatus) and washed in sterile distilled water at 4 °C. Cells from the BFC cultures were collected by a suction system from the surface of each Erlenmeyer flask once the velum was fully formed and then centrifuged and washed in the same conditions than cells from NBFC. In both conditions, for triplicates, three aliquots for proteomic analysis were carried out.

Proteome analysis from sub-cellular fraction of the *S. cerevisiae* flor strain using 3100 OFFGEL Fractionator (Agilent Technologies, Palo Alto, CA) and an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a nano LC Ultimate 3000 system (Dionex, Germany) for protein identification was previously reported in detail by Moreno-García et al. [5,6,22].

A database search was performed with Proteome Discoverer 1.0 (Thermo Fisher Scientific Software, San Jose, CA. USA) against Uniprot including fixed modification (*viz.*, carbamidomethylation in Cys) and proteome results were statistically analyzed with the same software. The score of proteins was calculated by summing Xcorr values from each peptide. After identification, proteins involved to stress responses (lack of fermentable carbon source, ethanol, acetaldehyde and oxidative stress) were selected and sorted from both conditions attending to mutant phenotypes, gene ontology terms (GO terminology by using the Gene Ontology section on the *Saccharomyces* Genome Database; http://www.yeastgenome.org) or references using YeastMine tool from SGD and Uniprot databases. Also, a search has been carried out for proteins involved in the response to other different stresses through their genetic mutant phenotypes.

The amounts of proteins measured under both types of conditions were compared via the exponentially modified protein abundance index (emPAI; [23]), which is calculated as follows:

$$\text{emPAI} = 10^{\text{PAI}} - 1$$

The PAI value for each specific protein was obtained by dividing the number of peptides observed (with provision for charge state and missed cleavages) into that of observable peptides, which was taken by using the software MS Digest (http://prospector.ucsf.edu/). Fragmentation spectra matching the same peptide sequence but with different charge or modification state, or containing a maximum of 3 missed cleavage sites, were counted separately. Protein relative contents under each condition were calculated from:

$$\text{Protein content (weight \%)} = \frac{\text{emPAI} \times \text{Mr}}{\Sigma(\text{emPAI} \times \text{Mr})} \times 100$$

where Mr is the protein molecular weight.

To simplify the discussion, subcellular localization and biological processes in which proteins are involved according to SGD and Uniprot databases, were shown in Tables 1 and 2 in Ref. [24]. Further, the tool "GO Term finder" from SGD was used to determine the *p*-value for each annotation, this is the probability or chance of seeing at least "*x*" number of ORFs out of the total "*n*" ORFs in the list annotated to a particular GO term, given the proportion of genes in the whole genome that are annotated to that GO Term. GO Terms with *p*-values lower than 0.1 have been highlighted through Tables 1 and 2 in Ref. [24].

3. Results and discussion

A total of 413 proteins were identified under BFC while 611 under NBFC. Viable cell concentration at 29 days in BFC was $33.24 \pm 12.08 \times 10^6$ cells/mL and $21.76 \pm 1.64 \times 10^6$ cells/mL at 12 h in NBFC. For a better discussion, it has to be mentioned that cells at 12 h under NBFC perform a respire-fermentative metabolism hence can be considered as "semi-anaerobic" condition [18,19].