Phenotypic analysis, genome sequencing and annotation of the non-conventional wine yeast *Saturnispora diversa*

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Abstract

Interest in the use of non-*Saccharomyces* strains as co-adjuvants in winemaking has emerged in the last few decades, given their capacity to produce aromatic molecules that *S. cerevisiae* does not produce. However, the lack of knowledge concerning the genetics and physiology of these species limits their more extensive utilization. The present work is focused on the genomic exploration of a *Saturnispora diversa* strain, MJT240, retrieved from grape must. Karyotyping of *S. diversa* MJT240, based on Pulse Field Gel Electrophoresis, showed the genomic DNA totals 9.68 Mb distributed along five chromosomes. The genomic sequence was obtained using Illumina and de novo assembled using SPAdes to yield 9.86 Mb, along 1150 contigs. Using manually curated ab initio gene detection, annotation of *S. diversa* MJT240 genome resulted in a predicted ORFeome of 5070 ORFs. Metabolic reconstruction revealed *S. diversa* is equipped with all genes for central carbon metabolic pathways, but does not contain key enzymes necessary for catabolism of galactose nor does it encode full biosynthetic pathways for the synthesis of thiamine and biotin. Enzymes necessary for the synthesis of the volatile aromatic compounds higher alcohols, ethyl esters, acetaldehydes were identified. Proteomic comparative analysis with species *Hanseniaspora guilliermondi, Torulaspora delbrueckii, Lachancea fermentati* and *Saccharomycodes ludwigii* revealed 1584 proteins for which no robust orthologue was identified, including five enzymes for the synthesis of glycosylphosphatidylinositol (GPI) proteins. Finally, the potential of *S. diversa* MJT240 to inhibit the growth of the spoilage yeast *S. ludwigii* UTAD17 was herein studied using a co-culture setup, having resulted in *S. ludwigii* growth inhibition by 99.998%.

Introduction

Grape must is a rich nutrient and sugar environment with low pH, where a wide variety of yeasts prosper, living on the berry surfaces during the grape development[1]. The composition of the microbes present in grape must, generally defined as the microbiome, depends on numerous factors including the climate conditions (temperature, humidity, etc), application of agrochemical treatments, geographical location and grapevine cultivar[2, 3]. Against what might be expected, *S. cerevisae*, the leading species in vinification, isn't abundant in the grape's must. In fact, the most abundant yeasts in the must are several non-saccharomyces yeasts (NSY), especially in the early stages of grape processing and alcoholic fermentation[4]. Nowadays, NSY are being considered to possibly contribute to better wine's flavour and other characteristics. That will depend on the microbial interactions and what metabolites these yeasts can produce and in what concentration, which may be affected by the degree of activity of the yeasts in the must. However, to date, their viability tends to decrease significantly as the fermentation progresses. The reasons underlying this decrease are complex and yet to be fully clarified (especially because many will show a species-dependence) although their reduced competitiveness due to more limited metabolic capabilities, the reduction in oxygen availability and the increase in the concentrations of ethanol and SO_2, have been pinpointed as more relevant[5]. Nevertheless, in mixed fermentations with *S. cerevisiae*, either with co-inoculation or sequential inoculation, some of the negative features of NSY described above can be altered or not expressed, as the yeasts do not coexist passively, but instead interact, both physiologically

and metabolically. Thus, some of those traits are modulated by interaction with *S. cerevisiae* and stagnation of fermentation is also avoided[6]. Since these yeasts have specific oenological characteristics that are not incorporated in *S. cerevisiae* commercial strains, controlled mixed cultures can have an additive impact on the wine quality, in terms of an aromatic and analytical profile. Still, the knowledge on NSYs physiology and behaviour at lab scale and afterwards, at industrial scale wine fermentation is very scarce, as is their dynamics of metabolic and physiological interactions with *S. cerevisiae*[7, 8].

In this work we progress this understanding of genomics in wine yeasts, this time focusing on an isolate that was retrieved during a survey of microbial profiling of wine musts produced in the Lisbon area, in 2019. This isolate, identified as belonging to the *Saturnispora diversa* species, is herein characterised and its genomic sequence obtained, annotated (including manual curation) and further explored.

Methods and materials

Yeast Strains and Culture media

The yeast *S. diversa* MJT240, a strain retrieved from grape must obtain from Muscat grapes harvested in the Lisbon area and the yeast *S. ludwigii* UTAD17[9], an autochthonous Douro Wine Region strain, were both isolated in our laboratory and used in this study. Yeasts were routinely maintained at 4 °C on Yeast Peptone Dextrose agar plates (YPD) which contains, per litre, 20 g of glucose, 10 g of peptone, 5 g of yeast extract, and 20 g of agar from stocks stored at −80 °C. Prior to use, the yeasts were transferred to a new slant of YPD and incubated for 24–48 h at 28 °C, unless otherwise stated. Strains were also cultivated in liquid YPD medium (whose composition is identical to the one described above without agar), in selective minimal medium plates (20g/L of carbon source, 20g/L of agar, 2,67g/L of ammonium sulphate, 1.7g/L of YNB w/out amino acids nor ammonium sulphate) and in YPD plates supplemented with 6 mM SO_2. For that, a stock solution of SO_2 0.1M, previously sterilized by filtration, was used. Strains were also cultivated in synthetic grape juice medium (GJM), which was produced as described by Henschke et al.[10], which contains, per litre, 100g of glucose, 100 g of fructose, 3g of malic acid, 0.2g of citric acid, 1.14g of K2HPO4, 1.23g of MgSO4.7H2O, 0.44g of CaCl2.2H2O, 0.504g of diammonium potassium and 5g of tartrate potassium, 100 mL of vitamin solution 20x, 1mL of mineral solution 10000x, 0.5 mL of ergosterol solution 10x and 22.12mL of amino acid solution 25x. WL medium[11, 12] was also prepared for use of WL agar plates in viable cell plate counting. Finally, YPD + SO_2 6mM was prepared as described above for YPD solid medium. Additionally, after medium sterilisation, a determined volume of SO_2 solution mother (0.1M) was added using a syringe + filter setup.

Assessment of *S. diversa* MJT240 and *S. ludwigii UTAD17* growth parameters during growth in synthetic grape juice medium.

S. diversa MJT240 and *S. ludwigiii UTAD17* were profiled for their growth rates in synthetic grape juice medium. For both assays, a pre-inoculum of the strains was performed overnight in GJM medium at 30ºC with orbital agitation of 250rpm in 50mL shake flasks containing 25mL of medium. On the next day, an appropriate volume of the culture was inoculated in fresh medium, for the corresponding assay, yielding a cell suspension with an OD600nm of 0.1. These cultures were then incubated at 30ºC and 250rpm for 48-72h until stationary phase was reached, and OD600nm and viable cell plate counting were measured in appropriate time intervals.

Using the values of OD600nm from the exponential phase, the specific growth rates (μ) and doubling time (Dt) were calculated for *S. diversa MJT240* and *S. ludwigiii UTAD17*.

Growth of *S. diversa* MJT240, alone or in combination with *S. ludwigii* UTAD17, in liquid GJM medium.

Growth trials in synthetic GJM were conducted by inoculating (1) a single culture of *S. ludwigii* UTAD17 (U17), (2) a single culture of *S. diversa* MJT240 (Sd240) and 3) a mixed culture with *S. ludwigii* UTAD17 and *S. diversa* MJT240 inoculated simultaneously, using the procedure described above for growth parameters determination. The growth of yeasts was followed by measuring the increase of CFUs in appropriate time intervals until the stationary phase was reached. CFU measurement of *S. diversa* MJT240 and *S. ludwigii*

UTAD17 were performed, respectively, by plating 100 µL of determined diluted samples in solid WL medium and YPD medium + 6mM SO_2. CFU measurement in solid YPD medium was performed as control of *S. diversa* growth in the first 24h.

Yeast Karyotype analysis with PFGE (Pulse Field Gel Electrophoresis).

Separation of *S. diversa* MJT240 chromosomal DNA was carried out as described by Sipiczki et al. [13] and as modified by El Hage & Houseley[14]. Briefly, yeast chromosomes were separated in 1% agarose gels in 0.5 xTBE buffer cooled at 12 °C in a BioRad CHEF-DRIII electrophoresis apparatus (Bio-Rad, Hercules, CA, USA). Electrophoresis was conducted in different conditions. For separation of low size chromosomal bands system conditions were voltage gradient of 6 V/cm for 26 h, switch interval 60-120s and angle 120º. For separation of higher size bands system conditions were, in a first attempt, voltage gradient of 2 V/cm for 72 h, switch interval of 1800s and angle 106º. After optimization, system conditions were voltage gradient of 2 V/cm, with three consecutive run blocks: block 1, with a run time of 24h with angle 96º and switch interval 1200s; block 2 with run time of 24h with angle 100º and switch interval 1500s and block 3, with run time of 24h with angle 106º and switch interval 1800s. The CHEF-DNA size markers used to calculate the molecular sizes of *S diversa* MJT240 chromosomal bands were *S. Pombe* (chromosome bands 3.5 to 5.7 Mbp) and *S. cerevisiae* (chromosome bands 0.225- 2.2 Mbp). The molecular sizes for *S. diversa MJT240* chromosomes were calculated through a calibration curve (band distance vs molecular size) for each marker. The image result of gels after PFGE were taken in an image acquisition system, model ALLIANCE 4.7, from UVITEC Cambridge.

Genome sequencing, assembly and annotation of *S. diversa* MJT240.

Genomic DNA extraction of *S. diversa* MJT240, as well as subsequent Illumina sequencing, were performed as a paid service at CD Genomics (Shirley, New York, United States). Genomic DNA of *S. diversa* MJT240 was extracted using Quiagen Magattract HMW kit according to the manufacturer's instructions. After Illumina sequencing, 43659976 reads were obtained with approximately 150 bp on average and were de novo assembled in SPAdes. The annotation of the resulting contigs was performed in the Geneious software framework (version 2019.2.3) using as a gene finder Augustus trained in *Saccharomyces, S. pombe* and *Candida tropicalis*. Manual curation of the three predictions performed was based on BLASTP using the predicted protein sequences as input for searches across the entire dataset of proteins described at NCBI. Only those models for which an orthologue in other species could be identified were considered valid. Using the predicted ORFeome of *S. diversa* MJT240, a putative metabolic reconstruction was performed making use of the KEGG BlastKoala annotation tool and choosing Fungi as the taxonomic group and enabling Koala to search against the family_eukaryotes.pep KEGG database. To further improve this functional annotation the eggNOG-mapper set at the default parameters was also used. For the comparative proteomic analysis between *S. diversa* MJT240 and *S. cerevisiae* EC1118*, H. guilliermondii* UTAD222*, H. uvarum* AWRI3580*, T. delbrueckii* CBS1146*, B. bruxellensis* UCD 2041*, Z. bailii* clib213 and *L. fermentati* CBS6772 pairwise BLASTP analyses were performed using the sets of proteins publicly available at UNIPROT and NCBI for each strain. Two proteins from the different yeast species under analysis were considered highly similar whenever identity associated with the pairwise alignments was above 50% had an associated e-value below e−50. Whenever protein pairwise alignments resulted in identities between 30 and 50% with an associated e-value below e−20, the corresponding proteins were considered similar. In all other cases, the protein pairs were considered dissimilar.

Results and discussion

S. diversa MJT240 karyotyping and overview of Whole-genome sequencing and corresponding functional annotation.

To establish the structure of *S. diversa* MJT240 genome karyotyping, pulse-field gel electrophoresis (PFGE) was performed. This technology was selected based on the successful prior experience of the team in analysing the genome of other Non-*Saccharomyces* wine yeasts.

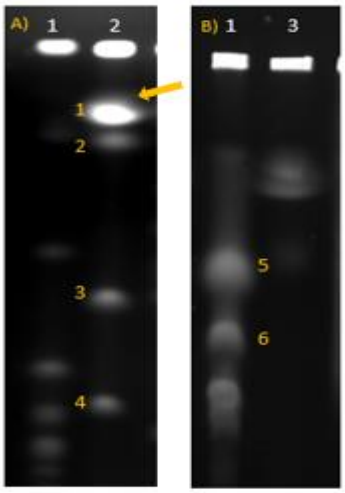

Figure 1- Gel image results after PFGE were performed. Left image (**A**) refers to the result of PFGE in adjusted conditions of *S. cerevisiae* PFGE and right image (**B**) refers to the result in the following conditions. Column 1 refers to *S. cerevisiae*, 2 to *S. diversa* and 3 to *S. pombe*. Separated chromosomal bands are named from A1 to A4 (left) and B1-B2 (right). Band sizes were calculated using a pattern for calibration curves two different markers: *S. cerevisiae* marker[21] for bands 2-4; *S. pombe* marker[22] for bands 5 and 6. 2- 2164 kb; 3- 1386 kb; 4- 1027 kb; 5- 2933 kb; 6- 2173kb.

Two different experimental setups were made (as detailed in materials and methods), with a change of several system conditions- run and switch time, voltage gradient, buffer temperature, angle and number of blocks – to separate the bands with smaller sizes (**Fig. 1A**) and the higher size bands (**Fig. 1B**), respectively, as the chromosomal band **1** didn't separate properly. The results obtained revealed the presence of 5 chromosomal bands, ranging from 1 to 2.9Mb, totalling 9.68 Mb. Both the number of chromosomes and total DNA size have already been described in other wine yeasts, such as C. zemplinina and H. vinae[15] [16]. Genomic sequencing was performed with Illumina sequencing, which resulted in a total of 43659976 reads, with an average size of 150 bp. The de novo assembly of these reads was performed resorting to two different algorithms, the one embedded in CLC Genomics Workbench software and SPAdes assembler[17], as plugin in Geneious software.

Table 1- Parameters of the result of de novo assemble of *S. diversa* MJT240 reads, performed by two different algorithms, CLC and SPAdes.

Parameters	CLC	SPAdes
Number of contigs	579	1150
% GC	33.8	34.0
Contig minimum size (bp)	200	78
Contig maximum size (bp)	1,021,160	970,026
N50 (excluding scaffolded regions) (bp)	272,524	374,723
Sum of assembled contigs (bp)	9,773,418	9,861,525

Table 2- General features of *S. diversa* MJT240 genome annotation performed in Geneious software framework, after manual curation.

Annotation features	Value
Nºof genes	5070
max length (amino acid)	4980
min length (amino acid)	34
average length (amino acid)	484
GC %	34.5
nº of introns	160
% Introns	3.16
genes with 2 exons	150
genes with 3 exons	5
genes with +3 exons	5

Features of both *assemblies* are presented in **Table 1**. Comparative analysis of the results obtained with both algorithms involving BLASTN analysis revealed no significant differences in the information included in contigs assembled in each algorithm and, therefore, taking all parameters into account, for the annotation procedure we decided to go with the contigs provided by SPAdes since these had a bigger N50 and therefore are larger, a trait that facilitates gene finding. The annotation of the 195 contigs was performed at the Geneious computational framework which allows ab initio gene prediction using various gene finders. In this work we resorted to the utilization of the Augustus[18] gene finder plugin and used three

different reference datasets- *Saccharomyces*; *Schizosaccharomyces pombe* and *C. tropicalis.*

Figure 2- Visualization of the annotation set up in Geneious software, using Find genes with Augustus tool and as reference datasets *S. Pombe, S. cerevisiae and C. tropicalis*. Green lines refer to the gene, red lines refer to mRNA and yellow lines refer to the coding sequence (CDS). Each gene model predicts one gene and corresponding mRNA and CDS.

Using this analysis a prediction is made, for every genomic region, of three gene models (see **Figure 2**) out of which only one was selected and confirmed as a possible *S. diversa* gene. For that selection, manual curation of the gene models was performed resorting to BLASTP analysis with proteins described at the NCBI protein database. Only those gene models having a described orthologue at NCBI were considered valid, regardless of the reference dataset they were identified from. After an exhaustive manual curation, a total of 5070 protein-encoding sequences (CDS) were predicted (features of annotation can be seen in **Table 2**). From this set, the incidence of introns is ~3.16%, which is in line with the low percentages obtained in other yeasts, such as *S. kluyvery*, *Z. rouxii* and *H. guilliermondi* [19, 20]. More specifically, there were identified 150 genes with two exons, 5 with three exons and 5 with more than three exons.

Metabolic reconstruction of *S. diversa* MJT240.

To obtain a metabolic reconstruction network of *S. diversa* MJT240 genome, the predicted ORFeome was uploaded to the BlastKoala tool[23], which performs in silico prediction of a biological function for the proteins, distributing them in functional classes and, particularly, among the pathways that are described in the database. This analysis shows that *S. diversa* MJT240 is equipped with all the central metabolic pathways (glycolysis, gluconeogenesis, pyruvate oxidation, citrate cycle, glyoxylate cycle, pentose phosphate pathway [PPP], oxidative phosphorylation and fatty acid catabolism). Importantly, it was determined the yeast is not equipped with a biosynthesis pathway for thiamine nor biotin. Other wine yeasts, like those belonging to the *Hanseniaspora* genus, were also found to be auxotrophic for thiamine and biotin biosynthesis, this being suggested to underlie their lower fermentation rates[20]. The absence of these pathways could be a determining factor for a low growth rate of *S. diversa MJT240* in a highly competitive environment like the grape must use for alcoholic fermentation. It was also determined the yeast is equipped with genes for catabolism of starch, sucrose, xylose and mannose, but not with genes for catabolism of galactose nor acetate. These findings were later confirmed by executing *S. diversa* MJT240 growth assays in solid YPD medium, with those sugars as sole carbon sources or sodium acetate as acetate source, as shown in **Fig. 3**. Only with xylose as the source of carbon, the result was contradictory, as *S. diversa* MJT240 cells didn't grow. This can be explained by the absence of orthologue genes encoding for a non-specific aldose reductase, part of the cofactor associated with xylitol formation, necessary for xylose assimilation, nor for xylose isomerase.

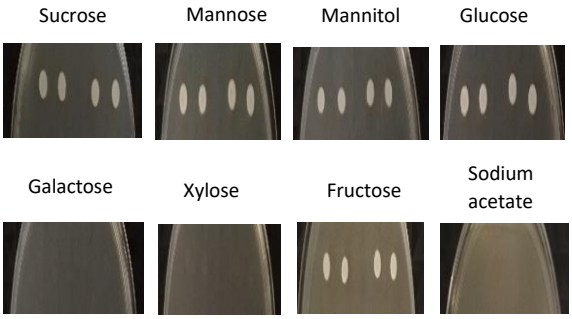

Figure 3- Growth of *S. diversa* MJT240 in YPD agar plates with different sugars as sole carbon sources,

using as technique spot assays containing *S. diversa* (DO=0.02). Replicates were used for each spot. Sodium acetate was tested as a source of acetate.

Regarding nitrogen metabolism, it was uncovered the yeast cannot assimilate nitrate nor nitrite as nitrogen source, similarly to yeasts belonging to genera *Schizosaccharomyces* and *Saccharomyces*[24], as there are no orthologue genes encoding for the necessary enzymes nitrate and nitrite reductase. Furthermore, assimilation of urea as a nitrogen source is also not possible, since no orthologue genes encoding for urea carboxylase were uncovered. Nevertheless, nitrogen assimilation is performed via the assimilation of amino acids. Accordingly, genes encoding for enzymes required for the biosynthesis of all the proteogenic amino acids were found.

As for sulphur metabolism, the yeast contains all the genes necessary for the assimilatory sulphate reduction pathway, as also for the methionine salvage pathway, that is not present in some wine yeasts, such as *Hanseniaspora* yeasts and *S. ludwigii* strains[20, 25, 26]. One of the functions of the methionine salvage pathway is to supply precursors for the synthesis of polyamines, such as putrescine, spermidine and spermine[20]. Genes encoding for enzymes involved in the synthesis of these polyamines, with the exception to spermine, were all found. The yeast's ability to synthesize spermidine is very interesting since the latter has been demonstrated to be an essential nutrient for *S. cerevisiae* growth[27], and could be a determining factor for a high growth rate in *S. diversa* MJT240.

Comparative analysis of the predicted ORFeome of *Saturnispora diversa* MJT240 with other wine yeast species.

In order to determine the extent at which the ORFeome of this *S. diversa's* strain is similar to the proteomes described for other wine yeast species, a comparative proteomic analysis was performed. For that, the sequences of proteins predicted to be encoded by *S. diversa* MJT240 were compared, using BLASTP, with those described to be encoded by *H. uvarum, H. guilliermondi* and *S. ludwigii* from the *Saccharomycodeacea* family, the *S. cerevisiae* strain EC1118, *T. delbrueckii, L. fermentati*, all from the family *Saccharomycetacea*. The spoilage yeasts *Brettanomyces bruxellensis* and *Z. bailli* were also selected for this analysis. The results obtained, presented in **Fig. 4**, showed that the lowest degree of similarity was obtained with the *H. guilliermondii* and *H. uvarum* proteomes, that goes accordingly to previously performed comparison analysis [25]. Thus, it is suggested that these *Hanseniaspora* species belong to a subgroup, as even *S. ludwigii,* that belongs to the same family, showed a considerably higher degree of identity with *S. diversa*, similarly to other yeasts. Accordingly, all the proteomes of *S. ludwigii, Z. bailii* and the yeasts of *Saccharomycetacea* family shared a close degree of similarity, from which *L. fermentati* had the most proteins with high similarity. Interestingly, the yeast *B. bruxellensis* was by far the one with higher degree of similarity towards this *S. diversa* strain, a result that was unexpected.

To capture more specific features of *S. diversa MJT240*, the proteins considered dissimilar from those found in the four yeast species used for the comparative proteomic analysis were compared resulting in the Venn plot showed in **Fig. 5**. Thereby, it was possible to identify the set of *S. diversa* proteins that were dissimilar from those found in the other yeast species. From the original 5070 annotated ORFs, 1584 were only found in *S. diversa* MJT240. A substantial part of these are hypothetical proteins or have poorly characterized function. It will now be interesting to compare this set of proteins with those found in *B. bruxellensis*, in order to identify which could actually be the set of *S. diversa* more "specific" proteins. It was interesting to find the set of ORFs that were found in S. diversa, but not in *T. delbrueckii, L. fermentati, H. guilliermondi* and *S. ludwigii*, eight enzymes involved in protein glycosylation: two oligosaccharyltransferases (Sd48_g3 and Sd5_g14), involved in the first step of protein glycosylation - transfer of a glycan to an asparagine residue-; a beta-1,4-N-acetylglucosaminyltransferase (Sd3_g98), linked to generation of N-acetyl-D-glucosamine-beta-1,4-O-D-mannosylprotein; a phosphatidylinositol N-acetylglucosaminyltransferase subunit P (Sd2_g29),

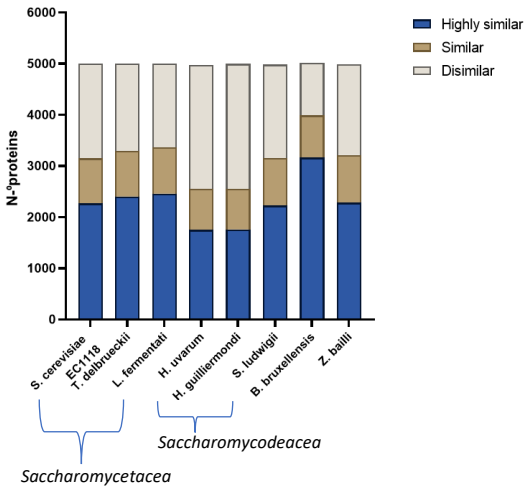

Figure 4– Comparative analysis of *S. diversa* MJT240 Proteome with other wine yeasts using pairwise Blastp alignments. The graphic shows proteins with high similarity (E-value < 10^{-50}), similarity (E-value 10^{-50} > x < 10^{-20}) and dissimilarity (E-value > 10^{-20}). Wine yeast to whom *S. diversa 240* was compared are *S. cerevisiae EC1118, T. delbrueckii, L. fermentati,* from *Saccharomycetacea* family, *H. gluilliermondi, H. uvarum and S. ludwigii*, from *Saccharomycodeacea* family, and spoilage wine yeasts *B. bruxellensis* and *Z, bailii clib213*.

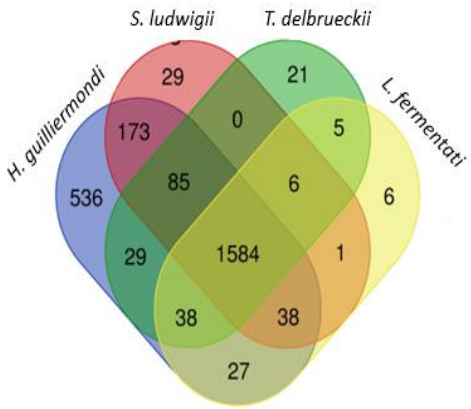

Figure 5– *S. diversa* MJT240 proteins considered to be dissimilar compared to others found in yeast species *T. delbrueckii, H. uvarum, S. ludwigii, C. glabrata* and *L. fermentati* were compared to determine the set of proteins specific to *S. diversa* MJT240. The results are shown in the Venn's plot. A set of 1584 proteins were determined to be unique for *S. diversa* MJT240.

involved in the first step of GPI biosynthesis; a GPI-anchor transamidase subunit S (Sd3_g259) and a GPI ethanolamine phosphate transferase subunit F (Sd27_g6). Also, it was uncovered the presence of a dolichol-phosphate mannosyltransferase (Sd5_g238), whose function is to form dolichol phosphate mannose, the mannosyl donor in pathways that lead to N-glycosylation and a dolichol phosphate-mannose regulatory protein (Sd1_g45_B), that regulates the function of dolichol-phosphate mannosyltransferase and is also involved in the first step of GPI biosynthesis. Interestingly, an ethanolamine kinase (Sd36_g17) was also identified in the set of proteins specifics to *S. diversa*, this being an enzyme that catalyzes the synthesis of phosphatidylethanolamine, a pivotal compound for GPI biosynthesis, as it is involved in several steps of this pathway. Further studies will now be required to investigate whether these proteins are also absent from the proteomes of other wine yeast species, and, more importantly, what could be their physiological function in the context of cell physiology. Seven proteins related to oxidative phosphorylation metabolism, namely in Complex I - NADH dehydrogenase, were also identified in the set of S. diversa proteins but not in *T. delbrueckii, L. fermentati, H. guilliermondi* and *S. ludwigii*. Notably, these enzymes are also not present in *S. cerevisiae*[29]. However, it is not clear whether the presence of this proteins has any effect in oxidative phosphorylation, as Complex I is composed of 42 subunits and there are several genes encoding for necessary proteins that were not identified, in *S. diversa* ORFeome, mainly those related to NADH dehydrogenase alpha and beta subcomplex.

The predict "Flavoroma" genes in *S. diversa* MJT240 genome.

Several Non-*Saccharomyces* wine strains have shown potential to improve wine aroma by producing aromatic molecules that are not produced, or that are produced in very low concentrations, by *S. cerevisiae*. At least one study has reported the usefulness of *S. diversa* in this context[30]. Therefore, although it is not known whether such a beneficial effect could also be observed for the strain used in this work, the genome sequence was searched for genes encoding

for enzymes involved in production of volatile aroma compounds. Reconstruction of *S. diversa* MJT240 metabolic network showed the yeast is equipped with genes required for synthesis of higher alcohols, ethyl esters, acetaldehydes, volatile compounds that have a positive impact in wine aroma. However, the predicted proteome didn't include important enzymes such as beta-glucosidases, related to production of terpenes, and alcohol acetyltransferases, involved in production of acetate esters.

These findings are consistent with the aroma profile derived from fermentation of Touriga nacional musts with *S. diversa* species[30], in which an intense aroma was detected in comparison with other wine from other yeasts. Specifically, the associated volatile compounds to the aroma descriptors that had more impact in those wines (nutty and dried fruits), benzaldehyde, esters and isopentyl alcohol, are synthesized by the enzymes above described to be encoded by *S. diversa*, thus suggesting the species may produce volatile aroma compounds with impact in wine aroma.

Effect of the presence of *S. diversa* MJT240 in growth of the contaminating yeast *Saccharomycodes ludwigii* UTAD17 during fermentation.

Considering the previously described potential of *S. diversa* MJT240 as a bio-control agent of several phytopathogenic fungal species[31-34], including those that target grape vines, it was herein decided to examine whether the strain in use in this thesis could also show an interesting biocontrol potential against the contaminating yeast *Saccharomycodes ludwigii* UTAD17, in synthetic GJM. Single culture growth assays were firstly performed to determine growth kinetics of both yeasts in GJM (results shown in **Table 3**). Afterwards, co-culture growth assays were carried on, for which a method was devised consisting in CFU cell counting to determine growth of yeasts: *S. ludwigii* cells were plated and grown in YPD+ 6mM SO_2, as it was established that *S. diversa* cells didn't grow in such SO_2 concentration in medium, whereas *S. ludwigii* grew normally; and *S. diversa* cells were plated and grown in WL medium, in which yeast species can be distinguished on the basis of morphology and colour of colonies.

Table 3- Growth kinetics of *S. diversa* MJT240 and *S. ludwigii* UTAD17: specific growth rate, μ, presented in h^{-1} and min^{-1}, and doubling time, Td (min).

Sd240		**U17**	
μ (h-1)	0.6267	**μ (h-1)**	0.2820
μ (min-1)	0.01040	**μ (min-1)**	0.00470
Td (min)	66.4	**Td (min)**	147

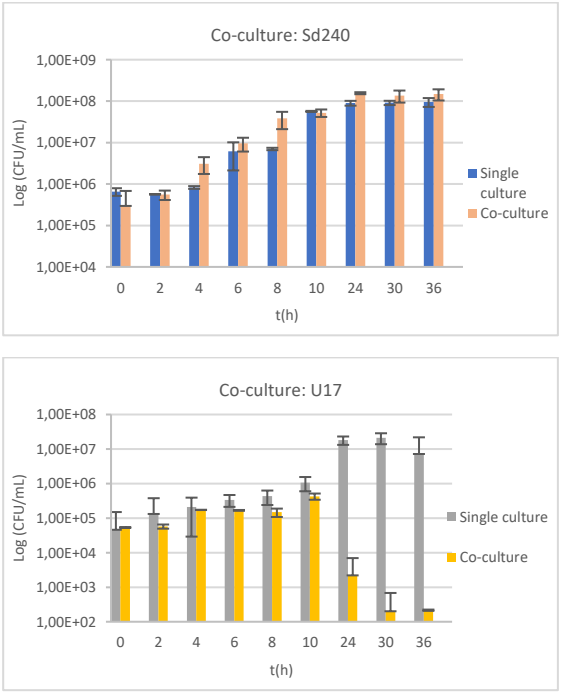

Figure 6- Graphic representation of *S. diversa* MJT240 *(*Sd240) and *S. ludwigii's* growth (U17) in co-culture assays along time (h) using CFU counting as growth measuring tool. Data is presented in logarithmic scale. Sd240 cells were plated in WL medium, whereas U17 cells were plated in YPD+ 6mM SO2 medium. Single culture growth assay is presented for comparison.

The results obtained show no significant differences in growth of *S. diversa* MJT240 while in single or in co-culture (**Fig.24**), thus, there was little effect in this species by the presence of *S. ludwigii* UTAD17. Differently, in the case of *S. ludwigii* a marked reduction in cellular viability was registered after 10h of co-cultivation with the viability decreasing significantly, compared to the values observed in single culture (**Fig.25**)- from $1.82x10^7$ CFU/mL to $2.02x10^2$ CFU/mL, a percentage loss of 99.998%.

This loss of viability was very interesting and appears to point in the direction towards a biocontrol activity by the *S. diversa* strain, however, further assays should be performed to determine that. It is possible that the reduction in *S. ludwigii* viability results from a competition of nutrients since *S. diversa* has a doubling time much lower than UTAD17 and, therefore, consumes sugar at a much faster rate. However, this alone shouldn't account for a reduction in cellular viability that appears more to result from a toxicity mechanism that may involve, for example, the production by *S. diversa* of an antimicrobial peptide or a compound whose accumulation is deleterious for *S. ludwigii*.

Conclusions

In this work, the first genomic sequence, dully annotated, of a strain belonging to the species Saturnispora diversa, MJT240, was determined. With the genomic sequence determined, it will now be possible to perform a more extensive metabolic analysis of the yeast, for instance, facilitating the execution of Proteome and Transcriptome assays. As for future perspectives, it will be important to evaluate whether the volatile aromatic compounds described before are in fact produced by Saturnispora diversa MJT240 and if the impact on the aroma profile is significant. Concerning its phenotypic behaviour, it will also be essential to determine whether the phenotype of biocontrol by S. diversa MJT will also be observed in real musts and not only in GJM. It will also be interesting to determine if S. diversa MJT240 presence has the potential to inhibit the growth of other spoilage yeasts, namely B. bruxellensis and Z. baili. And, necessarily, it will be essential to further detail the underlying inhibitory mechanism, something that the availability of the genomic sequence now turns possible to inspect, for example, at the transcriptomic level.

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