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Genomic insights into the glutathione metabolism of the wine yeast *Starmerella bacillaris*

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ABSTRACT

Glutathione (GSH) is an antioxidant molecule of great technological interest due to its wide range of applications in the food and beverage industry. In winemaking, although glutathione is produced during fermentation, its addition is possible for the control of oxidative spoilage of wine. Recently, to improve wine quality, mixed fermentation has been proposed by introducing a selection of non-*Saccharomyces* yeasts as complementary starters to the oenological species *S. cerevisiae*. Among them, *Starmerella bacillaris*, an osmophilic and high glycerol producer yeast, has been extensively studied.

In the present study, the genomes of two *S. bacillaris* strains were compared with *S. cerevisiae* to identify the GSH metabolic pathway. The results showed that GSH biosynthesis includes the *GSH1* and *GSH2* genes in both species. The identification of a new transcription factor which binds sites in the promoter region of these genes underlined differences in the transcriptional regulation of both species. Additionally, between *S. bacillaris* strains, a high number of polymorphisms was found in genes involved in GSH redox balance. Preliminary laboratory scale fermentations revealed marked differences in the cell glutathione content of the two *S. bacillaris* strains. By comparing genomes, it was possible to gain a better understanding of the genes involved in the GSH metabolism pathway in *S. bacillaris*.

KEYWORDS

Saccharomyces cerevisiae, *Candida zemplinina*, glutathione, *GSH1*, *GSH2*

Supplementary data can be downloaded through: https://oeno-one.eu/article/view/4374

INTRODUCTION

During alcoholic fermentation, sugars present in grape must are transformed into ethanol. This process is generally carried out by a single *Saccharomyces cerevisiae* strain added to the grape must as a starter culture. Many non-*Saccharomyces* yeasts are present on the grape surface and, therefore, in the grape must. In the past, they were considered undesirable spoilage microorganisms, because they were often isolated from stuck or sluggish fermentations, or from wines with anomalous analytical compositions or negative sensorial profiles (Jolly *et al.,* 2013; Ciani and Comitini, 2015). Throughout the past decade, they have become popular in the wine industry and their role has been re-considered, as many species contribute to wine fermentation and may positively affect wine quality (Ciani and Comitini, 2015; Ivit and Kemp, 2018). Indeed, there has been an increasing number of claims that wines made with *Saccharomyces* starter cultures are more standardised. The yeast *Starmerella bacillaris* (synonym *Candida zemplinina*) is an osmophilic, fructophilic early-colonising wine yeast, which is preferentially isolated from high sugar grape musts (Sipiczki, 2003; Wang *et al.,* 2014). It is part of microbiota in the grape pomace, a main winemaking by-product (Bovo *et al.,* 2011). When used as a starter culture in sequential fermentation with *S. cerevisiae* strains, this yeast contributes to decreasing ethanol levels and increasing glycerol content while maintaining moderate volatile acid production (Jolly *et al.,* 2014; Bely *et al.,* 2013). Recently, this novel non-conventional wine yeast showed biocontrol activity against the fungal pathogens *Botrytis cinerea* and *Penicillium expansum* (Lemos Junior *et al.,* 2016; Nadai *et al.,* 2018).

Wine is sensitive to oxygen exposure, which can lead to a loss of aroma, the development of atypical aging characters, and undesirable colour changes. In winemaking, glutathione addition has been proposed for the control of oxidative spoilage of wine. Glutathione (L-g-glutamyl-L-cysteinyl-glycine, GSH) is a hydrosoluble tripeptide, an important molecule containing thiol residues that are responsible for its antioxidant property (Kiriyama *et al.,* 2013). GSH present in wine exerts a protective effect on various aromatic compounds (Ugliano *et al.,* 2011). Its concentration can change due to its release from yeast cells via autolysis (Lavigne *et al.,* 2007). Therefore, after fermentation, wine aging on yeast lees can potentially play a role in protecting wine from oxidation.

Moreover, as a result of the winemaking process in wineries, one ton of grapes generates approximately 0.06 t of lees (Oliveira and Duarte, 2014). Therefore, it is worth exploring how GSH recovered from lees can improve sustainability in winemaking.

In *S. cerevisiae*, genes involved in GSH production and regulation are well-known, as is the biological role of GSH. Indeed, GSH is involved in protection against oxidative stress and the elimination of heavy metals and toxic endogenous metabolites, and it is also a source of cysteine. Additionally, it can take part in nitrogen metabolism and other pathways generating sulphur compounds (Mezzetti *et al.,* 2014).

In *S. cerevisiae*, GSH is synthesised via two ATP-dependent steps (Figure 1).

γ-Glutamylcysteine synthetase (GSS1; coded by *GSH1*) catalyses the first and rate-limiting step, during which dipeptide γ-Glu-Cys is formed from glutamate and cysteine (Lisowsky, 1993). The second step is catalysed by glutathione synthetase (GSS2) coded by *GSH2*, which ligates γ-Glu-Cys with glycine (Grant *et al.,* 1997). GSH is degraded to form cysteinyl-glycine. Therefore, the cell content of γ-Glu-Cys precursor and cysteinyl-glycine catabolite can give information about GSH turnover in the cell (Penninckx, 2002). GSH activity is important during oxidative stress conditions as a cofactor for stress defence enzymes. Oxidative stress converts GSH to its oxidised disulphide form (GSSG) by ROS (reactive oxygen species) or in reactions catalysed by glutaredoxines (GRX). Eight related glutaredoxins have been identified in *S. cerevisiae* (coded by *GRX1-8* genes). *GRX1-2* are regulated via stress-responsive STRE elements. The same reaction is catalysed by Gto1–3 proteins as well. These enzymes are induced in response to oxidants under the control of Yap1p and STRE-responsive elements (Morano *et al.,* 2011). Reduced GSH from GSSG is regenerated in an NADPH-dependent reaction catalysed by a glutathione reductase (GRS) coded by *GLR1*. GSH can be conjugated to xenobiotics (RX) by glutathione S-transferases (GST), which comprise a major family of proteins involved in the detoxification of many xenobiotic compounds. GSH conjugates are transported to the vacuole by the Ycf1 GS-X pump. The GST family includes Gtt1–2 and Grx1–2 proteins, as well as Gto1-3, although the latter proteins are not active as GSTs

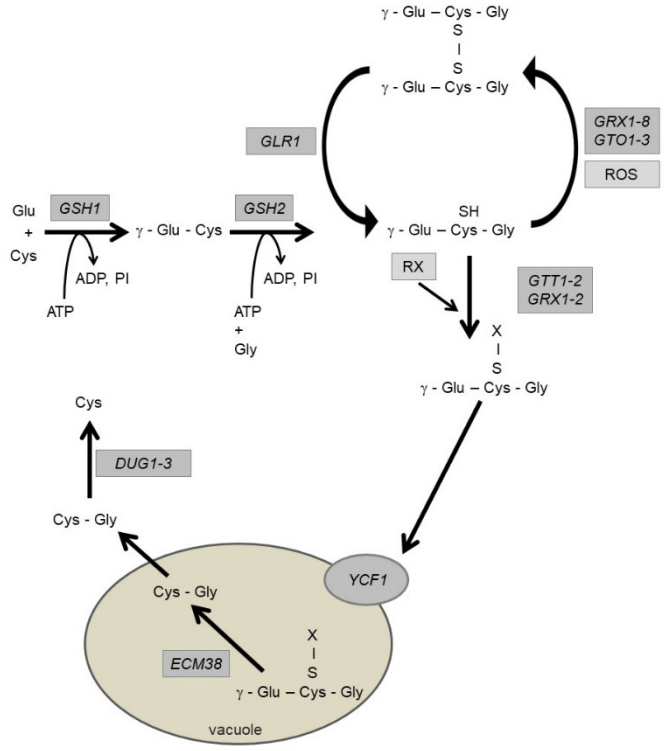

FIGURE 1. Gluthatione metabolism.
GSH1 Gamma glutamylcysteine synthetase, *GSH2* Glutathione synthetase, *GLR1* Glutathione oxidoreductase, *GRX1-8* Glutaredoxins, *GTO1-3* Glutathione transferases Omega-like, ROS Reactive oxygen species, RX Xenobiotics, *GTT1-2* Glutathione transferases, *GRX1-2* Glutaredoxins, *YCF1* Vacuolar glutathione S-conjugate transporter, *ECM38* Gamma-glutamyltranspeptidase, *DUG1-3* GSH degradosomal complex

with a xenobiotic compound, but, as already mentioned, as thioltransferases (glutaredoxins, GRX) (Lu, 2009). Gto1-3 proteins also function as glutathione peroxidases (GPX) and provide major enzymatic defence against oxidative stress caused by hydroperoxides (Barreto *et al.,* 2006). Moreover, specific Gpx proteins (coded by *GPX1-3*) are present and protect membrane lipids from peroxidation (Avery and Avery, 2001). GSH is a source of cysteine whose cellular concentration can be modulated by GSH degradation, which involves the gamma-glutamyl transpeptidase encoded by *ECM38* (Kumar *et al.,* 2003) or, with an alternative pathway, the Cys-Gly metallo-di-peptidase encoded by *DUG1* (Kaur *et al.,* 2012).

Knowledge about *S. cerevisiae*'s GSH metabolism has been used to investigate the possibility of increasing glutathione content using new *S. cerevisiae* wine starters during alcoholic fermentation in order to prevent wine oxidative spoilage (Mezzetti *et al.,* 2014). However, little information is available regarding genes involved in non-*Saccharomyces* yeast glutathione production and the contribution of these non-conventional starters to glutathione content in wines.

In the present study, two *S. bacillaris* strains whose genome were recently sequenced (Lemos Junior *et al.,* 2017a; Lemos Junior *et al.,* 2017b; Lemos Junior *et al.,* 2018) were considered.
By comparing the genomes of *S. bacillaris* and *S. cerevisiae*, *the* genes involved in the GSH metabolism pathway were identified and genomic variations of both *S. bacillaris* strains were investigated. Moreover, for both strains, the GSH cell content was determined in synthetic must, mimicking grape must composition.

MATERIALS AND METHODS

1. Bioinformatic analysis

The identification of single nucleotide polymorphisms (SNPs) was performed using SAMtools mpileup (Li *et al.,* 2009) and the output file was converted to a VCF format using BCFtools (https://github.com/samtools/BCFtools).
SNPs annotation in the genes of interest was conducted using SNPeff software (version 4.3g) (Cingolani *et al.,* 2012). The Neural Network Promoter Prediction (Reese, 2001) was chosen as the tool for the prediction of *GSH1* and *GSH2* promoter regions, whereas putative binding sites for transcriptional factors near promoter sequences

were identified with Yeastract (Teixeira *et al.,* 2013). The automated protein-structure homology-modeling server SWISS-MODEL (Biasini *et al.,* 2014) was used to carry out *GSH1* and *GSH2* modelling to compare with those obtained from *S. cerevisiae* EC1118 (reference templates). Ortholog analysis was carried out with SPOCS (Species Paralogy and Orthology Clique Solver) (Curtis *et al.,* 2013) to compare protein sequences related to the GSH pathway from *S. bacillaris* FRI751, *S. bacillaris* PAS13 and *S. cerevisiae* S288c. Protein multiple sequence alignment, visualisation and analysis were performed with Jalview v2.11 (Waterhouse *et al.,* 2009). Finally, a phylogenetic tree was constructed with crucial proteins involved in GSH biosynthesis using MEGA 7.0 (Kumar *et al.,* 2016). The Maximum Likelihood method based on the JTT matrix-based model with 1,000 bootstrap replicates was chosen. Due to its remarkable difference in terms of GSH content, *S. cerevisiae* EC1118 was included in all *in silico* analyses.

2. Yeast strains and growth conditions

Two *S. bacillaris* strains (FRI751 and PAS13) were isolated from dried grapes of the Raboso Piave variety, as described by Lemos Junior *et al.* (2016). *S. cerevisiae* EC1118 (Lallemand Inc., Montreal, Canada) was used as the control. The starter cultures were prepared from YPD agar plate (yeast extract 10 g/L, peptone 10 g/L, dextrose 20 g/L) to inoculate 5 mL of YPD broth in 15 mL tubes. A stationary phase culture with approximately 10^8 cells/mL was obtained after 24 hours of incubation at 30 °C; it was determined by OD measurements and confirmed by plate counts (CFU/mL).

3. Fermentations trials

Pre-cultures of each strain used in this work were prepared as described by Bovo *et al.* (2016). A suitable aliquot of each yeast culture, corresponding to a final cell concentration of 1.5 x 10^6 cells/mL, was used to inoculate 120 mL bottles, fitted with closures that enabled the carbon dioxide to escape, containing 100 mL of MS300 synthetic must according to Bely *et al.* (1990) with the following modifications: 100 g/L of glucose, 100 g/L of fructose and 6 g/L of malic acid, pH 3.3.

After yeast inoculation, the bottles were incubated at 20 °C. All experiments were performed in triplicate. CO_2 production was monitored by weighing the bottles twice a day and calculating the weight loss of each culture. The fermentations were terminated when the weight loss rate had dropped to under 0.05 g/day.

HPLC analysis was performed to determine the concentrations of residual glucose and fructose, acetic acid, glycerol and ethanol when the fermentations had been terminated, as described by Lemos Junior *et al.* (2019).

4. Thiols measurement

Yeast lees from synthetic wine were collected at the end of fermentation. The supernatant was discarded, and the pellet was washed twice (5 mL of 0.9 % NaCl). The pellet was weighed and re-suspended in 10 % w/v of 0.1 N HCl, 1 mmol/L Na2-EDTA. To prompt cellular lysis, glass beads were added and the suspension was vortexed for 3 min. Once cellular lysis had occured, 50 µL of supernatant was used for derivatisation with the fluorescent dye SBD-F, as described by Masi *et al.* (2002). 20 µl of filtered samples was analysed by reverse-phase HPLC separation. The HPLC instrument (Shimadzu, Tokio, Japan) was equipped with a refractive index detector fluorescent (excitation wavelength: 386 nm; emission wavelength: 516 nm) for the determination of cysteinyl-glycine, gGluCys, GSH. The chromatographic conditions were realised with the LC C18 100Å column (150 mm × 4.6 mm I.D., 5 µm particle size; Luna, Phenomenex, USA), running at a flow rate of 1 mL/min and at room temperature. The mobile phase was $NH4^+$ formate 50 mM pH 2.9 containing 3 % methanol.

5. Statistical Analysis

The statistical data analysis was performed using the XLSTAT software, version 2016.02 (Addinsoft, Paris, France). Data were subjected to a Student's t-test. Differences were considered statistically significant for a *p*-value of less than 0.05.

RESULTS AND DISCUSSION

1. Comparison of the genes involved in the GSH metabolism pathway in *S. bacillaris* strains and *S. cerevisiae*

To better understand the genes involved in the GSH metabolism pathway in *S. bacillaris*, the genomes of the two previously sequenced (Lemos Junior *et al.,* 2017a; Lemos Junior *et al.,* 2017b; Lemos Junior *et al.,* 2018) strains were analysed and compared to *S. cerevisiae* EC1118, a well-known commercial wine strain.

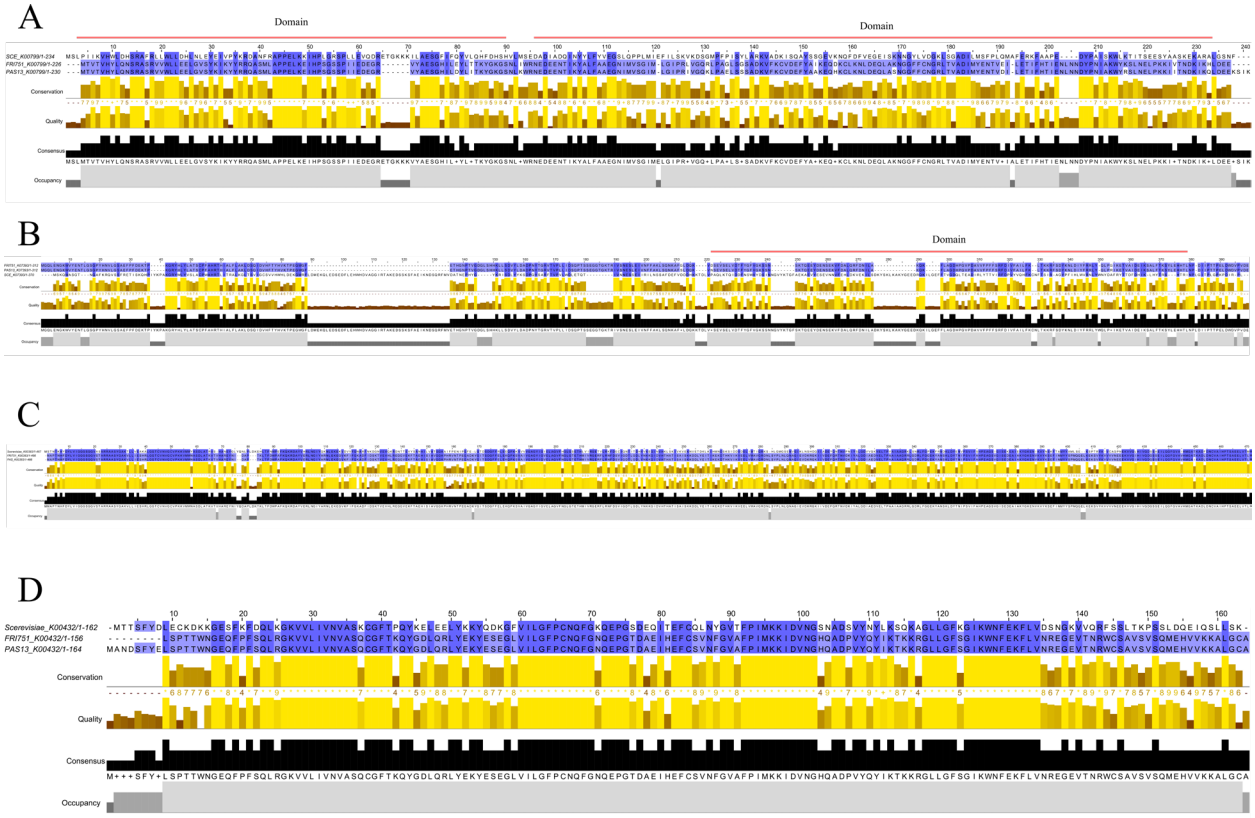

FIGURE 2. Different sequence similarities displayed by four GSH-related proteins as a result of amino acid substitution and/or deletion in S. bacillaris strains associated with genomic variants. A-GTT1 (K00799); B-ECM4 (K07393); C-GSR (K00383); D-GPX (K00432).

The conventional GSH biosynthetic pathway (Figure 1) was evaluated by comparing *S. bacillaris* strains and *S. cerevisiae* EC1118/S288c in terms of enzymatic classes, localisation of genes across the genome (synteny), presence of transcriptional factors binding sites in promoter regions, and the putative effect of SNPs on GSH-related genes.

KEGG pathway analysis was performed using a data-set of 24 protein-coding genes (Supplementary Datasheet 1) obtained after ortholog prediction between *S. bacillaris* FRI751, *S. bacillaris* PAS13, and *S. cerevisiae S288c*. Proteins that belong to the enzymatic classes involved in GSH metabolism were found, such as GSS1 (γ-Glutamylcysteinesynthetase), GSS2 (glutathione synthetase), GRX (glutaredoxin), GST (glutathione S-transferase), GPX (glutathione peroxidase) and GSR (glutathione reductase). Among the three GST-coding genes, two out of three are in a tandem array separated by an intergenic region of 1,698 bp on the chromosome sequence in both *S. bacillaris* strains. Additionally, three genes encoding for components of the GSH degradosomal complex (*DUG1*, *DUG2,* and *DUG3*) were present, as well as two hydroxyacylglutathione hydrolases coding-sequences (*GLO1* and *GLO4*). The two hydrolases are involved the detoxification of methylglyoxal (a by-product of glycolysis; Inoue *et al.,* 2011). No genes related to GSH plasma-membrane transport or vacuolar GS–X pump were found, although a mitochondrial outer membrane protein (*POR1*) was identified.

To better understand whether genomic variants were present and their potential effects on protein primary structure, sequence comparison of the GSH-related proteins between the two *S. bacillaris* strains and their orthologs in *S. cerevisiae* was performed. Although the same enzymatic activities participate in the GSH-pathway in both fungal species, the comparison between *S. bacillaris* strains revealed a high number of SNPs able to impact the functioning of twenty genes (Table 1). According to Schacherer *et al.* (2007), the interpretation of biological assays can be facilitated by understanding the number and the position of genomic variants between strains that show different phenotypes. In a previous study conducted by Junior Lemos *et al.* (2018), the authors reported the existence of

TABLE 1. Annotation of twenty-four* proteins involved in GSH metabolism and number of variants for each impact category in *S. bacillaris* strains.

Reference pathway (KO)	Gene ID	Ortholog in *S. cerevisiae*	Enzyme Commission number (E.C.M)	SNPs			
				High	Low	Moderate	Modifier
K00432	S05_1418	HYR1	1.11.1.9	0	0	0	1
K00432	S03_775	GPX2	1.11.1.9	0	1	0	49
K15040	S07_1803	POR1	#	0	1	0	87
K00383	S10_2233	GLR1	1.8.1.7	0	0	1	0
K18802	S05_1468	DUG3	#	0	8	0	91
K15428	S09_2037	DUG1	3.4.13.-	0	1	0	33
K01070	S31_3701	YJL068C	3.1.2.12	0	3	2	122
K11204	S05_1424	GSH1	6.3.2.2	0	0	0	1
K00799	S32_3749	URE2	2.5.1.18	0	1	0	33
K01013	S09_2034	RDL1	2.8.1.-	0	0	0	31
K01069	S25_3474	GLO4	3.1.2.6	0	2	0	13
K01759	S17_3012	GLO1	4.4.1.5	0	0	0	2
K13566	S05_1296	NIT2	3.5.1.3	0	0	0	72
K01920	S25_3478	GSH2	6.3.2.3	0	0	0	11
K07393	S03_730	ECM4	#	0	5	3	127
K07232	S02_518	GCG1	4.3.2.7	0	1	0	5
K14262	S09_2045	DUG2	3.4.-.-	0	18	2	109
K03233	S01_244	TEF4	#	0	0	0	3
K00799	S06_1485	GTT1	2.5.1.18	0	6	1	78
K00799	S06_1486	GTT1	2.5.1.18	0	6	0	137
K00121	S11_2303	SFA1	1.1.1.284	0	0	0	0
K07390	S10_2243	GRX5	#	0	0	0	0
K03676	S15_2809	GRX2	#	0	0	0	0
K03386	S15_2788	PRX1	1.11.1.15	0	0	0	0

High (variant is assumed to have disruptive impact in the protein: stop codon gained and loss of function), low (harmless or unlikely to change protein behavior: synonymous codon), moderate (non-disruptive variant that might alter protein effectiveness: missense codon and inframe codon loss) and modifier (non-coding variants or variants affecting non-coding genes: exon variant and downstream gene variant) are parameters used by SnpEff to correlate SNPs and their putative impact on protein functioning.
* TEF4 and URE2: GST-like proteins. HYR – HYdroperoxide Resistance; GPX – Glutathione PeroXidase; POR – PORin; GLR – GLutathione Reductase; DUG – Deficient in Utilization of Glutathione; GSH – Glutathione; URE – UREidosuccinate transport; RDL1 – RhoDanese-Like protein; GLO – GLyOxalase; NIT2 – NITrilase superfamily; ECM4 - ExtraCellular Mutant; GCG – Gamma-glutamyl Cyclotransferase acting on Glutathione; TEF – Translation Elongation Factor; GTT – GlutaThione Transferase; SFA – Sensitive to FormAldehyde; GRX – GlutaRedoXin; PRX – PeroxiRedoXin.

33,771 high-quality variants in 1,146 genes in *S. bacillaris* FRI751 and PAS13. A similar content of genomic variants (37,424 SNPs) was identified in *S. cerevisiae* S288c and SK1 (Schacherer *et al.*, 2007), although SNP content can vary significantly (from 13,787 to 57,463) due to the reference strain of choice and the phylogenetic distance between the genomes of interest, as described in several studies (Borneman *et al.*, 2008; Otero *et al.*, 2010; Meijnen *et al.*, 2016).

At the amino acid level, substitutions were observed in two glutathione S-transferases (*ECM4* and *GTT1*), one glutathione peroxidase, and one glutathione reductase (Figure 2).

The enzymes annotated as glutathione S-transferases displayed the highest number of

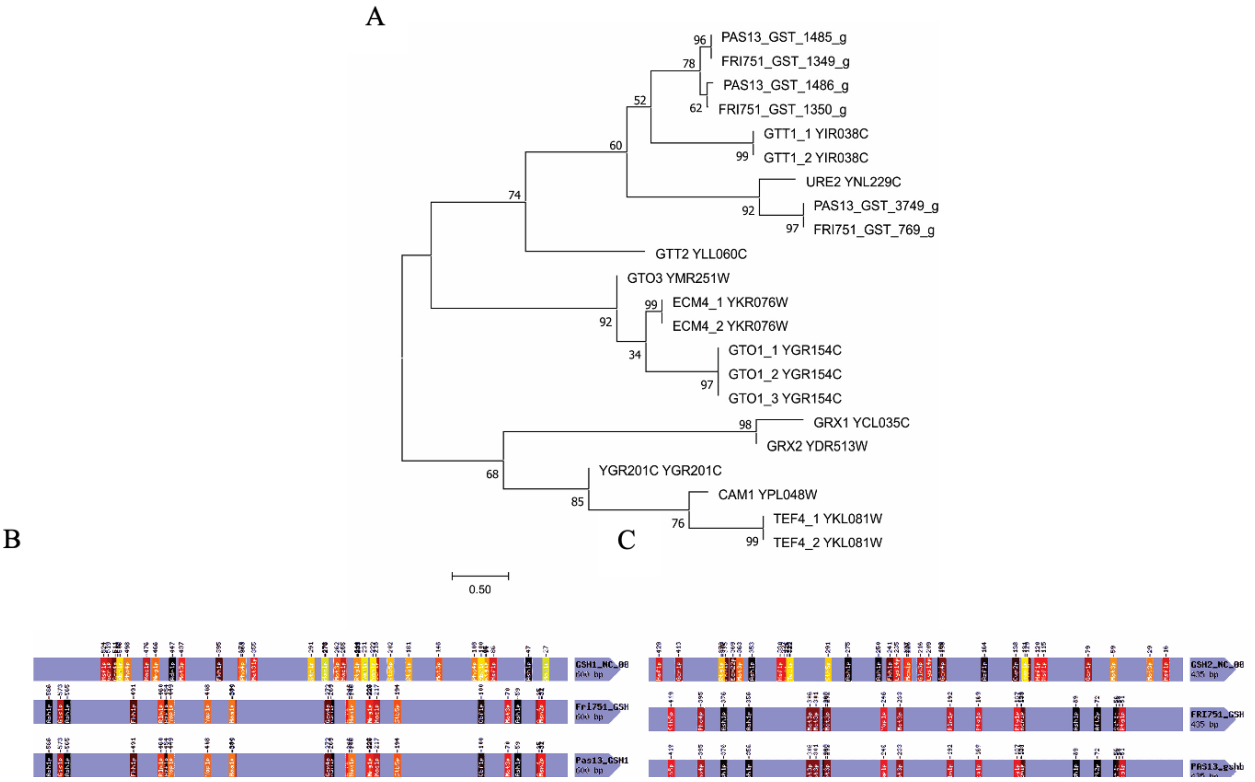

FIGURE 3. A-Molecular Phylogenetic analysis using the Maximum Likelihood method.
The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT (Jones Taylor-Thornton) matrix-based model (Jones *et al*., 1992). The tree with the highest log likelihood (-2135.54) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search was/were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 16 amino acid sequences of GSTs from *S. cerevisiae* and 6 from *S. bacillaris* FRI751 and PAS13. All positions containing gaps and missing data were eliminated. There was a total of 70 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016); B and C-Transcriptional factors prediction using upstream regions from *GSH1* and *GSH2* respectively. In each panel, sequences obtained from *S. cerevisiae* S288c, *S. bacillaris* FRI751 and *S. bacillaris* PAS13 are displayed from top to bottom respectively.

substitutions (K00799: S06_1486p.Thr80Ile, p.Ile93Lys, p.Leu127Ile, p. Arg131Lys, p.Gly135Glu, p. Gly137Ser, p. Asp153His, p. Lys169Ser, p. Glu191Asp, p. Lys216Arg, p. Val226Ile and p. His233Gln; K07393: p. Asp222Asn and p. Gly354Arg), and almost all of them were localised in domain regions (Figures 2a and 2b). Only one amino acid substitution was identified in the glutathione reductase enzymes (K00383: p. Thr461Ile) (Figure 2c), whereas one of the glutathione peroxidase enzymes (K00432; gene ID: S03_775) encoded by *S. bacillaris* FRI751 displayed a shorter protein length than *S. bacillaris* PAS13 and *S. cerevisiae* due to the deletion of the first eight amino acids (Figure 2d). Together, these results suggest that the described amino acid substitutions are not all neutral and they have a functional effect, evidencing a marked inter- and intra-strain variability that can be relevant to determine the differences in GSH production.

Since the three GST-coding genes showed a remarkable number of variants between *S. bacillaris* strains, reflecting substitutions at the amino acid level in two of them, further investigations have focused on these genes. According to Ma *et al.* (2009), seven proteins (Gtt1p, Gtt2p, Gto1p, Gto2p, Gtop3, Grx1p and Grx2p) display GST activity in *S. cerevisiae*. Phylogenetic analysis of GST amino-acid sequences for *S. cerevisiae* and *S. bacillaris* strains revealed that the two GST proteins encoded by genes in a tandem array are closely related to the paralogs encoded by *GTT1*, while the third is grouped with *URE2* (Figure 3a). *URE2* encodes a bifunctional protein that is not generally included in the glutathione pathway as it is involved in both nitrogen catabolite repression (Coschigano and Magasanik, 1991) and oxidative stress response (Rai and Cooper, 2005). The protein has been shown to exhibit glutathione peroxidase activity and can mutate to acquire GST activity (Coschigano and Magasanik, 1991;

Bai *et al.,* 2004). This finding confirms the presence of relevant differences in proteins involved in the GSH system between *S. cerevisiae* and *S. bacillaris*.

The regulation of the two genes involved in GSH synthesis, *GSH1* and *GSH2*, is crucial for determining the GSH level in the yeast cell. In *S. cerevisiae*, according to Murata and Kimura (1990), Gsh1p and Gsh2p enzymes are regulated through a feedback-loop by GSH and GSSG respectively, indicating that the step regulated by Gsh1p is rate limiting in GSH biosynthesis.

GSH1 expression is co-regulated by Met4p, a transcription activator that also controls the expression of genes involved in sulfur assimilation (Wheeler *et al.,* 2002). In addition, *GSH1* expression is induced by the levels of oxidants, such as hydrogen peroxide, and by heat shock. Both types of regulation involve the transcriptional factor Yap1p (Sugiyama *et al.,* 2000).

Bioinformatic analysis of transcription factor binding sites (TFBS) localised in the promoter region of *GSH1* of *S. bacillaris* revealed two proteins that can potentially be involved in its transcriptional regulation (Azf1p and Tec1p), while five other proteins (Aft2p, Aft1p, Gis1p, Pho4p and Rlm1p) are putatively involved in the regulation of *GSH2*. These TF binding sites were not identified in the corresponding regions of *S. cerevisiae* S288c (Figures 3b and 3c). Moreover, no Met4p or Yap1p analogs were found to regulate *GSH1* or *GSH2* in *S. bacillaris* strains. The TFBS predicted in the promoter region of *GSH1* is associated with diauxic shift under nutrient depletion and transformation of non-fermentable carbon source through oxidative metabolism (Azf1p, Gis1p, Pho4p). In these conditions, cells require protection against ROS and oxidative stress in general (Cherry *et al.,* 2011). Interestingly, Aft2p and Aft1p, putatively involved in the control of *S. bacillaris GSH2*, are involved in iron utilisation and homeostasis in *S. cerevisiae*. Although in this yeast no co-regulation has been found between GSH biosynthesis genes and iron metabolism, the involvement of glutathione in maturation Fe/S proteins has been demonstrated (Kumar *et al.,* 2011).

GSH1 and *GSH2* genes from *S. bacillaris* strains displayed 26 and 20 insertions or deletions (INDELs) respectively when compared to *S. cerevisiae* EC1118, but none were located in the *GSH2* regions of substrate binding (Table 2). Three amino acid substitutions were localised in three out of four domains in *GSH2*. Both *GSH* genes encode proteins with a reduced length in

TABLE 2. *GSH1* and *GSH2* comparisons between *S. bacillaris* strains and *S. cerevisiae.*

Parameters	GSH1			GSH2		
Strain	EC1118	FRI751	PAS13	EC1118	FRI751	PAS13
Length (a.a)	678	601	601	491	478	478
Mol. Weight (Da)	78253.60	68767.87	68767.87	55815.23	53662.36	53662.36
Isoelectric Point	5.87	5.30	5.30	5.50	6.85	6.85
Identity with *S. cerevisiae* (%)	-	47.25	47.25	-	42.55	42.55
*GMQE with *S. cerevisiae*	-	0.72	0.73	-	0.74	0.74
Numbers of INDELs by Swiss-Model	-	26	26	-	20	20

Substrate binding	GSH1 Amino acid sequence			GSH2 Amino acid sequence		
Region 1 (150 – 153)	-	-	-	VSVS	VSVS	VSVS
Region 2 (228 – 230)	-	-	-	ERN	ETN	ETN
Region 3 (285 – 479)	-	-	-	RTGY	RAGY	RAGY
Region 4 (478 – 479)	-	-	-	VA	IA	IA

Reduced length and molecular weight were found for both enzymes, however *GSH2* displayed high isoelectric point and substitutions on its active site.
*GMQE is the Swiss-Model global quality estimation and has a range of 0 to 1.

TABLE 3. a) Concentrations of residual glucose and fructose, of the main fermentation products and thiol content at 618 hours of fermentation in MS300 synthetic must; b) Statistical analysis (Student's t-test) results.

a)

Strain	Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Acetic acid (g/L)	Ethanol (%v/v)	Dry cell weight (g)	GSH (nmol/L)	GSH content (µg/g)	γGluCys content (µg/g)	CysGly content (µg/g)
EC1118	0.00±0.00	0.00±0.00	5.77±0.14	0.38±0.07	13.16±0.02	0.30±0.01	709.42±60.46	73±6	84±16	10±2
FRI751	78.22±2.41	24.50±1.84	5.72±0.20	0.38±0.05	4.53±0.11	0.05±0.00	119.44±13.11	80±4	17±6	1±0
PAS13	84.56±2.11	23.88±1.31	7.81±0.37	0.40±0.02	4.94±0.14	0.05±0.00	207.60±5.87	122±3	30±84	2±0

b)

Comparison	Glucose	Fructose	Glycerol	Acetic acid	Ethanol	Dry cell weight	GSH	GSH content	γGluCys content	CysGly content
EC1118 / PAS13	*	*	*	ns	*	*	*	*	*	*
EC1118 / FRI751	*	*	ns	ns	*	*	*	ns	*	*
FRI751 / PAS13	*	ns	*	ns	*	ns	*	*	*	*

Levels of glutathione (GSH) and GSH, γ-glutamylcysteine (γGluCys) and cysteinyl-glycine (Cys-Gly) content (µg/g dry cell weight) from total cell extract derivatised and quantified by reverse-phase HPLC. Data are expressed as the average of three replicates ± standard deviations. * indicate significant differences between values (Student's t-test, $p < 0.05$).

comparison to the *S. cerevisiae* ones. Additionally, a high isoelectric point was predicted for Gsh2p, evidencing great differences in the protein structure.

6. GSH production by *S. bacillaris* and *S. cerevisiae* in synthetic must

Glutathione is relevant in winemaking, due to its antioxidant property. With the aim of evaluating *S. bacillaris* potential contribution to glutathione production, a lab-scale fermentation trial was performed. GSH content was determined in yeast cells produced during a standard fermentation process that involved *S. bacillaris* as a starter. The industrial wine strain *S. cerevisiae* EC1118 was used as a control. The fermentations were run in MS300 synthetic must at 20 °C, mimicking winemaking conditions (Supplementary Figure 1). Due to the presence of GSH in natural musts (Lavigne *et al.,* 2007), the synthetic must was used to evaluate GSH content produced exclusively by yeasts. All the fermentations were stopped when *S. cerevisiae* had completed the sugar transformation. As reported in the literature, *S. bacillaris* strains were not able to consume all the sugars (Magyar and Tóth, 2011; Bely *et al.,* 2013; Lemos Junior *et al.,* 2016; Lemos Junior *et al.,* 2019). PAS13 and FRI751 showed 109.3 and 102.7 g/l of residual sugar respectively, and were confirmed to be fructophilic yeasts (Table 3a). Conversely, EC1118 fermentation showed no residual sugar.

The biomass produced (dry cell weight) by *S. bacillaris* during fermentation was much lower than *S. cerevisiae*. This could be due to the remarkable difference in the cell size of the two species (2.2-3.0 x 3.0-5.2 µm *S. bacillaris* (Sipiczki, 2003), 3-8x5-10 µm *S. cerevisiae* (Vincenzini *et al.,* 2005).

GSH is synthesised starting from glutamate and cysteine to form γ-glutamylcysteine (γ-GluCys), which is subsequently conjugated to glycine. Moreover, GSH is degraded to form cysteinyl-glycine. Therefore, the cell content of the two GSH-related molecules can give information about GSH turnover in the cell. At the end of fermentation, the concentration of the precursor, γGluCys, the catabolite cysteinyl-glycine and the GSH present in the yeast cells were all measured (Table 3a). Both *S. bacillaris* strains produced GSH at lower concentrations than *S. cerevisiae*. When the strain biomass was considered, the GSH cell content (mg/g dry cell weight) of *S. bacillaris* PAS13 was 67 % higher than EC1118, while the corresponding values for FRI751 were not significantly different from EC1118 (Table 3b). 61. At industrial-scale production, the cellular glutathione concentration of *Candida utilis* and *S. cerevisiae* strains, the most commonly used microorganisms for commercial glutathione production, is 0.1-1 % dry

cell weight (Li *et al.*, 2004). These concentrations are higher than those found in *S. bacillaris* strains PAS13 (0.012 %) and FRI751 (0.008 %), and *S. cerevisiae* EC1118 (0,007 %) in this study. In industrial production, however, growth conditions are optimised and they are very different from those of the oenological environment, mainly in terms of glucose and cysteine concentration in the medium. Interestingly, in the tested conditions, *S. bacillaris* PAS13 produces higher GSH concentrations than *S. cerevisiae* EC1118. These results are consistent with those of Gamero-Sandemetrio *et al.* (2018), who tested the GSH production of different non-*Saccharomyces* strains during the active dry yeast manufacturing process; among them, *C. stellata* (the previous taxonomic classification of *S. bacillaris*) was the only non-*Saccharomyces* species that produced higher GSH levels than *S. cerevisiae*. *S. cerevisiae* GSH precursor and catabolite contents were significantly higher than for *S. bacillaris* strains, suggesting a different GSH turnover. When the two *S. bacillaris* strains were compared, GSH and GSH-related molecules contents were higher in FRI751 compared to PAS13. Differences between strains were evidenced at a genomic level, involving genes related to GSH/GSSG balance and responsible for glutathione peroxidase, reductase and S-transferases activity.

CONCLUSIONS

In conclusion, the comparison of genomes makes it possible to gain a better understanding of the genes involved in the GSH metabolism pathway in *S. bacillaris*. Several orthologous genes were found to be involved in the synthesis, degradation and oxidation/reduction of GSH. No genes related to GSH plasma-membrane transport or vacuolar GS–X pump were identified. The gene comparison between both *S. bacillaris* strains revealed a significant number of SNPs present in four GSH-related genes, which directly impacted protein primary structure causing amino acid substitutions or deletions, which in turn can potentially impact protein function. These genes are mainly involved in a GSH/GSSG balance that contributes to modulating cell GSH content. The different GSH cell contents of the two *S. bacillaris* strains, determined at the end of fermentation, indicate a possible involvement of the genomic variations investigated in this study. Further studies are required to understand the role of these genomic variations in a large number of *S. bacillaris* strains. Moreover, in order to increase wine quality, *S. bacillaris* PAS13, which showed high GSH cell content, could be tested in mixed or sequential fermentations to evaluate the influence of high-producing strains in the GSH content of wines. Finally, our results can be considered as a starting point for strain selections focused on GSH production, in order to properly assess the efficacy of *S. bacillaris* as a source of glutathione.

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