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Genomic Analysis of an Excellent Wine-Making Strain *Oenococcus oeni* SD-2a

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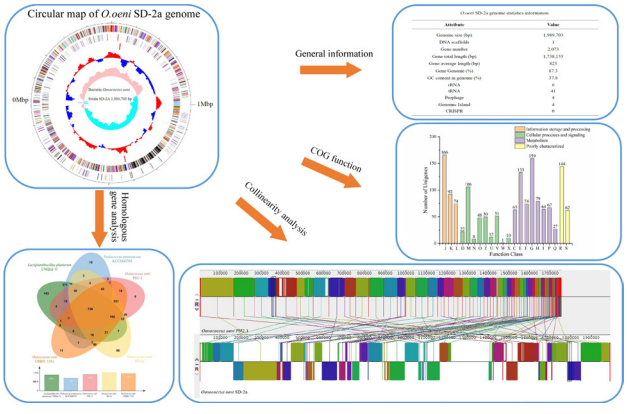
Abstract

Oenococcus oeni is an important microorganism in wine-making-related engineering, and it improves wine quality and stability through malolactic fermentation. Although the genomes of more than 200 *O. oeni* strains have been sequenced, only a few include completed genome maps. Here, the genome sequence of *O. oeni* SD-2a, isolated from Shandong, China, has been determined. It is a fully assembled genome sequence of this strain. The complete genome is 1,989,703 bp with a G+C content of 37.8% without a plasmid. The genome includes almost all the essential genes involved in central metabolic pathways and the stress genes reported in other *O. oeni* strains. Some natural competence-related genes, like *com*EA, *com*EC, *com*FA, *com*G operon, and *com*FC, suggest that *O. oeni* SD-2a may have natural transformation potential. A comparative genomics analysis revealed 730 gene clusters in *O. oeni* SD-2a homologous to those in four other lactic acid bacteria species (*O. oeni* PSU-1, *O. oeni* CRBO-11381, *Lactiplantibacillus plantarum* UNQLp11, and *Pediococcus pentosaceus* KCCM40703). A collinearity analysis showed poor collinearity between *O. oeni* SD-2a and *O. oeni* PSU-1, indicating great differences in their evolutionary histories. The results provide general knowledge of *O. oeni* SD-2a and lay the foundation for specific gene function analyses.



Key words: *Oenococcus oeni* SD-2a, genome, homologous genes, collinearity

Introduction

Wine-making has a long history and is still thriving in modern times (Bartowsky 2017). Oenological yeasts (like *Saccharomyces cerevisiae*) and lactic acid bacteria (LAB, like *Oenococcus oeni*) play important roles in the wine-making industry because these microorganisms complete alcoholic fermentation (AF) and the malolactic fermentation (MLF), respectively (Lonvaud-Funel 1999; Bartowsky 2017). MLF occurs during or after AF due to the metabolic activity of LAB, the decarboxylation of L-malic acid to L-lactic acid and CO_2, which exerts a significant impact on the organoleptic qualities wine (Bartowsky and Borneman 2011; Krieger-Weber et al. 2020). *Lactiplantibacillus*, *Pediococcus*, *Leuconostoc*, and *Oenococcus* are four LAB genera that perform MLF and are usually found in wine. However, identification by colony hybridization using total genomic DNA probes shows that species of *Lactiplantibacillus*, *Pediococcus*, and *Leuconostoc* progressively

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disappear during AF, while *O. oeni* is the only species identified when AF is finished (Wibowo et al. 1985; Lonvaud-Funel 1999; Liu 2002). Moreover, species of *Lactiplantibacillus* and *Pediococcus* can produce undesirable volatile compounds associated with wine spoilage (Bartowsky 2009). *O. oeni* is the most common species to perform MLF in wine-making, owing to its ability to grow and perform MLF in the low acidic, high ethanol, SO_2, nutrient-scarce, and low-temperature wine environment. Most species of *O. oeni* contribute positively to sensory wine characters (Bartowsky 2009; Grandvalet 2017).

O. oeni is a Gram-positive, facultative anaerobic bacterium that organizes into chains or pairs of circular to ellipsoidal cells (Grandvalet 2017). *O. oeni* is present at extremely low to undetectable levels on intact grapes or other fruits in the natural environment, with wine seemingly representing the exclusive niche of this bacterium (Franquès et al. 2017). Based on the 16S rRNA sequence, *O. oeni* was reclassified into a new genus from the previous *Leuconostoc oenos* (Dicks et al. 1995). To reveal the mechanisms of *O. oeni* specifically adapted survival in the wine-making environment, various omics techniques like genomics, transcriptomics, and proteomics are used for many species from this genus. Genome sequencing of different strains of *O. oeni* showed that the bacterium has a relatively small genome, mostly ranging from 1.7 to 2.2 Mb, which most likely results from the specialization of this microbe in the relatively narrow ecological niche of a similar wine environment (Makarova et al. 2006; Lorentzen et al. 2019). Another striking feature of the *O. oeni* is that it lacks the *mutS-mutL* system involved in DNA mismatch repair, making *O. oeni* a "hypermutable" species (Mills et al. 2005). It plays a significant part in the phylogenetic diversity and adaptation of *O. oeni* to its niche (Marcobal et al. 2008). Sequence-based phylogenetic trees of *O. oeni* strains showed that they are mainly divided into four clades named groups A, B, C, and D according to the wine environment they lived in (Lorentzen et al. 2019). Strains from group A were found only in wine. Group B and C contained strains isolated from wine and cider, while group D consisted of strains isolated from kombucha, a low-alcohol beverage (Lorentzen et al. 2019). Transcriptomics and proteomics are usually used for the characterization of wine-related stress responses in *O. oeni*, especially in ethanol shock and acid shock environments. After ethanol shock or transferring *O. oeni* strains to a wine-like medium, the abundance of the protein-encoding genes of citrate metabolism, MLF, and molecular chaperones increased, while genes involved in carbohydrate or lipid transport and metabolism, as well as coenzyme transport and metabolism, decreased (Olguín et al. 2015; Margalef-Català et al. 2016a). The induced molecular chaperones include *danJ*, *danK*, and *grpE* in early responses and *clpP* in the late response (Costantini et al. 2015; Margalef-Català et al. 2016a). The Hsp20 mRNA was lower in the presence of 12% (v/v) ethanol but induced in the presence of 8% (v/v) ethanol in the late response (Costantini et al. 2015). Margalef-Català et al. (2016a) speculated that at a higher concentration, such as 12% (v/v), cells require the involvement of the molecular chaperones GroEL and GroES with the ability to repair damaged proteins.

Acid shock leads to the overexpression of Hsp20, malate transport, and malate permease-encoding genes (Liu et al. 2017), while the combined acid and ethanol shock increased the expression levels of the *recN*, *recO*, *mutT* genes involved in DNA repair, spermidine and putrescine transport-encoding genes, and the stress-relevant genes *hsp20*, *clpP*, *trxA*, *ctsR*, and *uspA* (Zhao et al. 2020; Yang et al. 2021). Onetto et al. (2021) recently characterized the transcriptional response of *O. oeni* to SO_2. They found that the *hsp20* gene and the glutaredoxin protein-encoding gene *nrdH* were up-regulated at the SO_2 concentration equal to 5 mg/l, while down-regulated genes were involved in transcription regulation, oxidative stress replication, and cell wall assembly. These studies indicated that *O. oeni* could effectively adjust the gene expression strategies to better adapt to the wine environment.

Genome sequencing can quickly and comprehensively provide genetic information on species and lay the foundation for performing other omics technologies. Until April 2022, 247 genome sequences of *O. oeni* strains were available from the National Center for Biotechnology Information (NCBI), and some genome sequences information on different *O. oeni* strains was analyzed by researchers (Borneman et al. 2010; Bridier et al. 2010; Borneman et al. 2012; Capozzi et al. 2014; Dimopoulou et al. 2014; Lamontanara et al. 2014; Campbell-Sills et al. 2015; Jara and Romero 2015; Mendoza et al. 2015; Sternes and Borneman 2016; Costantini et al. 2020). However, only five complete genomes of *O. oeni* strains have been reported (Mills et al. 2005; Iglesias et al. 2018; Lorentzen et al. 2019; Onetto et al. 2021). Overviews of genome sequencing, phylogenetic analyses, and genomic differences among *O. oeni* strains have been reported, but systematic genomic analyses of specific strains of *O. oeni* are rare. The first detailed genomic analysis of *O. oeni* was *O. oeni* PSU-1, which has also been a most studied strain and has always been taken as a reference by researchers (Mills et al. 2005). We report the complete genome map of one *O. oeni* strain isolated from Shandong, China. In addition to the basic genome map-related information, the genes involved in several critical metabolic pathways, like MLF, citrate metabolism, stress-related processes, and natural transformation

processes, were also analyzed. Comparative genomics analysis of five LAB strains isolated from wine or wine-like environments, *O. oeni* SD-2a, *O. oeni* PSU-1, *O. oeni* CRBO-11381, *Lactiplantibacillus plantarum* UNQLp11, and *Pediococcus pentosaceus* KCCM40703, and a collinearity analysis between *O. oeni* SD-2a and *O. oeni* PSU-1 were also performed. The results increase our understanding of how *O. oeni* strains adapt to the specific wine environment and lay a foundation for the further study of specific gene functions.

Experimental

Materials and Methods

Strain purification and culture. The *O. oeni* strain SD-2a was isolated from Shandong, China. *O. oeni* SD-2a, stored at −80°C, was streaked on solid FMATB plate, and a single colony was selected and inoculated into a liquid FMATB medium. When it reached middle logarithmic growth ($OD_{600} \approx 1$), the appropriate volume of fresh bacterial solution was used for subsequent genomic DNA extraction.

Extraction and quality of genomic DNA. The genomic DNA was extracted following the instructions of the TIAnamp bacterial DNA kit (TIAGEN, China). Qualified genomic DNA was used as the starting material for sequencing and library construction. DNA purity testing was assayed using a micro-ultraviolet spectrophotometer (BioDrop, UK). An $OD_{260/280}$ in the range of 1.8 to 2.0 was required for further use. A Qubit 3.0 Fluorometer (Thermo Fisher, USA) was used to accurately quantify the starting DNA, with a total DNA amount of ≥ 5 μg being required. The genomic integrity of the DNA was detected by 1% agarose gel electrophoresis.

Whole-genome sequencing and assembly. In this study, Illumina HiSeq combined with the third-generation sequencing technology (single-molecule sequencing, PacBio) was used to complete the genome map of the strain. An Illumina PE library and a PacBio library (8–10 kb) were constructed. After quality control, the Illumina sequencing data were preliminarily assembled using SOAPdenovo (v2.04) (Li et al. 2010). Then, the sequencing data of PacBio were compared using BLASTR, and the single-molecule sequencing data were corrected based on the comparison results. The purpose was to reduce the errors resulting from single bases, insertions or deletions in the long single molecule sequences. Finally, the corrected single-molecule sequencing data were used for assembly. The assembly principle was similar to that of the first-generation sequencing technology, in which the overlap relationships between sequences are used to connect scaffolds. The Celera Assembler 8.0 software was used for the subsequent assembly (Powers et al. 2013). After all the scaffold connections were completed, they were verified using the Illumina data. In addition, GapCloser v1.12 software was used for gap closing (Koren et al. 2012). Finally, a gap-free circular genome sequence was assembled. To provide a more comprehensive and intuitive understanding of the characteristics of the genome, Circos v0.64 (http://circos.ca) software was used to construct the genome's circular map and comprehensively display the relevant information. The samples' Illumina sequencing data volume requirement was > 1 GB, and the PacBio sequencing data volume requirement for samples was > 50×. The sequencing was completed by the Beijing CapitalBio Technology Co., Ltd.

Gene annotation and analysis. The open reading frame (ORF) sequences in the *O. oeni* SD-2a genome were predicted and analyzed using Glimmer version 3.02 (http://cbcb.umd.edu/software/glimmer) software. The ORF sequences predicted by BLAST 2.2.28+ were compared against the NR (non-redundant database in NCBI) (Feng et al. 2018), Genes (http://www.genome.jp/kegg), and String (http://string-db.org) databases to obtain annotations for the predicted genes. The ribosomal RNA (rRNA) and transport RNA (tRNA) contained within the genome were predicted using the software RNAmmer-1.2(v1.2, http://www.cbs.dtu.dk/services/RNAmmer) and tRNAscan-SE (v1.3.1, http://lowelab.ucsc.edu/tRNAscan-SE). The genomic island sequences of the strain's genome were predicted using Island Viewer 3 and IslandPick software and presented in a circular diagram (Dhillon et al. 2015). The nucleic acid sequence of the prophage integrated into the host genome was determined using PHASTER (PHAge Search Tool Enhanced Release). The CRISPRFinder online tool (http://crispr.u-psud.fr) was used to predict CRISPR sequences present in the genome.

Comparative genomic analysis. To further study important functional genes of the *O. oeni* SD-2a genome, four other LAB strains (*O. oeni* PSU-1, *O. oeni* CRBO-11381, *L. plantarum* UNQLp11, and *P. pentosaceus* KCCM40703) having complete genome sequences were selected for a follow-up comparative genomics analysis. The whole-genome sequences of these four LAB strains were downloaded from the NCBI database. OrthoMCL (v2.0.3) software was used for homologous gene analysis (Gross et al. 2013). OrthoMCL software compared the amino acid sequences of the five strains. Appropriate thresholds were selected (BLASTP e-value ≤ $1\ e^{-5}$, MCL inflation = 1.5) for similarity clustering to obtain the homologous gene families (Li et al. 2010). The collinearity analysis of *O. oeni* SD-2a and *O. oeni* PSU-1 genomic sequences was performed using the Mauve software (http://darlinglab.org/mauve/mauve.html).

Results and Discussion

Genome overview of *O. oeni* SD-2a. The whole-genome map of *O. oeni* SD-2a was obtained through genome sequencing and bioinformatics analyses. A circular genome was constructed by sequencing data assembly (Fig. 1). The quality control of DNA samples and the sequencing process are shown in Fig. S1, S2 and S3 and Tables SI and SII. The total length of the *O. oeni* SD-2a genome sequence was 1,989,703 bp, including 2,073 genes having an average length of 823 bp. There is no plasmid in this strain. The total length of sequences encoding genes is 1,738,155 bp, approximately 87.3% of the whole genome. The G+C content is approximately

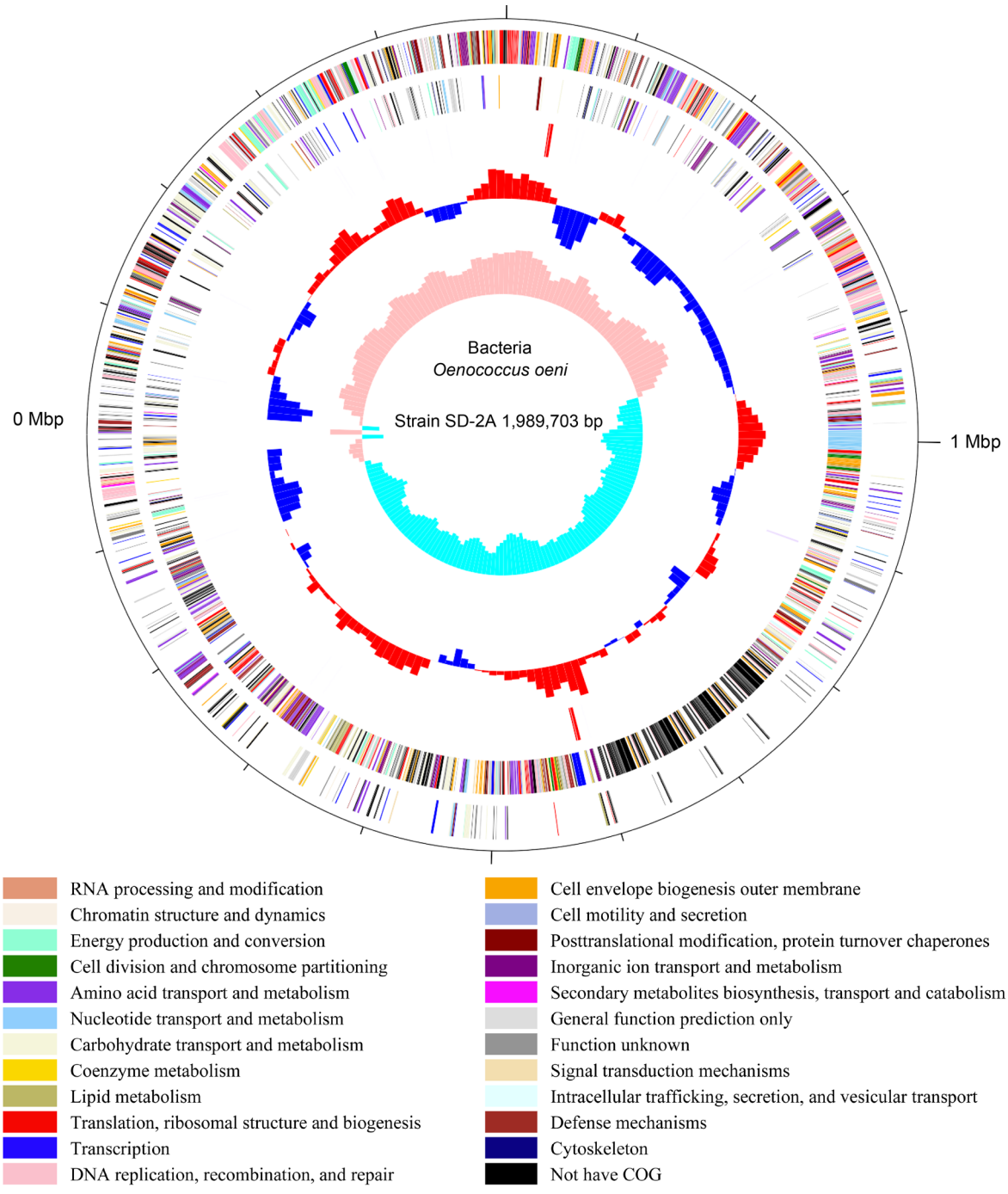


Fig. 1. Circular map of *Oenococcus oeni* SD-2a genome. The outermost circle of the circle graph is an indicator of the size of the genome. The second and third circles are genes on positive and negative chains; different colors represent the functional classification of COGs with different genes. The fourth circle is rRNA and tRNA. The fifth circle is GC content; the outward red part indicates that the GC content in this region is higher than the average GC content of the whole genome, the inward blue part indicates that the GC content in this region is lower than the genome-wide average GC content, the higher the peak value, the greater the difference from the average GC content. The innermost circle is the GC skew value; the outward red part indicates that the value is positive, the inward blue part indicates that the value is negative.

Table I
O. oeni SD-2a genome statistics information.

Attribute	Value
Genome size (bp)	1,989,703
DNA scaffolds	1
Gene number	2,073
Gene total length (bp)	1,738,155
Gene average length (bp)	823
Gene/Genome (%)	87.3
GC content in genome (%)	37.8
rRNA	6
tRNA	41
Prophage	4
Genomic Island	4
CRISPR	0

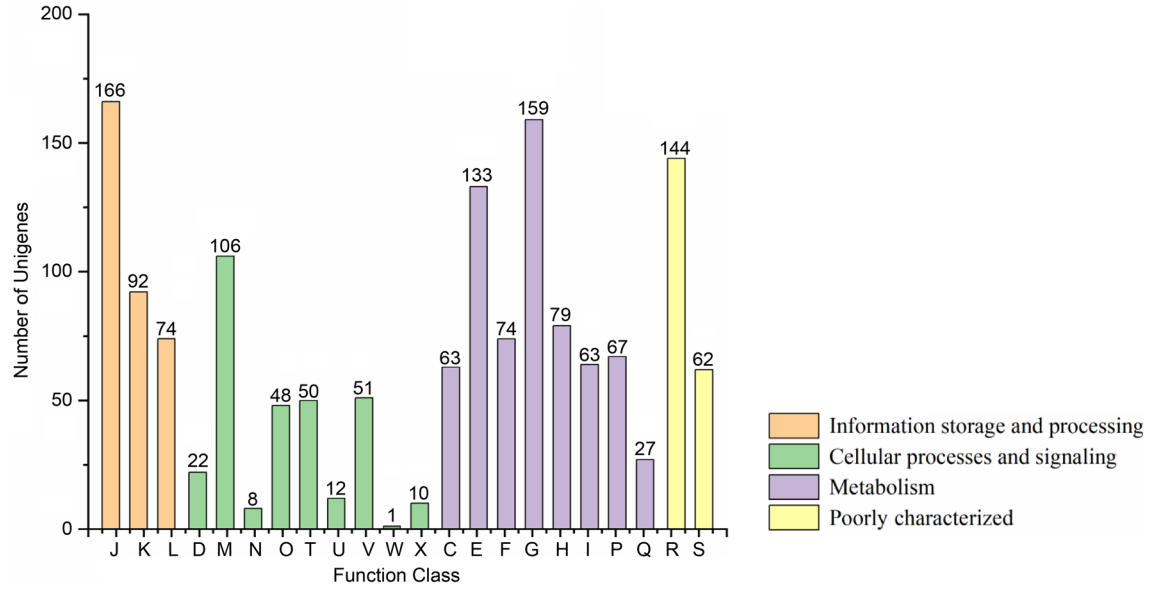
37.8%, similar to other *O. oeni* strains. The genome of *O. oeni* SD-2a contains 41 tRNA genes and six rRNA genes, and two copies of the 5S-16S-23S rRNA genes. Four potential genomic islands were identified in the genome, with three being longer than 21 kb. These sequences mainly encoded hypothetical genes. Four complete prophage sequences of 54.7, 54.5, 54.5, and 46.8 kb were also identified in the *O. oeni* SD-2a genome. No CRISPR sequences were found in the genome. The basic genomic information is presented in Table I.

The total length of the *O. oeni* SD-2a genome is a medium-size among *O. oeni* strains, which mostly have genomes ranging from 1.7 to 2.2 Mb (Makarova et al. 2006; Lorentzen et al. 2019). It is a relatively small size among bacteria, and it most likely results from their nutrient-rich wine living environment. Many functional genes, such as the genes related to nutrient anabolism and catabolism, appear to be lost during the specialization of the species (Lorentzen et al. 2019). There are 2,073 ORFs in *O. oeni* SD-2a, much more than in *O. oeni* PSU-1of 1701, and a relatively high number among the wholly sequenced *O. oeni* strains. The ORFs numbers of the other four completed genome sequences of *O. oeni* strains are between 1858–1970 (Iglesias et al. 2018; Lorentzen et al. 2019; Onetto et al. 2021). *O. oeni* SD-2a has six rRNA genes and two copies of 5S-16S-23S rRNA, similar to other sequenced *O. oeni* strains. The number of rRNA genes may be related to the protein synthesis capabilities. *O. oeni* SD-2a has 41 tRNAs, which is fewer than 43 tRNA in *O. oeni* PSU-1 (Mills et al. 2005). The large numbers of tRNAs may play essential roles in the accurate transmission of genetic information.

Four complete prophage sequences of 54.7, 54.5, 54.5, and 46.8 kb were found in the genome of *O. oeni* SD-2a. It is unlike *O. oeni* PSU-1, in which no complete prophage sequences were found (Mills et al. 2005). In fermentation engineering, phage contamination is a common threat, and it is the main reason for the slowing or termination of milk fermentation (Papadimitriou et al. 2016). The phage contamination also occurs in the MLF process during winemaking. However, due to the complexity of the process, it does not have a significant impact on the MLF fermentation results (Mills et al. 2005). Prophage sequences can confer host immunity or exclusion and protect carrier strains from double infections (Gentile et al. 2019). Therefore, the prophage sequences of *O. oeni* SD-2a may help avoid the infection by other phages.

Clusters of orthologous groups of proteins (COG) functional classifications of predicted *O. oeni* SD-2a proteins. The ORF sequences of *O. oeni* SD-2a were placed in a string database (v9.05) and analyzed using BLASTP (BLAST 2.2.28+). Then, the COG annotations corresponding to the genes were obtained. The proteins were classified based on the COG annotations, as shown in Fig. 2. 1,314 protein sequences out of 2,073 genes were classified into four main COG functional categories, accounting for 63.4% of the predicted proteins. It suggests that *O. oeni* SD-2a may have unique biological processes, and many hypothetical proteins with unknown functions need to be investigated. Among those annotated proteins, proteins involved in metabolism account for the most significant proportion, including 159 in carbohydrate transport and metabolism, 133 in amino acid transport and metabolism, 79 in coenzyme transport and metabolism, 74 in nucleotide transport and metabolism, 67 in inorganic ion transport and metabolism, 64 in lipid transport and metabolism, 63 in energy production and conversion, and 27 in secondary metabolites biosynthesis, transport, and catabolism. It is similar to *O. oeni* PSU-1. Indeed, these metabolism-related genes are also the most differentially expressed in wine under different experimental conditions. For example, after ethanol or acid shock, treatment with 5 mg/l SO_2, or transfer into wine-like medium or non-*Saccharomyces*-fermented wines, the genes involved in carbohydrate transport and metabolism, amino acid transport and metabolism, and some other metabolisms related genes accounted for the largest proportion in all differentially expressed *O. oeni* genes (Costantini et al. 2015; Olguín et al. 2015; Margalef-Català et al. 2016a; Liu et al. 2017; Onetto et al. 2021; Balmaseda et al. 2022). It suggests that these metabolisms-related genes are critical for *O. oeni* strains to survive in the wine environment.

Another category that accounts for many *O. oeni* SD-2a proteins is information storage and processing. Proteins involved in this category are mainly divided into three small categories, including 166 in translation, ribosomal structure, and biogenesis, 92 in transcription, and 74 in replication, recombination, and repair. These processes involve transcription, translation, and



C – Energy production and conversion
D – Cell cycle control, cell division, chromosome partitioning
E – Amino acid transport and metabolism
F – Nucleotide transport and metabolism
G – Carbohydrate transport and metabolism
H – Coenzyme transport and metabolism
I – Lipid transport and metabolism
J – Translation, ribosomal structure and biogenesis
K – Transcription
L – Replication, recombination and repair
M – Cell wall/membrane/envelope biogenesis
N – Cell motility
O – Posttranslational modification, protein turnover, chaperones
P – Inorganic ion transport and metabolism
Q – Secondary metabolites biosynthesis, transport and catabolism
R – General function prediction only
S – Function unknown
T – Signal transduction mechanisms
U – Intracellular trafficking, secretion, and vesicular transport
V – Defense mechanisms
W – Extracellular structures
X – Mobilome– prophages, transposons

Fig. 2. COG function classification of predicted *Oenococcus oeni* SD-2a proteins. Genes are classified into four main COG functional categories. The number of genes in each category and specific notes on categories are indicated.

regulation and are necessary for cells to respond to external changes and make corresponding changes. Proteins involved in cellular processes and signaling are distributed in small categories, including 106 proteins engaged in cell wall/membrane/envelope biogenesis, 51 in defense mechanisms, 50 in signal transduction mechanisms, 48 in posttranslational modification, protein turnover, chaperones, 22 in cell cycle control, cell division, chromosome partitioning, ten in mobilome of prophages and transposons, eight in cell motility, and one in extracellular structures. These proteins act on various relatively independent and closely related cell activities and play an essential role in maintaining the normal activities of cells in different environments. In addition to the above three main categories of proteins, 144 general function prediction only proteins and 62 function unknown proteins are classified as poorly characterized. These proteins may act on unknown pathways and need to be further studied. The COG functional classification provides a general understanding of protein functions in *O. oeni* SD-2a.

Carbohydrate transport and metabolism. Like other heterofermentative LAB (Van Vuuren and Dicks 1993; Mills et al. 2005), *O. oeni* SD-2a has almost all the genes required for utilizing hexose and pentose through heterotypic lactic acid fermentation and phosphoketolase pathway. The COG functional classification revealed 159 genes involved in carbohydrate transport and metabolism in *O. oeni* SD-2a, suggesting that this strain has a relatively complete carbohydrate transport and metabolism system. Borneman et al. (2012) reported the potential L-arabinose and L-xylulose utilization profiles (including the arabinose polymer arabinan) in fourteen *O. oeni* strains. The utilization of these two sugars may be strain-dependent. They found that all fourteen strains have the three enzymes encoding genes of L-arabinose isomerase, L-ribulose-5-phosphate 4-epimerase, and L-ribulokinase involved in utilizing L-arabinose. Only *O. oeni* BAA-1163 and *O. oeni* AWRIB418 strains have alpha-L-arabinofuranosidase, which were predicted to degrade the arabinose polymer arabinan. Nine of the fourteen strains have another three enzymes required to convert L-xylulose to D-xylulose-5-phosphate. *O. oeni* SD-2a has six of the genes encoding enzymes mentioned above but lost the L-ribulokinase encoding gene, suggesting it has the potential to utilize arabinan and L-xylulose. Whether it can utilize L-arabinose, needs further study.

Many genes in the genome of *O. oeni* SD-2a are related to a carbon source transport system and are mainly divided into four categories: PTS sugar transporter, sugar: proton symporter, sugar ABC transporter, and other carbohydrate transport-related genes, as shown in Table SIII. Several genes, including those represented by ORF00178 (*celC*, PTS cellobiose transporter subunit IIA), ORF00191 (*fruB*, PTS fructose transporter subunit IIA), ORF00301 (*manX*, PTS mannose transporter subunit IIB), ORF01618 (*ulaB*, PTS lactose transporter subunit IIB), ORF00708 (*msmG*, ABC-type maltose transporter permease), and ORF01922 (sucrose phosphorylase) encode proteins that may transport or modify different sugars. These genes allow *O. oeni* SD-2a to obtain carbohydrates from the environment and metabolize them. It has been reported that *O. oeni* PSU-1 can use sucrose, lactose, maltose, and raffinose as the sole carbon source; however, later studies have shown that it lost this ability, perhaps because crucial genes have been lost or mutated (Mills et al. 2005). The genes that may transport these sugars were all found in the genome of *O. oeni* SD-2a, suggesting that *O. oeni* SD-2a may use these sugars as carbon sources. Further studies are needed to confirm this hypothesis.

Malate metabolism. Malolactic fermentation is an important metabolic pathway to metabolize malic acid in *O. oeni* and a vital process in improving wine quality. Malate decarboxylase (*mle*A) and malate permease (*mle*P) are the two key genes involved. ORF01700 and ORF01699 are two ORFs encoding *mle*A and *mle*P in *O. oeni* SD-2a. Next to these two genes is the LysR family transcriptional regulator-encoding gene, represented by ORF01701, which may regulate the expression of the other two genes. The arrangement of these three genes is widespread in many *O. oeni* strains, indicating that this gene cluster is relatively conserved in the strain. Like in *O. oeni* PSU-1, both *mle*A and *mle*P have homologous genes in the genome of *O. oeni* SD-2a, as shown in Table SIV. ORF00337 (*mae*A, malate dehydrogenase) is homologous to *mle*A, whereas ORF01907, ORF00338, ORF01100, and ORF01583 are homologous to *mle*P. Comparisons to other known sequenced LAB strains revealed that *O. oeni* had most genes of the MleP protein family (Mills et al. 2005). It may be due to the high malic acid content in grape must or wine where *O. oeni* lives, whereas *mle*P can effectively transport malic acid in an environment with higher malic acid content. ORF00337 and ORF00338 are located in a citrate lyase gene cluster in the genome, which suggests that these two genes may be involved in the citrate metabolic pathway. The specific functions of these genes need to be further studied.

Citrate metabolism. Like other LAB, *O. oeni* can utilize citric acid as a carbon source, an auxiliary way of metabolizing besides glucose metabolism. *O. oeni* does not use citrate as a sole carbon source; however, co-metabolizing citrate with glucose increases the biomass of *O. oeni* compared with growth on glucose only (Salou et al. 1994). The *O. oeni* citrate metabolic pathway is shown in Fig. S4. Citrate may be transported through citrate permease (CitP). Decarboxylation then occurs by citrate lyase (CitE), which catalyzes the decarboxylation of citric acid to oxaloacetate. Oxaloacetate can then be catalyzed to pyruvate by oxaloacetate decarboxylase. Malate dehydrogenase (MaeA) may be involved in this reaction. The pyruvate can be converted to several metabolites through different pathways. Pyruvate can be catalyzed to L-lactate by L-lactate dehydrogenase (Ldh). Pyruvate can also react with Coenzyme A (CoA) and be converted to acetyl-CoA and CO_2 by pyruvate dehydrogenase (Pdh). In this pathway, acetyl-CoA was catalyzed to acetyl-P and acetate. In another pathway, pyruvate was catalyzed to α-acetolactate by α-acetolactate synthase (AlsS), then to acetoin by α-acetolactate decarboxylase, and finally to 2,3-butanediol. During this process, α-acetolactate may be catalyzed to diacetyl, and diacetyl is then catalyzed to acetoin. A citrate lyase gene cluster, including *cit*R, *mae*A, *mae*P, *cit*C, *cit*D, *cit*E, *cit*F, *cit*X and *cit*G, was found in *O. oeni* SD-2a genome. Genes in this cluster mainly function in converting citrate to oxaloacetate and pyruvate. The gene arrangement of this cluster is very similar to those of *O. oeni* PSU-1 and some other LAB strains (Mills et al. 2005), indicating that this gene cluster is highly conserved. Besides this cluster, the other enzymes encoding genes shown in Fig. S4 are also found in *O. oeni* SD-2a genome. Table SV listed all genes involved in citrate metabolism that were found in the genome of *O. oeni* SD-2a. Like the citric acid metabolic pathway shown in Fig. S4, in *O. oeni* SD-2a strain, citrate was transported into the cell and converted to oxaloacetate, pyruvate, and then a mixture of lactate, acetate, diacetyl, acetoin, and 2,3-butanediol through the catalysis of various enzymes. These metabolites are essential for the wine flavor. For example, diacetyl is an aromatic compound that influences the buttery aroma of wine (Bartowsky and Henschke 2004). Thus, citrate metabolism and MLF, have significant impacts on the organoleptic qualities of wine in *O. oeni* SD-2a and other *O. oeni* strains.

Amino acid synthesis, transport, and metabolism. The Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis classifies 65 genes in the genome of *O. oeni* SD-2a into the amino acid biosynthetic pathways. The analysis of key genes in each amino acid synthetic pathway indicated that *O. oeni* SD-2a might synthesize seven amino acids: leucine, valine, cysteine, proline, serine, threonine, and glycine. *O. oeni* SD-2a lost essential genes in arginine, glutamate, and tyrosine synthesis pathways. It differed *O. oeni* SD-2a from *O. oeni* PSU-1, which might synthesize eight amino

acids: alanine, aspartate, asparagine, cysteine, glutamine, lysine, methionine, and threonine (Mills et al. 2005). This phenomenon is not surprising because different *O. oeni* strains often present different patterns of amino acids auxotrophy. It may be attributed to their different living environments and available nutrients. *O. oeni* strains may have adapted to the specific environment in which they lived during evolution. For example, if some amino acids are plentiful in an environment, genes related to their synthesis may be lost during evolution. Whereas amino acids that are not easily obtained from the environment are in great demand, the genes may be retained. Cysteine is an essential amino acid for the growth of *O. oeni*, but its content is shallow in grape must or wine (Mills et al. 2005). Jin et al. (2014) studied the amino acid concentrations before and after the MLF processes in four *O. oeni* strains isolated in China. After MLF, in the *O. oeni* SD-2a experimental sample, the leucine, valine, serine, threonine, glycine, glutamate, alanine, arginine, lysine, and methionine contents increased significantly, with arginine and valine increasing the most (Jin et al. 2014). However, the genomic analysis revealed that *O. oeni* SD-2a could not synthesize glutamate, alanine, arginine, lysine, or methionine. *O. oeni* SD-2a may have unclear amino acid synthetic pathways.

In the *O. oeni* SD-2a genome, there are many amino acid transport-related proteins, including ten amino acid permeases, three branch-chain amino acid permeases, six ABC amino acid transporters, and two APC amino acids transporters. Many of these proteins transport amino acids that *O. oeni* SD-2a cannot synthesize. There are also 13 ABC-type oligo/dipeptide permease transporter systems and 20 peptidases in the genome of *O. oeni* SD-2a, suggesting that the bacterium may metabolize amino acids from the proteins and peptides in wine. Liu et al. (2010) have reported the essential role of peptidases for bacterial growth and survival as they are encoded in all LAB genomes, such as PepC, PepN, PepM, PepX, and PepQ. The genome of *O. oeni* SD-2a also contains all of these peptidases-encoding genes. Margalef-Català et al. (2016a) showed that after transferring *O. oeni* strains to the wine-like medium, several peptidases were up regulated at both gene and protein levels. Balmaseda et al. (2022) also showed that after transferring *O. oeni* strains to *Saccharomyces*- or non-*Saccharomyces*-fermented wines, several proteins associated with peptidase activity and peptide transport increased their abundance, indicating these proteins are helpful for *O. oeni* to adapt wine-like conditions. The genes mentioned above ensure the smooth uptake from the environment and transport of peptides or amino acids required for growth and survival.

Amino acid metabolism plays an important role in carbon and nitrogen balance. Arginine metabolism is important because it is a potential energy source that can be coupled to cellular growth owing to ATP formation (Arena et al. 1999). The arginine deiminase pathway, which converts arginine to ornithine, CO_2, and NH_3, exists in many LAB species (Arena et al. 1999; Jiao et al. 2014). This pathway involves three enzymes, arginine deiminase, ornithine transcarbamylase, and carbamate kinase (Arena et al. 1999). Homologous genes encoding these proteins are located in *O. oeni* SD-2a genome. Determining whether these three enzymes function in *O. oeni* SD-2a requires further study. Metabolic intermediates produced in the arginine deiminase pathway, like citrulline and carbamyl phosphate, should be investigated because these two intermediates may react with ethanol to form ethyl carbamate. It is a multisite carcinogen in experimental animals (Jiao et al. 2014; Capozzi et al. 2021). The formation of ethyl carbamate is affected by many factors in the fermentation process (Jiao et al. 2014). Other undesired metabolic intermediates of amino acids are biogenic amines, which are produced from the decarboxylation of their corresponding amino acids by decarboxylases (Barbieri et al. 2019; Capozzi et al. 2021). No amino acid decarboxylase enzyme-encoding genes were found in *O. oeni* SD-2a genome, which suggests that the production of biogenic amines by *O. oeni* SD-2a during the fermentation process can be neglected.

Stress-related genes. Like other *O. oeni*, *O. oeni* SD-2a can survive in the harsh wine-making environment and start the MLF process. There must be a variety of stress-response mechanisms that help *O. oeni* SD-2a adapt to the environment. As shown in the Table SVI, many stress-related genes have been reported that provide resistance to various stress conditions in other *O. oeni* strains (Tonon et al. 2001; Bourdineaud et al. 2003; Fortier et al. 2003; Beltramo et al. 2004; Silveira et al. 2004; Grandvalet et al. 2005; Olguín et al. 2009; Guzzo 2011; Seydlová et al. 2012; Olguín et al. 2015; Cafaro et al. 2016; Margalef-Català et al. 2017a; Zhao et al. 2019). These genes have homologs in the genome of *O. oeni* SD-2a, and the corresponding encoding gene numbers are also provided in the Table SVI. It suggests that *O. oeni* SD-2a also has similar adversity-related adaptive pathways. Bonomo et al. (2018) summarized several main stress-response mechanisms, including MLF-related proton motive force generation and pH maintenance, membrane proton-extruding ATPase activation, stress-related protein induction and synthesis, and membrane fluidity changes. The proteins listed in Table SVI mainly function in these pathways. The most well-known stress-related proteins are molecular chaperones, including Hsp20 (also referred to as Lo18), GroESL, DnaK, ClpC, ClpX. These proteins can help other proteins to fold normally, prevent their aggregation under stress conditions, or guide

protease to degrade denatured proteins (Bech-Terkilsen et al. 2020). In *O. oeni* strains, the expression level of *hsp20* increased significantly after acid shock, ethanol shock (at 8% v/v ethanol condition), combined acid and ethanol shock, or treatments with 5 mg/l SO_2 (Costantini et al. 2015; Liu et al. 2017; Yang et al. 2021; Onetto et al. 2021).

The expression of Hsp20 can also respond to the yeast inoculation strategy used. The use of non-*Saccharomyces* yeast (such as *Torulaspora delbrueckii* and *Metschnikowia pulcherrima*), usually combined with *S. cerevisiae*, reduced the abundance of Hsp20, which could mean a less stressful wine-like condition for *O. oeni* (Balmaseda et al. 2022). Hsp20 possibly protects intracellular proteins from aggregation and loss of activity in these conditions. Maitre et al. (2014) also made a model for the involvement of Lo18 in both membrane stabilization and protein protection, which hypothesized that Hsp20 stabilizes the membrane short-term and facilitates modification of the fatty acid concentration. For example, cyclopropane fatty acid synthase (Cfa) increases cyclopropane-fatty acids in the membrane. It reduces the affinity of Hsp20 to the membrane, thus, facilitating its release as oligomeric molecular chaperones (Maitre et al. 2014).

Darsonval et al. (2016) further confirmed the hypothesis that Hsp20 was responsible for the rigidification of the membrane following ethanol-induced fluidization. They also showed GroESL might also involve in membrane stabilization in *O. oeni* (Darsonval et al. 2016). CtsR regulates the expression of GroESL and DnaK in *O. oeni*, which is different from other Gram-positive bacteria that use *hrcA* as a transcription regulator of stress proteins (Grandvalet et al., 2005).

CtsR is also a heat sensor that can interact with ClpL1 in *O. oeni* (Darsonval et al. 2018). It is also reported that much more ATP was spent on the *O. oeni* strains living in a wine environment than in optimal growth conditions (Mendoza et al. 2017). It is because most of this ATP was involved in the extrusion of protons by (F_1F_0) H^+-ATPases, rather than growth (Mendoza et al. 2017). The ATPase F_1F_0 β-subunit-encoding gene *atp*B was located adjacent to several other ATP synthase-encoding genes (*atp*E, *atp*F, *atp*G, *atp*H, *atp*A, *atp*G, *atp*D, and *atp*C), forming an F_0F_1 ATPase system. This arrangement is similar to *O. oeni* PSU-1 (Mills et al. 2005). ATPases aid in responses to acidic stress and help cells maintain intracellular pH stability. The wine environment is also prone to producing high oxidative stress. Hence, proteins and molecules involved in the antioxidant system like thioredoxin, thioredoxin reductase, and glutathione help *O. oeni* adapt to wine. The thioredoxin system in *O. oeni* PSU-1 was recently characterized, including thioredoxin reductase (*trx*B), ferrodoxin reductase (*fdr*), and three thioredoxin genes (*trx*A1, *trx*A2 and *trx*A3) (Margalef-Català et al. 2017a). *O. oeni* SD-2a has only two *trx*A genes compared to *O. oeni* PSU-1. The most probably absent gene is *trx*A1, which was horizontally transferred from *Lactiplantibacillus* to *O. oeni* and was absent in a large number of *O. oeni* strains (Margalef-Català et al. 2017a). Margalef-Català et al. (2017b) also showed that though *O. oeni* cannot synthesize glutathione (GSH), it can uptake GSH and contains several genes related to its utilization, such as glutathione reductase, glutathione peroxidase, and glutaredoxin-like proteins. What is more, *O. oeni* PSU-1 cells grown with GSH improved survival against ethanol shock (14%, v/v), and GSH addition also increased biomass production during the adaptation to wine stress conditions (Margalef-Català et al. 2016b). Genes that are used for GSH metabolism in *O. oeni* PSU-1 are also located in the genome of *O. oeni* SD-2a, implying *O. oeni* SD-2a can also use GSH to adapt to wine. In general, *O. oeni* SD-2a was found to contain different stress-related genes, which may explain how it can resist the harsh external wine-making environment at the genetic level.

Natural competence-related genes. Many microorganisms can transfer DNA from outside into the appropriate naturally competent cells. This ability is helpful for horizontal gene transfer and plays a vital role in bacterial adaptation, new species formation, and evolution (Chen and Dubnau 2004; Johnsborg et al. 2007). It is also the basis of genetic manipulation. In Gram-positive bacteria like *O. oeni*, a series of proteins is involved in the uptake of extracellular DNA, including DNA receptors (ComEA), transmembrane pores (ComEC), transformation pili (ComGC), ATP-dependent translocases (ComFA), and some other proteins encoded in the *com*G operon (Sternes and Borneman 2016). These genes help cells capture DNA from the outside and then transfer it into cells upon consuming energy. In the genome of *O. oeni* SD-2a, *com*EA (ORF00855), *com*EC (ORF00856), *com*FA (ORF01229), *com*G operon, and *com*FC (ORF01230) were identified. The *com*FC gene encodes amidophosphoribosyl transferase, but the exact function of this protein in the natural transformation process is still unknown. *com*GB, *com*GB, *com*GD, *com*GF, and a hypothetical protein are located in the *com*G operon, lacking *com*GA. Whether this hypothetical protein can transfer NTPs needs to be investigated. Thus, *O. oeni* SD-2a has most of the genes involved in natural transformation, suggesting that it may be a naturally competent cell. However, this conclusion needs to be confirmed through further experimental research.

Otherwise, the mechanisms and biological functions of natural competence are not precise. Pressure-related stimulation is the main reason for the induction of natural bacterial competence. Whether natural competence plays a role in stress response mechanisms

also needs further investigation. Sternes and Borneman (2016) revealed that the genes related to the natural competence of *O. oeni* came from the same ancestor. However, with evolution, *O. oeni* gradually lost the ability to induce natural competence (Sternes and Borneman 2016). There are few reports on the successful genetic manipulation in *O. oeni*. Whether *O. oeni* SD-2a can be successfully genetically manipulated under induction conditions is worth of further study.

Comparative genomics analysis. Venn diagrams were constructed to understand the homologous genes and specific genes of *O. oeni* SD-2a compared to other LAB strains performing MLF, *O. oeni* PSU-1, *O. oeni* CRBO-11381, *L. plantarum* UNQLp11, and *P. pentosaceus* KCCM40703 were chosen for the comparison. These strains have complete genome sequence data, which are available in the NCBI database, and they are all isolated from wine-similar environments. As shown in Fig. 3, 730 homologous gene clusters were identified in five strains, indicating that these gene clusters were highly conserved. These gene clusters are mainly classified as being involved in the basic internal functions of cells. It is logical because these genes are relatively conserved among the different species. In total, 1,562, 1,628, 1,001, and 823 gene clusters in *O. oeni* SD-2a have homologs in *O. oeni* PSU-1, *O. oeni* CRBO-11381, *L. plantarum* UNQLp11, and *P. pentosaceus* KCCM40703, respectively. There were 66 (*O. oeni* SD-2a), six (*O. oeni* PSU-1), 11 (*O. oeni* CRBO-11381), 143 (*L. plantarum* UNQLp11), and 19 (*P. pentosaceus* KCCM40703) specific gene clusters in each strain, accounting for 3.7%, 0.37%, 0.66%, 9.3%, and 1.5% of their total gene clusters involved in homologous gene analysis, respectively. In the *O. oeni* SD-2a genome, 66 gene clusters do not have homologs in the other four LAB strains. This number is more significant than *O. oeni* PSU-1, *O. oeni* CRBO-11381, and *P. pentosaceus* KCCM40703. Gene ontology (GO) analysis results of these particular gene clusters in *O. oeni* SD-2a are shown in Fig. S5. These clusters are mainly enriched

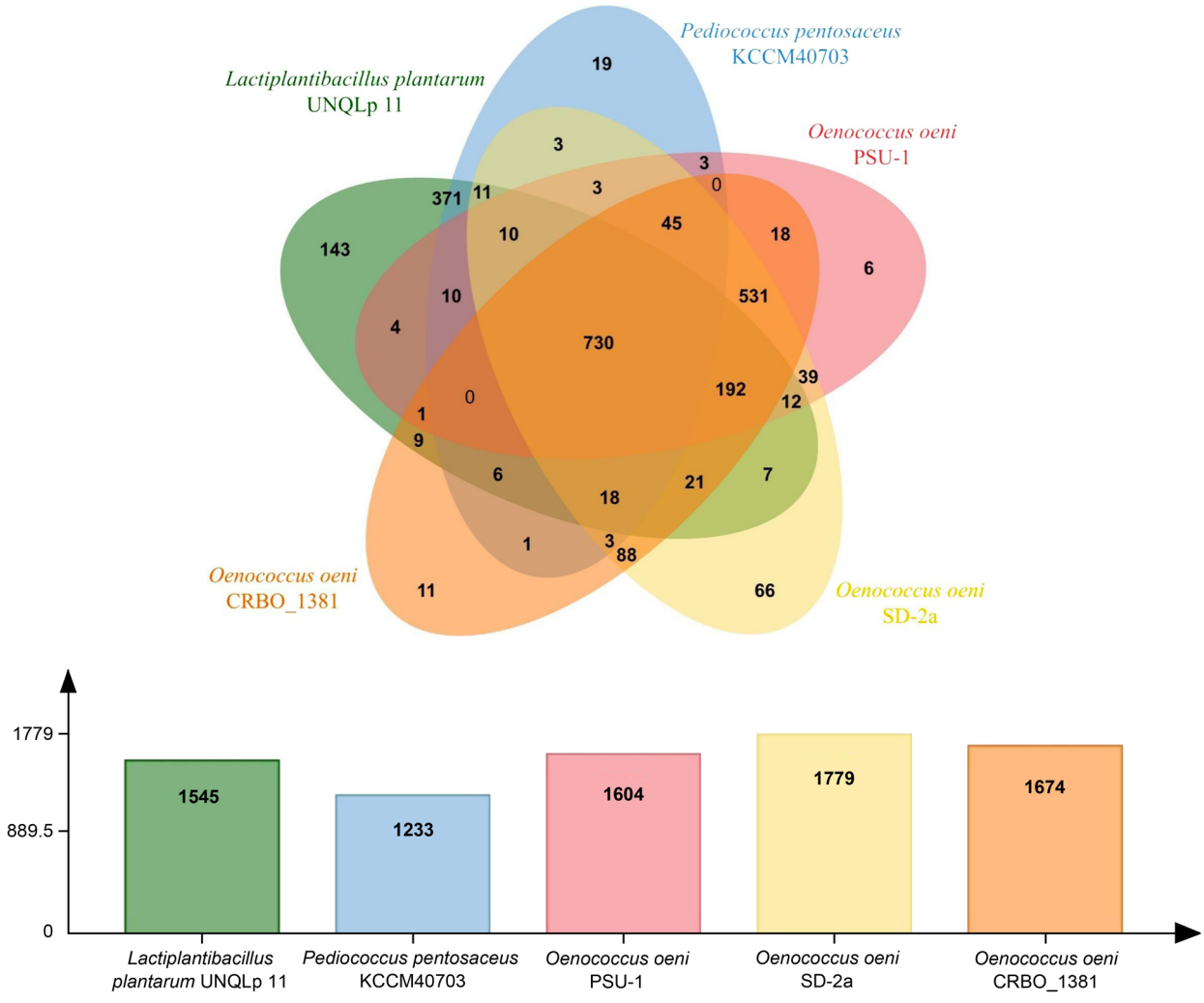


Fig. 3. Venn diagram showing the number of unique and homologous gene clusters in *Oenococcus oeni* SD-2a, *Oenococcus oeni* PSU-1, *Oenococcus oeni* CRBO-11381, *Lactiplantibacillus plantarum* UNQLp11, and *Pediococcus pentosaceus* KCCM40703. The histogram shows each strain's total gene clusters involved in homologous gene analysis.

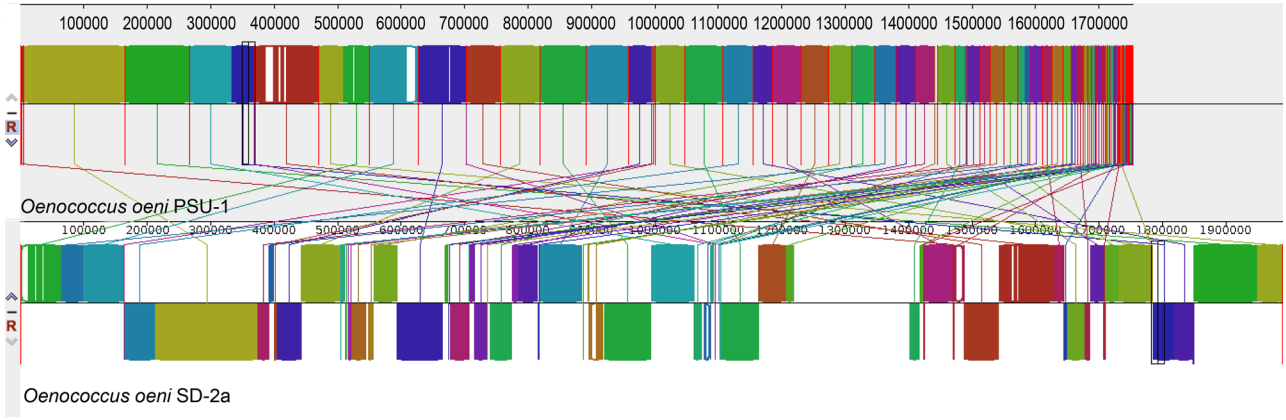


Fig. 4. Analysis of collinearity between *Oenococcus oeni* SD-2a and *Oenococcus oeni* PSU-1.

in biological processes, including carbohydrate and other substrates transport, DNA modification, carbohydrate, a nitrogen compound, and other metabolic processes. These gene clusters possibly help *O. oeni* SD-2a to better adapt to the wine environment. A collinearity analysis between *O. oeni* SD-2a and *O. oeni* PSU-1 was also performed, as shown in Fig. 4. There was poor collinearity between *O. oeni* SD-2a and *O. oeni* PSU-1. Compared with *O. oeni* PSU-1, the genome of *O. Oeni* SD-2a showed obvious fragmented insertions, deletions, inversions, and translocations. The result suggests that although the two strains belong to *O. oeni*, there are significant differences in their evolutionary histories. It may be related to their long-term independent evolution in different microenvironments. However, the essential genes involved in adapting to the harsh wine-making environment have been retained in both strains. More in-depth analyses of the completely sequenced genome of *O. oeni* strains and investigating the specific functions of important genes are necessary for the future.

Conclusion

This study sequenced the whole genome of *O. oeni* SD-2a strain, used for wine-making in China. The detailed genomic analysis provides a more comprehensive and accurate understanding of this strain. The complete genome information of *O. oeni* SD-2a lays a foundation for transcriptome research and the study of specific gene functions. It also provides a good foundation for further studies on the evolution, biological characteristics, environmental adaptability, stress-response mechanisms, and even commercial applications of *O. oeni* SD-2a.

Availability of data and material

The whole-genome sequence of *O. oeni* strain SD-2a has been deposited at GenBank under the accession number CP087569.

Author contributions

H.W. and L.X.L. planned the experiments. L.X.L., S.P. and H.Y.Z. performed the experiments. L.X.L., S.P., W.Y.S., H.L. and H.W. wrote the manuscript.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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