

# Hsp12p and PAU genes are involved in ecological interactions between natural yeast strains

Damaríz Rivero,<sup>1</sup> Luisa Berná,<sup>2</sup> Irene Stefanini,<sup>3</sup>  
Enrico Baruffini,<sup>4</sup> Agnes Bergerat,<sup>5</sup>  
Attila Csikász-Nagy,<sup>3†</sup> Carlotta De Filippo<sup>3</sup> and  
Duccio Cavalieri<sup>1\*‡</sup>

<sup>1</sup>Department of Neurosciences, Psychology, Drug Research and Child Health, University of Florence, Florence, Italy.

<sup>2</sup>Molecular Biology Unit, Institut Pasteur, Montevideo, Uruguay.

<sup>3</sup>Centre for Research and Innovation, Fondazione Edmund Mach, San Michele all'Adige, Trento, Italy.

<sup>4</sup>Department of Life Sciences, University of Parma, Parma, Italy.

<sup>5</sup>Department of Pathology, Boston University School of Medicine, Boston, USA.

## Summary

**The coexistence of different yeasts in a single vineyard raises the question on how they communicate and why slow growers are not competed out. Genetically modified laboratory strains of *Saccharomyces cerevisiae* are extensively used to investigate ecological interactions, but little is known about the genes regulating cooperation and competition in ecologically relevant settings. Here, we present evidences of Hsp12p-dependent altruistic and contact-dependent competitive interactions between two natural yeast isolates. Hsp12p is released during cell death for public benefit by a fast-growing strain that also produces a killer toxin to inhibit growth of a slow grower that can enjoy the benefits of released Hsp12p. We also show that the protein Pau5p is essential in the defense against the killer effect. Our results demonstrate that the combined action of Hsp12p, Pau5p and a killer toxin is sufficient to steer a yeast community.**

Received 19 January, 2015; accepted 6 June, 2015. \*For correspondence. E-mail [duccio.cavalieri@fmach.it](mailto:duccio.cavalieri@fmach.it); Tel. (+39) 0461 615 153; Fax (+39) 0461650872. Present addresses: <sup>†</sup>Randall Division of Cell and Molecular Biophysics and Institute of Mathematical and Molecular Biomedicine, King's College London, London SE1 1UL, UK; <sup>‡</sup>Computational Biology Department, CRI Centre for Research and Innovation, Fondazione Edmund Mach, Via E. Mach 1, 38010 San Michele all'Adige, Trento, Italy.

## Introduction

Few studies have examined the genetics lying at the basis of cooperation and competition in *Saccharomyces cerevisiae*. Cooperation has been investigated using genetically modified strains to act as cheaters in the *SUC* genes system involved in the metabolism of sucrose (Greig and Travisano, 2004). Nevertheless, a recent work demonstrated that this model is not properly capturing a naturally emerging cooperation–competition relationship (Bozdag and Greig, 2014). Regarding natural competition, the best-known competitive strategy amongst *S. cerevisiae* strains is the killer phenomenon. Killer yeasts secrete extracellular toxins which are lethal to sensitive strains (Schmitt and Breinig, 2006). Nevertheless, little is known about the genes regulating cooperation and competition among *S. cerevisiae* strains in ecologically relevant settings.

To understand the genetics of ecological interactions, we isolated eight yeast strains from a single vineyard and examined the fitness in single-clone strain cultures of these natural isolates in synthetic wine must (SWM) (Landry *et al.*, 2006) simulating their natural environment (Riou *et al.*, 1997). We found great differences in the fitness of the eight strains, thus we performed genome-wide analyses in order to identify the genes involved in the establishment of their coexistence in the vineyard (Sinclair, 2014). The results highlight the existence of an unexpected altruistic behaviour, which mainly depends on a released heat-shock protein, Hsp12p. The altruistic strain also punishes close relatives non-producers by contact-dependent transmission of a killer toxin, while a membrane protein (Pau5p) can provide resistance against this toxin. The reward–punishment loop created by the combined action of Hsp12p, Pau5p and killer toxin is sufficient to steer a yeast community and ensure the survival of a cooperating strain.

## Results

### *Natural yeast strains have different growth rates*

Albeit isolated from the same source and geographical region, different strains of *S. cerevisiae* showed different phenotypes when cultured in conditions mimicking natural must fermentations. We characterized the fitness of eight natural isolates, monitoring the cell density by cell

counting with a Bürker chamber, in SWM. The strains were isolated from Sangiovese grapes collected in the same vineyard, in the Chianti region (Italy), during the years indicated in Table S1. The strains were distinguished by phenotypic and genotypic traits observed after their collection (Casalone *et al.*, 2005; Landry *et al.*, 2006; Stefanini *et al.*, 2012).

When the strains were inoculated at low cell densities ( $2 \times 10^4$  cell ml<sup>-1</sup>), they showed different doubling times, even if all began to grow in the early hours of culturing (Fig. S1 and Fig. 1A). We were indeed able to categorize strains as 'fit' and 'unfit' (showing a short or long doubling time respectively). Albeit the medium (SWM) herein used reproduced the natural conditions, we also considered the coexistence of different strains in the same fermentation to effectively mimic the natural environment. This coexistence could affect the fitness of the single strains, we thus analysed in depth the interaction of the fittest (Sgu165) and of an unfit strain (Sgu421). When the strains were grown in SWM, the growth rate of Sgu421 was lower with respect to that of Sgu165 (Fig. S1 and Fig. 1A). However, a statistically significant improvement of the fitness was evident when the strain Sgu421 was inoculated in a cell-free spent medium of the fittest strain Sgu165 (Fig. 1A). The strain growth differences are condition dependent, being evident only in SWM, not in the rich medium yeast extract peptone dextrose (YPD) in which the strains present almost identical growth rates (Fig. 1B).

In order to study if the effect of the spent medium on the fitness of Sgu421 depends on the Sgu165 cell concentration, we prepared the spent medium by using both high density and low density cultures of this strain (Fig. S2). The spent media were named Sgu165 high-density spent medium (Sgu165\_HDSM) and Sgu165 low-density spent medium (Sgu165\_LDSM) respectively. We observed a recovery of the growth of Sgu421 only when grown in Sgu165\_HDSM (Fig. 1A). We used the high-density spent medium of the same strain (Sgu421\_HDSM) as a control and it failed to contribute to growth recovery (Fig. 1A).

#### *Hsp12p presence in media increases the fitness of natural yeast strains*

Since the fitness variation of Sgu421 is obtained by simply using the spent medium of the Sgu165, we decided to study the molecules, released by Sgu165 and able to improve the fitness of Sgu421. We thus dissected the spent media compositions through mass spectrometry analyses of Sgu165\_HDSM, Sgu421\_HDSM and Sgu165\_LDSM. We did not find significant differences in glucose and metabolite content of Sgu421\_HDSM with respect to Sgu165\_HDSM (Table S2). Since Sgu421\_HDSM does not improve the fitness of the unfit strain, we concluded that glucose or the metabolites are not decisive on the growth

rescue. For this reason, we focused on the comparison of protein content of Sgu165\_HDSM and Sgu165\_LDSM (Table 1 and Table S3). We found cell wall, ribosomal-related and heat shock proteins (HSPs) (Eno1p, Hsp12p, Tdh3p, Ssb1p) in much higher levels in Sgu165\_HDSM compared with Sgu165\_LDSM, with Hsp12p showing the highest ratio (3.9) between the two samples (Table 1 and Table S3). Considering that HSPs act in response to different cellular stresses (Welker *et al.*, 2010), such as those present in our fermentation conditions in SWM, we focused our attention on Hsp12p which shows the largest difference in its level in Sgu165\_HDSM versus Sgu165\_LDSM (Table S3) and is known to be weakly expressed in exponentially growing cultures but is induced several hundred-fold during the entry into stationary phase (Welker *et al.*, 2010).

To test if the Hsp12p present in the medium holds an important role in the modulation of Sgu421 growth, we studied its fitness in a cell-free high density spent medium prepared from Sgu165-*HSP12* deletion mutant (Sgu165\_Δ*hsp12*\_HDSM) (Fig. S2). We did not observe an improvement of the fitness of Sgu421 (Fig. 1C). In addition, we study if the overexpression of *HSP12* modified the fitness of the unfit strain. We observed that Sgu421 transformed with the centromeric plasmid YCp80 containing *HSP12* cloned from the strain Sgu165 improved its growth rate in SWM (Fig. 1D), indicating that Hsp12p holds a very important role in the fitness of a naturally isolated yeast strain.

Since the presence of Hsp12p in wines has been explained by yeast autolysis (Marchal *et al.*, 2011), we performed LIVE/DEAD Analysis in order to understand if the Hsp12p is released by dying cells in high density cultures. Sgu165 showed statistically significant increase in cell death in high-density cultures with respect to the low-density ones, while such density difference had no effect on cell death in Sgu421 or Sgu165\_Δ*hsp12* (Fig. S3). Based on our results, we propose that the releases of Hsp12p are dependent on the amount of dying cells and speculate that it is due to cell lysis.

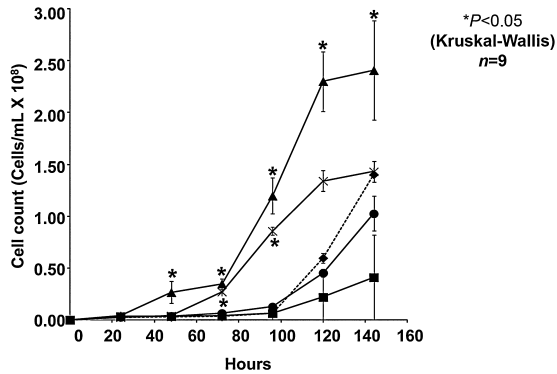
#### *Role of PAU genes in yeast fitness during alcoholic fermentation*

Next, we performed transcriptional analyses, by comparing cells of Sgu421 grown in SWM (Sgu421/SWM) with respect to the same cells grown in Sgu165\_HDSM (Sgu421/Sgu165\_HDSM), searching for genes allowing the Sgu421 fitness improvement in Sgu165\_HDSM. The analysis was done 48 h after inoculation, because at this time, the cultures showed almost the same cell density, right before they started to differ (Fig. 1C). Therefore, we were able to capture differences in growth rates of cells cultivated in Sgu165\_HDSM with respect to those grown

**A**

**Description**  
 Sgu165: fit strain in synthetic wine must (SWM)  
 Sgu421: unfit strain in SWM  
 /SWM: strain grown in SWM  
 /Sgu165\_HDSM: strain grown in the high density spent medium of Sgu165  
 /Sgu165\_LDSM: strain grown in the low density spent medium of Sgu165  
 /Sgu421\_HDSM: strain grown in the high density spent medium of Sgu421

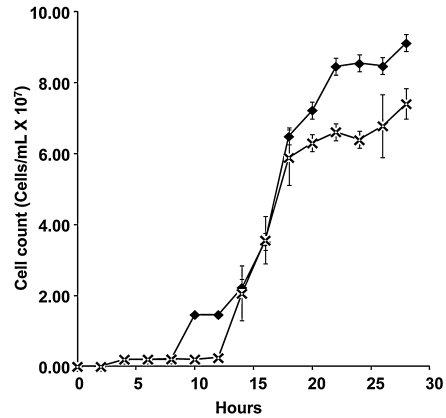
▲ Sgu165/SWM    ✕ Sgu421/Sgu165\_HDSM    ■ Sgu421/Sgu421\_HDSM  
 ◆ Sgu421/SWM    ● Sgu421/Sgu165\_LDSM



**B**

**Description**  
 Sgu165: fit strain in synthetic wine must (SWM) grown in YPD  
 Sgu421: unfit strain in SWM grown in YPD

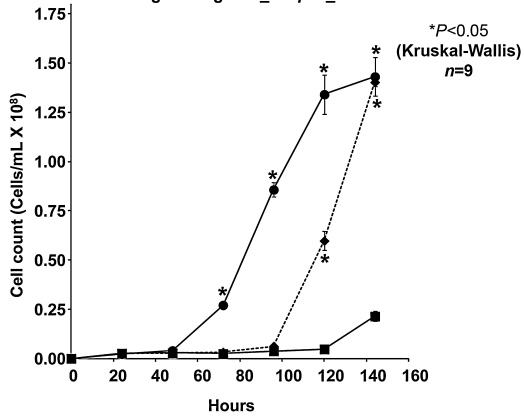
◆ Sgu421    ✕ Sgu165



**C**

**Description**  
 Sgu421: unfit strain in SWM  
 /SWM: strain grown in SWM  
 /Sgu165\_HDSM: strain grown in the high density spent medium of Sgu165  
 /Sgu165\_Δhsp12\_HDSM: strain grown in the high density spent medium of the deletion mutant of HSP12

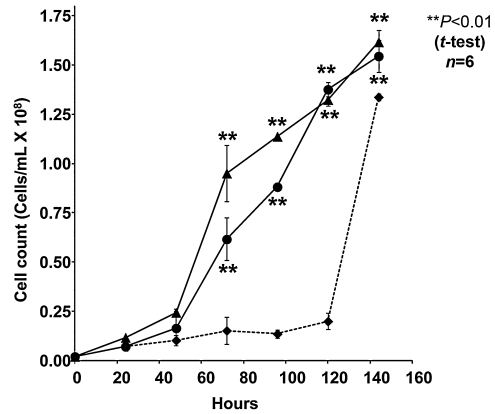
◆ Sgu421/SWM    ● Sgu421/Sgu165\_HDSM  
 ■ Sgu421/Sgu165\_Δhsp12\_HDSM



**D**

**Description**  
 Sgu421-YCp80-HSP12: unfit strain in SWM transformed with the plasmid YCp80 containing the HSP12 gene  
 Sgu421-YCp80-PAU5: Sgu421 transformed with the plasmid YCp80 containing the PAU5 gene  
 Sgu421-YCp80: Sgu421 transformed with the plasmid YCp80 (negative control)

▲ Sgu421-YCp80-HSP12    ● Sgu421-YCp80-PAU5    ◆ Sgu421-YCp80



Strain	Hours						
	0	24	48	72	96	120	144
Sgu421/SWM	2.0±0.155	260±8.84	281±19.4	325±1.77	624 ±5.30	5975±476	14000±198
Sgu421/Sgu165_HDSM	2.0±0.0546	247±10.6	407±194	2710±74.2	8570 ±354	13400±990	14300±990
Sgu421/Sgu165_Δhsp12_HDSM	2.0±0.132	268±17.7	289±10.6	252 ±24.7	379 ±19.4	478±32.1	2140±257

**Fig. 1.** Growth rate of different yeast strains. Overnight cultures of the indicated strains were diluted; (A) into fresh SWM at  $2 \times 10^4$  cell/ml and grown for 6 days. Sgu421 was also diluted in cell-free Sgu421 HDSM, Sgu165\_HDSM and Sgu165\_LDSM at the same cellular concentration; (B) into fresh YPD at  $2 \times 10^4$  cell/ml and grown for 28 h; (C) into fresh SWM at  $2 \times 10^4$  cell/ml, cell-free Sgu165  $\Delta hsp12$  HDSM and in cell-free Sgu165 HDSM and grown for 6 days; (D) into fresh SWM at  $2 \times 10^6$  cell/ml and grown for 6 days. At indicated time points, the cell density was monitored by cell counting with a Bürker chamber.

**Table 1.** Proteins detected in cell-free Sgu165 HDSM and LDSM. Proteins precipitated from 50-fold concentrated HDSM or LDSM were subjected to HPLC-MS analysis.

Systematic name	Standard name	Coverage (%) <sup>a</sup>		Cellular component <sup>b</sup>	Description <sup>c</sup>
		LDSM	HDSM		
Yjl160cp		–	2.8	PM/S	Cell wall protein PIR5
Ylr390w-ap	Ccw14p	10.1	18.1	CW/M/S	Cell wall protein
Yjl158cp	Cis3p	–	4.4	PM/S	Covalently-linked cell wall protein
Ygr189cp	Crh1p	5.9	8.3	CW	Cell wall protein
Ykl096wp	Cwp1p	6.3	13.8	CW	Cell wall protein
Ybr078wp	Ecm33p	6.0	9.5	PM/CW/M/S	Cell wall protein
Ygr254wp	Eno1p	5.0	12.1	C	Enolase
Yor393wp	Err1p	–	4.1	C	Enolase-related protein
Ylr300wp	Exg1p	2.5	5.6	CW/S	Exo-1,3-beta-Glucanase
Ymr307wp	Gas1p	8.2	14.7	PM/CW/M/S	Glycophospholipid-Anchored Surface protein
Ydl223cp	Hbt1p	–	2.9	C/M	Substrate of the Hub1p ubiquitin-like protein
Yfl014wp	Hsp12p	10.1	39.4	C/E/N/PM	Heat shock protein
Yjl153cp	Ino1p	–	4.1	C	Inositol-3-phosphate synthase
Ylr044cp	Pdc1p	–	2.7	C/N	Pyruvate decarboxylase
Ymr008cp	Plb1p	1.7	3.2	PM/M	PhosphoLipase B
Ygl008cp	Pma1p	–	1.1	PM/M/Mi	Plasma membrane ATPase
Yfr031c-ap	Rpl2ap	–	4.7	C	Ribosomal protein
Yer056c-ap	Rpl34ap	–	22.3	C	Ribosomal protein
Yol040cp	Rps15p	–	9.2	C	Ribosomal protein
Yel026wp	Snu13p	–	19.0	N/No	Ribonucleoprotein-associated protein
Yhr008cp	Sod2p	37.7	53.2	Mi	Superoxide dismutase
Ydl229wp	Ssb1p	–	2.0	C/M	Heat shock protein
Yjl052wp	Tdh1p	13.0	18.4	C	Triose-phosphate dehydrogenase
Yjr009cp	Tdh2p	–	18.4	C/M	Glyceraldehyde-3-phosphate dehydrogenase
Ygr192cp	Tdh3p	–	18.4	C/M	Triose-phosphate dehydrogenase

a. Coverage refers to the percentage of the database protein sequence covered by matching peptides.

b. Cellular component are based on *Saccharomyces* Genome Database (SGD) (<http://www.yeastgenome.org/>) and uniprotkb (<http://www.uniprot.org/>) annotations: CW: cell wall, M: membrane, S: secreted, C: cytoplasm, PM: plasma and membrane, E: endosome, N: nucleus, No: nucleolus, Mi: mitochondrion.

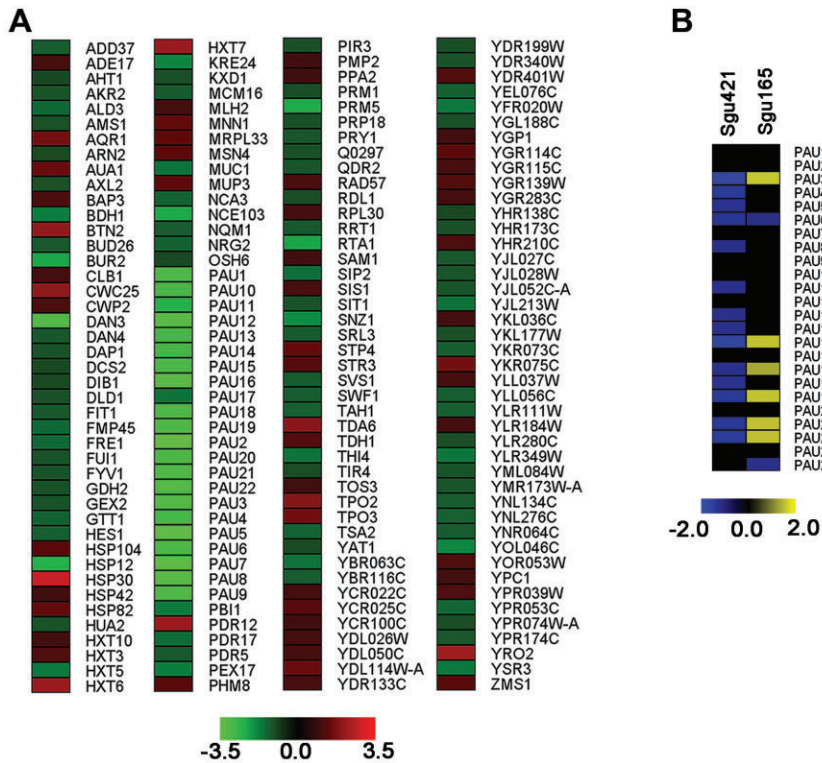
c. Most important characteristics of the proteins based on SGD and UniProtKB information. The heat shock proteins are highlighted in grey.

in SWM without being mistracked by the differences in population densities. A large part of differentially expressed genes (DEGs) (Table S4), both down and upregulated encode HSPs or other genes involved in the response to different types of cellular stresses (*HSP42*, *HSP104*, *HSP82*, *HSP30*, *MSN4*, *HSP12*). In addition, almost all genes of the *PAU* family (22 out of 24) were also found consistently downregulated in Sgu421/Sgu165\_HDSM with respect to Sgu421/SWM (Fig. 2A). To investigate whether the Sgu421 disadvantage when growing in SWM was due to genomic variations, we performed comparative genomic hybridization (CGH) analyses (Brachmann *et al.*, 1998) to investigate if there were differences in the genes copy number of Sgu421 with respect to those of Sgu165. In effect, we detected that with respect to the reference strain By4743, Sgu421 shows 14 out of 24 genes of the *PAU* family down-represented (Fig. 2B and Table S5). On the contrary, Sgu165 has a significantly higher number of copies of six of the *PAU* genes (Fig. 2B and Table S6). In addition, we associated CGH results with the whole genome sequence of the two strains, and we concluded that despite their genetic differences both of them belong to the natural wine strain group (Fig. S4).

Since *PAU* genes were found among the DEGs in transcriptional analyses and taking into account that Sgu421 bears the *PAU* genes down-represented in CGH assays, we investigated the role of *PAU* genes in the fitness of Sgu421. By cloning *PAU5*, the member of *PAU* family mostly upregulated under stress condition (Rachidi *et al.*, 2000), in the centromeric plasmid YCp80 and transforming Sgu421 with this plasmid, we observed that the transformant expressing *PAU5* improves its growth rate in SWM (Fig. 1D). This data agree with the results of Luo and van Vuuren (2009) who proposed that *PAU* genes are involved on the adaptation of the yeast to certain environmental stresses and therefore on the fitness of yeast during alcoholic fermentation. The results also suggest that the slow growth of Sgu421 is partially caused by the decreased genomic level of *PAU* genes in this strain.

#### Multiple interactions between yeast strains in co-cultures

After the study of the fitness in single cultures of the natural strains, to fully explore what happens in natural situations, we examined the fitness of Sgu421



**Fig. 2.** Effect of PAU genes on the fitness of *S. Cerevisiae*.

**A.** Heat map of DEGs using  $\pm 1$  as fold change (FC) threshold. Coloured spots indicate genes significantly ( $P < 0.05$ ) upregulated (red) or downregulated (green).  
**B.** Heat map of CGH analyses of the strain Sgu421 and Sgu165 using By4743 as reference strain. Coloured spots indicate significantly ( $P < 0.05$ ) up-represented (yellow) or down-represented (blue) genes.  
**C.** Overnight cultures of the strains Sgu421H11 and Sgu165E10 with or without (cured) killer capacity, were diluted and cultivated, alone or in competition, into fresh SWM or cell-free Sgu165\_HDSM. The cultures were grown for 6 days and then genomic DNA of the culture was purified. Real-time PCR were performed using specific primers for the tags. C(t) values were measured, and the amount of genomic DNA of each strain in the culture was calculated.

in co-culture with Sgu165 looking for an interaction among the cells. We labeled the strains with 20 mers genomic sequences (Fig. S5) in order to genetically recognize and quantify them by real-time polymerase chain

reaction (RT-PCR). Sgu165 was tagged with the 20 mers sequences A10-B10 (Table S7) to give the strain Sgu165E10. Sgu421 was tagged using the sequences A11-B11 (Table S7) to give the strain Sgu421H11. Since

Sgu165\_HDSM improved the fitness of Sgu421 in SWM, we expected that the co-culturing of the two strains would also improve the Sgu421 fitness. However, contrary to this hypothesis, Sgu421 DNA content of the mixed culture was lower with respect to that of the Sgu421 single culture, indicating that the Sgu421 strain was affected by the presence of competitive Sgu165 living cells (Fig. 2C).

We wondered if Sgu165\_HDSM could improve the fitness of Sgu421 also in the case when living cells of Sgu165 were present. We thus cultivated Sgu421H11 and Sgu165E10 in Sgu165\_HDSM and observed a higher Sgu421 DNA concentration, with respect to that obtained in the co-culture in SWM, suggesting a growth recovery of this strain, thanks to the Sgu165\_HDSM despite the presence of competing Sgu165 cells (Fig. 2C).

Since we previously observed that the overexpression of PAU5p improves the Sgu421 growth rate, we tested whether PAU genes were involved in the Sgu421 growth recovery observed in presence of Sgu165\_HDSM even in the presence of Sgu165 cells. Aiming at this, we cultivated the Sgu421H11 with YCp80-PAU5 in the presence of Sgu165E10 in SWM. In this case, the deoxyribonucleic acid (DNA) concentration of Sgu421H11 was higher than that of Sgu165E10 (Fig. 2C). These results suggest that PAU genes not only are involved on the fitness of yeast during alcoholic fermentation but are also involved in determining their fitness during competitive interactions. Since Pau5p has been described as an integral membrane protein (Luo and van Vuuren, 2008), we suggest that the subcellular localization of this protein could collaborate in its action.

#### *Sgu165 competitive action is based on a contact-dependent killer behaviour*

Using transwell plates, a well-known approach to show cell to cell communication non-mediated by contact (Cervantes *et al.*, 1996; Cheng *et al.*, 2010), we determined whether the Sgu165 interference on Sgu421 growth depends on molecules released into the culture medium or on the contact between the cells. We cultivated for 6 days Sgu421H11 on the basal surface of 24 transwell plate and on the upper side Sgu165E10 in SWM (Fig. 3A). Real-time PCR DNA analysis showed that when the strains were grown separated by the permeable membrane, Sgu421H11 was able to grow better with respect to when grown in contact with Sgu165E10. This suggests that Sgu165 needs to be in direct contact with Sgu421 cells to inhibit their growth.

With the aim to further investigate the nature of this interference of Sgu165 on the growth of Sgu421, we performed a killer phenotypic characterization to identify

if the interference might come from the presence of a killer toxin (Schmitt and Breinig, 2006) in Sgu165 cells. We observed that Sgu421 was not able to grow (showing clear zones of growth inhibition) around the spots of the control K2 killer strain (EX73) as well as of Sgu165 strains (Fig. 3B). Genotypic characterization showed that Sgu165, as well as EX73, carried two nucleic acid molecules, similar in size to the double-stranded (ds)RNA genome of the L-A virus (4.6 kb) and to the M viruses genome (1.5 to 2.3 kb) (Schmitt and Breinig, 2006) (Fig. 3C). These results indicate that the interactions in the co-cultures are based on killer competitive behaviour. We followed the same approach to characterize further the effect of PAU genes on the fitness of Sgu421 during co-culture. We tested the killer phenotype upon induction of the expression of the PAU genes, growing the cells in YPE (7% ethanol, 0.5% glucose) (Luo and van Vuuren, 2008), using the Sgu165-YCp80 as killer and Sgu421-YCp80 and Sgu421-YCp80-PAU5 as strains tested for sensitivity to the killer toxin, in these conditions the halo of growth inhibition was reduced, thus showing a protective effect of PAU genes in Sgu421 strain (Fig. S6).

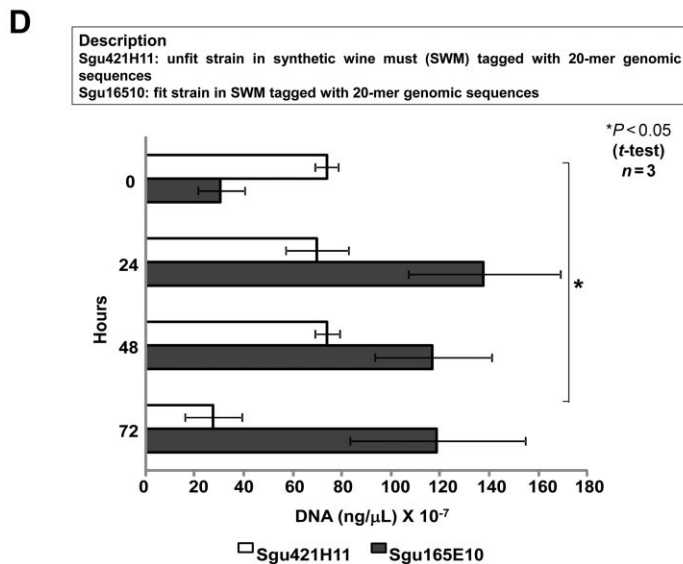
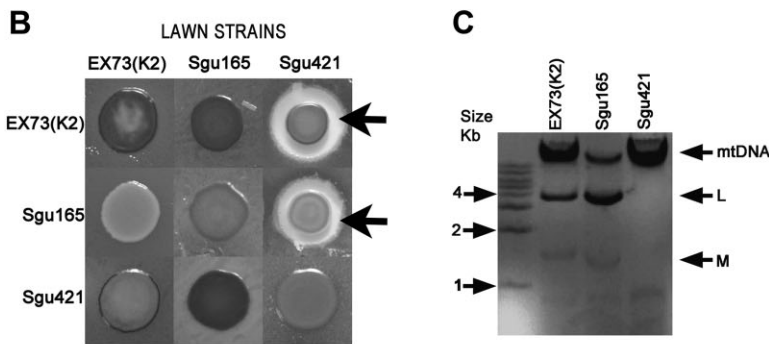
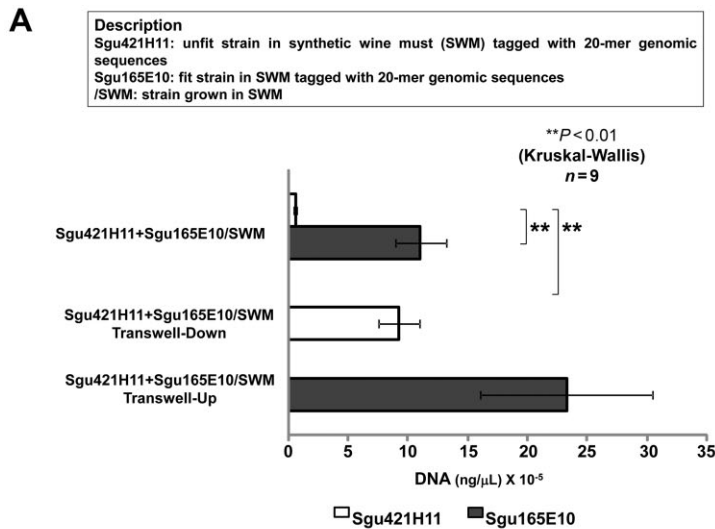
Taken together, these results suggest that Sgu421 and Sgu165, when grown in co-culture, establish competitive social interactions based on the contact-dependent killer phenomenon. In addition, we can conclude that the protein Pau5p helps Sgu421 cells to resist the killer effect.

Although several of the conditions of natural fermentation were reproduced *in vitro*, we tried to better simulate a fermentation process, considering the media oxygenation, performing a static co-culturing of the strains Sgu165E10 and Sgu421H11. The DNA concentration during 72 h of static cultures (Fig. 3D) confirmed what we observed in mixing conditions, with a progressive decrease in Sgu421H11 DNA concentration upon co-culturing. This suggests that the inhibition of Sgu421 by the killer effect of Sgu165 could be relevant also in natural fermentation settings.

## Discussion

Natural strains of *S. cerevisiae* showed different growth rates in wine must (Fig. S1). We wondered what can cause these differences and whether a fitter strain could produce some molecules that help the community.

Our findings demonstrate that variations in PAU genes copy number are important for the differences in strains fitness in SWM (Fig. 2B). However, we believe that the real advantage of the fitter strain is its cooperative behaviour. Sgu165 can use molecules like Hsp12p, liberated during cell lysis of altruistic cells (Ackermann *et al.*, 2008) into the medium, to communicate and improve the



**Fig. 3.** Killer competitive behaviour of Sgu165.

A. The indicated strains were cultivated in transwell plates, alone or in competition into fresh SWM for 6 days. We cultivated 500  $\mu$ l of Sgu421H11 at  $2 \times 10^6$  cell/ml on the basal surface, 100  $\mu$ l of Sgu165E10 at  $0.5 \times 10^4$  cell/ml on the upper side or were cultivated together as a control (400:1 ratio). Genomic DNA was purified, and the DNA concentration of each strain was determined by RT-PCR. B. Killer phenotypic characterization:  $1 \times 10^7$  cells of the indicated strains were seeded in MBA. Clear zones (indicated with black arrows) of growth inhibition around the spot of the killer strains are shown. C. Killer genotypic characterization: Nucleic acids of indicated strains were obtained. Three bands were clearly identified: the mitochondrial DNA (mtDNA), the dsRNA of the L-A helper virus (L) and the dsRNA of the toxin-coding (M) killer virus. D. Static co-cultures: the indicated strains were diluted in SWM and cultivated in competition (1:1 ratio) without shaking. At indicated time points, genomic DNA of the culture was purified and the DNA concentration of each strain was determined by RT-PCR.

survival of the group. Since this behaviour was observed only at high cellular density (Fig. 1A), we propose that it could be caused by a quorum sensing-like process (Chen and Fink, 2006). The fact that the cell-free spent medium of Sgu165 improves the growth of Sgu421 (Fig. 1A), but

that the co-culture of both strains induces a decrease of its growth, can be ascribed to a controlled policy system regulating cooperative behaviour. There are many examples of self-destructive cooperation in nature involving several organisms, from insects to bacteria (Wireman and

Dworkin, 1975; Paton, 1996; Voth and Ballard, 2005; Bourke, 2008). We describe for the first time this behaviour on *S. cerevisiae*.

The cooperative strain extends access to the advantage described above only to genetically identical younger cells, and has implemented a policy strategy to prevent close relative non-producers from exploiting this potential advantage by using contact mediated killing (Fig. 3A and B). Indeed, a cooperative strain cured of its killer property presents a competitive disadvantage (Fig. 2C). These findings agree with the results published recently (Jousset *et al.*, 2013) where the maintenance of cooperation is controlled by antagonism against phylogenetically related defectors (Fig. S4).

Defense against yeast toxins is provided by Pau5p indicating, in agreement with Luo and van Vuuren (2008), that *PAU* genes have a significant protective effect against stress in particular in competition phenomena.

Regarding heat shock proteins (HSPs), they could be released and have important effects in the extracellular environment, as described also in the mammalian immune system (Oppenheim and Yang, 2005; Giuliano *et al.*, 2011). Yeast extracellular HSPs, in particular Hsp12p, could act as local 'danger signals' to activate the stress response in surrounding cells. Based on the use of Hsp12p by Sgu421 for survival, we propose it as a public good (Samuelson, 1954) in the Sgu165\_HDSM since it helps Sgu421 but not hurts Sgu165, thus it might not be limiting for any cells. We speculate that the combined action of Hsp12p, Pau5p and killer toxins is sufficient to create a reward–punishment loop, regulating production and utilization of public goods and policing against insurgence of opportunistic strains.

The interpretation of microbial behaviours in a social evolutionary framework is an argument under debate (Rainey *et al.*, 2014). Yet so far, the indications on the interplays possibly occurring among *S. cerevisiae* strains have been derived from experiments carried out in laboratory strains, selected and modified, in non-natural settings. Here, we have mimicked the natural environment, by culturing natural strains in conditions resembling grape fermentation. Albeit the Sgu421 and Sgu165 were isolated in different years, they were found in the same grapes collected in the same vineyard. The flux of microorganisms in the vineyard environment has been shown to occur (Schuller and Casal, 2007), the isolation of multiple *S. cerevisiae* strains on grapes skin in the vineyard (Polsinelli *et al.*, 1996) and the recent observation of the ability of wasps to maintain such yeasts all year long (Stefanini *et al.*, 2012) allows the assumption that these strains may have effectively cohabited the same environment. In this way, we ensure that the observed sociological interactions might indeed happen in the wild, and the observed phenotypic traits are not laboratory forced.

Further studies will be necessary to understand better the interaction between gene expression and ecological behaviours in more complex environments including interspecific interactions. Our findings show a simple, two-strain example of a complex system with multiple levels of interactions, so it will be a real challenge to understand more complex systems with multiple species.

## Experimental procedures

### *Strains, growth media and cell viability*

Subcloning Efficiency DH5 $\alpha$  *Escherichia coli* Competent Cells (Invitrogen, Life Technologies Italia, Monza, Italy) were used for selection and amplification of plasmids. All *S. cerevisiae* strains used in this study are described in Table S1.

Luria-Bertani (LB) broth with ampicillin (LBA) (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 100  $\mu\text{g ml}^{-1}$  ampicillin) was used for *E. coli* DH5 $\alpha$  transformants selection. Yeast strain were grown in YPD (1% yeast extract, 2% peptone, 2% glucose), yeast extract peptone ethanol (YPE) (1% yeast extract, 2% peptone, 7% ethanol, 0.5% glucose) or in SWM, simulating fermentation conditions (Landry *et al.*, 2006). Synthetic wine must contains 0.17% yeast nitrogen base without amino acids and (NH<sub>4</sub>)SO<sub>4</sub>, 0.15% casamino acids, 0.05% NH<sub>4</sub>Cl, 0.60% DL-malic acid, 0.02% citric acid, 0.15% L-tartaric acid, 21% glucose and 0.20% anaerobic factors ergosterol–Tween 80 (total 10 mg L<sup>-1</sup> ergosterol, 0.5 ml L<sup>-1</sup> Tween 80). The pH was adjusted to 3.3 with sodium hydroxide. Yeast extract peptone dextrose with adenine (YPAD) was YPD supplemented with 75 mg L<sup>-1</sup> adenine. Yeast extract peptone dextrose with geneticin (YPDGen) plates were prepared with solid YPD (YPD with 2% agar) supplemented with 150  $\mu\text{g ml}^{-1}$  geneticin and used for tagged strains selection. Yeast extract peptone dextrose with hygromycin (YPDHg) plates (solid YPD supplemented with 300  $\mu\text{g ml}^{-1}$  hygromycin B) were used for YCp80 transformant selection. Methylene blue agar (MBA) plates were prepared with solid YPD at pH 4.7, adjusted with phosphate-citrate buffer, and 0.003% methylene blue (Ambrona *et al.*, 2005).

Yeast cultures were prepared by dilution of an overnight pre-culture of the strains into fresh medium (SWM or YPDGen) at either  $2 \times 10^6$  cell ml<sup>-1</sup> (YCp80 transformants) or  $2 \times 10^4$  cell ml<sup>-1</sup> (the other cells). Five millilitres of the cultures were grown into 25 ml conical flask at 27°C for 6–7 days in a shaker at 200 r.p.m. The growth rate of yeast strains was monitored by cell counting with a Bürker chamber at different times.

Cell-free HDSM and LDSM were prepared from an overnight pre-culture of the indicated strains in YPD. The cells were centrifuged at 4500  $\times$  g for 10 min, washed twice with sterile water, then re-suspended in SWM at  $1 \times 10^8$  cells ml<sup>-1</sup> (HDSM) or  $2 \times 10^4$  cells ml<sup>-1</sup> (LDSM) and left to grow for 6 days. After centrifugation, the supernatant was filtered using a 0.2  $\mu\text{m}$  sterile syringe filter (Merck Millipore S.p.A., Milan, Italy) (Fig. S2). For the growth of the strains using the spent medium, it was diluted in a 1:3 ratio with fresh SWM in order to ensure the minimum of nutrients necessary for the growth.

Cell viability was determined using the LIVE/DEAD Yeast Viability Kit (Life Technologies).



### Plasmid construction and HSP12 gene disruption

For the construction of the plasmid YCp80 (Baruffini *et al.*, 2009) (YCp80-PAU5 and YCp80-HSP12), the genes were amplified from genomic DNA of Sgu165 using the primers described in Table S7 (see 'Supporting Protocols' for more detail).

For the disruption of *HSP12* gene, the KanMX4 cassette, flanked by the upstream and downstream regions of *HSP12*, was PCR amplified from strain BY4742 *hsp12::KanMX4* (Euroscarf collection, University of Frankfurt, Germany) with the primers HSP12D-Fw and HSP12D-Rv. Eight micrograms of amplified cassette were used for transformation of Sgu165. Selection was performed on YPDGen medium, thus obtaining strain Sgu165 *hsp12::KanMX4*. The KanMX4 cassette was switched to HphMX cassette through transformation with 15 µg of *SacI-SalI*-digested plasmid pAG32 (Goldstein and McCusker, 1999) and selected on YPDHyg, thus obtaining strain Sgu165 *hsp12::HphMX*. This strain was further transformed with 8 µg of *hsp12::KanMX4* cassette and selected on YPD supplemented with both antibiotics, thus obtaining the null strain Sgu165 *hsp12::KanMX4 hsp12::HphMX*.

### Transformation of yeast strains

Natural yeast strains were transformed by lithium acetate protocol (Gietz and Woods, 2002). After transformation, cells were re-suspended in 1 ml YPAD, and plated on YPDHyg or YPDGen after 2 h of adaptation time.

### Tagging of the strains

The tagging of the strains was achieved by using a PCR-based gene deletion strategy previously described (Baudin *et al.*, 1993; Wach, 1996). The *HO* gene, involved in mating-type switching and shown to be neutral for gene replacement (Baganz *et al.*, 1997), was replaced by the KanMX6 cassettes flanked in 5' and in 3' by two pairs of 20 mers. The two external 20 mers sequences, UI and D1 (Fig. S5) were common to the tagged strain. The two internal Ai and Bi 20 mers tag sequence (in which 'i' is a different number for each sequence) were unique identifier of the strains (Table S7) chosen from a list previously established (Shoemaker *et al.*, 1996).

The Kan disruption cassettes were generated as described in the legend of Fig. S5. The PCR steps were performed as described in Wach (1996) and the transformation of yeast as described previously. The geneticin-resistant transformants were checked for a proper integration by few rounds of PCR. The location and the substitution were tested by a qualitative PCR between the primers P3 and P5 (Table S7) that give a pair of fragments of 2556 bp and 2454 bp corresponding respectively to the wild chromosome carrying the *HO* region and the chromosome carrying the KanMX6 cassette. The presence of the tags was tested by a qualitative PCR between the tags and the KRT-PCR1 and by sequencing the regions between P3 and KRT-PCR2 and between P5 and KRT-PCR3 (Fig. S5).

Sgu165 was tagged with the 20 mers sequences (Table S7) A10-B10 to give the strain Sgu165E10. Sgu421

was tagged using the sequences A11-B11 to give the strains Sgu421H11.

### Competitive cultures

Overnight cultures of Sgu421H11 and Sgu165E10 were diluted to  $2 \times 10^6$  cell ml<sup>-1</sup> and  $0.5 \times 10^4$  cell ml<sup>-1</sup>, respectively, and cultivated alone or in competition in a 400:1 (Sgu421H11:Sgu165E10) ratio, into fresh SWM or cell-free Sgu165\_HDSM. The cultures were grown for 6 days, and then the genomic DNA of the culture was purified and used in RT-PCR analysis.

For the study of the role of the contact among cells in competition cultures, we used the permeable supports 24 Corning Transwell plates (Corning, Sigma-Aldrich S.r.l., Milan, Italy). We cultivated 500 µL of Sgu421H11 at  $2 \times 10^6$  cell ml<sup>-1</sup> on the basal surface, and on the upper side, we added 100 µL of Sgu165E10 at  $0.5 \times 10^4$  cell ml<sup>-1</sup> in SWM (400:1 ratio).

For static co-culturing, Sgu165E10 and Sgu421H11 were diluted in SWM and cultured at the same concentration without shaking. The genomic DNA of the culture was studied for 72 h by RT-PCR analysis.

### Nucleic acid preparation

The genomic DNA used in both CGH analyses and real-time (RT) PCR experiments was extracted following the protocol described by Hoffman and Winston (1987). Ribonucleic acid extraction for microarray analysis was carried out as previously described (Stefanini *et al.*, 2010). For killer genotypic characterization, dsRNA mini preps were performed following the protocol described by Rodriguez and colleagues (2011).

### RT-PCR

Real-time PCR was performed using the QuantiTect SYBR Green PCR Kit (QIAGEN S.r.l., Milan, Italy) on Sgu165E10 and Sgu421H11 genomic DNA. We performed calibration curves using a maximum of 2 ng of genomic DNA per reaction. We used 0.5 µM of the specific 20 mers primer A10 to amplify the label of Sgu165E10 and A11 for Sgu421H11. We also used 0.5 µM of the return primer (KRT-PCR1) common to all the reaction and located on the KanMX6 cassette. Using the Applied Biosystems 7900HT Fast Real-Time PCR (Applied Biosystems, Life Technologies), the samples were incubated 15 min at 95°C and amplified 40 cycles with 15 s at 94°C, 30 s at 55°C and 30 s at 72°C. The threshold cycle values were determined from semi-log amplification plots (log increase in fluorescence versus cycle number) and compared with the calibration curves. The amplification product was 144bp.

### Transcriptional microarray hybridization

Overnight cultures of Sgu421 were diluted into fresh SWM, or in cell-free Sgu165\_HDSM, at  $2 \times 10^4$  cell ml<sup>-1</sup>. After 48 h of culture, cells were collected and frozen in liquid nitrogen. We extracted the RNA and performed hybridization onto Agilent 015072 Yeast Oligo Microarray 4x44K 60mer oligonucleotide

arrays (G2519F, Agilent, Agilent Technologies Italia S.p.A., Milan, Italy). Total RNA (300 ng) was processed with the Agilent QuickAmp Labeling Kit, according to the manufacturer's instructions. Transcriptional analyses were performed by comparing Sgu421 grown in Sgu165\_HDSM (Cy5-red), respect to Sgu421 grown in SWM (Cy3-green). Fluorescent complementary DNA bound to the microarray was detected with a GenePix 4000B microarray scanner (Axon Instruments, Molecular Devices, Berkshire, UK), using the GENEPIXPRO6.1 software package to quantify microarray fluorescence. The control microarray spot quality required were described previously (Stefanini *et al.*, 2010). Data were normalized using the limma R package (Smyth, 2004), and differentially expressed genes (DEGs) were identified with the RANKPRODUCT R package (Anders and Huber, 2010), using a *P*-value < 0.05 and applying  $\pm 1$  as the fold change threshold.

#### Comparative genomic hybridization

In a CGH, the genes copy number could be increased (ratio > 1), decreased (that is, deletion, differences in the sequences or less gene copies) (ratio < 1) or unchanged (ratio = 1); respect to the reference (Pollack *et al.*, 1999). We used BY4743, diploid derivative of S288c, as reference strain for the hybridization. For these experiments, overnight cultures of Sgu421, Sgu165 and BY4743 in YPD were harvested, and cellular pellets were conserved at  $-20^{\circ}\text{C}$ . The extracted genomic DNA was concentrated using the filter Microcon YM-30 (Millipore) and fragmented by sonication. The labeling of the DNA and the microarray hybridization were performed as previously described (Giuntini *et al.*, 2005).

Microarray slides were fabricated in the Bauer Center for Genomics Research at Harvard University (<http://sysbio.harvard.edu/csb/>). The slides were constructed using the *S. cerevisiae* Genome Oligo Set (Operon Technologies, CA, USA) composed of 6240 optimized oligonucleotides (70mers) each representing one yeast gene. We followed the same procedure applied for transcriptional microarray data for the normalization of CGH. Transcriptional and CGH raw and normalized data were submitted to Gene Expression Omnibus (GEO), under National Center for Biotechnology Information (NCBI) accession number GSE49551.

#### Killer activity

Killer activity was tested on MBA (Ambrona *et al.*, 2005). The plates were seeded ( $1 \times 10^7$  cell) with a standard K2 killer strain (EX73), Sgu165 or Sgu421, as lawn strains. Tested strains ( $1 \times 10^7$  cell) were spotted after the lawn strains were dried. After incubating the plates for 3–5 days at  $27^{\circ}\text{C}$ , yeast strains incapable of conferring functional immunity to killers showed a sensitive phenotype, resulting in a cell-free growth inhibition zone, whereas resistant strains did not (Melvydas *et al.*, 2007). Curing of killer activity was performed using cycloheximide (Fink and Styles, 1972).

#### Fluorescence microscopy

An Olympus BX63 microscope coupled to CELLSENSE DIMENSION SOFTWARE (Olympus, MI, Italy) was used for cell death

studies. The cells were observed with 40X magnification using epifluorescence. Pictures were processed with ADOBE PHOTOSHOP CS5 (64 Bit).

#### Mass spectrometry analyses

The spent media (Sgu421\_HDSM, Sgu165\_HDSM and Sgu165\_LDSM) compositions were dissected through mass spectrometry analyses. We performed solid phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS) evaluation of volatile compounds and Nano-liquid chromatography Nano-electrospray ionization mass spectrometry (Nano LC- nano ESI-MS/MS) identification of tryptic peptides for protein identification (see 'Supporting Protocols' for more detail).

#### Sequencing of DNA and phylogenetic analysis

Complete genome sequences of the natural strains SG60, Sgu90, Sgu165 and Sgu421 were performed on a Genome Analyzer II (GA II), and base called with ILLUMINA RTA (REAL-TIME ANALYSIS) VERSION 1.8.70.0 (Illumina, Illumina Inc, San Diego, USA). Sequences were compared with *S. cerevisiae* pre-edited sequences downloaded from the Sanger institute (Liti *et al.*, 2009) and from the *Saccharomyces* Genome Database (SGD) (Cherry *et al.*, 2012) websites, plus the re-sequenced reference strain S288C (S288Cr). Phylogenetic analysis and tree drawing were carried out as previously described (Ramazzotti *et al.*, 2012).

#### Statistic

Statistical significance was evaluated using the Kruskal–Wallis test for samples with  $n \geq 9$  and a paired *t*-test for samples with  $n \leq 8$ . Results, expressed as *P*-value, are reported for each graph.

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## Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

**Fig. S1.** Growth rate of *S. cerevisiae* natural isolated. Overnight cultures of eight natural yeast strains were diluted into fresh SWM at  $2 \times 10^4$  cell mL<sup>-1</sup> and grown for 6 days. At indicated time points, the cell density was monitored by cell counting with a Bürker chamber A. In B, we showed a detail of the growth curves in the first 120 h.

**Fig. S2.** Growth rate of the cultures used to prepare cell-free spent media. Overnight pre-culture of the strains in YPD were centrifuged at 4500 × g for 10 min, washed twice with sterile

water and re-suspended in SWM at  $1 \times 10^8$  cells mL<sup>-1</sup> (HD) to prepare cell-free HDSM or  $2 \times 10^4$  cells mL<sup>-1</sup> (LD) to prepare cell-free LDSM. The cultures were left to grow for 6 days. At indicated time points, the cell density was monitored by cell counting with a Bürker chamber. The sixth day, the cultures were centrifuged and the supernatant was filtered.

**Fig. S3.** Cell death percentage of the cultures at high and low cellular density. YPD overnight cultures of Sgu165, Sgu421 and Sgu165\_Δhsp12 yeast strains were diluted into fresh SWM at  $1 \times 10^8$  cell mL<sup>-1</sup> (HDC) and grown for 6 days after which the percentage of cell death was evaluated using LIVE/DEAD Yeast Viability Kit (Life Technologies). The cultures were diluted again into fresh SWM at  $2 \times 10^4$  cell mL<sup>-1</sup> (LDC), grown for another 6 days and re-evaluated the percentage of cell death.

**Fig. S4.** Genome-wide phylogenetic tree of the natural strains SG60, Sgu90, Sgu165 and Sgu421. The genome-wide sequences of four natural strains were compared with 40 *S. cerevisiae* pre-edited sequences downloaded from the Sanger institute and from the SGD websites, plus the re-sequenced reference strain S288C (S288cr). Neighbour-joining phylogenetic tree was obtained by calculating the genetic distances (Kimura two parameters) based on the single nucleotide polymorphisms (SNPs) differences obtained for the whole genomes. The clades were found through whole genome analysis (Liti et al., 2009).

**Fig. S5.** Schematic view of the three steps PCR synthesis of disruption cassettes used for the labeling of *S. cerevisiae* strains. First step: the sequences flanking the *HO* gene in 3' and 5' were amplified with the primers, RP3-RU1 and P3, and RD1-RP5 and P5 (Table S7). These primers were also designed to introduce at the end of each arm, the 20 mers sequences, UI and D1 that were common to all the cassettes. Second step: a specific tag (named Ai or Bi for each arm of a given pair) and 20 bp homologues to the KanMX6 cassette were fused to the arms by PCR using the primers P3 and RU1-RAI-RU2 for one arm, and P5 and RD2-RBi-RD1 for the other. Third step: two arms were used as primers to amplify the KanMX6 cassette and produced the disruption cassettes.

**Fig. S6.** Protective effect of *PAU* genes in *S. cerevisiae* strains. Killer phenotypic characterization:  $1 \times 10^7$  cells of Sgu421-YCp80 grown in YPD and Sgu421-YCp80-PAU5 grown in YPD or YPE (7% ethanol, 0.5% glucose) were seeded in MBA. The strain Sgu165-YCp80 was spotted to study killer effect. Clear zones of growth inhibition around the spot of the killer strain are shown. The halo was reduced when the lawn strain were grown in YPE (indicated with black arrows).

**Table S1.** Complete list of the *S. cerevisiae* strains used in the study. The source, genotype, origin and collection data are reported for each strain.

**Table S2.** Glucose and metabolites detected in Sgu165 and Sgu421 cell-free HDSM. The results (mean ± SD) are showed in concentration (v/v% or ppm).

**Table S3.** Proteins detected by high-performance liquid chromatography–mass spectrometry (HPLC-MS) analysis in cell-free Sgu165 HDSM and LDSM. Protein coverage (%) refers to the percentage of the database protein sequence covered by matching peptides.

**Table S4.** Differentially expressed genes in Sgu421/Sgu165\_HDSM respect to Sgu421/SWM at 48 h.

**Table S5.** Comparative genomic hybridization results: Differentially represented genes (DRGs) in Sgu421 respect to By4743.

**Table S6.** Comparative genomic hybridization results: Differentially represented genes (DRGs) in Sgu165 respect to By4743.

**Table S7.** Oligonucleotides used in this study. \*RAi and RBi stands, respectively, for the inverse complementary sequences of the primers Ai and Bi and 'i' the number of the tag.

**File S1.** Supporting protocols.