Oxidative stress tolerance, adenylate cyclase and autophagy are key players in yeast chronological life span during winemaking

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Abstract

Most grape juice fermentation takes place when yeast cells are in a non dividing state, called the stationary phase. In such circumstances, we aimed to identify the genetic determinants controlling longevity, known as chronological life span. We identified commercial strains with both short (EC1118) and long (CSM) life spans in laboratory growth medium, and compared them under diverse conditions. Strain CSM shows better tolerance to stresses, including oxidative stress, in the stationary phase. This is reflected during winemaking, when this strain has an increased maximum life span. Compared to EC1118, CSM overexpresses a mitochondrial rhodanese-like gene, *RDL2*, whose deletion leads to increased ROS production at the end of fermentation, and a correlative loss of viability at this point. EC1118 shows faster growth and a higher expression of glycolytic genes, and this is related to greater PKA activity due to the up-regulation of the adenylate cyclase gene. This phenotype has been linked to the presence of a δ element on its promoter, whose removal increases life span. Finally, EC1118 exhibits a higher level of protein degradation by autophagy, which might help achieve fast growth at the expense of cellular structures and may be relevant for long-term survival under winemaking conditions.

Introduction

Yeast growth during grape juice fermentation develops through the typical growth phases on batch: lag phase, exponential growth, stationary phase and death phase (6, 39). The majority of sugar fermentation takes place after cells enter the non dividing state; that is, the stationary phase. Therefore, the viability and vitality of *Saccharomyces cerevisiae* in the stationary phase are key factors in successful vinification. The yeast death phase during winemaking is still a poorly understood process that has been exclusively linked to the toxicity of the high ethanol concentration reached during fermentation (14). Some authors claim that cells start dying when sugar is still present (39), while others state that cells only start dying when sugars are consumed (6). In our experience, both situations can take place depending on the environment. The same yeast strain grown in the same grape juice is highly viable at the end of fermentation at a low temperature, but the death phase starts in the presence of sugars when the temperature rises (9). Cell death leads to loss of cell integrity and to the release of cell contents, which could influence other microorganisms' growth; for instance, lactic acid bacteria, which carry out malolactic fermentation, can be inhibited (via medium chain fatty acids) and stimulated (via nitrogenous components) by yeast lysis products (3). Spoilage microorganisms can likewise be affected. Besides, certain wines age in the presence of dead yeast (aging on lees), which confers wine chemical and color stability (25). Cell autolysis is central in these increasingly popular forms of winemaking. The impact of autolysis on sparkling wines has been studied in detail (12).

Survival in the stationary phase has been referred to as chronological life span (CLS) and is one of the standard ways of studying aging in *S. cerevisiae* (22, 29). CLS is highly variable in natural isolates and tends to be shorter than in laboratory strains

76 (37). Molecular causes of aging have been widely studied in several model organisms,
77 including yeast (29). Traditionally, aging has been explained to be caused by an
78 accumulation of molecules damaged by free radicals, mainly produced upon electron
79 transport events in mitochondria (5). The impact of reactive oxygen species (ROS),
80 such as superoxide, on CLS has been studied in yeast (19). Antioxidant proteins like
81 superoxide dismutase Sod2p are essential for life span. The high sugar concentration of
82 grape juice is believed to cause a repression of the oxidative phosphorylation
83 machinery. However in wine yeast grown on a synthetic medium with a high sugar
84 concentration, ROS are produced and oxidative damage takes place (30). In the last few
85 years, many authors have found that nutrient-sensing pathways, such as Sch9/TOR and
86 Ras/cAMP/PKA, have a considerable impact on life span (24). For instance, the
87 mutations in adenylate cyclase *CYR1*, an enzyme involved in cAMP synthesis which
88 activates protein kinase A (PKA), extend CLS in laboratory yeast strains (23). This
89 result is related to the fact that a low-nutrient diet without malnutrition (dietary
90 restriction or DR) is an effective way of extending life span. This situation would
91 diminish the activity of these pathways, leading to less growth, lower metabolism rates
92 and develop mechanisms that extend life span, such as stress resistance and autophagy.
93 Autophagy consists in the sequestration of part of the cytoplasm by a double membrane
94 that fuses to vacuoles to release nutrients (33). This phenomenon has also been
95 described in sparkling wine production (12). Work done under laboratory conditions has
96 linked aging to two relevant metabolites in winemaking: ethanol and acetic acid. The
97 down-regulation of alcohol dehydrogenase 2 by deacetylase Sir2p leads to higher
98 ethanol levels which shorten CLS (20). Acetic acid has been identified as a pro-aging
99 agent under laboratory conditions because of its ability to acidify media (8).

In this work, we aimed to identify the genetic determinants of stationary phase viability and CLS. To go about this, we compared the CLS of a large number of industrial wine yeasts, and we isolated model short-lived (EC1118) and long-lived (CSM) strains. We compared their stress resistance, vinification performance, autophagy and transcriptome to find differences in their behavior. CSM's greater oxidative stress in stationary phase tolerance correlates with its longer CLS, and we found that rhodanese-like proteins Rdl1/2p are players in this response. The higher metabolic rate of strain EC1118 also seems to be a determinant for its short life span. Finally, we also suggest that it is at least partially dependent on a particular allele of adenylate cyclase gene *CYR1*.

Materials and methods

Yeast strains and growth media.

Table S1 lists the industrial wine yeasts used in this work. Haploid strain C9 was a gift from M. Walker (47). Gene disruptions were performed by using recyclable selection marker *loxP-kanMX-loxP* from plasmid pUG6 following the protocol of Güldener et al. (28). The marker was eliminated by transforming with the cre recombinase-containing plasmid YEp351-cre-cyh according to Deleneri et al. (17). Table S2 lists the oligonucleotides employed to amplify deletion cassettes and to check transformants.

For yeast growth, YPD medium (1% yeast extract, 2% bactopeptone, 2% glucose) and SC medium (0.17% yeast nitrogen base, 0.5% ammonium sulphate, 2% glucose and 0.2% drop-out mix with all the amino acids) were used (2). Selective plates

125 contained 20 μg mL^{-1} geneticine or 0.1 μg mL^{-1} cycloheximide. Red grape juice (Bobal
126 variety) was a gift from Bodegas Murviedro and was sterilized overnight with 500 μg/L
127 of dymethyldicarbonate. Synthetic grape juice MS300 was prepared as described by
128 Riou et al. (40), but with an equimolar amount of glucose and fructose at 10%.
129
130 **Yeast growth, chronological life span measurements and stress conditions**
131
132 Under laboratory conditions, the CLS experiments were adapted from Fabrizio
133 and Longo (22) and were performed as follows: precultures of the selected strains were
134 grown overnight on YPD and inoculated in SC media at an OD_{600} of 0.1. From day 3 of
135 growth at 30ºC, aliquots were taken, diluted and plated. Colonies were counted and
136 percentage of survival was calculated by taking day 3 of growth as 100% survival.
137 For the microvinification experiments, cells from 2-day cultures in YPD were
138 inoculated at a final concentration of 10^6 cells/mL in filled-in loosely closed conical
139 centrifuge tubes with 50 mL of grape juice. Incubation was performed with very low
140 shaking, at 22ºC for natural juice and at 24ºC for synthetic grape juice. Evolution of
141 vinifications was followed by determining cell viability and sugar consumption, as
142 previously described (49). Survival plots were drawn by taking the highest cell viability
143 point (around 2-4 days) as 100% survival.
144 For the stress tests, cells were either grown in YPD for two days to test cells
145 under stationary phase conditions or diluted in fresh YPD at OD600=0.1 and grown to
146 0.5 when testing the exponential conditions. Before applying stress, an aliquot of the
147 culture was diluted and plated, and its viability was considered 100%. The stress
148 condition was applied to the remaining culture. Ethanol was added at 10%(V/V) for 1h;
149 for hyperosmotic stress, cells were spun and placed in YPD containing 20% glucose for

90 min, and heat shock was achieved by incubating for 1 h at 42ºC. For oxidative stress, 5 mM H_2O_2 were added to the culture and cells were incubated for 30 min if they were growing exponentially, or for 1 h in the case of stationary phase cells.

Microscopy methods

For propidium iodide staining, 500 µl of cells were washed in PBS buffer. Then 5 µl of a 1 mg/mL stock solution of the dye were added to be incubated in darkness for 30 min. Cells were washed in PBS and visualized. Dihydrorhodamine 123 was added at 5 µg/mL from a 2.5 mg/mL stock solution in ethanol. Cells were viewed after a 2-hour incubation at room temperature in the darkness. Cells were visualized with a rhodamine filter under a Nikon eclipse 90i fluorescence microscope.

Metabolite and lipid peroxidation determinations

Reduction of sugars during fermentation was measured by their reaction to DNS (dinitro-3.5-salycilic acid) following a modified version of Miller's method (41). Ethanol and acetic acid were measured with the kits provided by r-Biopharm following the manufacturer's instructions.

Quantification of lipid peroxidation was carried out by the reaction of thiobarbituric acid with the malondialdehyde (MDA) product of oxidized fatty acid breakage (18). Cells were collected and then extracted by vortexing with one volume of glass beads in 0.5 ml of 50 mM sodium phosphate buffer, pH 6.0, 10% trichloroacetic acid (TCA) with FastPrep 24. After centrifugation, 300 µl of supernatants were mixed to 100 µl of 0.1 M EDTA and 600 µl 1% thiobarbituric acid in 0.05 M NaOH to be then

incubated at 100°C for 15 min. Malondialdehide was measured by absorbance at 535 nm.

Microarray analysis and Northern blot

RNA isolation was carried out as previously described (10). cDNA was synthesized by combining 20 μg of total RNA and 1 μg of oligo (dT) (Roche), dithiothreitol (0.1M), dNTPs mixture (10mM each; Invitrogen), aminoallyl-dUTP 50mM (Fermentas), RNase inhibitor and Superscript III reverse transcriptase (Invitrogen). Aminoallyl-cDNA was purified using the MinElute PCR Purification Kit (Qiagen) following the manufacturer's instructions. A labeling reaction was produced by incubating 1.5-2 mg of aminoallyl-cDNA with 3 mL of the Cy3 and Cy5 fluorophores (Amersham) at a basic pH (0.2M Na2CO3, pH 9) for 2 h at room temperature. Labeled cDNA was purified using the aforementioned kit. Yeast 6.4Kv7 microarray slides from the Microarray Center of University Health Network (Canada) were used. Microarrays were prehybridized for 45 min at 42ºC in 3xSSC (0.15M NaCl and 0.015M sodium citrate), 0.1% (w/v) sodium dodecyl sulphate (SDS) and 0.1 mg/mL bovine serum albumin. Before hybridization, equal amounts of Cy5- and Cy3-labeled cDNA, 50% (v/v) formamide, 5xSSC and 0.1% (w/v) SDS were mixed and denatured for 1 min at 95ºC. The mixture was applied to the prehybridized microarrays, covered with a Hybri-slip (Sigma-Aldrich) and hybridization took place for 16 h in a humidified chamber in a water bath at 42ºC. The hybridized microarrays were washed for 5 min at 42ºC in 2xSSC, 0.1% (w/v) SDS for 20 min at room temperature in 0.1xSSC, 0.1% (w/v) SDS, for 6 min at room temperature in 0.1xSSC, and for 1 min at room temperature in 0.01xSSC. After washing, microarrays were immediately scanned with an Axon 4100A scanner at a resolution of 10 mm and the data were analyzed using

the GENEPIX PRO 6.1 software package (Axon Instruments). Functional categories were identified using Funcassociate 2.0.

The array experiments were carried out in triplicate and the data were deposited under GEO accession number GSE33068.

Total RNA was separated in formaldehyde agarose gels, blotted on nylon membranes and hybridized in accordance with Sambrook and Russell (43).

Western blotting

For the Western blot analysis, cells were broken with a volume of glass beads in a buffer containing Tris-HCl 0.1 M pH 7.5, NaCl 0.5 M, $MgCl_2$ 0.1 M, NP40 1% (v/v), PMSF 10 mM and protease inhibitors (complete Mini, EDTA-free from Roche). Protein concentration was measured by the Bradford method (7) using the Bio-Rad Protein assay following the manufacturer's instructions. Extracts were diluted in loading buffer (Tris-HCl 240 mM pH 6.8, SDS 8% (p/v), glycerol 40%, β-mercaptoethanol 10%).

To conduct the Western blot analysis, SDS-PAGE were done in an Invitrogen mini-gel device and were blotted onto PVDF membranes. The anti-ALDH antibodies were obtained from Rockland (Gilberstville, USA). The ECL Western blotting detection system (Amersham) was used following the manufacturer´s instructions.

Results

Study of chronological life span in wine yeast

In order to gain a better understanding of the genetic determinants of longevity in industrial yeasts, we first compared a set of industrial strains of different origins (see Supplementary Table 1) under standard laboratory conditions (22) to identify the long- and short-lived strains to be used as aging models. To go about this, a preculture was grown overnight in YPD and inoculated to an O.D. $_{600}$=0.1 in synthetic complete medium SC. After 3 days (time 0), aliquots were taken, diluted and plated to obtain cell viability. The time 0 value of cfu/mL was taken as 100% survival (Fig. 1A), this being a commonly used time point in life span experiments (22). On day 3, all the strains had stopped growing and their total cell numbers were similar, ranging from 76 to 156 x10^6 cfu/mL (see Fig. S1a). In general, we observed a wide variety of death profiles. The viability of a group of strains (EC1118, UCLM 5235, L2056, Zymaflore and BM45) rapidly declined. EC1118 and UCLM5235 presented the shortest life span and a constant decrease in longevity. The other three strains displayed a different death profile with an extended long-term life span after initial sudden loss of viability. Of the long-lived strains, Fermichamp exhibited high viability after the first 5 days after which it sharply fell, while CSM presented the most remarkable long-term life span. We included a strain devoted to the biological aging of Sherry wines (C Jerez). Despite its highly different physiology, it exhibited an intermediate life span. Therefore, we chose a short-lived strain (EC1118) and a long-lived one (CSM) to be compared at several levels in the following experiments. Upon plotting the day that cells reached a viability of 50% in relation to oxidative stress resistance (measured as the inhibition diameter caused by H_20_2), we found a correlation between both parameters (Fig. S1b), be it a

poor one. This indicates that oxidative stress tolerance is an important factor for survival, but not the only one. To understand the differences between our model strains, we first tested their stress tolerance under standard laboratory conditions; i.e., applying stress conditions to those cells growing exponentially in rich medium YPD (Fig. 1B). Under these conditions, we found no difference in sensitivity to ethanol, but CSM was more sensitive to hyperosmotic (produced by a large amount of glucose), oxidative (caused by hydrogen peroxide) and heat shock (at 42ºC) stresses. Unexpectedly, the long-lived strain had a lower basal stress response than the short-lived one. Next we tested the stresses that could prove relevant at the end of fermentation, such as high ethanol and oxidative stress, in the stationary cells grown in YPD for two days (Fig. 1C). We found that CSM was more tolerant and matched the generally accepted role of stress tolerance as a positive factor of life span.

Differential behavior of strains EC1118 and CSM during winemaking

We compared the behavior of strains EC1118 and CSM under winemaking conditions during red grape juice fermentation (Fig. 2A). Cell viability displayed a very distinct pattern between both strains. EC1118 grew faster and reached higher cell density, but also went through a fast cell death phase which started when sugars were about to be finished. CSM showed a totally different pattern: it reached lower cell density, but cells remained viable over a longer time. Therefore during winemaking, CLS correlated with the results observed under laboratory conditions. Regarding glucose consumption, EC1118 underwent a faster pace of metabolism, although both strains finished vinification to dryness efficiently and produced a similar amount of final ethanol (data not shown). We repeated the experiment in synthetic grape juice and

obtained similar results (Fig. S2); in this case, however, the growth and glucose metabolism rates were slower, with greater differences between both strains. The colony-counting method detected cells which were able to re-enter a proliferative state. To determine whether there was also a difference in cell integrity, we stained cells with propidium iodide, which is only taken by damaged cells (15). We compared EC1118 and CSM after glucose consumption in grape juice fermentation (taken as time 0). We found that the damaged cells in strain EC1118 also quickly increased if compared to CSM (Fig. 2B), which correlates with the viable cell count. Hence at least a partial lysis took place during the aging process under our winemaking conditions. The amount of ethanol during this process did not change and was similar between strains (data not shown); thus, mortality is dependent on neither different production nor ethanol consumption.

Oxidative stress is a key factor for cell aging. To determine potential differences in oxidative damage in our model strains, we measured reactive oxygen species (ROS) at the end (13 days) of fermentation in synthetic must MS300 (Fig. 2C). We used this medium to avoid interference of florescence with red grape juice components. We observed a significantly higher incidence of ROS in EC1118. As a marker of oxidative damage, we measured lipid peroxidation ten days after fermentation completion (Fig. 2D). Once again, EC1118 accumulated greater oxidative damage than the CSM strain. Therefore, EC1118 seemed less prepared to detoxify ROS under winemaking conditions, leading to increased damaged that may contribute to loss of viability. This correlated with a higher sensitivity to H_2O_2 as seen under laboratory conditions (Fig. 1C).

A well-known mechanism required for long-term survival is autophagy. A marker of nitrogen deficiency-induced autophagy is cytosolic aldehyde dehydrogenase

301 Ald6p (36). Using an anti-ALDH antibody, we detected Ald6p and mitochondrial Ald4p
302 (used as an internal loading control under laboratory conditions) during fermentation in
303 synthetic grape juice (Fig. 3A). Ald4p remained relatively stable throughout
304 fermentation and showed a similar pattern between strains. Surprisingly, Ald6p
305 disappeared quickly in strain EC1118, while CSM showed a lower degree of autophagy
306 as the Ald6p band was visible in later vinification stages. According to the yeast
307 producer, EC1118 had low nitrogen requirements, while CSM had high ones
308 (www.lallemandwine.us/products/yeast_chart.php). EC1118 seemed to have a better
309 protein turnover, which may lead to enhanced nitrogen supply and, therefore, to growth
310 at the expense of long-term survival. Despite its good autophagic mechanism, EC1118
311 reacted to the nitrogen levels in the medium, and nitrogen starvation extended CLS (Fig.
312 S3), as previously observed with another commercial strain: L2056 (H. Orozco, E.
313 Matallana and A. Aranda, submitted).

314 As Ald6p was not only the main cytosolic aldehyde dehydrogenase, but also the
315 main enzyme involved in acetate production during winemaking (38), we measured the
316 amount of acetic acid at the end of the fermentation (when sugars were under 2 g/L)
317 carried out on synthetic grape juice (the conditions used in Fig. 3A) and on natural
318 grape juice (as in Fig. 2A). In all cases, we found that acetic acid reached higher levels
319 in the CSM strain when compared to EC1118. Therefore, acetic acid production during
320 fermentation may be modulated by the autophagic degradation of Ald6p. It is worth
321 mentioning that acetic acid under winemaking conditions is not a key player in life span
322 (no correlation was found between higher acetic acid accumulation and shorter CLS).
323 This is possibly because of the highly buffered nature of grape juice due to the presence
324 of a large amount of tartaric, malic and citric acids, which allows pH to remain stable
325 throughout fermentation.

Comparative global analysis between strains EC1118 and CSM

In order to find genetic determinants of life span under winemaking conditions, we compared the transcriptome of our reference strains in the stationary phase of fermentation on day 6 of growth in synthetic grape juice (see Fig. S2). Thirty-five genes were up-regulated by a factor of 2 or more in CSM if compared to EC1118. Both the functional categories of aryl alcohol dehydrogenase (*AAD* genes) and the thiamine biosynthetic process (*THI* genes) were enriched (Table 1). Thiamine is a cofactor of several enzymes, like pyruvate dehydrogenase and decarboxylase, involved in the carbon metabolism. This could potentially confer CSM a selective advantage in late fermentation stages. *THI* and *AAD* are families of highly homologous and subtelomeric-located genes (16). We measured the telomere length of both strains during fermentation (Fig. S4) and found that CSM had slightly shorter telomeres than EC1118, which may cause a different chromatin structure, leading to a higher expression of these genes. As previously shown with laboratory strains, telomere length did not correlate with life span (4) as the strain with the longest telomeres (EC1118) showed a shorter life span. Genes with oxidoreductase activity were relatively abundant, including the genes in the ergosterol biosynthesis pathways (*ERG5* and *ERG11*), which may confer strain CSM better tolerance to oxidative stress (31). Two homologous genes, *RDL1* and *RDL2,* were among the genes that do not belong to any functional category, which could be relevant in antioxidant protection given their mitochondrial location (26); we go on to study them in the next section.

Regarding the relatively up-regulated genes in strain EC1118, one remarkable finding was that the abundance of the functional categories related to metabolism in the 87 genes that were induced by 2-fold or more (Table 1B). Enrichment was noted in

351 glycolysis and the amino acid metabolism (particularly sulphur amino acids) genes.
352 This stronger metabolism could explain this strain's faster growth and glucose
353 consumption rate if compared to CSM. The other genes found may also influence
354 EC1118 metabolism; for instance, plasma membrane proton pump gene *PMA1* was
355 induced, together with the two protein kinases *HRK1* (2.37-fold) and *PTK2* (1.65-fold),
356 which stimulate activity (27). This may lead to the use of ATP, produced during
357 enhanced glycolysis, to create a higher membrane potential, which would facilitate
358 transport and, therefore, growth. The relative up-regulation of adenylate cyclase gene
359 *CYR1* (2.45-fold) would activate PKA, thus stimulating growth and reducing life span
360 (23), which will be further studied later.
361
362 **Rhodanese-like protein Rdl2p and adenylate cyclase are relevant for life**
363 **span under winemaking conditions.**
364
365 In strain CSM, two genes of rhodanese-like proteins (*RDL1*/*2*) were up-regulated
366 (Table 1A). Rhodanese enzymes are able to catalyze the transfer of sulphane sulphur
367 from thiosulphate to cyanide (26). Rhodanese has been shown to play a role in anti-
368 oxidant defense in bacteria (13) and mammals (34), and to be age-related in humans
369 (48). To test their role in oxidative stress in wine yeast, we deleted *RDL1*, *RDL2* and
370 both the genes in the haploid wine strain C9 derived from strain L2056 (47). We tested
371 their viability after H_2O_2 shock in stationary cultures in YPD (Fig. 4A). We saw
372 increased sensitivity in both single mutants, although the *rdl2*Δ mutant was more
373 affected. The double mutant was no more affected than the single ones, suggesting that
374 both proteins could act in the same protection pathway. To investigate the role of these
375 proteins, we chose the *rdl2*Δ mutant, the most stress-sensitive one, to carry out

376 fermentation in synthetic grape juice (which enabled us to measure ROS). We saw that
377 the mutant strain grew slightly more than the parental one and started to metabolize
378 sugars faster. However, its viability sharply declined in later fermentation stages, which
379 is probably the cause of the slower sugar assimilation rate noted at this point (Fig. 4B).
380 Note that viability dropped at around day 6 of fermentation in synthetic grape juice,
381 which corresponds to the point at which the *RDL2* gene was more highly expressed in
382 strain CSM if compared to strain EC1118, suggesting that this protein is relevant for
383 maintaining viability at this fermentation stage. However, it displayed an extended life
384 span once fermentation finished, indicating a physiological change upon saturation after
385 which the Rdl2p function becomes important. Another change occurs once glucose is
386 consumed, after which point Rdl2p seems to play a negative role. We measured ROS
387 during fermentation and observed that oxidatively stressed cells progressively increased
388 in the parental strain, and vastly increased in the *rdl2*Δ mutant strain at the point where
389 viability dropped (Fig. 4C). Therefore, Rdl2p plays a role in diminishing oxidative
390 damage at the end of fermentation when mitochondria seem to play a role despite the
391 still fermentative metabolism.

392 As mentioned in the previous section, gene *CYR1*, which codes for adenylate
393 cyclase, was up-regulated in strain EC1118. cAMP overproduction, which would cause
394 PKA induction, may explain its shorter CLS. While the EC1118 genome was sequenced
395 (35), we searched for distinct properties of this gene. In the promoter, 117 bp upstream
396 from the transcription start site (32), we found a 336-bp δ element, a long terminal
397 repeat (LTR) which marked an ancient transposition event by a Ty2 transposon (Fig.
398 5A). This element was not present in the S288c laboratory strain background, which
399 was used in the initial genome sequencing project. By PCR, we tested the presence of
400 this δ element in the industrial strains described in Figure 1 using oligonucleotides c and

e, and by also checking the original allele by employing oligonucleotides d and e (Fig. 5A). Most of them presented a canonical sequence, but strains UCLM 5235, ICV46 and Sherry strain C (depicted in gray in Fig. 1) also had the δ element inserted into the *CYR1* promoter. UCLM5235 had the second shortest CLS, so this element could be common in strains with an extremely short life span. This element contained a promoter, but its transcription ran in the opposite direction to *CYR1*. Thus, the higher *CYR1* expression could be due to changes in chromatin structure, or to the elimination or distancing of a repressor element. To gain a better understanding of its function in, and its influence on, EC1118 life span, we deleted the two copies of the δ element using a recyclable *kanMX* cassette (28). This would replace the LTR with a shorter 71 bp *loxP* element. We measured the *CYR1* mRNA of this mutant and its parental strain together with strain CSM (Fig. 5B). We found higher levels in strain EC1118 when compared to CSM which, given the array data, was expected. The δ element deletion lowered the levels of this transcript, suggesting that the shorter length of *loxP* restores, at least partially, the canonical arrangement or the chromatin structure of the *CYR1* promoter. We carried out vinifications in grape juice with EC1118 and EC1118 δΔ to find that the mutant strain grew at a slightly slower rate, although it reached similar cellular density to start a less pronounced death phase (Fig. 5C). Regarding glucose consumption, the mutant strain displayed a slower metabolic rate, although it finished fermentation efficiently. All these aging and metabolism results fit with a decreased PKA activity in the δΔ mutant given its lower *CYR1* expression.

Discussion

This paper studies determinants of longevity in the stationary phase in wine yeast. Our approach involved screening a collection of industrial wine yeasts, most of which are commercial, with a view to identifying strains with extreme life spans under standard laboratory conditions of incubation in synthetic medium SC (Fig. 1A). We identified EC1118, a widely used commercial strain, as our short-lived strain model. On the other hand, CSM exhibited an extremely long life span. This behavior noted in the laboratory medium correlated with the behavior observed during natural grape juice fermentation. As expected, we observed an intermediate behavior of other strains with an intermediate CLS, such as L2056 or T73, during winemaking (data not shown). Life span was measured by the ability to re-enter the cell cycle. However, we noticed that cell death during winemaking is linked to increased cell lysis, indicating that cellular content was leaked to media (Fig. 2B), and that may potentially affect malolactic bacteria or spoilage microorganisms' growth. Therefore, the CLS studies done under laboratory conditions are a good approach to study aging in a large number of strains. The comparison made between these two strains indicates that oxidative stress resistance and metabolism control are factors which contribute to CLS under winemaking conditions.

As in laboratory strains, stress tolerance is a key factor in aging (21). Strain CSM has increased tolerance to stress, but only under stationary conditions (Fig. 1B), while the basal stress tolerance of EC1118 in the exponential phase is greater. The global stress response has been shown to change during the growth phase in a strain-dependent manner (42). CSM's greater tolerance to ethanol in the stationary phase may prove positive for survival after finalizing fermentation. It is worth mentioning that both

strains produce a similar amount of final alcohol. Under laboratory conditions, strain CSM shows enhanced tolerance to oxidative insult by hydrogen peroxide (Fig. 1B). During winemaking, we found that ROS production, and the concomitant damage measured as lipid peroxidation, correlate with EC1118's lower oxidative stress tolerance and shorter life span (Fig. 2). However, such greater ROS production may be caused by the larger number of dying cells. At this point, we are unable to state if ROS production is the cause or the result of cell death. This differential stress tolerance does not seem to be based on transcriptional regulation as no significant up- or down-regulation of the oxidative stress genes was noted when comparing the transcriptome of both strains (Table 1). Nevertheless, the up-regulation of the two genes (*RDL1* and *RDL2*) related to the mitochondria in strain CSM was striking. Both proteins belong to a family of enzymes called rhodaneses that are able to transfer sulphur from thiosulphate into cyanide (26). There is no evidence that cyanide detoxification is relevant during vinification, but we believe that these proteins are probably involved in other processes. Deletion of both genes in a wine yeast strain, particularly *RDL2*, produces enhanced sensitivity to oxidative stress (Fig. 2). The short-term viability of mutant strain *rdl2*Δ diminishes during winemaking, although long-term CLS is extended. In the last fermentation stages, we see increased ROS production in the mutant, indicating a defective free radical metabolism or its increased production. This increase in oxidative damage may be behind the drop in the mutant strain's viability, but surviving cells may activate the antioxidant mechanism to cause elongation in their long-term life span. Rhodanese RhdA from *Acetobacter* is required for an oxidative stress response and has been linked to the repair of Fe-S clusters of certain proteins. An isoform of mitochondrial bovine rhodanese is able to oxidize thioredoxin by acting as a detoxification mechanism of the intramitochondrial reactive species (34).

Life span is closely linked to metabolic activity. EC1118 is a more vigorous strain that grows and consumes glucose faster than CSM, and may be caused by the relative up-regulation of the genes involved in carbon (particularly glycolysis) and nitrogen metabolisms (Table 1). This higher metabolism rate can cause unbalances, leading to greater ROS production in this strain. This difference in the metabolism rate may be related, at least partially, to the higher expression of adenylate cyclase gene *CYR1* (Table 1). Higher cAMP levels possibly lead to the activation of protein kinase A, an inductor of a transcriptional program linked to growth and cell division which also stimulates glycolysis (44). High PKA activity has been linked to necrosis in mammals, and deletion of regulatory PKA subunit *BCY1* induces autolytic phenotypes in yeast under sparkling wine second fermentation conditions (46). We found that this difference may be caused by the presence of an ancient retrotransposition event in some wine yeasts which leavesa δ element in the promoter of *CYR1* (Fig. 5). Replacing it with a shorter *loxP* sequence extends life span, but lowers the glucose metabolic rate. Therefore, this allele might be a good marker to identify those strains displaying rapid growth and sugar consumption with a potentially shorter life span and increased cell lysis.

This work also identifies another molecular difference between our model strains. EC1118 displays a higher degradation of autophagy marker Ald6p (Fig. 3). Cytosolic aldehyde dehydrogenase is degraded when nitrogen is low, but only by autophagy (36). This higher protein turnover may cause its lower nitrogen requirements if compared to CSM. Ald6p degradation is also a way of controlling acetic acid production, whose impact on wine organoleptic quality is negative. Studying autophagy with simpler markers, such as alkaline phosphatase, could be a good test to select yeast with low nitrogen requirements and potentially low acetic acid production. Autophagy

usually tends to be required to achieve a full life span (29), but could prove detrimental to cells under certain conditions. For instance the *csc1-1* allele, which causes a constitutive autophagy phenotype, displays accelerated autolysis (11). Therefore, balanced autophagy is vital for an optimal life span. Autophagy may degrade a protein or an organelle, which may prove useful for survival under winemaking conditions. Autophagy is repressed by the nutrient-sensing pathways PKA and TOR (45, 46). In this case, EC1118 shows increased autophagy and Cyr1p; thus PKA does not seem to be a determinant in wine strains under grape juice fermentation conditions. This may relate to the fact that PKA responds to carbon sources and that grape juice fermentation takes place in an environment where glucose is not limiting. Nitrogen is the limiting nutrient during vinification and TOR responds to nitrogen levels (24), hence this pathway is likely the key factor in controlling autophagy under such conditions. Therefore, finding inhibitors of TOR could be a good way of increasing autophagy and of obtaining better adaptation to low nitrogen fermentation with potentially lower acetate production due to Al6p degradation. Finally, GRAS food additive caffeine is able to inhibit TOR and has been proposed to be a good candidate for this very purpose (1).

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672 **Figure legends**

673

674 FIG. 1. Comparative chronological life span and stress response between strains. A)
675 CLS analysis of industrial yeast strains in the SC medium. The assays were performed
676 by making serial dilutions, plating an aliquot and counting colonies. CFU (colony-
677 forming units)/mL were done in relation to the values obtained from day 3 post-
678 inoculation, and were considered 100% survival. Stress tolerance of strains EC1118 and
679 CSM grown exponentially (B) or to the stationary phase (C) in YPD. Stress was
680 applied, and serial dilutions were made and plated. Cell viability numbers were made
681 relative to viability before the stress conditions. Experiments were done in triplicate,
682 and the mean and standard deviation are provided. *$p<0.05$, **$p<0.001$, unpaired t-test,
683 two-tailed.

684

685 FIG. 2. Behavior of strains EC1118 and CSM during grape juice fermentation. A)
686 Natural grape juice fermentation. Cell viability measured as CFU/mL and total sugars
687 are indicated over time. B) Cell integrity measured by propidium iodide staining after
688 grape juice fermentation had finished. The percentages of the non stained cells count
689 under a fluorescence microscope are shown. C) ROS production. Cells containing ROS
690 were detected on day 13 of fermentation by fluorescent probe dihydrorhodamine-123
691 and the % of stained cells is shown. D) Lipid peroxidation measured as pmol of
692 malondialdehyde per mg of cells 10 days after glucose consumption. Experiments were
693 done in triplicate, and the mean and standard deviation are provided. *$p<0.05$,
694 **$p<0.001$, ***$p<0.005$, unpaired t-test, two-tailed.

695

FIG. 3. EC1118 and CSM have a different autophagy and acetic acid production levels. A) Western blotting of aldehyde dehydrogenase (Ald) during synthetic grape juice fermentation in both strains. Alcohol dehydrogenase (Adh) was used as a loading control. B) Acetic acid production after fermentation in synthetic grape juice (MS) and natural grape juice (GJ) for both strains. Experiments were done in triplicate, and the mean and standard deviation are provided. **$p<0.001$, ***$p<0.005$, unpaired t-test, two-tailed.

FIG. 4. Rhodanese Rdl2p plays a role in oxidative stress response, ROS levels and life span. A) Viability after oxidative stress caused by H_2O_2 of stationary cultures in YPD of *rdl1*Δ, *rdl2*Δ and *rdl1*Δ*rdl2*Δ mutants and their parental strain C9. B) Synthetic grape juice fermentation of *rdl2*Δ mutant and C9. Cell viability measured as CFU/mL and total sugars are indicated over time. C) ROS production measured during fermentation described in B). Experiments were done in triplicate, and the mean and standard deviation are provided. *$p<0.05$, ***$p<0.005$, unpaired t-test, two-tailed.

FIG. 5. A δ element in the *CYR1* promoter affects longevity. A) Scheme of the LTR present in the *CYR1* promoter of some industrial yeast strains. The oligonucleotides used to detect it by PCR are depicted. B) Northern blot analysis of the *CYR1* gene expression in strains CSM and EC1118, and a mutant with both copies of the δ element replaced with a *loxP* site, EC1118 Δδ at six days of fermentation in synthetic grape juice MS300. rRNA was used as a loading control. C) Natural grape juice fermentation of strain EC1118 and the mutant strain Δδ. Cell viability measured as CFU/mL and total sugars are indicated over time.

Table 1. Comparative microarrays analysis of the gene expression between strains CSM and EC1118. A) Functional categories overrepresented in the genes up-regulated by a factor of 2 or more in strain CSM. B) Functional categories overrepresented in the genes up-regulated by a factor of 2 or more in strain EC1118. The induction level is shown for each gene in brackets. In both cases, all the genes up-regulated more than 2.5-fold have been included.

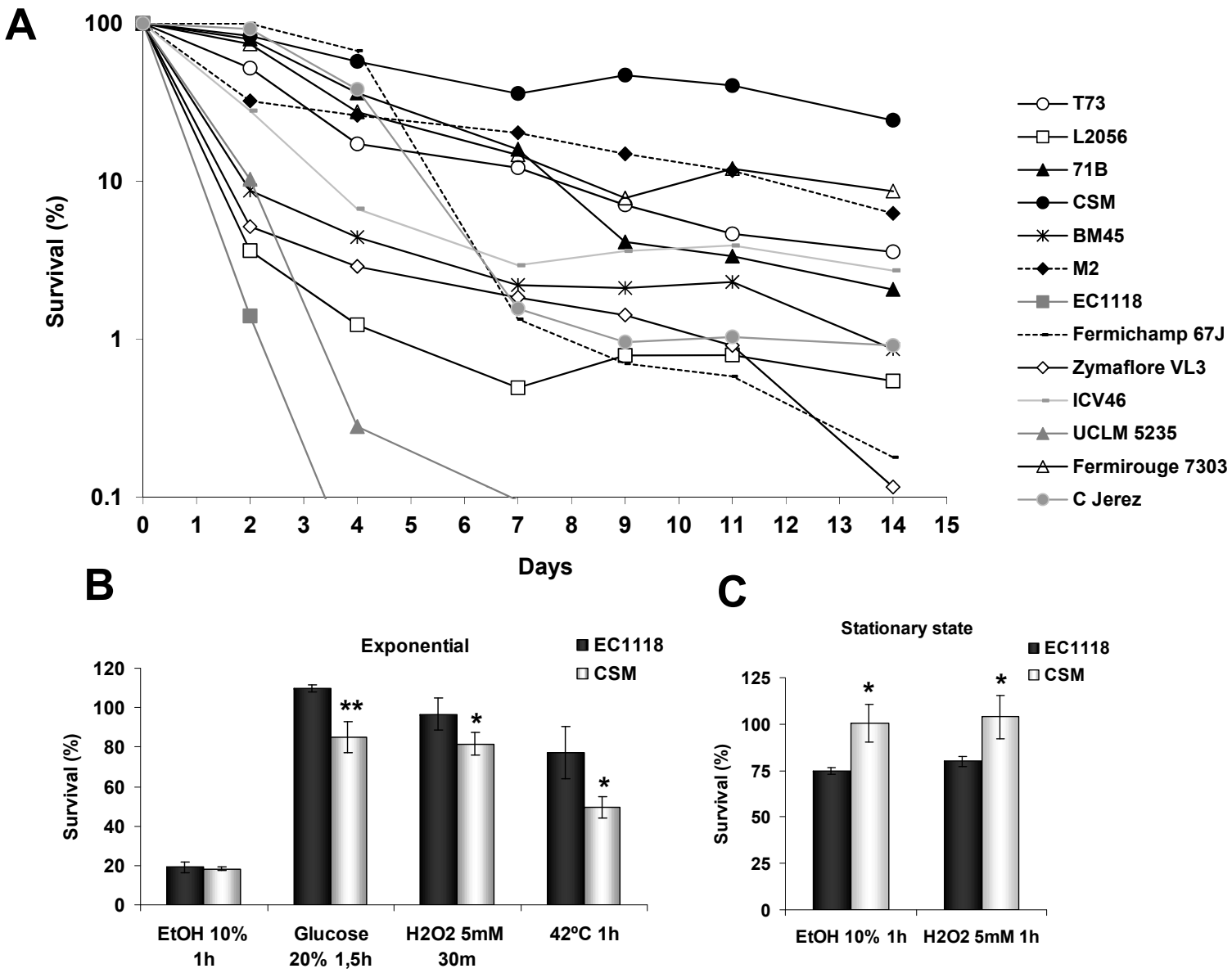
A)

Categories	**Genes up-regulated in strain CSM (fold induction)**
Thiamine biosyntetic process (p= 1.04e-6)	*THI12* (10.88), *THI11* (8.44), *THI13* (6.71), *THI5* (2.65)
Aryl alcohol dehydrogenase (p= 5.19e-13)	*AAD15* (3.42), *AAD10* (2.86), *AAD6* (2.30), *AAD14* (2.24), *AAD4* (2.05), *AAD3* (2.04)
Oxidoreductase activity (p=4.82e-10)	*ERG11* (2.70), *ERG5* (2.66), *FRE7* (2.28), *MET12* (2.22), *GTT1* (2.15), *COX8* (2.05)
Others	*HPA2* (4.16), *RDL1* (3.92), *SPG4* (3.55), *RIM4* (2.97), *YOR348c* (2.94), *RDL2* (2.82)

B)

Categories	**Genes up-regulated in strain EC1118 (fold induction)**
Glucose metabolic process (p= 4.1-e12)	*ENO2* (4.55), *TDH3* (3.65), *PFK1* (3.38), *ENO1* (2.98), *TDH2* (2.91), *PGK1* (2.74), *TPI1* (2.54)
Amino acid metabolic process (p= 8e-14)	*ADH3* (4.50), *ARG4* (4.09), *ADH5* (4.06), *MET16* (3.86), *MET17* (3.71), *SER33* (3.37), *CYS3* (3.35), *CPA1* (3.31), *BAT1* (3.04), *MET22* (2.92), *ARO3* (2.59), *MET14* (2.55)
Others	*YIL060w* (3.74), *YCT1* (3.43), *SPT4* (3.00), *PMA1* (2.88), *YCR023c* (2.79), *POL4* (2.63), *HXT3* (2.62), *JLP1* (2.60), *ROG3* (2.56), *PDR12* (2.54), *CYR1* (2.49), *HRK1* (2.37)

Fig 1


A
100
10
1
0.1
Survival (%)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days
T73
L2056
71B
CSM
BM45
M2
EC1118
Fermichamp 67J
Zymaflore VL3
ICV46
UCLM 5235
Fermirouge 7303
C Jerez
B
Exponential
EC1118
CSM
120
100
80
60
40
20
0
Survival (%)
**
*
*
EtOH 10% 1h
Glucose 20% 1,5h
H2O2 5mM 30m
42ºC 1h
C
Stationary state
EC1118
CSM
125
100
75
50
25
0
Survival (%)
*
*
EtOH 10% 1h
H2O2 5mM 1h

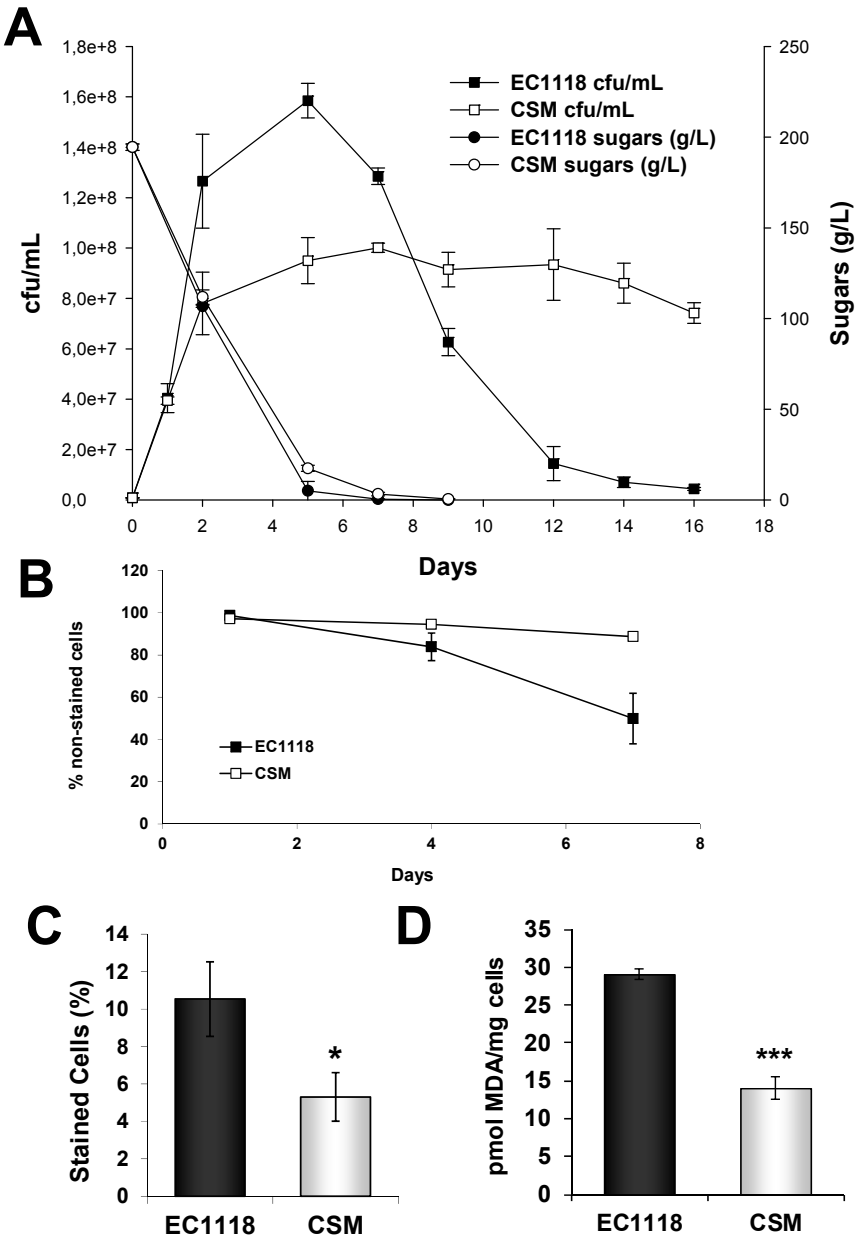
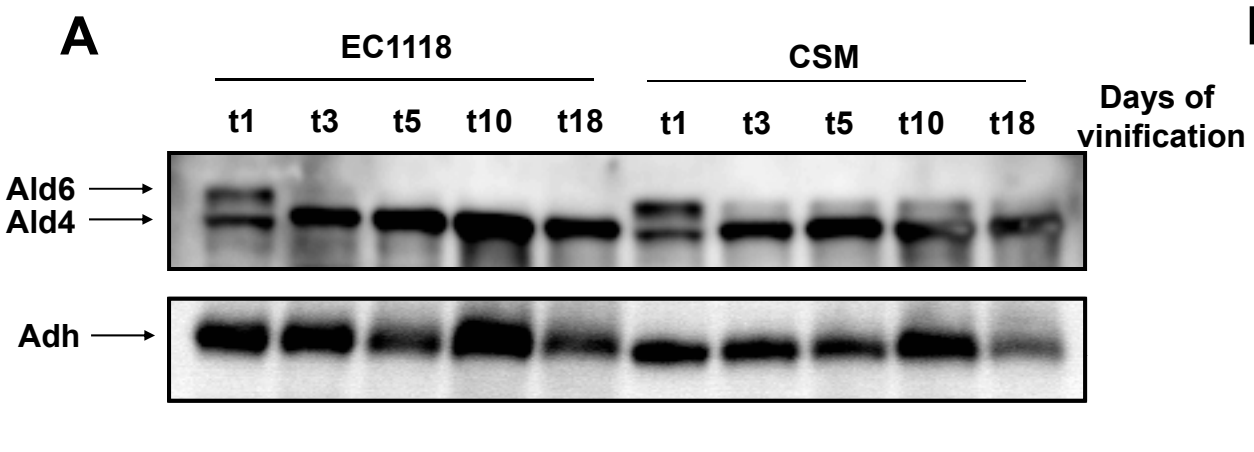
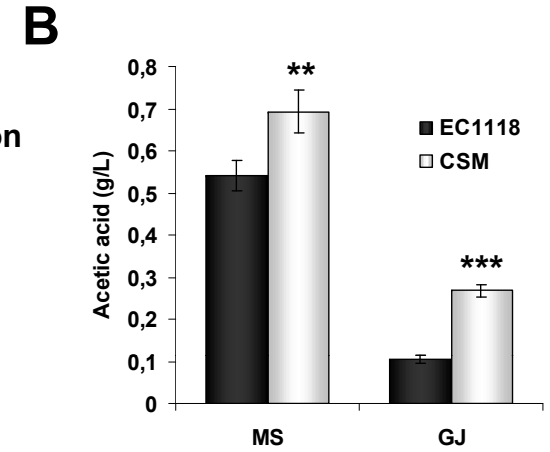
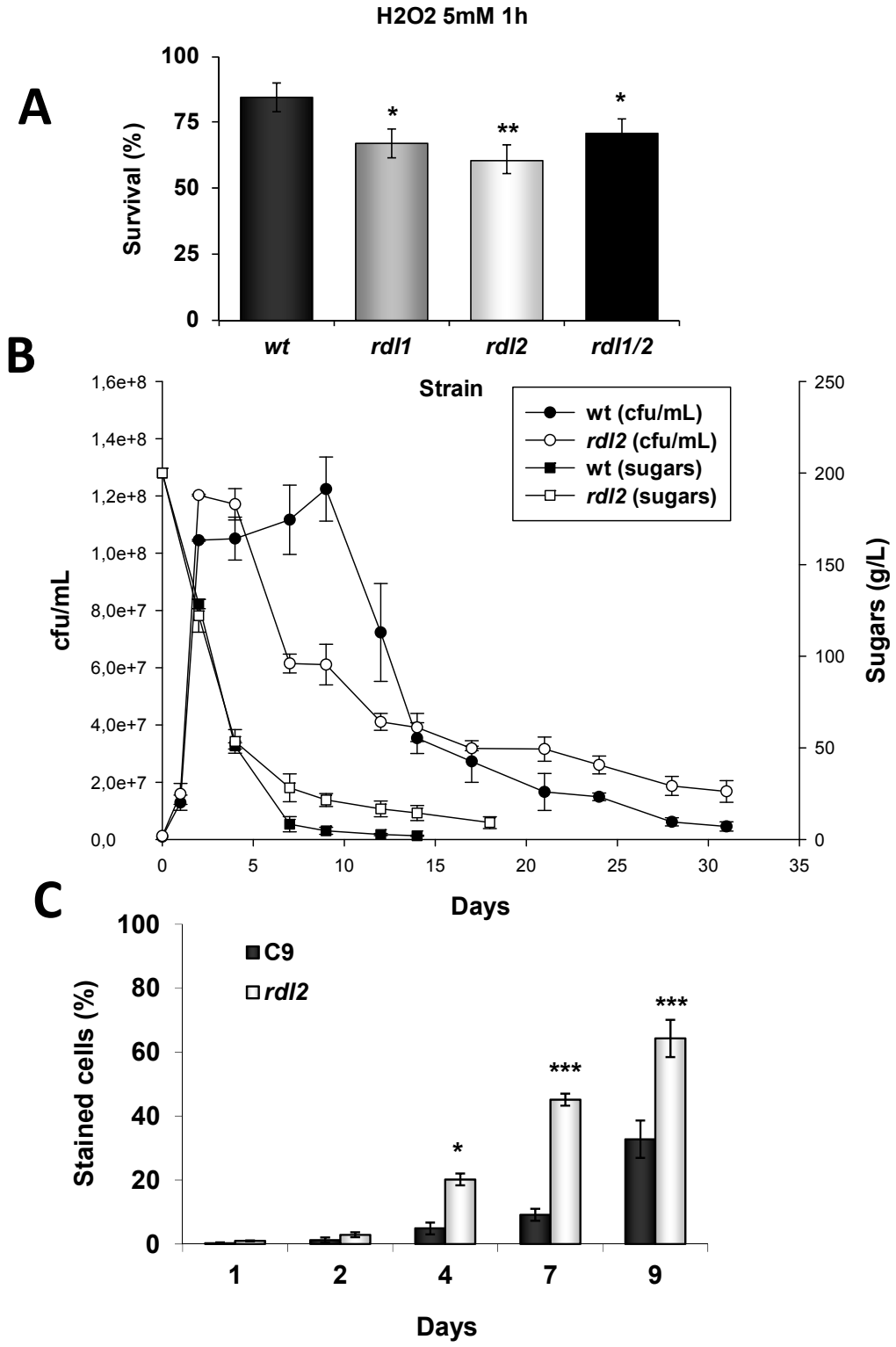
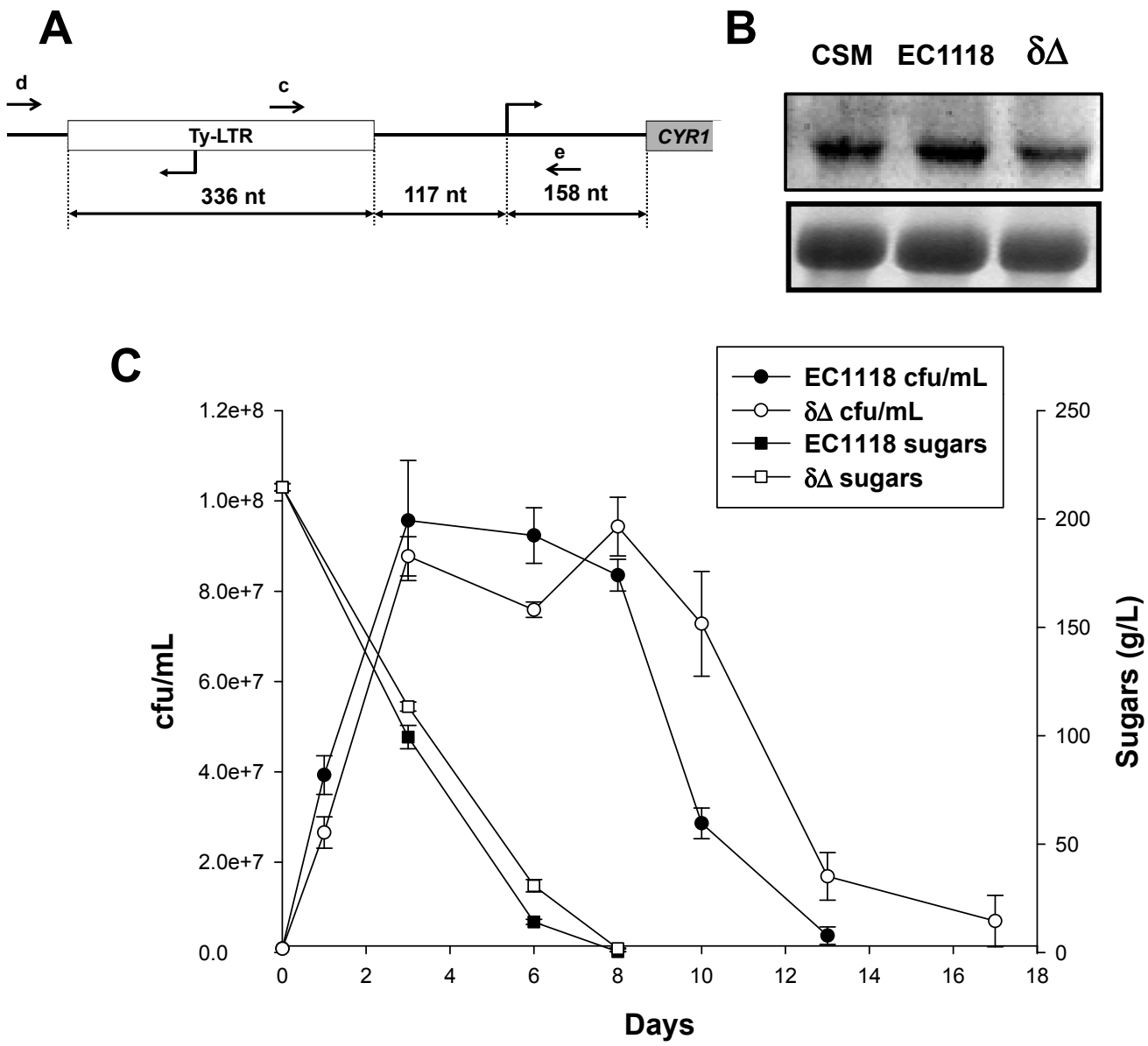

A
1,8e+8
1,6e+8
1,4e+8
1,2e+8
1,0e+8
8,0e+7
6,0e+7
4,0e+7
2,0e+7
0,0
cfu/mL
250
200
150
100
50
0
Sugars (g/L)
0 2 4 6 8 10 12 14 16 18
Days
EC1118 cfu/mL
CSM cfu/mL
EC1118 sugars (g/L)
CSM sugars (g/L)
B
120
100
80
60
40
20
0
% non-stained cells
0 2 4 6 8
Days
EC1118
CSM
C
Stained Cells (%)
14
12
10
8
6
4
2
0
*
EC1118
CSM
D
pmol MDA/mg cells
35
30
25
20
15
10
5
0
***
EC1118
CSM

Fig 3


A
EC1118
CSM
t1
t3
t5
t10
t18
t1
t3
t5
t10
t18
Days of vinification
Ald6
Ald4
Adh


B
Acetic acid (g/L)
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
**
***
EC1118
CSM
MS
GJ


A
H2O2 5mM 1h
Survival (%)
100
75
50
25
0
*
**
*
wt
rdl1
rdl2
rdl1/2
B
Strain
wt (cfu/mL)
rdl2 (cfu/mL)
wt (sugars)
rdl2 (sugars)
cfu/mL
Sugars (g/L)
1,6e+8
1,4e+8
1,2e+8
1,0e+8
8,0e+7
6,0e+7
4,0e+7
2,0e+7
0,0
250
200
150
100
50
0
0
5
10
15
20
25
30
35
Days
C
C9
rdl2
Stained cells (%)
100
80
60
40
20
0
*
***
***
1
2
4
7
9
Days


A
d
c
Ty-LTR
CYR1
e
336 nt
117 nt
158 nt
B
CSM
EC1118
δΔ
C
EC1118 cfu/mL
δΔ cfu/mL
EC1118 sugars
δΔ sugars
cfu/mL
Sugars (g/L)
Days
1.2e+8
1.0e+8
8.0e+7
6.0e+7
4.0e+7
2.0e+7
0.0
250
200
150
100
50
0
0
2
4
6
8
10
12
14
16
18