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Minireview

MINIREVIEW

Lager-brewing yeasts in the era of modern genetics

Arthur R. Gorter de Vries[†], Jack T. Pronk[§] and Jean-Marc G. Daran[*,‡]

Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629 HZ Delft, The Netherlands

[*]**Corresponding author:** Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629 HZ Delft, The Netherlands. Tel: +0031-1-52-78-24-12; E-mail: J.G.Daran@tudelft.nl

One sentence summary: *Saccharomyces pastorianus* is a hybrid yeast that has been domesticated since the 16th century. The review presents the slough of lager yeast research under the influence of genome science.

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[†]Arthur R. Gorter de Vries, http://orcid.org/0000-0002-0841-6583
[‡]Jean-Marc G. Daran, http://orcid.org/0000-0003-3136-8193
[§]Jack T. Pronk, http://orcid.org/0000-0002-5617-4611

ABSTRACT

The yeast *Saccharomyces pastorianus* is responsible for the annual worldwide production of almost 200 billion liters of lager-type beer. *S. pastorianus* is a hybrid of *Saccharomyces cerevisiae* and *Saccharomyces eubayanus* that has been studied for well over a century. Scientific interest in *S. pastorianus* intensified upon the discovery, in 2011, of its *S. eubayanus* ancestor. Moreover, advances in whole-genome sequencing and genome editing now enable deeper exploration of the complex hybrid and aneuploid genome architectures of *S. pastorianus* strains. These developments not only provide novel insights into the emergence and domestication of *S. pastorianus* but also generate new opportunities for its industrial application. This review paper combines historical, technical and socioeconomic perspectives to analyze the evolutionary origin and genetics of *S. pastorianus*. In addition, it provides an overview of available methods for industrial strain improvement and an outlook on future industrial application of lager-brewing yeasts. Particular attention is given to the ongoing debate on whether current *S. pastorianus* originates from a single or multiple hybridization events and to the potential role of genome editing in developing industrial brewing yeast strains.

Keywords: *Saccharomyces pastorianus*; strain improvement; hybrid heterosis; whole genome sequencing; genome editing

EMERGENCE AND INDUSTRIALIZATION OF LAGER BREWING

Beer brewing is tightly intertwined with human culture. Archaeological remains from the 12th millennium BC indicate that microbial fermentation of cereals may predate the agricultural revolution (Liu *et al.* 2018). Chemical archaeology and pictographic evidence show that beer brewing was customary as early as in the 4th millennium BC (Michel, McGovern and Badler 1992; Sicard and Legras 2011). Lager-style beer emerged only in 16th century Bavaria under the influence of novel regulations to standardize the brewing process and to improve quality. For example, the well-known 'Reinheitsgebot' of 1516 restricted ingredients used for brewing to water, barley and hops (Hornsey 2003). When, in 1553, beer brewing was legally restricted to winter months, bottom-fermenting yeast emerged as a consequence of the lower fermentation temperatures (Unger 2004). In contrast to the top-fermenting yeasts used at higher temperatures for brewing ale-type beers, bottom-fermenting yeast form flocs that sediment at the end of the fermentation (Oliver and Colicchio 2011). Bottom-fermenting yeasts were initially used to brew a

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dark brown beer, which was stored to enable consumption during the summer months. This beer was designated as lager, in reference to the German 'lagern' which means 'to store' (Meussdoerffer 2009). In 1842, Bavarian brew master Josef Groll, working in the Bohemian city Pilsen, started brewing a pale style of lager beer with fruity Saaz-type hops, which became known as Pilsner beer (Meussdoerffer 2009). The advent of Pilsner coincided with rapid technological advances that enabled industrialization of beer brewing. The discovery that yeast is responsible for fermentation (Pasteur 1876) and the isolation of pure lager-brewing strains (Hansen 1883; Moritz and Morris 1891) enabled inoculation of beer fermentation processes with pure cultures, resulting in more consistent quality. Moreover, the invention of the steam engine and ammonia refrigeration enabled industrial mass production (Appel 1990; Poelmans and Swinnen 2011a). Finally, the invention of bottle production using iron molds, of crown corks and of beer filtration improved product stability and enabled exportation (Painter 1892; Kunze 2004; Lockhart 2007). As a result of these innovations, global beer production soared to 17.7 billion liters in 1899 (Michel 1899), and further increased to 193 billion liters in 2015, of which 89% was lager-type beer (Brickwedde *et al.* 2017).

THE LAGER-BREWING YEAST *SACCHAROMYCES PASTORIANUS*

Lager beers are fermented with *S. pastorianus* strains. These hybrids of *Saccharomyces cerevisiae* and *Saccharomyces eubayanus* have only been encountered in brewing-related contexts (Libkind *et al.* 2011). *S. cerevisiae* has a long history of use in bakery, wine fermentation and brewing of ale-type beers and has been intensively studied for well over a century (Gallone *et al.* 2016). In contrast, *S. eubayanus* was discovered only in 2011 (Libkind *et al.* 2011). First isolated in South America, *S. eubayanus* was subsequently isolated from oaks and other deciduous trees in North America, Asia and Oceania (Bing *et al.* 2014; Peris *et al.* 2014; Gayevskiy and Goddard 2016). Despite efforts of many European research groups, isolation of wild *S. eubayanus* strains has remained unsuccessful in Europe so far. While DNA from *S. eubayanus* was detected in samples from oak and spruce trees in Europe by ITS (Internal Transcribed Spacer) sequencing (Alsammar *et al.* 2018), this DNA does not prove the presence of wild *S. eubayanus* strains, as the DNA may also originate from hybrids such as *S. pastorianus* or *Saccharomyces bayanus*. Currently, Tibetan isolates of *S. eubayanus* have the highest degree of genetic identity to the *S. eubayanus*-derived genome sequences of *S. pastorianus* strains (Bing *et al.* 2014; Salazar *et al.* 2019). Based on this observation, trade along the Silk Road has been hypothesized to have enabled migration of *S. eubayanus* from Asia to the European birthplace of lager brewing (Bing *et al.* 2014). Alternatively, a now extinct or as yet undiscovered European *S. eubayanus* wild stock may be the ancestor of current *S. pastorianus* strains. In recent studies, hybrids between *S. cerevisiae* and *S. eubayanus* that were created in the laboratory were shown to outcompete their parental strains in lager-brewing related environments by combing the fermentative vigor of *S. cerevisiae* with the low temperature optimum of *S. eubayanus* (Hebly *et al.* 2015; Krogerus *et al.* 2015; Mertens *et al.* 2015). These observations are consistent with the emergence of *S. pastorianus* by spontaneous hybridization between an ale-brewing *S. cerevisiae* strain and a wild *S. eubayanus* contaminant, as well as with its subsequent dominance in lager-beer production. In this review, we will refer to lager yeast derived from spontaneous hybridization as *S. pastorianus* and to laboratory-made lager hybrids as *S. cerevisiae* × *S. eubayanus*.

In contrast to the genomes of laboratory-made hybrids, *S. pastorianus* genomes are extensively aneuploid, with 45 to 79 chromosomes instead of the allodiploid complement of 32 chromosomes (Fig. 1) (Dunn and Sherlock 2008; Nakao *et al.* 2009; Walther, Hesselbart and Wendland 2014; Van den Broek *et al.* 2015; Okuno *et al.* 2016). Based on genetic differences, two *S. pastorianus* subgroups were identified, Group 1 strains ('Saaz') and Group 2 strains ('Frohberg'), which show marked differences in chromosome copy numbers (Liti *et al.* 2005; Dunn and Sherlock 2008). While both groups have an approximately diploid *S. eubayanus* chromosome complement, the *S. cerevisiae* chromosome complement is incomplete in Group 1 strains and diploid or higher in Group 2 strains (Fig. 1) (Dunn and Sherlock 2008; Van den Broek *et al.* 2015; Okuno *et al.* 2016). Genome-sequence comparison revealed group-specific genes, substantial differences in subtelomeric regions and different frequencies of synonymous nucleotide variations between both groups (Liti *et al.* 2005; Baker *et al.* 2015; Monerawela *et al.* 2015). While Group 1 strains display superior growth kinetics at low temperatures, they generally show limited maltotriose utilization, resulting in an overall inferior brewing performance relative to Group 2 strains (Gibson *et al.* 2013b).

EVOLUTIONARY HISTORY OF *S. PASTORIANUS*: MULTIPLE HYBRIDIZATION EVENTS OR MAN-MADE POPULATION BOTTLENECKS?

Based on their phenotypic and genotypic differences, Group 1 and 2 strains were initially hypothesized to have emerged from two independent hybridization events (Fig. 2A) (Rainieri *et al.* 2006; Dunn and Sherlock 2008). Indeed, distinct haploid and diploid *S. cerevisiae* ancestors could explain the ploidy of Group 1 and 2 strains, respectively (Krogerus *et al.* 2016). However, identical recombinations between *S. cerevisiae* and *S. eubayanus* chromosomes were found at the *ZUO1*, *MAT*, *HSP82* and *XRN1/KEM1* loci in all Group 1 and 2 strains (Hewitt *et al.* 2014; Walther, Hesselbart and Wendland 2014; Okuno *et al.* 2016). When evolved under lager-brewing conditions, laboratory-made *S. cerevisiae* × *eubayanus* hybrids acquired a diverse range of interchromosomal recombinations, but these did not include those present in *S. pastorianus* strains. While differences between the parental genomes of *S. cerevisiae* × *S. eubayanus* hybrids and *S. pastorianus* may affect likeliness of individual recombinations, the diversity of recombinations obtained in individual *S. cerevisiae* × *S. eubayanus* isolates and the complete lack of recombinations shared with *S. pastorianus* indicate that recombination patterns emerge mostly serendipitously and point toward a common hybrid ancestry of all current *S. pastorianus* strains (Gorter de Vries *et al.* 2019b).

Two theories have been forwarded to reconcile the evidence for a common ancestry of Group 1 and Group 2 strains with their genetic differences (Fig. 2): (2B) Group 1 and 2 strains shared an initial hybridization event, with Group 2 strains resulting from a subsequent hybridization between the initial hybrid and a distinct *S. cerevisiae* strain, or (2C) Group 1 and 2 strains resulted from the same hybridization event involving a heterozygous *S. cerevisiae* ancestor, after which different paths of loss of heterozygosity and loss of genetic material caused the two Groups to diverge (Okuno *et al.* 2016). Long-read nanopore sequencing and comparative genome analysis indicated that the *S. cerevisiae* genetic material is highly similar in both groups, thereby

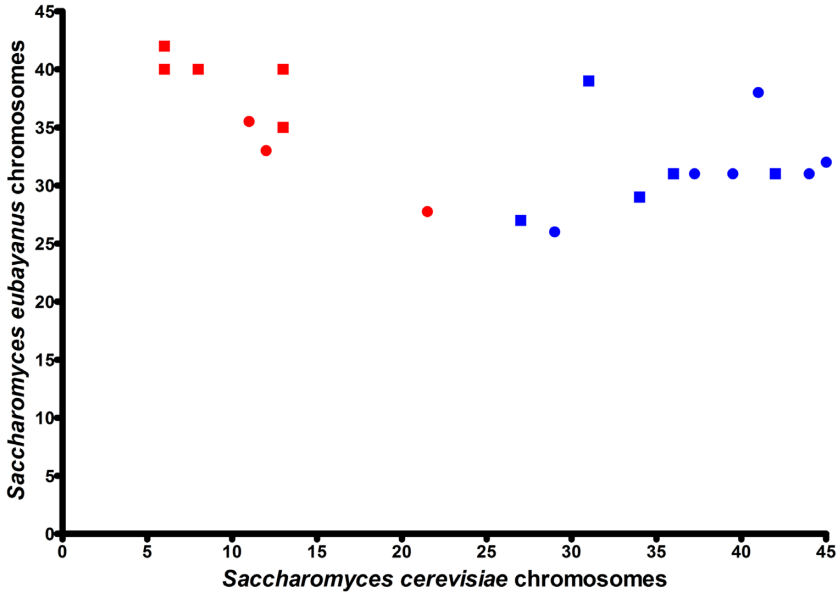

Figure 1. Estimated chromosome copy numbers in *S. pastorianus* strains as determined by whole-genome sequencing. Chromosome copy number estimates of various Group 1 (red) and Group 2 (blue) strains were estimated from short-read sequencing data published by Van den Broek *et al.* 2015 (circles) and Okuno *et al.* 2016 (squares) (Van den Broek *et al.* 2015; Okuno *et al.* 2016). For each strain, the estimated total number of chromosomes derived from *S. eubayanus* is plotted against the estimated total number of chromosomes derived from *S. cerevisiae*. Due to copy number differences within individual chromosomes, copy number estimates should be interpreted as indicative.

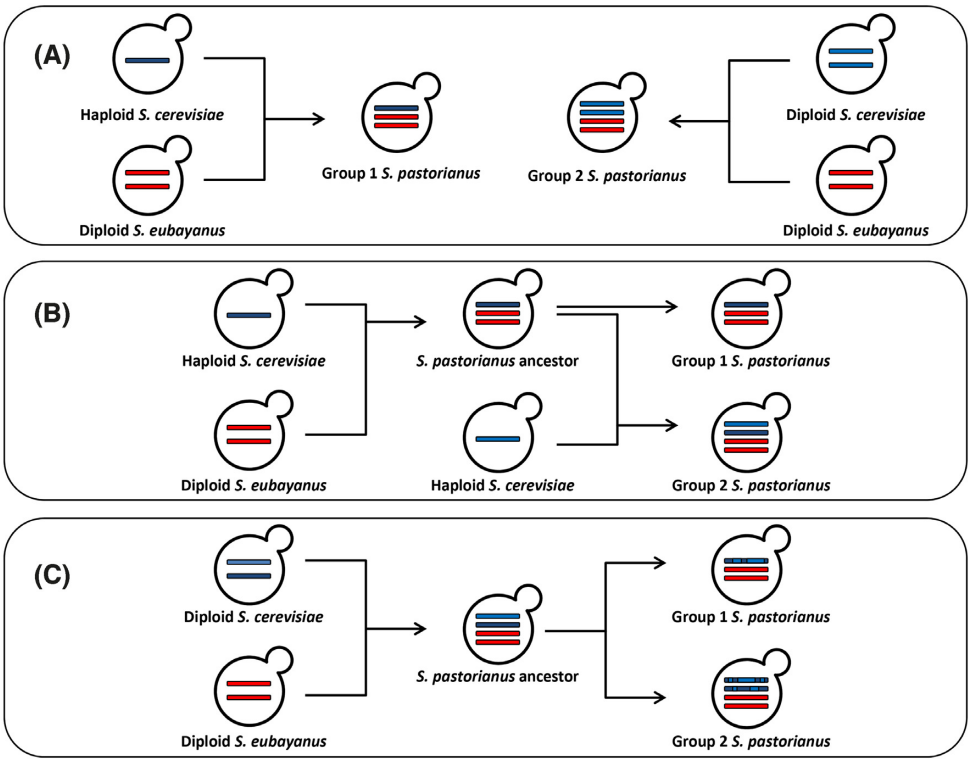

Figure 2. Theories formulated about the emergence of Group 1 and 2 *S. pastorianus* strains. **(A)** Emergence by two independent hybridizations (Dunn and Sherlock 2008). While both groups shared a similar *S. eubayanus* ancestor, Group 1 emerged from hybridization with a haploid *S. cerevisiae* while Group 2 emerged from a diploid *S. cerevisiae*. **(B)** Emergence by two successive hybridizations (Okuno *et al.* 2016). *S. pastorianus* emerged from an initial hybridization between a haploid *S. cerevisiae* and a diploid *S. eubayanus*. Group 1 strains evolved directly from this ancestor, while Group 2 strains emerged from a subsequent hybridization between the *S. pastorianus* ancestor and a haploid *S. cerevisiae* strain of different origin. **(C)** Emergence by a single hybridization followed by different evolutionary trajectories (Okuno *et al.* 2016; Salazar *et al.* 2019). *S. pastorianus* emerged from the hybridization of a heterozygous diploid *S. cerevisiae* strain and a mostly homozygous diploid *S. eubayanus* strain. Group 1 and 2 strains both evolved from this ancestor. However, Group 1 and Group 2 strains were affected differently by loss of heterozygosity and by loss of *S. cerevisiae* genome content. As a result, Group 2 strains are more heterozygous than Group 1 strains and their *S. cerevisiae* subgenomes differ despite common ancestry.

reducing the likelihood of multiple hybridization events (Salazar *et al.* 2019).

Domestication has been shown to stimulate rapid genetic adaptation and diversification in widely different genetic contexts (Arnold 2004; Bachmann *et al.* 2012; Gibbons *et al.* 2012; Gibbons and Rinker 2015; Gallone *et al.* 2016; Peter *et al.* 2018). In hybrids such as *S. pastorianus*, genetic plasticity is exacerbated by an increased incidence of (segmental) aneuploidy and loss of heterozygosity (Delneri *et al.* 2003; Pérez Través *et al.* 2014; Peris *et al.* 2017; Gorter de Vries *et al.* 2019b). Therefore, genetically divergent *S. pastorianus* populations likely emerged during the centuries of extensive subsequent batch cultivations across Europe. Due to the sterility of *S. pastorianus*, the absence of genetic admixture through sexual reproduction enabled genetic diversification even within yeast populations of individual breweries. However, the industry practice of replacing locally evolved brewing strains by strains from successful breweries, as illustrated by the Bavarian origin of the Carlsberg strain isolated by Hansen (Meussdoerffer 2009), is likely to have expanded successful populations at the expense of genetic diversity. Even narrower bottlenecks may have occurred when Hansen isolated the first Group 1 strain at Carlsberg in 1883 and Elion isolated the first pure Group 2 strain at Heineken in 1886 (Hansen 1883; Struyk 1928). These isolates likely spread as other European breweries increasingly implemented pure-culture brewing, thereby replacing previously used mixed starter cultures. Furthermore, in the 19th and early 20th centuries, small breweries commonly used yeast starter cultures sold by large breweries such as Carlsberg and Heineken, thereby further reducing the diversity of industrial strains (Mendlik 1937). Rather than reflecting different origins, the differences between Group 1 and 2 strains may therefore reflect genetic divergence during domestication, followed by severe population bottlenecks caused by anthropological selection (Fig. 2C).

COMPLEXITY OF *S. PASTORIANUS* GENOMES

S. pastorianus genomes are alloaneuploid, with varying, strain-dependent copy numbers of homologous and homeologous chromosomes. This chromosome copy number variation affects the phenotype due to two general mechanisms: (i) a general aneuploidy-associated stress response, encompassing growth defects, genetic instability and low sporulation efficiency, and (ii) chromosome-specific copy-number effects, resulting from the cumulative impact of copy number differences of individual genes harbored by the affected chromosomes (Gorter de Vries, Pronk and Daran 2017b). In *S. pastorianus*, genetic differences between the *S. cerevisiae* and *S. eubayanus* subgenomes present an additional degree of complexity (Fig. 3). During genome evolution, recombinations between both subgenomes can create new genetic complexity, for example by creating novel, hybrid open-reading frames (Fig. 3A) (Dunn *et al.* 2013; Hewitt *et al.* 2014; Brouwers *et al.* 2019b). Since gene complements of the two subgenomes differ (Salazar *et al.* 2017; Brickwedde *et al.* 2018), genes and gene products that do not occur together in either of the parental genomes can interact in hybrids to generate novel, difficult to predict phenotypes (Fig. 3B). For example, protein subunits encoded by different subgenomes can assemble into novel, chimeric protein complexes (Fig. 3C) (Piatkowska *et al.* 2013), while non-specificity of regulatory elements can cause cross-talk of transcriptional regulation networks (Fig. 3D) and of protein modification (Fig. 3E) (Tirosh *et al.* 2009; Vidgren and Gibson 2018). Moreover, functional differences between homeologous genes (Fig. 3F) (Yamagishi *et al.* 2010; Bolat *et al.* 2013), as well as gene dosage-effects (Fig. 3G) (Ogata, Kobayashi and Gibson 2013; Yao *et al.* 2013), can result in complex interactions. Expression levels of homeologous genes generally differ, resulting in stronger expression of one of the two versions (Fig. 3H) (Gibson *et al.* 2013a; He *et al.* 2014). Overall, understanding the complex interactions between subgenomes is critical, as they underlie the synergistic phenomenon of heterosis (Lippman and Zamir 2007; Chen 2013; Shapira *et al.* 2014), which enables hybrids such as *S. pastorianus* to outperform their parental species (Belloch *et al.* 2008; Hebly *et al.* 2015; Krogerus *et al.* 2016). For example, in *S. pastorianus*, interaction between maltotriose transporter genes from the *S. eubayanus* subgenome and the *MAL* regulator genes from the *S. cerevisiae* subgenome was shown to enable the trait of maltotriose utilization, which is critical to brewing performance (Brouwers *et al.* 2019a). The importance of subgenome interactions is consistent with the frequent loss of heterozygosity during evolution of *Saccharomyces* hybrids, since it facilitates elimination of non-beneficial genome content from the least adapted parental species (Smukowski Heil *et al.* 2017; Lancaster *et al.* 2019; Gorter de Vries et al. 2019b; Heil *et al.* 2019). The presence of mitochondrial DNA descending from *S. eubayanus* and the loss of mitochondrial DNA from *S. cerevisiae* in *S. pastorianus* strains may also have been beneficial for *S. pastorianus* strains (Rainieri *et al.* 2008; Baker *et al.* 2015; Okuno *et al.* 2016). Indeed, the loss of *S. cerevisiae* mtDNA was likely instrumental in the lager-brewing domestication process, as its replacement by *S. eubayanus* mtDNA enables improved growth at low temperatures (Baker *et al.* 2019).

Elucidation of the genetic complexity of *S. pastorianus* strains was initially limited by the accuracy of available genome assemblies (Brickwedde *et al.* 2017). The first *S. pastorianus* genome was published in 2009 and consisted of 25 Mbp divided over 3184 contigs (Nakao *et al.* 2009). While many more strains were sequenced since, short-read sequencing invariably yielded incomplete and fragmented genome assemblies with, at best, hundreds of contigs (Walther, Hesselbart and Wendland 2014; Van den Broek *et al.* 2015; Okuno *et al.* 2016). Short-read sequencing cannot resolve repetitive sequences, such as TY-transposons and paralogous genes within each subgenome, or homeologous gene pairs (Kim *et al.* 1998; Matheson, Parsons and Gammie 2017). As a result, subtelomeric regions, which are known hotspots of genetic plasticity and inter-strain diversity (Pryde, Huckle and Louis 1995; Liti *et al.* 2005; Brown, Murray and Verstrepen 2010; Bergström *et al.* 2014; Monerawela *et al.* 2015) and harbor many industrially-relevant genes (Teunissen and Steensma 1995; Denayrolles *et al.* 1997; Teste, François and Parrou 2010; Jordan *et al.* 2016), were poorly assembled.

Recent developments in long-read sequencing enabled the generation of chromosome-level *S. pastorianus* genome assemblies that include most telomeres (Salazar *et al.* 2019). *Saccharomyces* genome assemblies based on long-read sequencing typically capture up to 5% more genes than high-quality short-read assemblies (Goodwin *et al.* 2015; Giordano *et al.* 2017; Istace *et al.* 2017; Salazar *et al.* 2017; Brickwedde *et al.* 2018; Salazar *et al.* 2019). Such added genes were of particular interest due to their role in brewing-relevant traits; such as *FLO* genes involved in the calcium-dependent flocculation process that causes bottom fermentation of *S. pastorianus*, *MAL* genes encoding maltose and maltotriose transporters and hydrolases, and *HXT* genes encoding the uptake of glucose and other hexose sugars (Salazar *et al.* 2019). Despite the near-complete assembly of all its chromosomes, the first long-read *S. pastorianus* genome assembly captured only 23 Mbp of the 46 Mbp genome of strain CBS 1483 because assembled chromosomes were consensus

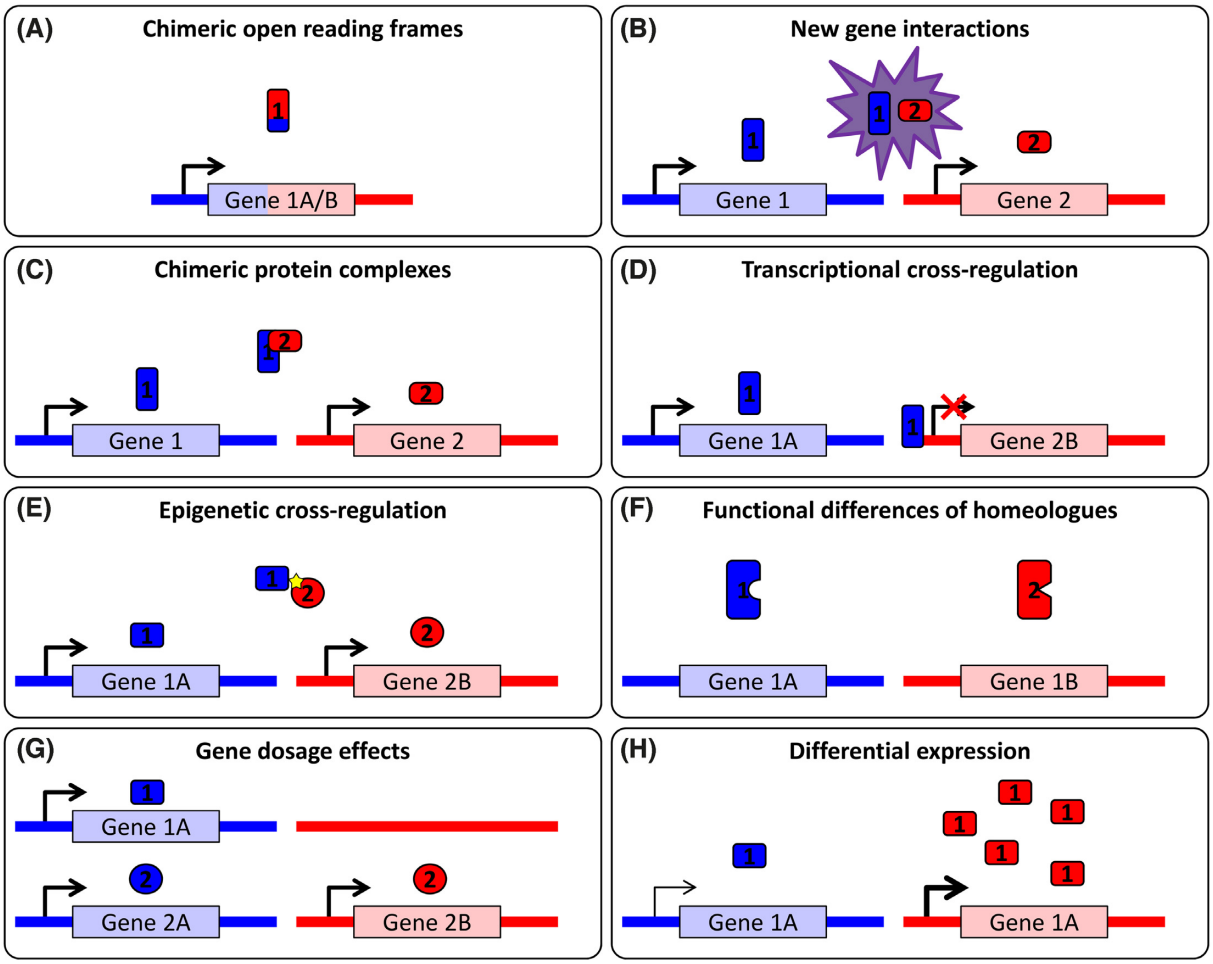

Figure 3. Mechanisms of subgenome interactions in hybrid organisms that can contribute to synergies between heterozygous genetic materials, a phenomenon referred to as heterosis. Components of the two subgenomes are shown in red and blue. **(A)** Generation of chimeric proteins due to recombinations within reading frames of (homeologous) genes from different subgenomes. **(B)** Interactions resulting from the simultaneous expression of subgenome-specific genes which were not expressed together in either parental genome. **(C)** Formation of chimeric protein complexes due to the assembly of subunits from different subgenomes. **(D)** Effects on transcription of genes from one subgenome by regulatory proteins from the other subgenome due to non-specificity of regulation. **(E)** Effects on the activity of proteins from one subgenome by regulatory proteins from the other subgenome due to non-specificity of regulation. **(F)** Functional differences between the homeologous genes of each subgenome, which can lead to subfunctionalization. **(G)** Effects due to differences in the relative copy number of different homeologous genes due to differences in gene composition of subgenomes. **(H)** Differences in transcription of homeologous genes, resulting in different contributions of each subgenome to the resulting phenotype.

sequences of all chromosomal copies, and intra-chromosomal variation of multi-copy chromosomes was not captured (Salazar *et al.* 2019). Nevertheless, alignment of short-read and long-read sequences allowed retrieval of sequence and structural heterozygosity (Okuno *et al.* 2016; Salazar *et al.* 2017; Salazar *et al.* 2019).

IMPROVEMENT STRATEGIES FOR LAGER-BREWING STRAINS

Industrial strain improvement typically relies on five pillars: exploration of existing diversity, mating, laboratory evolution, mutagenesis and selection and genome editing (Patnaik 2008; Steensels *et al.* 2014b). The complex genetics of *S. pastorianus* and, in particular, the lack of customer acceptance of genetic modification have restricted genetic modification for strain improvement of brewing yeasts (Gibson *et al.* 2017); therefore, development and potential of genetic modification are discussed in a separate section.

Compared to ale brewing *S. cerevisiae* strains, the genetic and phenotypic diversity of *S. pastorianus* is limited (Dunn and Sherlock 2008; Gibson *et al.* 2013b; Steensels *et al.* 2014a; Gallone *et al.* 2016; Okuno *et al.* 2016; Salazar *et al.* 2019). While diversity has been successfully expanded by crossing spores of an *S. pastorianus* strain with *S. cerevisiae* (Bilinski and Casey 1989; Sanchez, Solodovnikova and Wendland 2012), mating strategies are constrained by the low sporulation efficiency of alloaneuploid *S. pastorianus* strains (Gjermansen and Sigsgaard 1981; Liti, Barton and Louis 2006; Ogata *et al.* 2011; Santaguida and Amon 2015). As illustrated by the mating of an non-sporulating alloploid *S. bayanus* strain with beer-brewing *S. cerevisiae* strains (Sato *et al.* 2002), low sporulation efficiencies could be circumvented by using rare mating based on spontaneous or induced mating-type switching (Gunge and Nakatomi 1972; Alexander *et al.* 2016). Although labor- and time-intensive, non-sexual crossing methods such as spheroplast fusion can also be applied (Barney, Jansen and Helbert 1980).

The low mating efficiency of existing *S. pastorianus* strains was circumvented by mating different *Saccharomyces* species in

the laboratory to obtain novel *S. pastorianus*-like lager-brewing strains (Hebly *et al.* 2015; Krogerus *et al.* 2015). In addition to sharing the hybrid vigor of *S. pastorianus*, laboratory-made *S. cerevisiae* × *S. eubayanus* hybrids displayed phenotypic diversity depending on their ploidy and on the genetic background of parental strains (Mertens *et al.* 2015; Krogerus *et al.* 2016). Moreover, hybrids of *S. cerevisiae* with other cold-tolerant *Saccharomyces* species such as *S. arboricola*, *S. mikatae* and *S. uvarum* displayed similar fermentation performance at low temperature as *S. pastorianus* (Gonçalves *et al.* 2011; Nikulin, Krogerus and Gibson 2018). Laboratory hybrids are typically made by crossing strains with complementary selectable phenotypes and selecting hybrid cells which combined both phenotypes. In some cases, natural traits of the parental strains, such as growth at low temperature or the ability to utilize melibiose, can be used as selectable phenotypes (Sato *et al.* 2002). In the absence of such pre-existing selectable phenotypes, selectable genotypes can be introduced prior to mating. For example, uracil auxotrophy can be selected by growth in the presence of 5-fluoroorotic acid, lysine auxotrophy can be selected by growth in the presence of α-aminoadipate and respiratory-deficient strains can be obtained by growth in the presence of ethidium analogues (Chattoo *et al.* 1979; Fukunaga *et al.* 1980; Boeke *et al.* 1987). After crossing strains with different auxotrophies or deficiencies, hybrids can be isolated by selection on appropriate media (Krogerus *et al.* 2016; Magalhães *et al.* 2017; Krogerus, Holmström and Gibson 2018). Alternatively, selectable phenotypes may be introduced using genome editing, for example by introducing genes conferring antibiotic resistance (Jimenez and Davies 1980; Gritz and Davies 1983; Goldstein and McCusker 1999). By combining an uncommon auxotrophy and an introduced antibiotic resistance gene in one parental strain, it can be crossed with a large array of other strains without requiring any additional pre-existing or introduced selectable phenotypes (Hebly *et al.* 2015), however GM status of such strains complicates industrial application.

The requirement for phenotypic and genetic markers can be completely circumvented by staining parental strains with fluorescent dyes prior to mating and, subsequently, sorting double-stained cells using fluorescence-activated cell sorting. Indeed, a recent study shows how hybrids could be obtained with this method without the use of any selectable phenotype (Gorter de Vries *et al.* 2019a). Such laboratory hybrids generally display increased evolvability, which can be beneficial for strain improvement, as illustrated by faster and superior evolution of ethanol tolerance in hybrids during laboratory evolution under high-ethanol conditions (Krogerus, Holmström and Gibson 2018). Despite their increased plasticity, cultivation of laboratory-made *S. cerevisiae* × *eubayanus* hybrids under lager-brewing conditions during >100 repeated batches demonstrated that genetic instability was far more limited than it is in *S. pastorianus* and that phenotypic deterioration only occurred after far more brewing cycles than are customary in the lager-brewing industry (Gorter de Vries *et al.* 2019b). Overall, laboratory-made hybrids show high potential for brewing applications (Krogerus *et al.* 2017).

Both *S. pastorianus* strains and laboratory-made lager-brewing hybrids can been further improved by laboratory evolution and/or mutagenesis and selection (Table 1). Generation of novel phenotypes can occur by spontaneous acquisition of mutations during growth. Alternatively, the mutation frequency can be increased by mutagenesis using irradiation (such as ultraviolet light) or by exposure to mutagenic compounds (such as ethyl methanesulfonate (EMS), methyl benzimidazole-2-ylcarbamate (MBC), N-methyl-N'-nitro-N-nitroso-guanidine (MNNG)). Mutants of interest can be isolated by screening for desirable phenotypes, or by growth under conditions that confer a selective benefit to mutants with a desirable phenotype.

When growth under conditions favoring desired phenotypes is not only applied to select pre-existing mutants, but also to generate new mutants in the process, it is designated as laboratory evolution. This strategy has been successfully applied to select for lager-brewing-relevant phenotypes of *Saccharomyces* strains, including superior fermentation in 'high gravity' processes, increased ethanol tolerance, improved sugar utilization, increased performance under nutrient limitation, altered flocculation behavior and altered flavor profiles (Table 1). For an overview of relevant taste compounds in beer brewing and of relevant phenotypic properties of brewing yeast, we refer to recent reviews (Lodolo *et al.* 2008; Holt *et al.* 2019). Readers should keep in mind that strain improvement methods developed by commercial brewers are rarely published; therefore, the list in Table 1 is not exhaustive.

GENOME-EDITING TECHNIQUES IN *S. PASTORIANUS* AND THEIR POTENTIAL FOR INDUSTRIAL APPLICATION

Compared to the plethora of genome-editing techniques (also referred to as gene-, genetic- or genome engineering) in *S. cerevisiae* (DiCarlo *et al.* 2013; Nielsen *et al.* 2013; Jakočiūnas, Jensen and Keasling 2016; Nielsen and Keasling 2016), there are only very few accounts of targeted genome editing using cassette integration in *S. pastorianus* (Vidgren *et al.* 2009; Duong *et al.* 2011; Murakami *et al.* 2012; Bolat *et al.* 2013; Gorter de Vries *et al.* 2017a), supposedly due to limited homologous recombination efficiency (Gorter de Vries *et al.* 2017a). Even simple gene deletion studies were, until recently, complicated by the presence of several gene copies, which required repeated rounds of cassette insertion and marker removal. Instead, functional characterization often relied on expressing *S. pastorianus* genes in *S. cerevisiae* strains (Kobayashi *et al.* 1998; Yoshimoto *et al.* 1998; Kodama, Omura and Ashikari 2001; Salema-Oom *et al.* 2005; Bolat *et al.* 2013). While introduction of a double-strand break can drastically increase genome editing efficiency (Pâques and Haber 1999), Cas9 genome editing tools developed for *S. cerevisiae* were not immediately applicable in *S. pastorianus* strains (DiCarlo *et al.* 2013; Mans *et al.* 2015; Gorter de Vries *et al.* 2017a). However, polymerase-II-based expression of gRNAs flanked by self-cleaving ribozymes was successful in *S. pastorianus*, in laboratory-made *S. cerevisiae* × *S. eubayanus* hybrids and in both parental species (Gorter de Vries *et al.* 2017a; Brickwedde *et al.* 2018; Gorter de Vries *et al.* 2019b). While application of genetic modification (GM) to generate industrial strains is limited by customer acceptance issues (Akada 2002), non-GM strain improvement can also benefit from the prior application of efficient gene-editing techniques. The single-step deletion of all 9 copies of the *ATF1* and *ATF2* genes in *S. pastorianus* illustrated the potential of Cas9 to facilitate functional characterization by enabling fast and complete gene deletion (Gorter de Vries *et al.* 2017a). Furthermore, genome editing can be used to evaluate the desirability of mutations prior to the use of laborious non-GM techniques, as illustrated by the deletion of *FDC1* and *PAD1* genes in *S. eubayanus* prior to mutagenesis to obtain non-GM strains with low phenolic off-flavors (Diderich *et al.* 2018). In addition, when a phenotypic improvement is achieved through non-GM strain improvement methods such as laboratory evolution or mutagenesis, Cas9 can facilitate elucidation of the causal

Table 1. Non-GM mutagenesis, selection and/or laboratory evolution methods that resulted in lager-brewing-relevant phenotypic changes in *Saccharomyces* strains. For each method, the used *Saccharomyces* species, applied mutagenesis methods, applied selection and/or laboratory methods, and the selected phenotype are indicated. For mutagenesis methods, ultraviolet radiation (UV), ethyl methanesulfonate (EMS), methyl benzimidazole-2-ylcarbamate (MBC), N-methyl-N'-nitro-N-nitroso-guanidine (MNNG) are distinguished. RBS denotes the use of a repeated batch setup.

Application	Strain	Mutagenesis	Selection and/or laboratory evolution	Selected phenotype	Reference
Substrate utilization	*S. cerevisiae*	MNNG mutagenesis	Differential staining with Triphenyltetrazolium chloride	Crabtree-negative mutants	(Böker-Schmitt, Francisci and Schweyen 1982)
	S. cerevisiae	–	Growth on solid medium with 2-deoxyglucose	Loss of glucose repression	(Jones, Russell and Stewart 1986)
	S. cerevisiae	–	Growth on solid medium with glucosamine	Loss of glucose repression	(Hockney and Freeman 1980)
	S. eubayanus	–	RBS cultivations on synthetic medium with maltose and traces of glucose	Maltose utilization	(Baker and Hittinger 2018)
	S. pastorianus	–	Chemostat cultivation on maltotriose enriched mock-wort	Maltotriose utilization	(Brickwedde *et al.* 2017)
	S. eubayanus	–	RBS cultivations on synthetic medium with maltotriose and traces of glucose	Maltotriose utilization	(Baker and Hittinger 2018)
	S. eubayanus	UV mutagenesis	RBS cultivations on synthetic medium with maltotriose and chemostat cultivation on maltotriose-enriched wort	Maltotriose utilization	(Brouwers *et al.* 2019b)
	S. cerevisiae × *S. uvarum*	–	Chemostat cultivation under ammonium limitation	Increased fitness under nitrogen limitation	(Dunn *et al.* 2013)
	S. cerevisiae	–	Chemostat cultivation under nitrogen limitation	Increased fitness under nitrogen limitation	(Hong and Gresham 2014)
	S. cerevisiae × *uvarum*	–	Chemostat cultivation under carbon-, phosphate- and sulfate limitation	Increased fitness under nutrient limitation	(Smukowski Heil *et al.* 2017)
	S. cerevisiae	–	Chemostat cultivation under carbon-, phosphate- and sulfate limitation	Increased fitness under nutrient limitation	(Gresham *et al.* 2008)
Industrial performance	*S. pastorianus*	UV mutagenesis	RBS cultivations on high-gravity wort	High gravity fermentation	(Blieck *et al.* 2007)
	S. pastorianus	EMS mutagenesis	Fed-batch cultivation on high-gravity wort	High gravity fermentation	(Huuskonen *et al.* 2010)
	S. pastorianus	UV and EMS mutagenesis	Growth on solid medium with high ethanol concentrations	High gravity fermentation	(Yu *et al.* 2012)
	S. cerevisiae × *eubayanus*	–	RBS cultivation with high ethanol concentrations	High gravity fermentation	(Krogerus, Holmström and Gibson 2018)
	S. cerevisiae	MBC mutagenesis	Batch cultivation in high gravity medium in the presence of ethanol	High gravity fermentation	(Zheng *et al.* 2014)

Table 1. Continued

Application	Strain	Mutagenesis	Selection and/or laboratory evolution	Selected phenotype	Reference
	S. cerevisiae	–	Turbidostat cultivation with increasing ethanol concentrations	Increased ethanol tolerance	(Voordeckers *et al.* 2015)
	S. cerevisiae	–	RBS cultivations with increasing ethanol concentrations	Increased ethanol tolerance	(Dinh *et al.* 2008)
	S. cerevisiae	EMS mutagenesis	Turbidostat cultivation with increasing ethanol concentrations	Increased ethanol tolerance	(Stanley *et al.* 2010)
	S. uvarum	EMS mutagenesis	Turbidostat cultivation with increasing ethanol concentrations	Increased ethanol tolerance	(Brown and Oliver 1982)
	S. cerevisiae	–	Batch cultivations with intermittent exposure to 0.3-4.4 M of H_2O_2, 52 °C, 20–55 % ethanol and freeze/thawing cycles	Increased tolerance to oxidative-, temperature- , ethanol- and freezing–thawing stress	(Çakar *et al.* 2005)
	S. pastorianus	EMS mutagenesis	Repeated heat shocks at 55 °C	Increased heat shock tolerance	(James *et al.* 2008)
	S. cerevisiae	UV mutagenesis	Subjection to 200 freeze-thaw cycles	Increased freeze tolerance	(Teunissen *et al.* 2002)
	S. cerevisiae × *S. eubayanus*	–	RBS cultivations on wort	Increased flocculation	(Gorter de Vries *et al.* 2019b)
	S. cerevisiae	–	Chemostat cultivation	Increased flocculation	(Hope *et al.* 2017)
	S. cerevisiae	MNNG mutagenesis	RBS cultivation enriching for slow-sedimenting cells	Loss of flocculation	(Holmberg and Kielland-Brandt 1978)
	S. pastorianus	–	Batch cultivation in the presence of Ethydium Bromide	Loss of respiratory capacity	(Holmberg and Kielland-Brandt 1978)
	S. cerevisiae × *uvarum*	–	Chemostat cultivation at 15°C	Increased growth at low temperatures	(Heil *et al.* 2019)
Off-flavor reduction	*S. cerevisiae*	–	RBS cultivation with sulfate	Increased glycerol production	(Kutyna *et al.* 2012)
	S. cerevisiae	–	Batch cultivation with S-methyl-L-cysteine	Increased thiol production	(Belda *et al.* 2016)
	S. cerevisiae	UV mutagenesis	Screening for lack of coloration on lead plates	Decreased H_2S production, increased SO_2 production	(Chen *et al.* 2012)
	S. pastorianus	–	Growth on solid medium with ethionine, screening for coloration on lead plates	Increased SO_2 production	(Yoshida *et al.* 2008)
	S. cerevisiae	UV mutagenesis	Growth on solid medium with cadmium	Increased glutathione production	(Chen *et al.* 2012)
	S. pastorianus	UV mutagenesis	Growth on solid medium with disulfiram	Decreased acetaldehyde production	(Shen *et al.* 2014)
	S. pastorianus	EMS mutagenesis	RBS cultivation in the presence of chlorsulfuron	Decreased diacetyl production	(Gibson *et al.* 2018)
	S. eubayanus	UV mutagenesis	Screening for insensitivity to cinnamic acid	Decreased 4-vinyl guaiacol production	(Diderich *et al.* 2018)

Table 1. Continued

Application	Strain	Mutagenesis	Selection and/or laboratory evolution	Selected phenotype	Reference
Flavor modulation	*S. cerevisiae*	UV mutagenesis	Growth on solid medium with cerulenin	Increased fatty-acid synthesis	(de Araújo Vicente *et al.* 2006)
	S. pastorianus	–	Growth on solid medium with 5,5,5-trifluoro-DL-leucine	Increased Isoamyl alcohol and Isoamyl acetate production	(Strejc *et al.* 2013)
	S. cerevisiae	EMS mutagenesis	Growth on solid medium with isoamyl monochloroacetate	Increased isoamyl acetate production	(Watanabe, Nagai and Kondo 1995)
	S. cerevisiae	–	RBS cultivation in the presence of 1-farnesylpyridinium	Increased isoamyl acetate production	(Hirooka *et al.* 2005)
	S. cerevisiae	EMS mutagenesis	Growth on solid medium with econazole	Increased isoamyl acetate production	(Asano *et al.* 1999)
	S. cerevisiae	–	Batch cultivation in the presence of 8 mM Cu^{2+}	Increased isoamyl acetate production	(Hirooka *et al.* 2010)
	S. cerevisiae	EMS mutagenesis	Growth on solid medium with isoamyl monofluoroacetate	Increased isoamyl acetate production	(Watanabe *et al.* 1993)
	S. uvarum	–	Growth on solid medium with 5,5,5,-trifluoro-DL-leucine and fluoro-dl-phenylalanine	Increased isoamyl acetate and phenylethyl acetate	(Lee, Villa and Patino 1995)
	S. cerevisiae	–	Growth on solid medium with *p*-Fluoro-DL-phenylalanine	Increased β-phenethyl alcohol and β-phenethyl acetate production	(Fukuda *et al.* 1991)
	S. pastorianus	MNNG mutagenesis	Growth on solid medium with thiaisoleucine	Increased 2-methyl-1-butanol production	(Kielland-Brandt, Petersen and Mikkelsen 1979)

mutations by enabling rapid reverse engineering (Gorter de Vries *et al.* 2019b).

Regardless of recent advances in genetic accessibility, the lager-brewing industry does not currently use GM yeast for lager beer brewing. Many countries and trade blocks, including important beer markets such as the EU and the USA, tightly regulate use of GM technology in the food and beverages industry (Sprink *et al.* 2016). Historically, regulation was technology based: methods to modify genomes by non-targeted methods such as UV mutagenesis and chemical mutagenesis were not regulated, while any mutation introduced by targeted genetic engineering was subject to specific legislation (Nevoigt 2008). Recently, regulation appeared to be moving toward product- and risk-based evaluation, in which the type of mutation introduced determines regulatory status (Conko *et al.* 2016; Sprink *et al.* 2016). For example, Japan regulates genetic engineering less strictly when no foreign DNA is introduced ('self-cloning'). Similarly, in the USA, GM foods which only harbor single-nucleotide changes that might also have arisen after non-targeted mutagenesis, have been introduced into the market (Hino 2002; Ledford 2016; Waltz 2016). However, similar developments toward product- and risk-based regulation were recently blocked by legislative courts in the European Union. As a consequence, updating the GM regulations in the EU will now require a considerable political process (Eriksson *et al.* 2018).

Since, in the EU, food products only need to be labeled and regulated as GM if they contain >0.9% GM biomass, removal of GM yeast by filtration could, in principle, obviate the need for labeling the resulting beer as a GM product (Pérez-Torrado, Querol and Guillamón 2015). Moreover, already in 1990, a lager-brewing strain engineered for dextrin utilization was approved and used to brew a low-caloric beer in the UK (Hammond 1995; Akada 2002). As illustrated by the commercial failure of this GM beer, the application of GM yeasts for beer brewing is precluded primarily by customer acceptance—or by producers' concerns about consumer acceptance—rather than by insurmountable regulatory hurdles (Ishii and Araki 2016). However, recent regulatory developments have resulted in successful commercialization of foods based on targeted genetic modification, particularly on the US market (Waltz 2016; Ishii and Araki 2017). Moreover, Lallemand (Montreal, Canada) is currently concluding trials with a brewing yeast engineered to produce lactic acid, called Sourvisiae (Rice 2019).

Despite the current absence of large-scale industrial application, many possible genetic engineering strategies for lager-brewing yeasts are available, based on insights gained from laboratory studies and from analysis of strains obtained by classical strain improvement. Such strategies could rapidly and efficiently improve a vast array of yeast characteristics, including substrate utilization, general brewing performance and energy requirements for cooling, off-flavor and flavor profiles and, moreover, enable the introduction of novel flavors (Table 2). The relatively permissive legislation and relatively high consumer acceptance in countries such as Brazil, USA, Japan and Argentina may enable industrial application of GM yeast for lager beer brewing in the near future (Mertens *et al.* 2019).

OUTLOOK

Recent progress in genome sequencing and genome editing technologies has yielded chromosome-level genome assemblies and improved our understanding of the complex hybrid genomes of *S. pastorianus*. Ongoing developments in chromosome copy haplotyping and emerging assembly algorithms for haplotype phasing will further clarify the role of aneuploidy and heterozygosity in such genomes (Chin *et al.* 2016; He *et al.* 2018; Wenger *et al.* 2019). Furthermore, analogous to recent developments in *S. cerevisiae* and *S. eubayanus*, chromosome-level reference genomes will contribute to improved understanding of the complexity and plasticity of *S. pastorianus* genomes, and to simplifying and accelerating strain improvement strategies by mutagenesis and selection and/or laboratory evolution (Brickwedde *et al.* 2018; Mans, Daran and Pronk 2018; Brouwers *et al.* 2019b; Gorter de Vries *et al.* 2019b).

While the genetic diversity of *S. pastorianus* is limited by its reproductive isolation and, probably, by population bottlenecks during domestication, non-GM methods for the generation of interspecies hybrids create new opportunities to expand the diversity of lager-brewing strains (Mallet 2007; Mertens *et al.* 2015; Gallone *et al.* 2016; Nikulin, Krogerus and Gibson 2018; Salazar *et al.* 2019). Moreover, the emergence of Cas9 genome editing tools compatible with *S. pastorianus* enables the use of high-quality genome assemblies for functional characterization of genes (Gorter de Vries *et al.* 2017a), determination of targets for non-GM techniques (Diderich *et al.* 2018) and reverse engineering after non-GM strain improvement methods (Gorter de Vries *et al.* 2019b). Current developments in GM regulation outside the EU may lead to the direct applicability of genetically engineered strains, particularly when no heterologous DNA is introduced (Waltz 2016; Ishii and Araki 2017). Consolidations in the brewing industry during the 20th century have transformed brewing companies into international conglomerates with broad portfolios of beer brands (Poelmans and Swinnen 2011b; Howard 2014). Such conglomerates are unlikely to adopt GM yeasts for brewing, as customer acceptance backlash may not be restricted to a specific beer brand or customer market, but could result in decreasing sales of their entire brand portfolio over all markets. However, the 21st century saw a revitalization of the declining beer market, resulting in the emergence of many small new breweries, commonly referred to as craft- and micro-breweries (Carroll and Swaminathan 2000; Ellis and Bosworth 2015). Due to their small volumes and the presence of numerous competing beer brands, microbreweries generally strive toward clearly defined product identity to target highly specific customer segments (Thurnell-Read 2014; Maier 2016). GM-technology could be used to obtain characteristics which are popular in the microbrewery customer market, such as environmental sustainability and product uniqueness (Williams and Mekonen 2014; Carr 2017). For example, the use of GM yeast without diacetyl production could reduce the energy requirements of lager brewing by alleviating the need for lagering, which typically requires cooling during time periods of about two weeks (Duong *et al.* 2011). Similarly, introduction of genes for the production of hop flavors, could strongly reduce water, land and energy usage for hop production (Denby *et al.* 2018). The introduction of genes for the production of novel flavor compounds can generate novel products clearly distinct from other brands (Hansen *et al.* 2009), and fits into the recent commercial success of beers with fruity flavor additives, such as Radler or Shandy, which consist of beer mixed with non-alcoholic fruit-flavored beverages (Paixão 2015). While GM microbreweries could theoretically target progressive market segments with high GM acceptance specifically, technological and financial hurdles to generate and implement genetically modified yeast have been prohibitive. However, the development of efficient gene-editing tools has considerably lowered such hurdles and popularized genome editing, as illustrated in the extreme by the biohacking movement (Bennett *et al.* 2009; Yetisen 2018).

Table 2. Genetic engineering strategies that were successfully applied in *Saccharomyces* yeasts with potential application for the lager-brewing industry.

Application	Modification	Phenotype	Organism	Reference
Substrate utilization	*AGT1* overexpression	Increased maltose and maltotriose utilization	*S. pastorianus*	(Vidgren *et al.* 2009)
	Heterologous gene expression	Increased β-glucan degradation	*S. pastorianus*	(Penttilä *et al.* 1987)
	Heterologous gene expression	Increased dextrin utilization	*S. pastorianus*	(Cole *et al.* 1988; Perry and Meaden 1988; Sakai *et al.* 1989)
	PUT4 overexpression	Increased proline assimilation	*S. pastorianus*	(Omura *et al.* 2005)
Industrial performance	*GPD1* overexpression	Increased glycerol production, decreased ethanol production	*S. pastorianus*	(Nevoigt *et al.* 2002)
	FLO1, *FLO5* or *FLO11* overexpression	Increased flocculation	*S. cerevisiae*	(Govender *et al.* 2008)
	Stationary-phase *FLO1* overexpression	Stationary-phase flocculation	*S. pastorianus*	(Verstrepen *et al.* 2001)
	PEP4 disruption	Improved foam stability	*S. cerevisiae*	(Liu *et al.* 2009)
	LEU1 overexpression	Improved high gravity fermentation	*S. pastorianus*	(Blieck *et al.* 2007)
	FKS1 disruption	Improved anti-staling of beer due to reduced yeast autolysis	*S. pastorianus*	(Wang *et al.* 2014)
	MET10 disruption	Increased SO_2	*S. pastorianus*	(Hansen and Kielland-Brandt 1996)
	MET14 and *SSU1* overexpression	Increased SO_2	*S. cerevisiae*	(Donalies and Stahl 2002)
Off-flavor reduction	*HOM3* overexpression, *SKP2* disruption	Increased SO_2 and decreased H_2S production	*S. pastorianus*	(Yoshida *et al.* 2008)
	CYS4 overexpression	Decreased H_2S production	*S. cerevisiae*	(Tezuka *et al.* 1992)
	NHS5 overexpression	Decreased H_2S production	*S. pastorianus*	(Tezuka *et al.* 1992)
	MXR1 disruption	Decreased dimethylsulfide production	*S. cerevisiae*	(Hansen 1999)
	ILV5 overexpression	Decreased diacetyl production	*S. cerevisiae*	(Omura 2008)
	ILV6 disruption	Decreased diacetyl production	*S. pastorianus*	(Duong *et al.* 2011)
	Heterologous gene expression	Increased diacetyl degradation	*S. pastorianus*	(Sone *et al.* 1988; Fujii *et al.* 1990; Blomqvist *et al.* 1991; Yamano *et al.* 1994a; Yamano, Tanaka and Inoue 1994b)
	FDC1 disruption	Decreased 4-vinyl guaiacol production	*S. pastorianus*	(Mertens *et al.* 2019)
Flavor modulation	*LEU4* overexpression	Increased isoamyl acetate production	*S. cerevisiae*	(Hirata *et al.* 1992)
	ATF1 and *ATF2* overexpression	Increased acetate ester production	*S. pastorianus*	(Verstrepen *et al.* 2003)
	ATF1 and *ATF2* disruption	Decreased acetate ester production	*S. pastorianus*	(Verstrepen *et al.* 2003)
	ALD3 disruption, *ARO9* and *ARO10* overexpression	Increased 2-phenylethanol production	*S. cerevisiae*	(Kim, Cho and Hahn 2014)
	Heterologous gene expression	Increased ethyl hexanoate production	*S. cerevisiae*	(Han *et al.* 2009)
Introduction of new flavors	Heterologous gene expression	Hop monoterpene production	*S. cerevisiae*	(Denby *et al.* 2018)
	Heterologous gene expression	Hop lupulone production	*S. cerevisiae*	(Guo *et al.* 2019)
	Heterologous gene expression	β-ionone production	*S. cerevisiae*	(Beekwilder *et al.* 2014)
	Heterologous gene expression	Vanilin production	*S. cerevisiae*	(Hansen *et al.* 2009; Brochado *et al.* 2010)
	Heterologous gene expression	Valencene production	*S. cerevisiae*	(Asadollahi *et al.* 2008)
	Heterologous gene expression	Nootkatone production	*S. cerevisiae*	(Gavira *et al.* 2013)
	Heterologous gene expression	Raspberry ketone production	*S. cerevisiae*	(Beekwilder *et al.* 2007; Lee *et al.* 2016)

Overall, ongoing developments in genome sequencing, genome editing and interspecies hybridization methods are giving a new impulse to lager yeast strain improvement, and are likely to shape the lager beer market in the coming years.

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