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Brief report

Autophagy is required for sulfur dioxide tolerance in *Saccharomyces cerevisiae*

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Summary

Sulfiting agents are among the most widely used preservatives in the food and beverages industries, including winemaking, and one of their main functions is inhibition of spoilage microorganisms. We have used a whole genome quantitative fitness analysis in order to improve our knowledge on yeast tolerance to sulfites. Apart from the contribution of sulfite efflux to tolerance, results point to vesicle-mediated transport, autophagy and vacuolar activity as the main cellular functions required to survive sulfite challenges. The involvement of autophagic and vacuolar functions in sulfite tolerance was further confirmed by pairwise competition using a newly constructed *atg2*-defective strain, as well as by showing induction of *ATG8* expression by sulfite. Autophagy is required for the turnover of proteins and subcellular structures damaged by sulfite. In addition, the requirement for vacuolar functions might be related to its role in intracellular pH homeostasis. Finally, the involvement of the sulfite pump Ssu1 and the transcription factor Fzf1 in sulfite tolerance by *Saccharomyces cerevisiae* was confirmed; a result that validates the experimental approach used in this work. These findings have relevance for understanding sulfite toxicity and tolerance, as well as for the eventual design of strategies aiming to control yeast spoilage.

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Introduction

Sulfiting agents have been traditionally used in food preservation due to their antioxidant and antimicrobial activities. They can inhibit both non-enzymatic and enzymatic browning, as well as a wide range of other enzymes such us proteases, oxidases or peroxidases (Wedzicha, 1992). The most common sulfiting agents in winemaking are gaseous sulfur dioxide and potassium metabisulfite. Upon dissolution into grape must or wine, these compounds are converted to the same chemical species, depending on medium pH (Waterhouse *et al.*, 2016). In addition to prevention of the growth of unwanted bacteria and yeasts, and its antioxidant properties, sulfur dioxide combines reversibly or irreversibly with several compounds coming from either grapes or microbial metabolism. These reactions contribute to sulfur dioxide titration (Li and Mira de Orduña, 2017), as well as to the sensory properties of wines (Waterhouse *et al.*, 2016; Arapitsas *et al.*, 2018). However, the adverse effects (e.g. symptoms of allergic responses) observed for a small section of the population when exposed to sulfur dioxide (Gunnison *et al.*, 1987) prompted the current interest to reduce sulfite utilization in both wine and other foods (EFSA, 2014). A better understanding of the molecular basis of sulfite tolerance will help reduce sulfite content in foods.

Molecular sulfur dioxide is the only chemical species of sulfite possessing antimicrobial activity (Usseglio-Tomasset, 1992), due to the ability to cross microbial cell membranes. With a pK = 1.81 for the equilibrium between SO_2 and the bisulfite anion, this means that only a small fraction of the free SO_2 is available for its antiseptic role, with great variations in the narrow pH distribution found in wines and musts (Divol *et al.*, 2012). After reaching the intracellular space, the higher pH makes bisulfite the main sulfite species inside the cell (Divol *et al.*, 2012).

Sulfite shows reactivity with acetaldehyde (Waterhouse *et al*., 2016), disulfide bridges in proteins (Cecil and Wake, 1962), nucleotides, including NAD and flavin nucleotide cofactors in enzymes, vitamins or amino acids (Gunnison, 1981). It can also generate cross-linking of proteins and nucleic acids and initiate a free radical chain mechanism (Gunnison, 1981).

The best-known mechanisms developed by wine yeasts to endure the presence of SO_2 were reviewed by Divol *et al.* (2012). These involve sulfur reduction, acetaldehyde overproduction and active efflux. The main components of the later detoxification system are Ssu1, a sulfite pump and Fzf1, a transcription activator binding to the *SSU1* gene promoter. Chromosome rearrangements involving the promoter of *SSU1* are also responsible for the improved sulfite resistance shown by many *Saccharomyces cerevisiae* wine yeast isolates (Goto-Yamamoto *et al*., 1998; Pérez-Ortín *et al*., 2002; Zimmer *et al*., 2014; García-Ríos *et al*., 2019).

One interesting tool to explore the genetic determination of sulfite resistance in yeasts, beyond sulfite efflux, is competition experiments of the yeast knockout collection (Giaever *et al*., 2002; Pierce *et al*., 2007), coupled with Bar-seq analysis (Smith *et al*., 2010). This technology has been previously used to identify targets of several antimicrobial agents and drugs (Hillenmeyer *et al*., 2008). There are also previous examples of its use to get insight into oenologically related yeast features (Novo *et al*., 2013; Gonzalez *et al*., 2016). In this article, we used this experimental approach to discover cell functions required for proper tolerance to sulfur dioxide in *S. cerevisiae*, as a model for other yeast species commonly found in fermented food either as starter or as spoilage microorganisms.

Results and discussion

Determination of optimal sulfite concentration for competition experiments

The genetic background of the yeast knockout (YKO) strains used in this work is *S. cerevisiae* BY4743 (*MATa/alpha; his3Δ1/his3Δ1; leu2Δ0/leu2Δ0; met15Δ0/MET15; and LYS2/lys2Δ0; ura3Δ0/ura3Δ0*). This strain does not show chromosomal rearrangements or other modifications affecting the normal expression of *SSU1* (under the control of Fzf1). With the aim of performing competition experiments in conditions that are selective, but still allow growth of the parental yeast strain, a dosage experiment was performed in liquid minimal medium adjusted to pH 3.5. According to these results, potassium metabisulfite concentration was set at 60 mg L^{-1} for competitions. This is equivalent to around 34 mg L^{-1} of SO_2, and about 2% of it is expected to be in the molecular form at the beginning of the experiment, considering the medium initial pH. Using this concentration, growth of BY4743 was not completely arrested, but it was clearly inhibited, as compared to the control medium (Appendix S1). Parallel sequential batch competitions of all the strains in the YKO homozygous collection (about 4500) were run in triplicate, for around 10 generations, with or without selective pressure.

Bar-seq identification of genes required for sulfite tolerance

In order to identify the main genes whose activity is required in *S. cerevisiae* to survive in the presence of sulfite, we used the Bar-seq technique. This method allows to ascertain the relevance to cope with chemical or biological challenges for almost all non-essential genes in the genome. It consists in growing a pool of all homozygous barcoded YKO strains for several generations under the presence or absence of a growth inhibitor. In order to estimate the abundance of each deletion strain in the mix, after about ten generations of competition under the query and control conditions, the method takes advantage of the two 20 nt barcodes (up tag and down tag) associated with each deletion strain during the process of construction (Giaever *et al*., 2002). Those tags are PCR amplified with primers designed to help further analysis by NGS; and the frequency of each tag in each biological replicate is calculated with the aid of bioinformatic scripts. In this work, we followed the pipeline described by Gonzalez *et al.* (2016), in which edgeR was used to calculate logFC and FDR values for each gene. The sequence reads were deposited at the NCBI repository under the Sequence Read Archive SUB5584806, BioProject PRJNA541306. Results were filtered for FDR < 0.001. A great proportion of the gene deletions in the YKO collection resulted in severe growth impairment in the presence of sulfite (as compared to the control condition), with 98 mutants showing a logFC below -5 and about 550 strains showing logFC below -3 (Appendix S2).

The validity of the approach is supported by the result obtained for *SSU1* and *FZF1* that can be taken as positive controls of the experimental design. Both genes appear in the list of strains highly affected by sulfite and show logFC below -5 in our Bar-seq analysis (Appendix S2). As mentioned above, Ssu1 is the main plasma membrane sulfite pump, required for efficient sulfite efflux in *S. cerevisiae*. Susceptibility to sulfite is well reported for *ssu1* loss of function mutants (Xu *et al*., 1994). In addition, many wine yeast strains, showing increased tolerance to sulfite, as compared to isolates from other origins, carry a specific translocation and sequence repeat expansion, involving the promoter of *SSU1* and resulting in increased expression of this gene

(Pérez-Ortín *et al*., 2002). In turn, *SSU1* expression depends on the transcription factor Fzf1. The dominant gain-of-function allele *FZF1-4*, as well as the overexpression of the common *FZF1* allele, confers increased sulfite tolerance, while *fzf1*-defective strains are hypersensitive to sulfite (Avram and Bakalinsky, 1996).

GO term enrichment, of the 98 genes showing logFC below −5, was analysed by using the YeastMine database (Balakrishnan *et al*., 2012). GO terms showing Holm–Bonferroni corrected p-values < 0.05 were grouped by GO/Module (Yang *et al*., 2011). Results of this analysis for GO terms related to 'biological process' and 'cellular component' are shown in Tables 1 and 2 respectively. The first terms in Table 1 are 'vesicle-mediated transport' and 'macroautophagy'. All other biological processes highlighted in this analysis seem to be related with the above, including terms referring to autophagy, endosome, Golgi, protein transport or nitrogen starvation. Enriched 'cellular components' (Table 2) reinforce the image of the intracellular vesicular transport and autophagy as particularly relevant in this analysis. Indeed, 'endomembrane system' is represented by 36 out of the 98 genes and 'vesicle-mediated transport' by 24 of them. The interaction network or these 98 genes was visualized using STRING 11.0 (Franceschini *et al*., 2013). The result is shown in Appendix S3. The most compact and interacting subnetwork of the gene network points again to proteins related to vesicular transport in the cell, notably grouping *ATG* and *VPS* genes (for 'AuTophaGy related' and 'vacuolar protein sorting' respectively).

YKO competition experiments were previously used to identify genes required for sulfite tolerance in at least one instance (Hillenmeyer *et al*., 2008), together with many other drugs or stress conditions. Data for the homozygous collection were downloaded from the official web site (http://fitdb.stanford.edu) in order to compare with our own results. Surprisingly, we found only one gene in common among the top 100 genes in both data sets. Some minor differences between the experimental setup used in the different laboratories would not explain such a large divergence (e.g. we used minimal instead of rich medium, run the experiment for 10 instead or 5 generations, and used Bar-seq instead of microarray technology for analysis). However, there is a major difference that, considering the chemical properties of sulfur dioxide described in the introduction, would explain the divergent results. While we used media adjusted to pH 3.5, in order to keep sulfite in its active, antimicrobial ionization state, no adjustment in pH was reported by Hillenmeyer *et al.* (2008). The pH of yeast-rich media being usually close to neutrality, the fraction of added sulfite expected to be active in these experiments was almost null. Indeed, an indication of this comes from the observation that, in that analysis, deletion of *FZF1* showed almost no relative impact on survival to sulfite (or even positive, but with a non-significant p-value),

Table 1. 'Biological process' GO (gene ontology) enrichment for yeast knockout (YKO) strains showing impaired growth in the presence of sulfite.

GO IDs	GO terms	FDR	Sig.	GO-Module IDs
GO:0016192	Vesicle-mediated transport	1.22E-04	K	1
GO:0016236	Macroautophagy	9.15E-03	K	2
GO:0061919	Process utilizing autophagic mechanism	8.12E-03	K	3
GO:0016197	Endosomal transport	7.53E-04	K	4
GO:0072665	Protein localization to vacuole	4.49E-03	K	5
GO:0034067	Protein localization to Golgi apparatus	2.92E-02	K	6
GO:0006995	Cellular response to nitrogen starvation	2.92E-02	K	7
GO:0045053	Protein retention in Golgi apparatus	2.91E-03	K	8
GO:0045184	Establishment of protein localization	1.58E-02	K	9
GO:0042147	Retrograde transport, endosome to Golgi	1.02E-02	T	1;4
GO:0006914	Autophagy	2.57E-02	T	3
GO:0032258	Protein localization by the Cvt pathway	9.12E-03	T	3;5
GO:0015031	Protein transport	2.88E-02	T	9

Sig. stands for significance: true positive *P*-values are noted 'K' for local minimum, and 'T' for significant hierarchical descendants of local minimum. FDR stands for false discovery rate, estimated by the Holm–Bonferroni correction implemented in the YeastMine database.

Table 2. 'Cellular component' GO (gene ontology) enrichment for yeast knockout (YKO) strains showing impaired growth in the presence of sulfite.

GO IDs	GO terms	FDR	Sig.	GO-Module IDs
GO:0044433	Cytoplasmic vesicle part	1.77E-06	K	1
GO:0012505	Endomembrane system	3.93E-04	K	2
GO:0030904	Retromer complex	2.15E-04	K	3
GO:0019898	Extrinsic component of membrane	4.86E-04	K	4
GO:0000407	Phagophore assembly site	4.25E-02	K	5
GO:0031410	Cytoplasmic vesicle	1.32E-06	K	6
GO:0010008	Endosome membrane	7.82E-04	T	1
GO:0044440	Endosomal part	4.86E-04	T	1
GO:0005768	Endosome	3.75E-05	T	6

Sig. stands for significance: true positive p-values are noted 'K' for local minimum, and 'T' for significant hierarchical descendants of local minimum. FDR stands for false discovery rate, estimated by the Holm–Bonferroni correction implemented in the YeastMine database.

while deletion of *SSU1* showed an impact ten times below the top relevant genes in that analysis. In contrast, as discussed above, the results obtained for these two deletion strains, 5- and 10 logFC reduction in fitness for *FZF1* and *SSU1*, respectively (Appendix S2), clearly support the validity of the data presented in the present work. This illustrates that, while the use of highly multiplexed approaches undoubtedly offers interest to explore biological systems and broadly assess their function, specific experimental designs are required in order to reach sound conclusions when analysing particular growth conditions or individual gene functions.

Confirmation of autophagy requirement for sulfite tolerance with a newly constructed knockout strain

As described above, autophagy appeared in this analysis as one of the main cellular processes whose impairment results in increased sensitivity to sulfur dioxide. Considering that one of the main drawbacks of conclusions based on competition experiments with the yeast knockout collection is the accumulation over time of diverse types of mutations and genome rearrangements (Teng *et al*., 2013), we decided to confirm that result with a fresh autophagy-defective strain. To this end, the KanMX4 cassette was amplified from the *atg2* homozygous deleted strain (BY4743 background) from the YKO collection and transformed in both BY4741 (*MATa*) and BY4742 (*MAT*α). Mating both deleted strains allowed to construct a new *atg2* homozygous deleted strain. In parallel, untransformed BY4741 and BY4742 were also mated to generate a fresh BY4743 reference strain with identical genetic background.

Both newly generated diploid strains were submitted to a competition experiment in the conditions previously used for the competition of the whole YKO collection. In the control condition, proportions remained almost constant for three culture transfers, starting from about 50% abundance each strain (Fig. 1). This result excluded a general growth or survival impairment associated with this deletion in the absence of selective pressure, under these experimental conditions. However, the *atg2*-defective strain, easily identified based on the G-418 resistance phenotype, was gradually replaced batch after batch (Fig. 1). At the end of the experiment, this strain was not detected in two of the three replicate cultures and was also almost absent from the third one. The final average prevalence for the autophagy-defective strain was only 3% (Fig. 1).

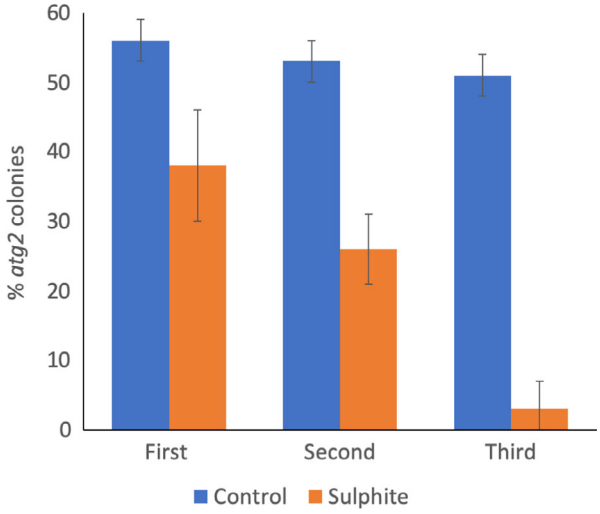

Fig. 1. Relative prevalence of the *atg2*-defective strain, in competition with the isogenic control strain, starting from 50%, in minimal medium (Yeast Nitrogen Base w/o amino acids or ammonium sulfate, 1.7 g L^{-1}; + $(NH_4)_2SO_4$ 5 g L^{-1}; glucose 20 g L^{-1}; inositol 18 mg L^{-1}; histidine 20 mg L^{-1}; leucine 60 mg L^{-1}; and uridine 20 mg L^{-1}) under non-selective and sulfite selective (potassium metabisulfite 60 mg L^{-1}) conditions. Each subculture (1st, 2nd and 3rd) corresponds to roughly + 3.3 generations, for a total of around 10 generations in the final sample. Values correspond to the percentage of G-418 colonies from random clones recovered without selective pressure.

Induction of autophagy by sulfite

Taken together, these analyses suggest that strains defective in autophagy show a special sensitivity to sulfite. We hypothesized that autophagy, in turn, might be induced by the presence of sulfite. Expression of many *ATG* genes is considered constitutive, i.e. not dependent on induction of autophagy. However, *ATG8* is an exception as it is upregulated upon induction of autophagy (He and Klionsky, 2009). We used qPCR on *ATG8* to confirm induction of autophagy by sulfite (Fig. 2). Interestingly, 15 min after sulfite addition, induction of *ATG8* was already evident and, despite differences in the induction kinetics, upregulation levels after 2 h were in the same order for *ATG8* and for the sulfite resistance-specific genes *SSU1* and *FZF1*. Induction levels of both latter genes are consistent with those described by Park and Hwang (2008).

Conclusions

According to the number of gene deletions showing very low fitness values in the presence of bisulfite, the diversity of metabolic pathways and biological processes that result in increased sulfite sensitivity when impaired appears to be relatively high. However, by focusing on the strains showing the greatest differential performance between control and sulfite growth conditions, autophagy and vacuole-related functions appear as specially enriched in this genome-wide analysis. Therefore, these would be the most relevant functions required for sulfite

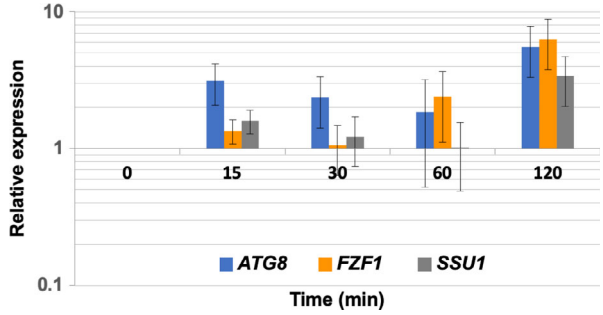

Fig. 2. Relative expression of *ATG8* at different times after addition of sulfite to cells growing in minimal medium (an exponential culture was refreshed to 0.24 OD_{600} in minimal medium with 60 mg L^{-1} potassium metabisulfite an samples withdrawn at the indicated times). Expression levels were quantified by quantitative real-time PCR of reverse-transcribed mRNA using a LightCycler thermocycler (Roche). *FZF1* and *SSU1* expressions are shown for comparison purposes. Expression levels were normalized with a combination of reference genes: *ACT1*, *TFA2*, *TFC1* and 18S, using the LightCycler 480 software 1.5.0 SP4.

tolerance revealed in this work (in addition to sulfite efflux). This conclusion is further supported by the severe impact of *ATG2* deletion in pairwise competition experiments in the presence of sulfite and indirectly by the induction of *ATG8* expression by sulfite. Requirement of vacuolar and autophagic functions can be related with turnover of proteins and subcellular structures damaged by sulfite. On the other side, vacuole activity is required for intracellular pH homeostasis (Carmelo *et al*., 1997), and given the strong dependence of sulfite toxicity on pH, this might also explain the dependence of cells on vacuolar function for survival to sulfite exposure.

While the impact these functions might be minimized on *S. cerevisiae* strains showing altered *SSU1* expression, it is to be expected that most yeast species will require normal autophagic and vacuolar functions to cope with sulfite challenge. Hence, there are potential implications of these findings for the design of industrial processes or to fight yeast spoilage. For example, it would be possible to combine sulfite supplementation with treatment conditions or growth inhibitors that target autophagy, like sorbic or benzoic acid (Abeliovich and Gonzalez, 2009), in order to attain a synergistic effect and to reduce the amounts of preservatives required to ensure the microbial stability of food products.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. Nephelometric monitoring of *S. cerevisiae* BY4743 growth in minimal medium containing different amounts of potassium metabisulfite. The concentration of 60 mg L^{-1} was considered to show a clear inhibitory effect, sufficient to run competition experiments of YKO strains in the BY4743 background.

Appendix S2. Genes whose deletion results in increased sensitivity to sulfite, according to result of the competition experiment. Sequential batch cultures were grown in triplicate, either without or with 60 mg L^{-1} potassium matabisulfite, until reaching between 1.5-3.0 OD_{600}, then 10% of the volume was transferred to fresh medium. The procedure was repeated up to three times (around 10 generations). Only genes with LogFC<-3 are shown.

Appendix S3. Known interactions among genes or gene products highlighted by strains showing impaired growth in the presence of sulfite. String 10.0 (Franceschini et al., 2013) was used to visualize known interactions between the genes (or their gene products) deleted in the strains highlighted by the pairwise comparisons. Analysis and visualization parameters were as follows. Confidence level: 0.700; view mode, evidence; prediction methods, all.

