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Adaptive Laboratory Evolution of Ale and Lager Yeasts for Improved Brewing Efficiency and Beer Quality

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Abstract

Yeasts directly impact the efficiency of brewery fermentations as well as the character of the beers produced. In recent years, there has been renewed interest in yeast selection and development inspired by the demand to utilize resources more efficiently and the need to differentiate beers in a competitive market. Reviewed here are the different, non-genetically modified (GM) approaches that have been considered, including bioprospecting, hybridization, and adaptive laboratory evolution (ALE). Particular emphasis is placed on the latter, which represents an extension of the processes that have led to the domestication of strains already used in commercial breweries. ALE can be used to accentuate the positive traits of brewing yeast as well as temper some of the traits that are less desirable from a modern brewer's perspective. This method has the added advantage of being non-GM and therefore suitable for food and beverage production.

BACKGROUND

Yeasts used in brewing have a specific suite of traits that contribute to the character of classic beer styles. These traits resulted from domestication processes that may have spanned several millennia (Gallone et al. 2016, Gonçalves et al. 2016). During this time, characteristic brewing yeast properties developed incrementally through a combination of natural and artificial selection to allow the production of beers that were clear, had positive flavor notes and minimal off-flavor, and could be prepared with recycled yeast because of the strains' tolerances to brewery-related stresses. The present-day beer market is, however, characterized by a demand for novel beer properties, and brewers are increasingly looking for new ways to differentiate their products (Aquilani et al. 2015). As yeast has such a significant impact on beer character, selecting or developing new yeast strains has the potential to introduce both diversity and functionality to beers. This strain development may be achieved through targeted genetic engineering, and one of the first genetically modified organisms to be cleared for use in the food industry was a brewing yeast expressing the glucoamylase-encoding *STA1* gene (Hammond 1995). Numerous studies have demonstrated the efficacy of genetic engineering for improving brewing performance and beer quality, but there remains a low level of public acceptance of genetically modified organisms for food production. The brewer is, for now, therefore restricted to using more "natural" means to improve yeast performance.

An indication of how the industry is changing is seen in the willingness of brewers to experiment with wild yeast species as fermentative or bioflavoring agents to diversify their product portfolios. A notable example is the industrial use of *Saccharomyces eubayanus* (Gibson et al. 2017, Hittinger et al. 2018). This species, first discovered in Patagonia (Libkind et al. 2011), was found to be the missing parent of the hybrid yeast *Saccharomyces pastorianus* (*Saccharomyces cerevisiae* × *S. eubayanus*) used in lager beer production. This genetic relatedness inspired studies focusing on its brewing potential. These studies revealed a number of relevant preadaptations in this species, chiefly the ability to use the main brewing sugar maltose, the generation of pleasant aroma profiles, and cold tolerance (Gibson et al. 2013), along with a number of less desirable traits, which, as is shown below, are amenable to mitigation or removal. There are, at present, eight recognized species in the *Saccharomyces* genus (Naseeb et al. 2018), and it remains to be seen which, if any, of the other wild species are suitable for application in brewing. Preliminary studies suggest that the ability to utilize maltose varies among the species and that the spicy/smoky phenolic off-flavor 4-vinylguaiacol is apparently produced by all wild *Saccharomyces* species (Nikulin et al. 2018). Recent reports have hinted at the potential for repurposing *S. cerevisiae* strains from other food systems in an effort to increase the diversity of yeast available to brewers. Strains have, for example, been isolated from sourdough cultures used for baking (Marongiu et al. 2015, Mascia et al. 2015). These strains have the natural advantage of being able to utilize maltose, and often maltotriose (the second most abundant wort sugar), in beer. Similarly, strains isolated from cachaça (sugarcane rum) fermentations, which presumably benefit from high ethanol tolerances, function efficiently as brewing yeasts (Araujo et al. 2018). The *S. cerevisiae* strain used in probiotic preparations, and commonly referred to as *Saccharomyces boulardii*, has been considered as a production strain for low-alcohol beers (because of its poor utilization of the main wort sugars) (Senkarcinova et al. 2019). Recently, traditional beers have also been used as a reservoir of new brewing strains. One such example is the kveik yeast group associated with Norwegian farmhouse ales, which, although clearly showing signs of domestication, appears to be genetically and phenotypically distinct from other brewing yeasts, presumably because of an extended period of isolation from those strains used in industrial breweries today (Krogerus et al. 2018b, Preiss et al. 2018). The inadvertent utilization of yeasts from alternative food and beverage environments in brewing may have occurred

frequently in the past; comprehensive analyses of the genomes of yeasts in brewing strain collections have uncovered the presence of some imposter strains, i.e., strains used in brewing that have crossed over from, for example, the wine industry (and vice versa) (Gallone et al. 2016).

Nonconventional yeasts with potential applications in brewing are not found only among the *Saccharomyces* species, and a diverse range of species has been utilized to introduce character to beer. The wild yeast *Lachancea thermotolerans* has, for example, been used to produce sour beers. An interesting characteristic of this species is its ability to acidify beer through the production of lactic acid while simultaneously producing ethanol (Domizio et al. 2016). This primary souring of beer obviates the need for lactic acid bacteria for beer souring and may be an attractive option for brewers concerned about introducing bacterial cultures into their facilities. The yeast is common in the natural environment and frequently found associated with wasps and other insects (Babcock et al. 2018). It is not typically associated with brewing environments and is a good example of how a bioprospecting strategy can help in the creation of new brewing practices (Cubillos et al. 2019). Other examples of yeasts with souring potential are *Lachancea fermentati*, *Hanseniaspora vineae*, and *Schizosaccharomyces japonicas* (Osburn et al. 2018). A common feature of wild yeasts is their variable ability to utilize wort sugars, with many species only capable of using the less abundant glucose and fructose. This limitation is a benefit to brewers seeking to produce low-alcohol beers. Potentially suitable species include *Cyberlindnera fabianii*, *Mrakia gelida*, *Pichia kluyveri*, *Pichia kudriavzevii*, *Scheffersomyces shehatae*, *Saccharomycodes ludwigii*, *Torulaspora delbrueckii*, *Wickerhamomyces anomalus*, *Zygosacchaormyces bailii*, and *Zygosacchaormyces rouxii* (Bellut & Arendt 2019). Although these species may display positive attributes for low-alcohol brewing or bioflavoring of beer, it should be noted that extra efforts may be needed to overcome some deficiencies caused by their nondomesticated nature. In some cases, overproduction of flavor compounds such as ethyl acetate may be observed, or poor flocculation may necessitate beer filtration/centrifugation. Consumer safety is also an issue. *Pichia kudriavzevii*, for example, has shown good potential for the production of low-alcohol beers but is synonymous with *Candida krusei*, a known cause of candidiasis in humans (Douglass et al. 2018).

Artificial hybridization has also gained popularity in recent years (Krogerus et al. 2015). Interspecies yeast hybrids can occur in natural environments, as evidenced by introgressions into *S. cerevisiae* from species such as *Saccharomyces paradoxus* (Duan et al. 2018, Peter et al. 2018). However, hybrids are encountered more frequently in fermentation environments. *S. cerevisiae* × *Saccharomyces kudriavzevii* and *S. cerevisiae* × *Saccharomyces uvarum* hybrids have, for example, been found in ale-brewing environments (González et al. 2008, Krogerus et al. 2018b, Peris et al. 2012), and the lager yeast *S. pastorianus* is a hybrid created by the crossing of *S. cerevisiae* and *S. eubayanus*. The common occurrence of hybrids in such environments indicates a fitness advantage that can be exploited by brewers. The creation of novel interspecies hybrids for brewing has been carried out several times in the past and has involved, for example, crosses between *S. cerevisiae* and *Saccharomyces bayanus* (Sato et al. 2002), and *S. cerevisiae* and *S. pastorianus* (Sanchez et al. 2012). However, the pace of such work increased rapidly after the discovery of *S. eubayanus* and the realization that this species was the missing second parent of the *S. pastorianus* lager yeast (Libkind et al. 2011). The creation of novel lager yeast strains through artificial crosses between *S. cerevisiae* and *S. eubayanus* has shown the added value of the association, especially in the context of low-temperature lager brewing. Improvements include greater tolerance of low temperatures, the ability to utilize maltotriose efficiently, and strong flocculation potential (Alexander et al. 2016; Brouwers et al. 2019a; Hebly et al. 2015; Krogerus et al. 2015, 2017; Mertens et al. 2015), all of which are necessary for successful lager fermentation. The ability to grow and ferment at low temperatures appears to be the main characteristic inherited from the cold-tolerant *S. eubayanus*. It should, however, be noted that, relative to *S. cerevisiae*, all other *Saccharomyces* species can be described as cold tolerant.

A study by Nikulin et al. (2018) demonstrated how *Saccharomyces* species other than *S. eubayanus* can be used in hybrid crosses with ale yeast to generate new lager yeast strains. Even a species such as *Saccharomyces mikatae* which has limited fermentative ability can be used successfully for lager yeast creation due to its ability to operate under low-temperature conditions (Nikulin et al. 2018). An important consideration when utilizing wild yeasts, or hybrids derived from wild yeasts, in brewing is that they can impart undesirable characteristics to beer. Such characteristics include phenolic or sulfurous off-flavors that need to be controlled (Diderich et al. 2018, Magalhães et al. 2016) if the yeasts are to be used effectively. Such problems are less pronounced in those strains that have undergone domestication in fermentation environments.

Compared with wild strains, strains isolated from fermentation environments (e.g., wine, beer, saké, baking) tend to possess traits that provide a fitness advantage in these same environments (Steensels et al. 2014). Of these yeast groups, the brewing yeasts are generally considered to be the most domesticated (Gallone et al. 2018). In addition to having a high degree of tolerance to ethanol and osmotic stress, they are able to use maltotriose (an abundant sugar in brewer's wort), lack phenolic flavor, and exhibit strong flocculation (Day et al. 2002, Gallone et al. 2016, Mukai et al. 2014). These traits, which offer no obvious fitness advantage in natural environments, may have emerged through artificial selection by humans. Whole-genome sequencing has also revealed that yeasts isolated from human-associated fermentation environments are genetically distinct from wild strains and cluster phylogenetically based on application (Barbosa et al. 2018, Gallone et al. 2016, Gonçalves et al. 2016, Legras et al. 2018, Peter et al. 2018). The commercially used brewing strains, for example, tend to cluster into one of two independently domesticated beer groups. These strains also possess genetic signatures of domestication, including loss-of-function mutations in genes related to phenolic off-flavor production (Gallone et al. 2016, Gonçalves et al. 2016, Mukai et al. 2014), increased copy numbers of genes related to maltose and maltotriose transport (Gallone et al. 2016, Gonçalves et al. 2016), and formation of a chimeric glucoamylase-encoding gene allowing extracellular maltotriose hydrolysis (Krogerus et al. 2019). The phenotypes and genotypes of brewing strains appear to have emerged naturally over centuries through processes that were likely a compromise between the requirements of the yeast for survival and the requirements of the brewer to produce a palatable product. Given that the discovery of yeast as the causative agent in fermentation did not occur until the nineteenth century, any artificial selection done on the brewer's part was inadvertent. With our greater understanding of yeast biology, it is now possible to adopt a rational, targeted approach to artificial evolution of yeast strains, thereby allowing for accelerated strain development. This strategy is applicable to not only existing brewing yeast strains but also wild yeasts that have not yet undergone any refinement through domestication but that may still possess traits of interest from a brewing perspective.

ADAPTIVE LABORATORY EVOLUTION

Like evolution in nature, adaptive laboratory evolution depends on genetic diversity within a population and a fitness advantage of a certain genotype under selective environmental conditions. Here, the environmental conditions are defined by the experimentalist to select for a certain phenotype.

In many ALE experiments, genetic diversity relies only on the spontaneous mutation rate of the organism. In *S. cerevisiae* cells, the rate for single-nucleotide mutations has been estimated to be $1.6–7.0 \times 10^{-10}$ per base and generation depending on the strain, e.g., its ploidy, and the environment (Liu & Zhang 2019, Sharp et al. 2018, Zhu et al. 2014). This rate can be increased by using physical mutagens such as UV radiation or chemical mutagens such as base alkylating agents or base analogs (**Figure 1*a***). Other possibilities are transposon mutagenesis or the use of

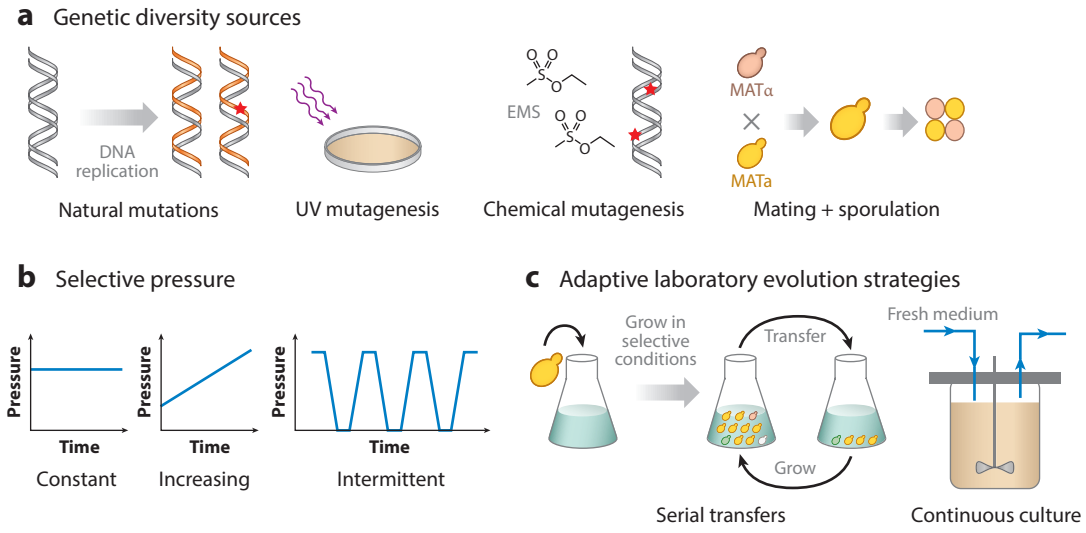

Figure 1

Overview of the main parameters that must be considered during adaptive laboratory evolution experiments. (*a*) Genetic diversity can rely on the natural mutation rate or be enhanced via measures such as UV mutagenesis, chemical mutagenesis, and mating. (*b*) The selective pressure can be constant throughout the experiment, gradually increased, or intermittent. (*c*) The most commonly applied cultivation strategies are either serial batch transfers or continuous cultures. Abbreviation: EMS, ethyl methanesulfonate.

so-called mutator strains that exhibit increased mutation rates due to genetic modifications that affect, e.g., DNA repair mechanisms (Serero et al. 2014). However, the optimal mutation rate for a given ALE experiment depends on the ploidy of the strain as well as the complexity of the trait to be selected for and may need to be adapted to the specific application. In addition to mutagenesis, various mating procedures, including direct mating, mass mating, and protoplast fusion (Steensels et al. 2014), can be integrated into ALE. The outcome may well depend on the strategy chosen to generate diversity. In a recent interlaboratory competition on the evolution of yeast at cold temperatures, strategies involving mating appeared to be more successful than others (Strauss et al. 2019).

In addition to genetic diversity, ALE requires a selectable trait. Directly selectable traits are, for example, an increased growth rate, a shortened lag phase, increased viability, improved substrate consumption, prototrophy for certain nutrients, buoyancy, flocculation, and sedimentation. ALE becomes more challenging if the phenotype to be improved is not directly selectable, e.g., the increased production of a certain product. In such cases, a smart experimental design or the introduction of genetic modifications can couple the nonselectable trait with a selectable one, e.g., product formation to growth rate. For example, alcohol acetyltransferase Atf2 is involved in the formation of volatile esters important in the brewing process. The same enzyme is also responsible for the detoxification of pregnenolone so that strains with high Atf2 activity can be selected for on media containing pregnenolone (Cauet et al. 1999). Furthermore, genome-scale metabolic models allow for the design of specific gene deletion strategies that couple the formation of the desired product with growth (von Kamp & Klamt 2017). Where complete genotype–phenotype relations remain unresolvable, genome-scale metabolic models simulate metabolic genotype–phenotype dependencies. Genome-scale metabolic models represent the complete biochemical conversion potential encoded in an organism's genome. Biochemical reactions are networked through shared reactants, and stoichiometries describe the mass conservation constraints of cellular metabolism.

Mass conservation constraints and additional constraints deriving from physicochemical laws set ultimate limits for metabolic phenotypes. Metabolic phenotype simulations have been used, for example, in predicting metabolic capabilities and designing metabolic engineering strategies (Castillo et al. 2019, O'Brien et al. 2015). Genome-scale metabolic model simulations also allow the rational design of substrate analog use for ALE and screening of desired traits (Cardoso et al. 2018, Jensen et al. 2019). Complex traits may require several mutations to arise. Such complex metabolic innovations become possible through sequential adaptation (Szappanos et al. 2016). Previous adaptation under conditions identifiable using genome-scale metabolic model simulations can predispose cells to adapt to new nutritional environments. Rationally designed, sequential ALE is a promising approach for developing complex flavor and aroma traits. In addition, a number of intracellular metabolite biosensors based on transcription factors have been developed in yeast and other organisms and can be coupled to the expression of essential genes and thus integrated into ALE experiments, as demonstrated, for example, in the production of muconic acid (Leavitt et al. 2017).

During the evolution process, the selective pressure can be either kept constant throughout or gradually increased, e.g., if the initial fitness of the strain is low (**Figure 1*b***). In some cases, an intermittent strategy with alternating cultivation under selective and nonselective conditions can be beneficial, as it may favor selection for constitutive adaptation mechanisms over induced adaptation. An example of this was the evolution to constitutive tolerance to acetic acid using dynamic selection pressure (González-Ramos et al. 2016).

The most commonly applied ALE cultivation regime is a serial batch transfer, in which strains are repeatedly cultivated in tubes or shake flasks (**Figure 1*c***). A subfraction of the culture is transferred into fresh media, typically during mid-exponential growth. This selects for cells with an increased maximum specific growth rate or potentially a shorter lag phase. In case the transfer occurs after cells have reached the stationary phase, cells with increased survival after nutrient depletion have a selection advantage. During the course of batch cultivation in shake flasks, many cultivation parameters change with time, including nutrient concentration, pH, oxygen availability, and media osmolarity, which can all influence the outcome of the evolution. To partially circumvent this, serial batches can also be performed in bioreactors where at least some of these parameters can be controlled. In addition, it is important to consider that the passage size, i.e., the amount of cells that are transferred from one vessel to the next, has an impact on the time frame in which a certain phenotype can be obtained (LaCroix et al. 2017).

An alternative to serial batch cultivations is continuous cultures in controlled bioreactors. These can be conducted as nutrient-limited chemostats at a fixed dilution rate (Brouwers et al. 2019b) or as turbidostats where the feed rate is controlled by the cell density, which allows for evolution at the maximum growth rate while keeping other parameters constant (Avrahami-Moyal et al. 2012, Gresham & Dunham 2014). Such a setup selects for cells with increased substrate affinity and/or utilization of mixed substrates. For both serial batch and continuous cultivations, automated systems have been developed that allow the operation of many evolution experiments in parallel (Strucko et al. 2018, Wong et al. 2018).

An ALE experiment is usually stopped after no, or only minor, additional increases in fitness are observed, typically after a few hundred generations. As the evolved population is heterogenic, single clones are isolated and characterized by their individual levels of fitness gain in comparison to the original strain. With decreased sequencing costs, it has become routine to analyze evolved clones using whole-genome sequencing to identify causal mutations (Caspeta et al. 2014). Sequencing of the entire population at different stages of the experiment can help in understanding evolution dynamics (Lang et al. 2013). Mutations occurring during evolution include single-nucleotide mutations, indels (insertion/deletions), deletions or duplications of larger chromosomal

regions, duplications of chromosomes, and changes in ploidy. Duplications or changes in ploidy are common early adaptation mechanisms under selective pressure but do not often exhibit long-term stability under nonselective conditions (Yona et al. 2012). If the improved phenotype is multifactorial, back-crossing and/or QTL analysis can benefit the identification of underlying mutations (González-Ramos et al. 2016). Identification of beneficial mutations can facilitate targeted strain engineering.

As outlined above, the design of an ALE experiment is crucial to its success, in accordance with "you get what you select for." Mutations that lead to increased fitness under the chosen conditions but have drawbacks in a different environment, i.e., trade-offs, are commonly observed (Caspeta & Nielsen 2015, Strucko et al. 2018). The probability of trade-off occurrence can be reduced through careful experimental design, e.g., by choosing conditions that are as close to the industrial fermentation process as possible.

CASE STUDIES

Although not traditionally used to improve brewing yeast strain properties, in recent years a number of studies have demonstrated the potential of ALE for improving the functionality of such yeasts, with potential benefits for both the brewer and consumer.

Stress Tolerance

During the brewing process, yeast cells are exposed to a range of stresses that can negatively affect their viability and fermentation performance. If the yeast is unable to resist these stresses during fermentation, beer quality and process consistency are negatively affected. Relevant stresses include ethanol toxicity, low oxygen availability, osmotic stress, CO_2 accumulation, nutrient limitation, and temperature shifts (Gibson et al. 2007). Ethanol and osmotic stress tolerance, in particular, have been frequent targets for brewing yeast adaptive evolution studies (Blieck et al. 2007, Ekberg et al. 2013, Gorter de Vries et al. 2019, Huuskonen et al. 2010, Krogerus et al. 2018a). From an experimental point-of-view, improved stress tolerance is relatively simple to select for, as exposing the yeast to various environmental stresses during growth naturally selects for cells with improved growth or survival in the stressful environment (Elena & Lenski 2003).

In an attempt to obtain variants with increased fermentation performance in high-gravity wort, Blieck et al. (2007) subjected an industrial lager yeast strain to UV mutagenesis and multiple consecutive 2-L-scale fermentations in very-high-gravity (VHG) wort. At the end of the fifth consecutive fermentation, an aliquot of the yeast was plated on complex media and individual colonies were selected. Two variants were shown to ferment faster or achieve higher attenuations in high-gravity wort at both the lab and pilot scales compared to the wild-type strain. The aroma profiles of the beers were similar, despite the differences in fermentation rate. Thirteen genes were differentially expressed in both variants compared to the wild type. Among these, *LEU1* (involved in leucine biosynthesis) was expressed at a higher ratio in both variant strains, and overexpression of *LEU1* in the wild-type strain resulted in faster fermentation in high-gravity wort. The reason for the improvement in fermentation following *LEU1* overexpression was not elucidated, but it was hypothesized that it helped overcome bottlenecks in amino acid metabolism and protein synthesis, as high-gravity fermentations are commonly nitrogen limited (Lei et al. 2012, 2013).

In a similar approach, Huuskonen et al. (2010) aimed to isolate variants of industrial lager yeast strains with improved fermentation performance in VHG wort through mutagenesis and selection in beer fermented from VHG wort. The mutagenized yeast population was inoculated into VHG wort, incubated anaerobically in the resulting beer, and fed with maltose or maltotriose for an

extended period. Individual colonies were isolated by plating aliquots of the beer on complex media once yeast viability had dropped below 10^{-4}. Multiple variants, selected at different time points, fermented faster and achieved higher attenuation levels at both the 2-L and 500-L scales compared to the wild-type strains. No significant differences in organoleptic properties were found by a trained sensory panel between the beers produced at the 500-L scale with the wild type and those produced with three variant strains. The genetic changes present in the variant strains were not elucidated in this study, but significant differences in fatty-acid composition were detected in the variant strains. The lipid composition of the plasma membrane has, for example, been shown to affect ethanol tolerance in yeast (Henderson et al. 2013, Vicent et al. 2015, You et al. 2003), and it is likely that the increased unsaturation index and fatty-acid chain length observed in some of the variants contributed to the improved fermentation performance in VHG wort.

In a follow-up study, Ekberg et al. (2013) aimed to improve the osmotolerance of both an industrial lager yeast and an ethanol-tolerant variant derived from it (Huuskonen et al. 2010), by repeated culturing in hyperosmotic conditions (4% maltose, 21% sorbitol) (Ekberg et al. 2013). A mutagenized population of the lager yeast and a nonmutagenized population of its ethanol-tolerant variant were cultured in at least 27 successive shake-flask cultivations in the sorbitol-supplemented media, after which aliquots were plated on complex media that was also supplemented with 21% sorbitol. The fastest-growing colonies were selected for further phenotypic testing. One osmotolerant variant fermented faster and reached higher attenuation levels during both 2-L-scale and 10-L-scale fermentations in high-gravity wort compared with the industrial lager yeast from which it was derived. Although fermentation rate increased, the flavor profile of the beer was slightly altered via higher concentrations of diacetyl. However, this was likely to be an effect of the shorter fermentation time rather than a change in yeast metabolism. Transcript analysis of the variant and wild-type strains revealed that genes encoding for α-glucoside transporters were expressed at higher levels in the osmotolerant variant, which could explain the increased fermentation rate (Kodama et al. 1995). In addition, the osmotolerant variant was shown to accumulate less trehalose and glycogen during fermentation. However, no differences in transcript levels of genes related to trehalose and glycogen metabolism were observed between the osmotolerant and wild-type strains.

Adaptive evolution has also been applied to improve the stress tolerance of de novo lager yeast hybrids. Krogerus and coworkers (2018a) attempted to generate ethanol-tolerant variants, with improved fermentation performance in wort, of three de novo lager hybrids and one industrial ale strain by culturing them in 30 successive batch fermentations in media containing 10% ethanol. Aliquots of the adapted populations were plated on media containing 10% ethanol, and the fastest-growing colonies were selected for a two-step screening process. Multiple variants were derived from the different hybrids, and the ale strain fermented faster during 2-L-scale wort fermentations compared to the strains from which they were derived. In addition, the majority of the adapted variants also produced increased amounts of desirable esters and exhibited increased ethanol tolerance. Whole-genome sequencing and flow cytometry revealed that the genomes of the variants had undergone changes at both the chromosome and single-nucleotide levels. The *S. cerevisiae*–derived chromosomes VII and XIV were duplicated in multiple variants. Mutations in *IRA2* and *UTH1* were also observed among the variant strains, and deletion of these genes has been shown to improve fitness and ethanol tolerance (Avrahami-Moyal et al. 2012, Sato et al. 2016, Venkataram et al. 2016).

Flavor-Active Compounds

Stress tolerance is a good selectable trait, but many yeast attributes that might interest the brewer are less amenable to selection. One such attribute is production of volatile flavor compounds,

where there is not necessarily a fitness advantage proffered to yeast cells due to the phenotype. In such situations, indirect methods are needed to adapt and select yeast cells. This has been done to modify carbonyl compound production (**Table 1**). Acetaldehyde, for example, is an intermediate product in the ethanol production pathway and is the most abundant carbonyl compound in beer. It can impart a flavor reminiscent of cut grass or under-ripe fruit and is generally recognized as one of the more common off-flavors in beers. In an effort to modify acetaldehyde production in brewing yeast, Shen and colleagues (2014) exposed UV-treated cells to high levels of the aldehyde dehydrogenase inhibitor disulfiram. Exposure prevented oxidation of acetaldehyde, leading to accumulation of the compound to toxic levels. Strains with reduced acetaldehyde production were selected under these conditions. Isolated variants were then exposed to media containing high concentrations of acetaldehyde as a sole carbon source, from which cell lines displaying rapid acetaldehyde catabolism could be isolated. Lower acetaldehyde production was observed during fermentation for selected strains, without any apparent detrimental effect on other volatile compounds.

Production of another carbonyl compound, diacetyl (2,3 butanedione), has been modified in a similar manner. This dicarbonyl is formed when α-acetolactate, released into wort by yeast during fermentation, undergoes spontaneous decarboxylation. The butter or butterscotch flavor of diacetyl can be regarded as a positive, even essential, component of the flavor profile in some beverages but is regarded as a major defect in lager beers, where it obscures the crisp and clean flavor notes associated with the style (Krogerus & Gibson 2013). Reduction of beer diacetyl is achieved during extensive maturation in cooled tanks at considerable cost to brewers. There is therefore an incentive to minimize production by the yeast of the diacetyl precursor. In an attempt to alter α-acetolactate production, Gibson and colleagues (2018) first exposed lager yeast cells to a chemical mutagen. The mutagenized population was then serially exposed to sublethal levels of chlorsulfuron, a specific inhibitor of the acetohydroxy acid synthase responsible for α-acetolactate production. After up to 30 transfers (~150 cell generations), cells were transferred to chlorsulfuron-supplemented agar plates, and individual colonies were selected based on growth. Variant strains produced less of the precursor during fermentation, and one in particular was especially effective, with 60% less total diacetyl detected in the beer at the end of fermentation. A number of genetic and chromosomal changes were observed in the best-performing variant, with one particular single-nucleotide polymorphism (SNP) in the gene responsible for acetohydroxy acid synthase production (*ILV2*) apparently causing the observed phenotype. Importantly, there were no consequences with respect to the fermentation performance of the yeast strains or aroma profile of the beer (Gibson et al. 2018). This outcome may be attributed to the avoidance of harsh conditions during adaptation that may have resulted in unwanted mutations accumulating in the test strain.

There is considerable potential for further customization of flavor profiles through the use of chemicals that disrupt specific steps in biochemical pathways. Although only rarely utilized for beer strain development (Lee et al. 1995), the approach has been used previously to modify production of aroma compounds by saké yeast strains. Examples of successfully targeted individual flavor compounds include the pear/banana flavor 3-methylbutyl acetate (Hirooka et al. 2005; Watanabe et al. 1993, 1995) and the apple/aniseed flavor ethyl hexanoate (Ichikawa et al. 1991).

Sulfur Metabolism

The production of sulfur compounds by *S. cerevisiae* is a function of the central sulfate reduction pathway responsible for the catabolism of sulfur compounds (i.e., sulfate and/or sulfite) and the anabolism of sulfur-containing amino acids (i.e., cysteine/methionine). Importantly, because of the

Table 1 Brewing yeast phenotypes manipulated through adaptive laboratory evolution

Brewing application	Phenotype	Species/strain	Evolutionary scheme	Selective pressure	Duration of evolution	Magnitude of improvement	Reference
VHG brewing	Fermentation performance	*Saccharomyces pastorianus* lager strain	UV mutagenesis and batch culture	VHG wort (22–28° Plato)	Five consecutive batch cultures	Fermentation rate in high-gravity wort was increased	Blieck et al. 2007
VHG brewing	Ethanol tolerance	*S. pastorianus* lager strain	EMS mutagenesis and continuous culture	Ethanol	Up to 59 days	Up to 65-h shorter fermentation time and improved attenuation in 25° Plato wort	Huuskonen et al. 2010
VHG brewing	Osmotolerance	*S. pastorianus* lager strain	EMS mutagenesis and batch culture	210 g/L of sorbitol	27+ consecutive batch cultures	3.5-day shorter fermentation time and improved attenuation in 15° Plato wort	Ekberg et al. 2013
VHG brewing	Ethanol tolerance	*Saccharomyces cerevisiae* × *Saccharomyces eubayanus* hybrid	Batch culture	10% ethanol	30 consecutive batch cultures	Up to 4-day shorter fermentation time and improved attenuation in 15° Plato wort	Krogerus et al. 2018b
Brewing efficiency	Maltotriose utilization	*S. eubayanus*	UV mutagenesis and chemostat cultures	Carbon-limited, maltotriose-enriched wort	NA	Maltotriose utilization was enabled	Brouwers et al. 2019a
Brewing efficiency	Maltotriose utilization	*S. eubayanus*	NA	SC with 2% maltotriose	100 transfers	Maltotriose utilization was enabled through formation of a chimeric transporter gene	Baker & Hittinger 2019
Brewing efficiency	Maltose and maltotriose utilization	*S. eubayanus*	NA	SC with 2% maltose	NA	Maltose utilization rates increased and maltotriose utilization was enabled	Baker & Hittinger 2019
Brewing efficiency	Maltotriose utilization	*S. pastorianus* lager strain	Chemostat culture	Maltotriose-enriched media	130 generations	4.75× higher maltotriose uptake rates, 5× less unfermented maltotriose	Brickwedde et al. 2017

(*Continued*)

Table 1 (*Continued*)

Brewing application	Phenotype	Species/strain	Evolutionary scheme	Selective pressure	Duration of evolution	Magnitude of improvement	Reference
Flavor control	α-Acetolactate production	*S. pastorianus* lager strain	Batch culture	Chlorsulfuron	30 consecutive batch cultures	60% lower diacetyl in green beer	Gibson et al. 2018
Flavor control	Acetaldehyde production	*S. pastorianus* lager strain	UV mutagenesis and continuous culture	Disulfiram and acetaldehyde	30-day exposure	60% lower acetaldehyde	Shen et al. 2014
Flavor control	H_2S production	*S. cerevisiae* wine strain	EMS mutagenesis, followed by batch culture and selection	Bismuth agar	One round of mutagenesis and selection	50–99% reduced H_2S, 1- to 5.6-fold increased SO_2	Cordente et al. 2009
Flavor control	H_2S production	*S. cerevisiae* ale strain	UV mutagenesis followed by batch culture and selection	Lead agar and cadmium sulfate	Two rounds of mutagenesis and selection	31% increased SO_2, 30% increased GSH, 75% reduced H_2S	Chen et al. 2012
Flavor control	SO_2 production	*S. cerevisiae* ale strain	UV mutagenesis followed by batch culture and selection	Lead agar and cadmium sulfate	Two rounds of mutagenesis and selection	31% increased SO_2, 30% increased GSH, 75% reduced H_2S	Chen et al. 2012
Flavor control	GSH production	*S. cerevisiae* wine strain	Hybridization of spores, batch culture, selection	Ammonium molybdate	One round of hybridization and selection	1.36- to 2-fold increased GSH	Mezzetti et al. 2014
Flavor control	GSH production	*S. cerevisiae* ale strain	UV mutagenesis, batch culture, selection	Lead agar and cadmium sulfate	Two rounds of mutagenesis and selection	31% increased SO_2, 30% increased GSH, 75% lowered H_2S	Chen et al. 2012
Clarification	Aggregation	*S. cerevisiae* ale strain	Continuous culture in tower-like fermentor, only flocculating cells retained	Sedimentation	14 days of continuous cultivation	Flocculation rate could not be determined in evolved isolates because cells could not be deflocculated	Conjaerts & Willaert, 2017
Clarification	Flocculence	*S. pastorianus* lager strain	Sequential batch cultivations	Sedimentation	Up to 900 generations	No value specified (flocculation demonstrated in pictures)	Oud et al. 2013
Clarification	Flocculence	*S. cerevisiae* × *S. eubayanus* hybrid	Sequential batch cultivations	Sedimentation	418 generations	Flocculation ability acquired	Gorter de Vries et al. 2019

Abbreviations: EMS, ethyl methanesulfonate; GSH, glutathione; NA, not available; SC, synthetic compete; UV, ultraviolet; VHG, very high gravity.

central nature of sulfur metabolism, all yeast strains produce the same sulfur compounds, including H_2S, SO_2, and glutathione. However, concentrations vary depending on the strains' genetic backgrounds and environmental conditions (Kumar et al. 2010, Linderholm et al. 2008). For example, the production of H_2S by different brewing and winemaking strains varies depending on allelic variation in sulfite reductase genes (*MET5* and *MET10*) (Linderholm et al. 2010) as well as under specific, stressful environmental conditions (Edwards & Bohlscheid 2007, Wang et al. 2003), e.g., low nitrogen, low temperature, and inadequate vitamins/trace nutrients. When sulfide is produced in excess, it diffuses out of the cell and forms H_2S in the acidified extracellular environment.

The management and/or remediation of H_2S is a major concern for the brewing and winemaking industries. Left unchecked, H_2S is a significant off-aroma that markedly lowers beer quality and its resultant value (Ferreira & Guido 2018). The presence of H_2S also forms secondary sulfur compounds (i.e., mercaptans and disulfides) that have aromas of cooked cabbage, cauliflower, garlic, onion, and burnt rubber (Kinzurik et al. 2015, 2016). Typically, H_2S production is managed by controlling environmental parameters, including nutritional supplementation (e.g., nitrogen and/or pantothenic acid) and reduction of temperature and/or osmotic stressors. However, H_2S remediation is typically conducted by lagering (maturation) of beer or sequestration with elemental copper, both of which add time, complexity, and cost to beer production (reviewed in Dzialo et al. 2017, Ferreira & Guido 2018). Alternatively, recent efforts in H_2S management have focused on biological engineering of yeast to reduce, or even prevent, H_2S production (discussed below). Although such efforts have not yet been actively applied to brewing yeast, there are notable examples of biological engineering and, more specifically, ALE being applied to wine yeast for this purpose (**Table 1**). It follows that these methodologies could be adapted for brewing yeast in the future.

Whereas the presence of H_2S is broadly regarded as a negative, the production of SO_2 can be desirable at moderate levels, as SO_2 has potent antioxidant and antimicrobial activity (reviewed in Guido 2016). Indeed, SO_2 functions to act as a reducing agent, scavenging free radicals and forming inert, nonvolatile, non-flavor-active adducts with various carbonyl compounds such as E-2-nonenal. Thus, in both beer and wine, SO_2 concentration is an important consideration for product quality, stability, and shelf-life (Guido 2016). However, for both beverages, there are regulated maximum allowable levels in finished products that must also be taken into consideration. Similar to SO_2, glutathione production by yeast is also desirable, as it plays a role in controlling oxidative spoilage in wine and beer, thereby enhancing product quality and shelf life. Glutathione is an important tripeptide, thiol-containing antioxidant in cells, and its presence in beer is known to improve flavor stability over time (Chen et al. 2012, Wang et al. 2010).

Several ALE approaches for modulating H_2S, SO_2, and glutathione have been employed (**Table 1**). For example, Cordente et al. (2009) utilized random ethyl methanesulfonate (EMS) chemical mutagenesis to isolate low-H_2S-producing variants of a commercial diploid wine yeast. In doing so, approximately 16,000 EMS-treated colonies were screened using BiGGY agar, a well-characterized bismuth-containing indicator that results in visibly dark colonies when H_2S is produced. Using this method, six yeast strains were identified that had H_2S production reduced by 50–99%. Importantly, these strains were shown to be methionine and cysteine prototrophs, indicating that the identified mutations in sulfite reductase were not complete loss-of-function (null) mutations; however, Cordente et al. (2009) did note that the isolated mutant strains produced significantly more sulfur dioxide than the parent yeast strain. A similar result has been observed for brewing strains engineered for increased SO_2 production, i.e., lower levels of H_2S, as well as other sulfur-containing off-flavors, were detected in beers (Ogata et al. 2013).

Alternatively, Mezzetti et al. (2014) utilized molybdate and chromate-resistance as a means to isolate low-H_2S variants of diploid wine yeast strains. Here, the authors used sexual reproduction

(hybridization of isolated spores) to generate novel biodiversity for sequential rounds of selection on molybdate and/or chromate (toxic sulfate analogs) (De Vero et al. 2011). In this way, a set of strains derived from four parents and unable to assimilate sulfate—presumably because of mutations in high-affinity sulfate permease genes—was obtained; accordingly, these strains did not produce H_2S during fermentation or overproduce sulfites relative to the parent strain. Interestingly, this approach—molybdate/chromate resistance—was also used to isolate strains producing between 1.36- and 2-fold more glutathione in wine fermentations (Mezzetti et al. 2014).

Similarly, Chen et al. (2012) used UV mutagenesis and multiple plate-based selection methods to isolate low-H_2S variants of a polyploid industrial brewing yeast, which were then further evolved for increased SO_2 and glutathione production. Using two rounds of mutagenesis and sequential selection on lead agar (similar to BiGGY described above) and then cadmium sulfate (similar to molybdate/chromate described above), the authors isolated one strain with 31% and 30% more SO_2 and glutathione, respectively, as well as 75% less H_2S. Importantly, these changes equated to a 33% improvement in staling resistance, relative to the parent brewing yeast (Chen et al. 2012).

Sugar Utilization

Present-day domesticated beer yeasts have evolved over the centuries through repeated inoculation into brewer's wort, a highly selective environment. To thrive in such conditions, a yeast must be able to tolerate high sugar concentrations, oxygen limitation, and high levels of ethanol and to grow and ferment starch-derived sugars. Maltotriose, in particular, is relatively rare in natural yeast environments; therefore, yeast had to develop the required mechanisms for its efficient utilization (Gallone et al. 2016).

The α-glucoside sugars maltose and maltotriose are the two most abundant sugars in brewer's wort. To utilize these sugars, the yeast must be able to transport them across the plasma membrane, thus requiring the presence of active transmembrane transporters (Serrano 1977, van den Broek et al. 1994). Therefore, the fermentation rate is highly dependent on the presence, nature, and quantity of α-glucoside transporters in the yeast cell (Rautio & Londesborough 2003, Magalhães et al. 2016, Vidgren & Londesborough 2018). Of the α-glucosidase transporters known, Malx1 transporters can only carry maltose, whereas Agt1 and Mtt1 can carry both maltose and maltotriose, with the latter favoring maltotriose (Chang et al. 1989, Dietvorst et al. 2005, Han et al. 1995, Salema-Oom et al. 2005, Stambuk et al. 1999).

In *Saccharomyces* yeast, maltose and maltotriose transporter genes are found in the subtelomeric regions (Alves et al. 2008, Baker et al. 2015, Dietvorst et al. 2005, Chang et al. 1989, Salema-Oom et al. 2005). Such regions are prone to increased rates of meiotic recombination and mutation, and small structural genome variations such as duplication, deletion, and recombination are frequent during evolution (Barton et al. 2008, Gallone et al. 2018).

As described earlier, the most common traits of interest for adaptive evolution of brewing yeast relate to stress tolerance, with the goal, in most cases, to improve fermentation performance in VHG worts (**Table 1**). In the aforementioned study by Ekberg et al. (2013), which is related to step-wise adaptation to ethanol toxicity and osmotic stress, a variant strain showed a higher fermentation rate and alcohol production. This was associated with decreased hexose transporter transcript levels and increased expression of *MALx1* and *MALx2*. Interestingly, Vidgren & Londesborough (2018) showed that transporter genes competing for plasma membrane space and increased expression do not necessarily lead to higher uptake, as not all transporter proteins reach the plasma membrane. Reducing the abundance of HXT transporter molecules could liberate plasma membrane space for α-glucoside transporters.

For yeast, maltotriose is generally the least preferred sugar in wort fermentations, and its use can be particularly challenging in VHG worts, where yeast is under additional pressure from high ethanol concentrations during the later stages of fermentation. As such, improved maltotriose utilization has been the subject of recent adaptation studies (**Table 1**). Brickwedde et al. (2017) challenged a lager strain in prolonged chemostat cultivations on maltotriose-enriched media, isolating a variant that leaves five times less maltotriose unfermented. Genome sequencing of the evolved strains showed no apparent variation in copy number of transporter genes. Genome analysis of hybrid strains remains challenging, particularly with short sequencing reads; it is also not possible to identify small structural variations at the gene level. Similarly, Krogerus et al. (2018a) showed that artificially generated *S. cerevisiae* × *S. eubayanus* hybrids showed improved performance that correlated with higher maltose and maltotriose utilization rates after adaptation to high ethanol concentrations. The improvement in α-glucoside consumption rate was apparently linked to chromosome duplications during the adaptation period. *Saccharomyces cerevisiae*–derived chromosome VII, which contains several maltose transporter genes (such as *MAL31* and *MAL11/AGT1*), was duplicated in adapted strains. Other than increased chromosome copy number, no SNPs, structural variations, or gene-level copy number changes were observed for the genes encoding α-glucoside transporters in the variants.

The hybrid nature of the lager yeast indicates that transporter genes can be inherited from both parents. However, thus far, all isolated *S. eubayanus* strains lack the ability to use maltotriose, although some maltotriose transporters in lager yeast are hypothesized to be of *S. eubayanus* origin (Brouwers et al. 2019a). Recent studies have aimed at enabling maltotriose utilization in *S. eubayanus* through ALE (Baker & Hittinger 2019, Brouwers et al. 2019a). Baker & Hittinger (2019) adapted a North American *S. eubayanus* isolate and Brouwers and colleagues (2019a) evolved the type strain of *S. eubayanus* (isolated in Patagonia) (Libkind et al. 2011). Both strains lack maltotriose transporters Agt1 and Mtt1, and each contains four open reading frames with similarity to *S. cerevisiae MAL31* genes (Baker et al. 2015, Baker & Hittinger 2019). These four gene products were shown to enable maltose utilization in strains deprived of maltose transporters but not maltotriose utilization (Brickwedde et al. 2018). In both experiments, maltotriose utilization was acquired as a result of the formation of chimeric genes between the maltose transporters. Additionally, in a study by Baker & Hittinger (2019), a Tibetan and a North Carolina isolate of *S. eubayanus* contained a gene with 99% and 95% homology, respectively, with the non–*S. cerevisiae AGT1* in lager yeast. Although the native strains did not grow on maltotriose and the growth was very poor in maltose, through adaptive evolution in maltose the North Carolina strain not only significantly improved its maltose utilization rates but also began taking up maltotriose as a result of the overexpression of the *AGT1* homolog. A more recent study resequenced two Tibetan *S. eubayanus* isolates [one being the same strain used by Baker & Hittinger (2019)] and showed that lack of growth on maltose and maltotriose was due to mutations in the transcription regulators (Brouwers et al. 2019b). The authors generated *S. cerevisiae* × *S. eubayanus* hybrids with complementary maltose metabolism genes; the hybrids demonstrated cross-regulation and as such were able to grow on maltose and maltotriose.

Flocculation

At the end of fermentation, lager yeasts exhibit flocculation, a process that results in the settling of the yeasts to the bottom of the fermentation tank, enabling easy removal and reuse. Ale yeasts, in contrast, flocculate and rise to the surface. Thus, flocculation is the tendency of yeast cells to aggregate, forming a multicellular mass that sediments at the bottom of the fermentation tank or rises to the surface. A selective advantage is likely to have played a role in the early domestication

of *S. pastorianus*, as sedimenting yeast remaining in the fermentation vessel was more likely to be used in a subsequent fermentation (Gorter de Vries et al. 2019). Still, many industrial brewing strains display poor flocculation, and ALE has been used in an attempt to improve the flocculation properties of yeast (**Table 1**).

In a study by Conjaerts & Willaert (2017), weakly flocculating industrial ale strain CMBSVM22 (CMBS) was evolved to a more pronounced aggregation phenotype. The original strain was characterized by a very low flocculation percentage (~4%). A continuous small-scale tower fermentor was constructed such that the selective pressure was based on gravity. Thus, during continuous culturing in these fermentors, only cells that formed aggregates were retained, and nonaggregating cells passed through the vessel. After 14 days of continuous cultivation, yeast flocs with a snowflake morphology were observed. Further characterization showed that the cell interactions in the aggregates were not the result of flocculin interactions but of changes in the mother–daughter separation process. A similar cell cluster morphology was observed when mother–daughter separation was impaired (Ratcliff et al. 2015). This phenotype was also seen in an ALE experiment with a haploid *S. cerevisiae* CEN.PK113–7D laboratory strain during long-term cultivation in sequential batch reactors (Oud et al. 2013). It was shown that mutations in *ACE2*, which encodes a transcriptional regulator involved in cell cycle control and mother–daughter cell separation, caused the snowflake phenotype. Further trials are required to determine whether this form of aggregation is compatible with brewery fermentations, where sedimentation is required only near the end of fermentation, when fermentable sugars have been exhausted and yeast in suspension has little further impact on fermentation.

In another study, an artificially created nonflocculent allodiploid hybrid of *S. cerevisiae* and *S. eubayanus* was evolved for up to 418 generations in industrial wort under simulated lager-brewing conditions in sequential batch bioreactors (Gorter de Vries et al. 2019). After each batch, bioreactors were partially emptied, leaving 7% of the culture volume as inoculum for the next fermentation cycle. Large phenotypic diversity and a large array of mutations were observed after 418 generations. Changes affecting the flocculation phenotype were observed in *SFL1*, encoding a transcriptional repressor of flocculation genes. The gene was present in both *S. cerevisiae*–type and *S. eubayanus*–type chromosomes. Strains with mutations in both *S. cerevisiae*–type and *S. eubayanus*–type *SFL1* showed rapid sedimentation, which was not observed if either gene was mutated alone. Resuspension in EDTA (ethylenediaminetetraacetic acid) eliminated flocculation, suggesting that in this case the process was flocculin-mediated.

CONCLUSIONS AND PERSPECTIVES

ALE is a powerful biological engineering method that leverages natural and/or induced genetic diversity in microbial populations to identify and isolate—by screening and/or selection methods—superior individuals in the population (Mans et al. 2018). As ALE is a method that can significantly affect brewing yeast phenotypes (without recourse to targeted genetic engineering), it is particularly suitable for brewing. A renewed interest in the technique has been inspired by the need to more effectively utilize natural resources. Improved brewing efficiency often involves intensification of the process in practices such as high-gravity brewing, which consequently increases the stresses to which the strains are exposed. Brewing yeast strains have evolved to adapt to brewing processes that predate these process changes. It can be assumed that the domestication processes that gave rise to the commercially used brewing yeast strains have essentially now been arrested because of the widespread practice of utilizing yeast from frozen stock cultures. There is therefore a need for strain development to keep pace with brewing process developments. Also, the beneficial properties of wild yeast are now being exploited by brewers, but these strains can also

have unwelcome traits. Laboratory domestication of these strains may help to fully realize their industrial potential. A clear example of this is the adaptation of *S. eubayanus* to utilize maltotriose during wort fermentation (Baker & Hittinger 2019, Brouwers et al. 2019b).

By virtue of its top-down, whole-organism approach to biological engineering, ALE has a number of strengths relative to other biological engineering methods. First, because ALE does not require knowledge of the genotype–phenotype mapping underlying a given trait, it is well suited for modulating complex, multigenic phenotypes such as stress tolerance and the output of metabolic pathways. Second, by employing phenotypic rather than genotypic screening/selection, ALE allows cellular systems to inherently self-select for compensatory, homeostasis-restoring combinations of genes/alleles that improve overall cellular fitness. Last, as a result of relatively modest genetic changes (relative to other classical strain improvement techniques, such as selective breeding), ALE may better preserve baseline and/or nontarget characteristics in improved individuals.

Despite the aforementioned strengths of ALE for the improvement of brewing yeast properties, the technique has a number of limitations as a standalone technique. For instance, because ALE drives changes in existing genetic material, it is typically limited to modulating existing traits already present in the phenotypic landscape of a species or strain. Additionally, because ALE employs phenotypic screening/selection methods, isolated individuals can often exhibit unintended consequences with bystander phenotypes not accounted for during screening/selection. Strains may also exhibit condition-specific improvements that are not generalizable outside of specific selective conditions. Both of these phenomena may then necessitate subsequent engineering to remedy. Finally, as mutation events in ALE are semirandom and occur relatively infrequently, significant time is often required to accumulate mutations that affect target phenotypes in the desired manner. This time requirement is even larger in the case of polyploid brewing yeast strains, with hundreds of generations or more often required under selective conditions.

Taken together, the relative strengths and weaknesses of ALE suggest that there is a considerable benefit to be realized by combining ALE with other biological engineering methods. Indeed, ALE has often been combined with targeted genetic-engineering approaches to improve the titer, rate, and yield of microbial cell factory strains that are metabolically engineered to produce various bulk and specialty chemicals (Baek et al. 2016, Guimarães et al. 2008, Leavitt et al. 2017, Otero et al. 2013, Reyes et al. 2014) and biofuels (Demeke et al. 2013, Diao et al. 2013, Lee et al. 2014, Peris et al. 2017, Qi et al. 2015). In this way, strains are engineered for novel functionality (e.g., production of a heterologous compound) and then optimized using ALE via screening/selection for improved performance. As metabolic engineering is not currently an option open to brewers, ALE may be combined with other top-down, biological engineering methods that exploit genetic recombination during reproduction. Such methods include hybridization, selective breeding, rare mating, and genome shuffling. Indeed, this approach may prove to be the most effective in terms of using ALE to improve complex phenotypes, as evidenced by a recent unbiased comparison of ALE methods for improving cold tolerance in yeast (Strauss et al. 2019).

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