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Targeted gene deletion in *Brettanomyces bruxellensis* with an expression-free CRISPR-Cas9 system

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Abstract

The ability to genetically manipulate microorganisms has been essential for understanding their biology and metabolism. Targeted genome editing relies on highly efficient homologous recombination, and while this is readily observed in the yeast *Saccharomyces cerevisiae*, most non-conventional yeast species do not display this trait and remain recalcitrant to targeted editing methods. CRISPR-based editing can bypass the requirement for high levels of native homologous recombination, enabling targeted modification to be more broadly implemented. While genetic transformation has been reported previously in *Brettanomyces bruxellensis*, a yeast with broad biotechnological potential and responsible for significant economic losses during the production of fermented beverages, targeted editing approaches have not been reported. Here, we describe the use of an expression-free CRISPR-Cas9 system, in combination with gene transformation cassettes tailored for *B. bruxellensis*, to provide the means for targeted gene deletion in this species. Deletion efficiency was shown to be dependent on homologous flanking DNA length, with higher targeting efficiencies observed with cassettes containing longer flanking regions. In a diploid strain, it was not possible to delete multiple alleles in one step, with heterozygous deletants only obtained when using DNA cassettes with long flanking regions. However, stepwise transformations (using two different marker genes) were successfully used to delete both wild-type alleles. Thus, the approach reported here will be crucial to understand the complex physiology of *B. bruxellensis*.

Key points
- *The use of CRISPR-Cas9 enables targeted gene deletion in Brettanomyces bruxellensis.*
- *Homozygous diploid deletions are possible with step-wise transformations.*
- *Deletion of SSU1 confirmed the role of this gene in sulphite tolerance.*

Keywords *Brettanomyces* · Wine · CRISPR · Gene deletion · Yeast

Introduction

Understanding the complex biology of microorganisms has been highly dependent on our ability to genetically manipulate them (Shapiro et al. 2018). Microorganisms, and particularly yeast, possess numerous biotechnologically relevant properties; however, for many yeast species, genetic manipulation is still challenging due to the serious lack of genetic editing tools (Cai et al. 2019).

A common strategy for stable gene modification in many yeast species is through the chromosomal integration of heterologous DNA cassettes. This integration can either proceed via non-homologous end joining (NHEJ), in which the cassette inserts randomly into the genome, or by homologous recombination (HR), where the cassette is targeted to a specific locus via homology to the site of interest (Löbs et al. 2017). HR-based integration is most often used to delete precise sections of DNA, which requires recombination between the genome and two separate HR-targeting domains that span the region to be removed. However, HR efficiency varies widely across yeast and fungi. While HR is the dominant

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mechanism in *Saccharomyces cerevisiae* and allows for very short stretches of homology (< 50 bp) to routinely target genomic loci (Fraczek et al. 2018), NHEJ is the dominant repair mechanism in most other yeast and filamentous species (Löbs et al. 2017). In situations of inefficient HR, much longer regions of homology (> 1 kb) can assist in increasing recombination efficiency; however, targeted gene modification in NHEJ-dominant yeast species remains extremely challenging.

Recently, the application of clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated protein 9 (CRISPR-Cas9) systems have revolutionized genome editing (Hsu et al. 2014; Jacobs et al. 2014; Kleinstiver et al. 2016; Mans et al. 2018). The double-stranded breaks that are induced during CRISPR-based editing have been shown to stimulate HR at the locus of interest, facilitating the introduction of precise genetic alterations in less tractable species (Shapiro et al. 2018). This has recently enabled genome editing techniques to be applied in many non-conventional yeasts, including several *Candida* species (Grahl et al. 2017; Zhang et al. 2019a; Zhu et al. 2019), the methylotrophic yeasts *Komagataella phaffii* (Gu et al. 2019; Liu et al. 2019), *Ogataea polymorpha* (Numamoto et al. 2017), and *Ogataea thermomethanolica* (Phithakrotchanakoon et al. 2018), the xylose catabolizing yeast *Scheffersomyces stipitis* (Cao et al. 2018), as well as *Aureobasidium pullulans* (Zhang et al. 2019b), *Issatchenkia orientalis* (Tran et al. 2019), *Kluyveromyces lactis* (Horwitz et al. 2015), *Kluyveromyces marxianus* (Juergens et al. 2018), *Pseudozyma antárctica* (Kunitake et al. 2019), *Rhodosporidium toruloides* (Jiao et al. 2019), *Schizosaccharomyces pombe* (Jacobs et al. 2014), and *Yarrowia lipolytica* (Ganesan et al. 2019).

Brettanomyces bruxellensis is a yeast species with significant industrial importance. It has a positive role during the production of Belgian lambic and gueuze ales (Vanbeneden et al. 2008), cachaça, a distilled spirit made from sugar cane (Parente et al. 2015) and bioethanol (Blomqvist et al. 2011; Galafassi et al. 2011). However, this species is best known for its role in the spoilage of fermented beverages including wine, beer, and cider (Curtin et al. 2015; Varela and Borneman 2017). In addition, *B. bruxellensis* has several traits relevant for biotechnological applications, including tolerance to high levels of ethanol and low pH (Conterno et al. 2006; Curtin et al. 2015), assimilation of a broad range of carbon sources, including chitin and cellobiose (Blondin et al. 1982; Curtin et al. 2012; Reis et al. 2014) and utilization of nitrate as a nitrogen source, which provides *B. bruxellensis* with a competitive advantage over *S. cerevisiae* (de Barros Pita et al. 2013; Neto et al. 2014).

Recently, genetic transformation cassettes have been developed for *B. bruxellensis*, which provide multiple drug-resistant markers and the ability to tag *B. bruxellensis* with different fluorescent proteins (Varela et al. 2018); however, the heterologous DNA that was introduced into the *B. bruxellensis* genome was shown to integrate randomly. In order to facilitate targeted genome editing in *B. bruxellensis*, the effect of combining these optimized selection cassettes with various lengths of flanking DNA and an expression-free CRISPR-Cas9 system were assessed for their ability to promote HR-based gene deletion.

Material and methods

Strain and media

B. bruxellensis AWRI2804 (UC Davis collection UCD2041) and *B. bruxellensis* AWRI1613 were obtained from the Australian Wine Research Institute (AWRI) Wine Microorganism Culture Collection (WMCC, Adelaide, SA, Australia). Cryogenically preserved (− 80 °C) strains were cultured and maintained on YMPG plates (3 g/L malt extract, 3 g/L yeast extract, 5 g/L peptone, 10 g/L glucose, 40 g/L $CaCO_3$, 20 g/L agar) and stored at 4 °C. Strains were grown in YPD medium (yeast extract 10 g/L, peptone 20 g/L, and glucose 20 g/L) or in minimal medium containing 5 g/L glucose and 6.7 g/L YNB (yeast nitrogen base) with amino acids pH 3.5 (MM5). Media for transformation and/or selection included YPD agar containing nourseothricin (clonNAT) at 50 μg/mL, YPD agar containing G418 at 200 μg/mL, YNB agar with and without uracil (20 mg/L), and YNB agar containing a limited amount of adenine (10 mg/L).

Construction of DNA deletion cassettes

Two different types of DNA cassettes were used to transform *B. bruxellensis* and delete specific genes. Cassettes containing short flanking regions (60 bp) were obtained by amplifying the *natMX* cassette encoding clonNAT resistance from the plasmid pMA-*TDH1pr-natMX* (Varela et al. 2018) using primers containing 60 bp flanking regions corresponding to regions up- and down-stream of the specific open-reading frame (ORF) being targeted. Cassettes containing long flanking regions (1.0 kb) were constructed in pUC19 using the method described by Gibson et al. (2009). Four PCR fragments were amplified; the first fragment contained 1.0 kb corresponding to the upstream region of the targeted gene and was obtained from *B. bruxellensis* AWRI2804 chromosomal DNA. The second fragment contained either the *natMX* or the *kanMX* cassette, amplified from plasmids pMA-*TDH1pr-natMX* (Varela et al. 2018) and pMK-T-*TDH1pr-kanMX* (Varela et al. 2018), respectively. The third fragment contained 1.0 kb corresponding to the downstream region of the targeted ORF and was obtained from *B. bruxellensis* AWRI2804 chromosomal DNA. The final fragment amplified plasmid pUC19. Fragments were assembled using the

NEBuilder HiFi DNA assembly kit (New England Biolabs, Ipswich, MA, USA) following the manufacturer instructions. All plasmids used in this study are listed in Supplemental Table S1, whereas all primers used in the construction of the cassettes containing short or long flanking regions are listed in Supplemental Table S2. For transformation, DNA cassettes containing long flanking regions and DNA cassettes without any flanking regions were amplified from the respective vectors using the primers M13-F and M13-R. PCR products obtained from all cassettes, cassettes without and with short and long flanking regions, were purified using the Wizard SV Gel and PCR clean-up system (Promega, Madison, WI, USA) and used for transformation as indicated below.

RNA guide design and formation of the ribonucleoprotein complex

RNA guides (gRNAs) were designed using the software sgRNAcas9 v2.0 (Xie et al. 2014). All RNAs were based on the genome sequence of *B. bruxellensis* AWRI2804 (Roach and Borneman 2020). gRNAs were synthesized by Integrated DNA Technologies (Coralville, IA, USA) as CRISPR RNAs (crRNAs, Table 1). crRNA is combined with the transactivating crRNA (tracrRNA) to form the gRNA. Formation of the gRNA and the ribonucleoprotein (RNP) complex was performed as described by Grahl et al. (2017). Briefly, 2 μL of crRNA and 2 μL of tracRNA were mixed, diluted to a final volume of 50 μL, and incubated 95 °C for 5 min to form the gRNA. Alt-R S.p. Cas9 nuclease 3NLS (60 μM) (Integrated DNA Technologies, Coralville, IA, USA) was diluted to 4 μM (1.5 μL of nuclease in 21 μL final volume). A volume of 3.6 μL of cooled gRNA was then mixed with 3 μL of diluted nuclease and incubated at room temperature for 5 min to form the RNP complex. For each transformation, 6.6 μL of RNP complex was used.

Transformation and confirmation of positive transformants

B. bruxellensis strains were transformed by electroporation following the protocol described by Miklenic et al. (2015). For each transformation reaction, 6 μL of DNA cassette (28–56 ng/μL) and 6.6 μL of RNP complex were mixed thoroughly with electrocompetent cells (50 μL) and incubated on ice for 5 min. After transformation and overnight incubation at 28 °C with shaking (140 rpm), samples were spread on plates containing the appropriate antibiotic. Plates were then incubated at 28 °C and transformants were visible after 5–7 days. Confirmation of positive transformants was performed by PCR with primers complementary to the DNA cassette and to the up- and down-stream sequences of the targeted ORF, as described previously (Kutyna et al. 2014). DNA was extracted from transformant colonies according to the method of Looke et al. (2011) using deep well 96-well plates. Transformants with a potentially deleted *URA3* gene were replica plated on minimal media with and without uracil, colonies not able to grow without uracil and which show a positive PCR confirmation were considered positive transformants. Similarly, transformants with a potentially deleted *ADE1* gene were replica plated on minimal media containing a limited amount of adenine, pink colonies showing a positive PCR confirmation were considered positive transformants. Transformants were confirmed by sequencing the corresponding gene locus. All strains generated in this study are listed in Supplemental Table S3.

Sulfite tolerance assay

Sulfite tolerance was assessed as described by Varela et al. (2019) in 96-well microplates sealed with Breathe-Easy sealing membranes (Diversified Biotech, Dedham, MA, USA). Briefly, strains were inoculated in a 96-well microplate containing 200 μL of YPD medium as a starter culture and incubated at 28 °C for 2 days. A volume of 5 μL of starter culture was then inoculated into 195 μL of MM5 and grown for 2 days at 28 °C. For the assay 5 μL of MM5 culture was inoculated into 195 μL of MM5 supplemented with different concentrations of a sterile potassium metabisulfite (PMS) solution (20 g/L). Plates were incubated at 28 °C and OD_{600} measured every 4 h for 3 days using a Tecan infinite M200 plate reader (Tecan Group Ltd., Zurich, Switzerland). Each strain was replicated four times per sulfite concentration. Free SO_2 was measured in minimal media supplemented with freshly prepared PMS using the aspiration/titration method (Rankine and Pocock 1970); these values were then used to estimate the concentration of molecular SO_2. Unless specified, SO_2 concentrations mentioned henceforth refer to molecular SO_2.

Genome sequencing and bioinformatic processing

Chromosomal DNA was isolated using the Gentra Puregene Yeast/Bact. kit (Qiagen, Hilden, Germany) with some modifications. After washing the cell pellet, cells were resuspended in cell lysis solution and transferred into screw cap tubes containing 200 μL of zirconia/silica beads (0.5 mm, Daintree Scientific, Tasmania, Australia). Cells were then disrupted in

Table 1 crRNAs designed in this study

Name	Target	Sequence
ADE1_S22	*ADE1*	5′-CCATCTACCAAAGCTGAATT-3′
URA3_S21	*URA3*	5′-GGAAACTACATCTGAACCTA-3′
SSU1_S33	*SSU1*	5′-CCAATAGGTATTCTCGGCCA-3′

a Precellys Evolution homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) at 8000 rpm with 6 cycles of 30 s of homogenizing with 30 s of rest between cycles. Samples were then incubated at room temperature for a few minutes and the Gentra Puregene Yeast/Bact. protocol resumed. Whole genome sequencing was performed using the MinION platform (Oxford Nanopore Technologies, Oxford, UK). Sequencing libraries were prepared using the Native Barcoding Kit 1D (EXP-NBD104) in combination with the Ligation Sequencing Kit (SQK-LSK109) and loaded into a FLO-MIN106 R9 flow cell. Fast5 files were base called and demultiplexed using Guppy v.3.2.1 (Oxford Nanopore Technologies, Oxford, UK) and the 'hac' model. Only reads with a minimum *q* score of 7 were retained for further analyses. Genome assembly of the AWRI2804 haploid transformant strains was performed using Canu v.1.7.1 (Koren et al. 2017) and polished with nanopolish v.0.11.2 (https://github.com/jts/nanopolish).

For the haplotype-phased assembly of the reference and transformant AWRI1613 strains, an initial assembly run with a corrected-out coverage of 200 and a corrected error rate of 0.15 was performed in order to obtain a smashed haploid assembly. Assemblies were then cleaned for secondary haplotigs using Purge Haplotigs (Roach et al. 2018) and phased using Longshot v.0.3.3 (Edge and Bansal 2019). The consensus tool of the BCFtools package v.1.9 (Li 2011) was used to obtain haplotype phased consensus sequences. Haplotypes were then combined and reads were mapped using Minimap2 v.2.17 (Li 2018) to obtain haplotype-specific reads. Reads mapping to each specific haplotype were extracted and re-assembled independently. Each haplotype assembly was then cleaned for minor secondary haplotigs using Purge Haplotigs (Roach et al. 2018) and polished with nanopolish v.0.11.2 (https://github.com/jts/nanopolish). The reference genome of AWRI1613 was finally polished with Illumina 2 × 100 bp reads using Pilon v.1.23 (Walker et al. 2014)

Genomes were annotated using AUGUSTUS v.3.2.3 (Stanke and Waack 2003). Coverage profiles were obtained by mapping reads back to the assemblies of the transformant strains and reference genomes using Minimap2 v.2.17 (Li 2018). Sequencing reads included in this study and the reference genome of AWRI1613 are available in NCBI under Bioproject PRJNA636495.

Results

CRISPR-Cas9 enables targeted gene deletion

CRISPR-based editing tools have been successfully applied for genome engineering in several non-conventional species, where they have been shown to stimulate HR via the production of double-stranded breaks (Cai et al. 2019; Shapiro et al. 2018). However, these systems generally rely on CRISPR components being produced in vivo (from plasmid-based expression systems), and the development of species-specific CRISPR plasmids remains a key hurdle in the genetic manipulation of many non-conventional species. Recently, an expression-free CRISPR system, which uses purified CRISPR protein and guide RNA, was used to modified the genome of several *Candida* species (Grahl et al. 2017), opening the way for the application of CRISPR in yeast species that lack well-defined plasmid expression systems.

B. bruxellensis is one such species that lacks a documented plasmid-based expression system. An expression-free CRISPR-Cas9 system was therefore used to target two genes that display prominent mutant phenotypes when deleted or disrupted, *URA3* (5-FOA resistance, uracil auxotrophy) and *ADE1* (pink coloration, adenine auxotrophy), as a proof-of-concept for the applicability of this technology in this species. Prior to transformation, RNA-protein complexes (ribonucleoprotein particles, RNPs) were formed by co-incubating the crRNA designed to the targeted ORF and the tracrRNA (Grahl et al. 2017). Targeted deletion of *URA3* and *ADE1* was assessed using DNA cassettes flanked by either short (60 bp) or long (1 kb) regions of homology and also with a marker cassette lacking flanking regions as a negative control. All DNA cassettes were used on their own, or in combination with RNPs (Table 2).

For both target genes, none of the colonies transformed only with the *natMX* cassette (without homologous flanking regions) displayed the combination of nourseothricin resistance (nat^R) and auxotrophy for either uracil (URA^-) or

Table 2 Transformation efficiency for *URA3* and *ADE1* deletions

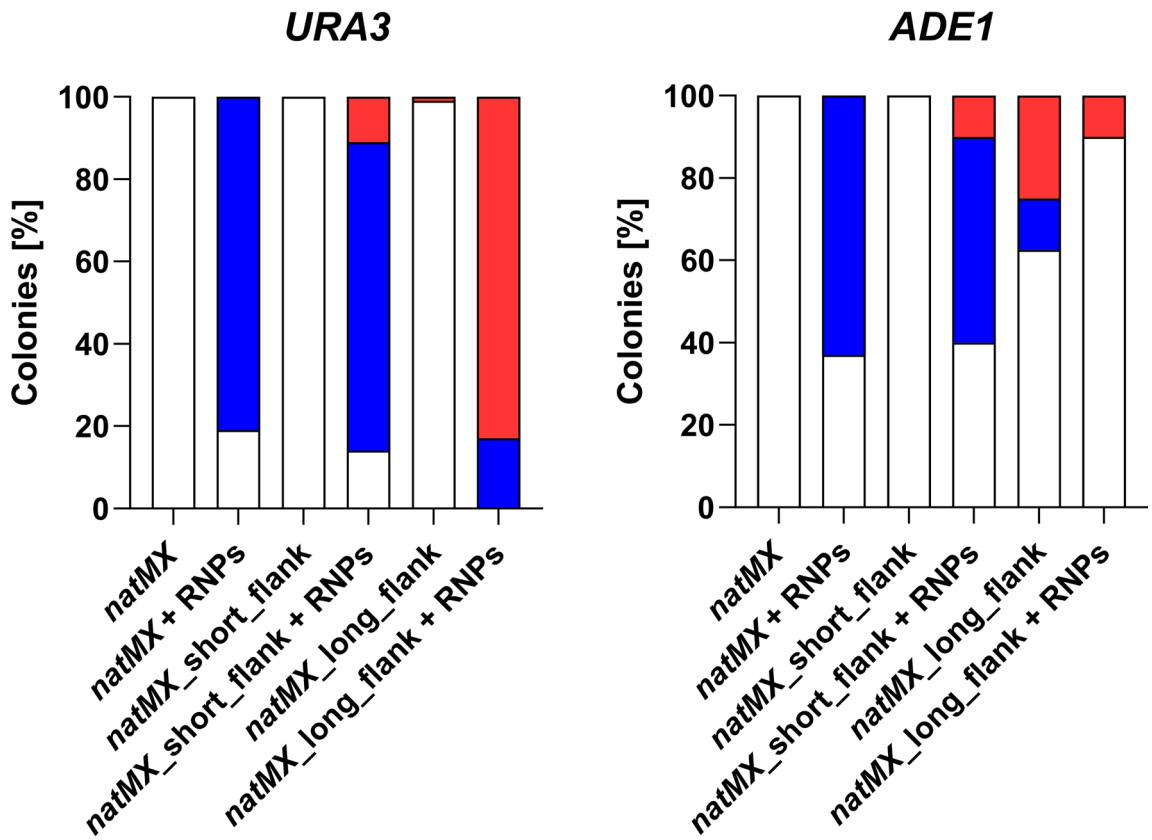
Gene	Cassette	RNPs	Positive colonies (positive/total)
URA3	*natMX*	−	0% (0/104)
URA3	*natMX*	+	0% (0/104)
URA3	*natMX*_short_flank[a]	−	0% (0/104)
URA3	*natMX*_short_flank	+	11% (11/104)
URA3	*natMX*_long_flank[b]	−	1% (1/101)
URA3	*natMX*_long_flank	+	83% (10/12)
ADE1	*natMX*	−	0% (0/99)
ADE1	*natMX*	+	0% (0/104)
ADE1	*natMX*_short_flank	−	0% (0/101)
ADE1	*natMX*_short_flank	+	10% (10/100)
ADE1	*natMX*_long_flank	−	25% (2/8)
ADE1	*natMX*_long_flank	+	10% (1/10)

[a] *natMX* cassette containing short flanking regions (60 bp) up- and downstream to the targeted ORF

[b] *natMX* cassette containing long flanking regions (1 kb) up- and downstream to the targeted ORF

adenine (*ADE*$^-$) (depending on the marker used), which is indicative of the cassette integrating ectopically in the *B. bruxellensis* genome via NHEJ (Fig. 1). Transformation with the *natMX* cassette and RNPs produced a high frequency of colonies exhibiting both nat^R and the expected auxotrophic phenotype (80% for *URA*$^-$ and 60% for *ADE*$^-$); however, none of these auxotrophic colonies contained the specific deletion of the corresponding ORF that would be produced by homologous recombination. Instead, these colonies had non-specific insertions and deletions in the targeted ORF, presumably due to imprecise repair of the double-stranded breaks that were induced by the RNPs, in addition to at least one copy of the *natMX* cassette integrated ectopically in the genome. Transformation with the *natMX* cassette flanked by short homologous regions alone also failed to produce any nat^R colonies that also showed the expected auxotrophic phenotypes.

These findings suggest that similar to other non-conventional yeasts (Klinner and Schafer 2004; Wang et al. 2001), *B. bruxellensis* favors NHEJ for DNA repair, making targeted gene deletion, which relies on HR, very challenging in these species. However, the combination of the specific short-flanking cassette and RNP together enabled the isolation of several colonies (11% for *URA3* and 10% for *ADE1*) showing a clean deletion of the ORF and with a DNA sequence consistent with replacement via homologous recombination. In addition to those colonies that had a clean replacement of the target gene, many also showed an auxotrophic phenotype



Clonat^R	*URA*- or *ADE*-	Seq+
+	-	-
+	+	-
+	+	+

Fig. 1 *B. bruxellensis* transformants after targeting the genes *URA3* and *ADE1*. Cells were transformed with the *natMX* cassette without flanking regions (*natMX*), with the *natMX* cassette containing short flanking regions (*natMX*_short_flank) or long flanking regions (*natMX*_long_flank). Colonies showed only resistance to the antibiotic marker indicating random integration, antibiotic resistance, and a clear phenotype indicating deletion or disruption of the targeted ORF, or antibiotic resistance, a clear phenotype and confirmed deletion of the ORF

due to partial deletion of the ORF, indicating that the homologous recombination machinery in *B. bruxellensis* is inefficient even when combined with the CRISPR-Cas9 system.

While very short homology (50 bp) is sufficient for HR in *S. cerevisiae* (Cai et al. 2019), very long stretches of homology (over 1 kb) are often needed in other species (Klinner and Schafer 2004; Löbs et al. 2017; Wang et al. 2001). For *B. bruxellensis*, transformation with the *natMX* cassette flanked by long homologous regions showed results that varied depending on the targeted gene (Fig. 1). For *URA3*, only one nat^R/URA^- colony (from 100 in total) showed a clean deletion of the ORF, whereas for *ADE1*, two of the nat^R/ADE^- transformants (from only 8 total) displayed the expected deletion. The long flanking regions therefore appear to provide some stimulation of endogenous HR in *B. bruxellensis.*

For *URA3*, most colonies transformed with combination of the long flanking cassette and specific RNP (10 out of 12 tested) showed a clean deletion of the gene, whereas only one colony (10 tested) showed a clean deletion of *ADE1*. Although more than 100 colonies were obtained per transformation for many of the treatments, only a limited number of strains were obtained for the long cassette transformation targeting *ADE1* and for the long cassette and RNP transformations targeting both genes (Table 2). The reason behind these phenomena is not clear, but it was reproducible under the transformation conditions tested.

Two nat^R/URA^- and two nat^R/ADE^- transformants were genome sequenced to confirm the structure of deletion locus, as well as ensuring against any additional ectopic copies of the cassette being present elsewhere in the genome. Long-read sequencing allowed a highly contiguous assembly of each strain, including a complete assembly of the region surrounding the targeted locus and confirmed a clean replacement of *URA3* (Fig. 2 a and b) and *ADE1* (Fig. 3 a and b) with the *natMX* cassette in all transformants. Read coverage of the *natMX* cassette locus confirmed that only one copy of the cassette was present in the genome of the nat^R/URA^- (Fig. 2 c and d) and nat^R/ADE^- (Fig. 3 c and d) transformants, although double coverage for the *TDH1*pr region (Figs. 2d and 3d) was observed due to the endogenous version of this promoter region that exists upstream of the native *TDH1* gene.

Targeted gene deletion of SSU1

As transformation with DNA cassettes containing short flanking regions and RNPs showed approximately 10% efficiency at the proof-of-concept stage, we decided to target *SSU1*, the main gene responsible for sulfite tolerance in yeast (Divol et al. 2012; Varela et al. 2019), following this approach in the haploid strain AWRI2804.

Following transformation, 30 nat^R colonies were assessed for the specific deletion of *SSU1* using PCR. Ten of these colonies showed the expected PCR fragments for gene deletion via HR and were considered positive transformants. Two of these were selected and were confirmed (by sequencing) as having a precise deletion of the ORF via integration of the *natMX* marker (Δ*SSU1*::*natMX*). Genome sequencing two Δ*SSU1*::*natMX* strains showed that both transformants had a clean deletion of *SSU1* and only one copy of the *natMX* cassette in the genome (Supplemental Fig. S1). Similar to *URA3* and *ADE1* deletants, double coverage for the *TDH1*pr region was also observed.

Given the importance of *SSU1* in tolerance to sulfite in other yeast species, the two Δ*SSU1*::*natMX* strains were evaluated in sulfite tolerance assays to determine if there was also a SO_2-sensitive phenotype that accompanied loss of this gene in *B. bruxellensis*. In media without SO_2, both the wild-type and Δ*SSU1*::*natMX* strains showed robust growth, although the parental strain AWRI2804 reached a slightly higher final OD_{600} value than either of the *SSU1* deletants (Fig. 4a). The presence of SO_2 in the medium resulted in significant inhibition in the proliferation of both Δ*SSU1*::*natMX* strains, with 0.09 mg/L mSO_2 completely inhibiting growth throughout the 60-h time course experiment (Fig. 4b). Consistent with previous reports, AWRI2804 was able to grow in media containing up to 0.24 mg/L mSO_2, well above the levels required to inhibit the Δ*SSU1*::*natMX* mutants (Supplemental Fig. S2) (Varela et al. 2019).

Targeted gene deletion in a diploid background

While *B. bruxellensis* AWRI2804 is a haploid strain, most strains of *B. bruxellensis* exist as either diploids or triploids (Avramova et al. 2018). To evaluate the possibility of simultaneously deleting multiple alleles in *B. bruxellensis*, targeted deletion of *URA3* was attempted in a diploid strain (AWRI1613). Transformation of AWRI1613 with the Δ*URA3*::*natMX* cassette flanked by short homology regions and RNPs failed to produce any colonies showing a nat^R/URA^- phenotype or the expected PCR results that would indicate correct integration of the cassette at the *URA3* locus. However, transformation with the combination of the *URA3* long-flanking cassette and RNPs delivered three nat^R colonies (96 tested), which also showed a PCR fragment pattern consistent with the correct integration of marker at *URA3* via HR. However, none of these three nat^R colonies also displayed uracil auxotrophy, suggesting that only one of the *URA3* alleles was targeted. Indeed, sequencing of the deleted *URA3* locus showed that two of the AWRI1613 Δ*URA3*::*natMX* transformants had a deletion in one allele of *URA3*, while the third transformant had the integration that occurs in the alternate *URA3* allele. These results indicate that while simultaneous multiple allele deletion in *B. bruxellensis* was not able to be demonstrated, heterozygous deletion was possible using a DNA cassette with long flanking regions. The fact that both

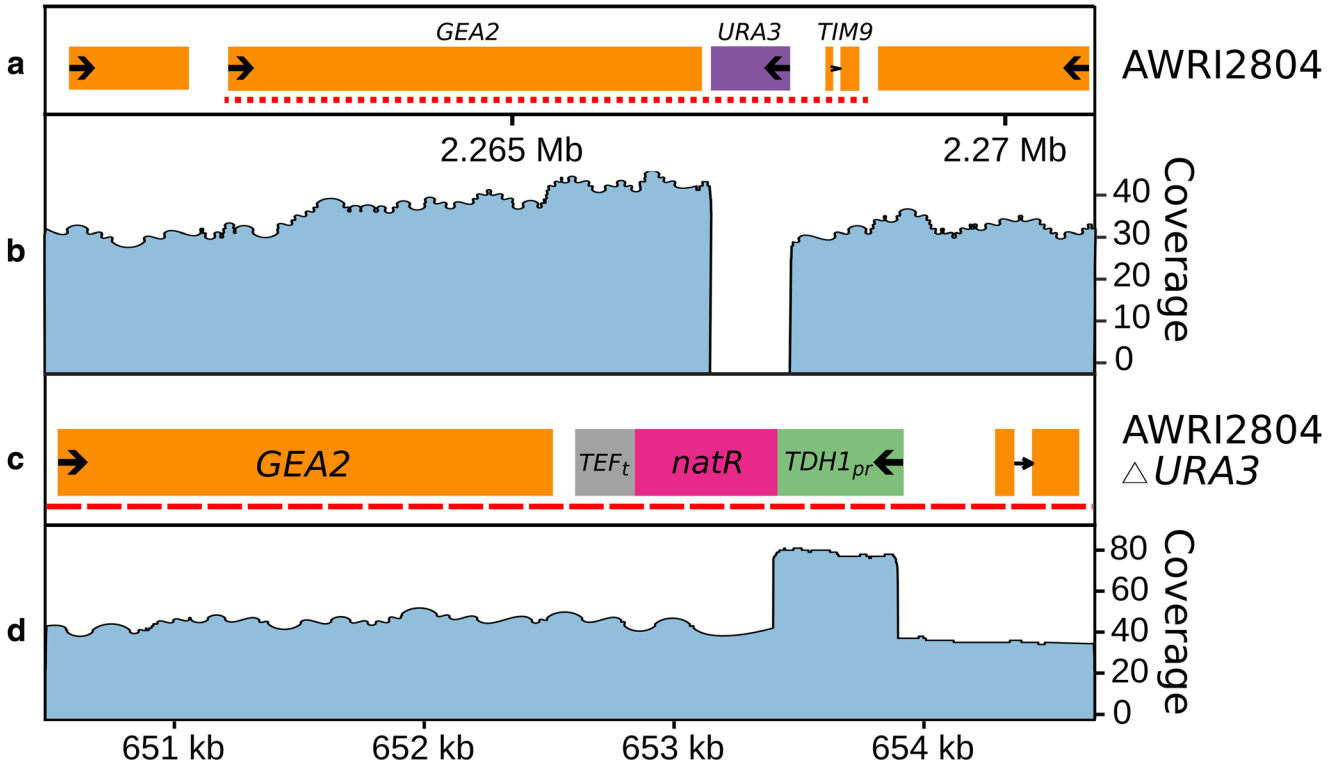


Fig. 2 Genome sequence for AWRI2804 and AWRI2804 Δ*URA3* transformants. **a** Genes near the *URA3* locus in AWRI2804, dotted line indicates genomic region in **c**. **b** Coverage of AWRI2804 Δ*URA3* genome sequencing reads mapped to region in **a**. **c** Location of the *natMX* cassette in the *URA3* locus for AWRI2804 Δ*URA3*. **d** Coverage of AWRI2804 Δ*URA3* genome sequencing reads mapped to region in **c**

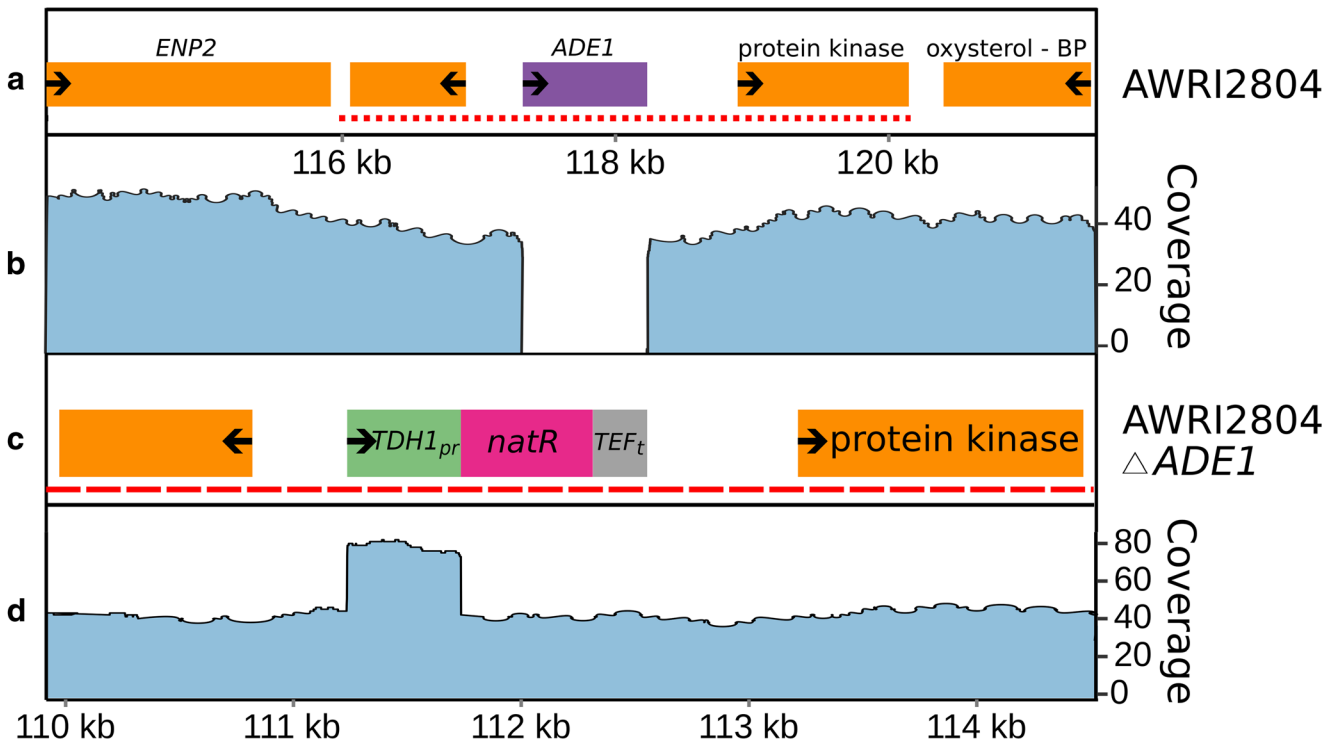


Fig. 3 Genome sequence for AWRI2804 and AWRI2804 Δ*ADE1* transformants. **a** Genes near the *ADE1* locus in AWRI2804, dotted line indicates genomic region in **c**. **b** Coverage of AWRI2804 Δ*ADE1* genome sequencing reads mapped to region in **a**. **c** Location of the *natMX* cassette in the *ADE1* locus for AWRI2804 Δ*ADE1*. **d** Coverage of AWRI2804 Δ*ADE1* genome sequencing reads mapped to region in **c**

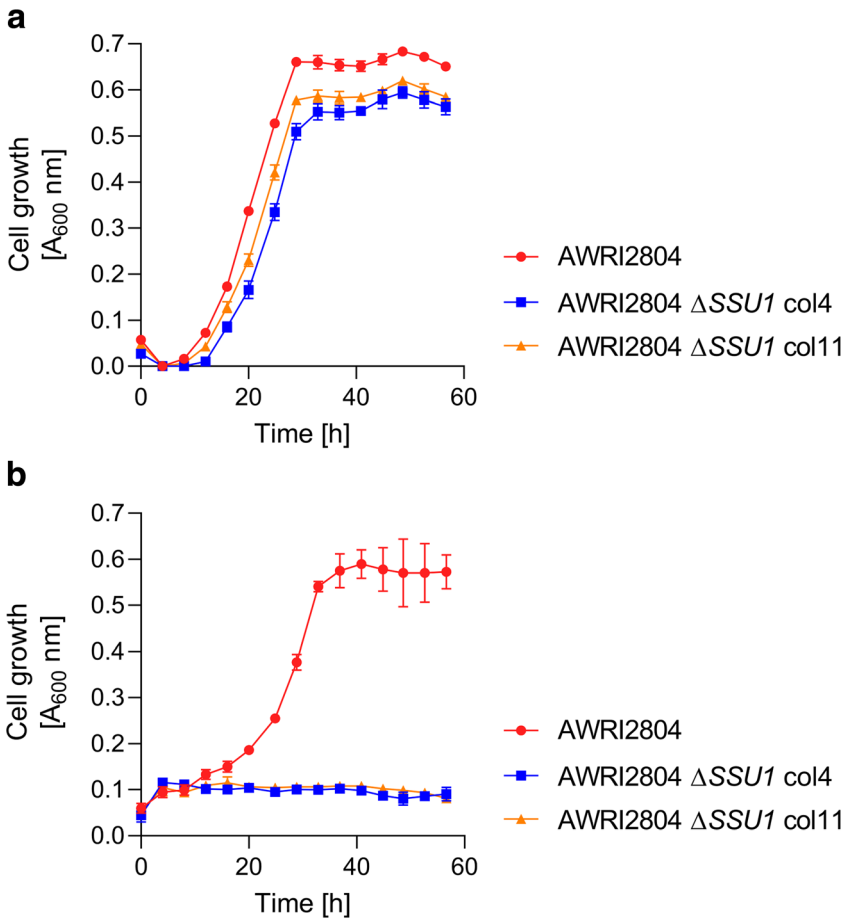


Fig. 4 Sulfite tolerance for AWRI2804 and two transformants lacking the gene *SSU1*, AWRI2804 Δ*SSU1* col4 and AWRI2804 Δ*SSU1* col11 in media without SO_2 (**a**) and with 0.09 mg/L mSO_2 (**b**)

URA3 haplotypes were deleted in the different AWRI1613 Δ*URA3* transformants also suggests that there was no bias for any specific haplotype, despite nucleotide variation between the alleles. Genome sequencing two AWRI1613 Δ*URA3*::*natMX* transformants confirmed the clean deletion of the different *URA3* alleles and one integrated copy of the *natMX* cassette (data not shown).

While it was not possible to simultaneously target both alleles in AWRI1613, as multiple selectable markers exist for *B. bruxellensis* (Varela et al. 2018), the possibility of stepwise deletion of the two alleles remained as an alternative methodology. A homozygous deletion of the *URA3* locus was therefore attempted by transforming one of the heterozygous AWRI1613 Δ*URA3::natMX* strains with the combination of a long-flanking Δ*URA3::kanMX* marker cassette and RNPs. Nearly 20% of the transformants (81 tested) were shown to be phenotypically $G418^R/URA^-$ and also had the correct integration of the *kanMX* cassette at the remaining wild-type allele of *URA3* (as assessed by PCR). Genome sequencing of two of the potential double deletion strains confirmed a clean replacement of the second copy of *URA3* with the *kanMX* cassette, while leaving the original Δ*URA3::natMX* unchanged (Fig. 5). No additional copies of either the Δ*URA3::natMX or* Δ*URA3::kanMX* were found in the genome. However, several SNPs were observed 500 bp up- and downstream of the *URA3* ORF when comparing the double deletion strains and AWRI1613, particularly for *URA3* haplotype 1 (H1). This reflects the divergence of this haplotype compared to the sequence of AWRI2804, which was used for designing the long homology regions of the integration cassette, with the AWRI2804 flanking sequence being integrated along with the marker gene via the homologous recombination process.

Discussion

Despite the increasing biotechnological potential of *B. bruxellensis* and the significant economic losses caused by this yeast for fermented beverages industries, targeted genome editing tools have not been reported for this species. Here, we report the use of an expression-free CRISPR-Cas9 system to perform targeted gene deletion in *B. bruxellensis*. In a haploid strain, deletion frequency of the genes *URA3* and *ADE1* depended on the targeted ORF and the length of the regions flanking the ORF used in the integration cassette. Similar deletion frequency was observed for both genes when using a DNA cassette containing short flanking regions and

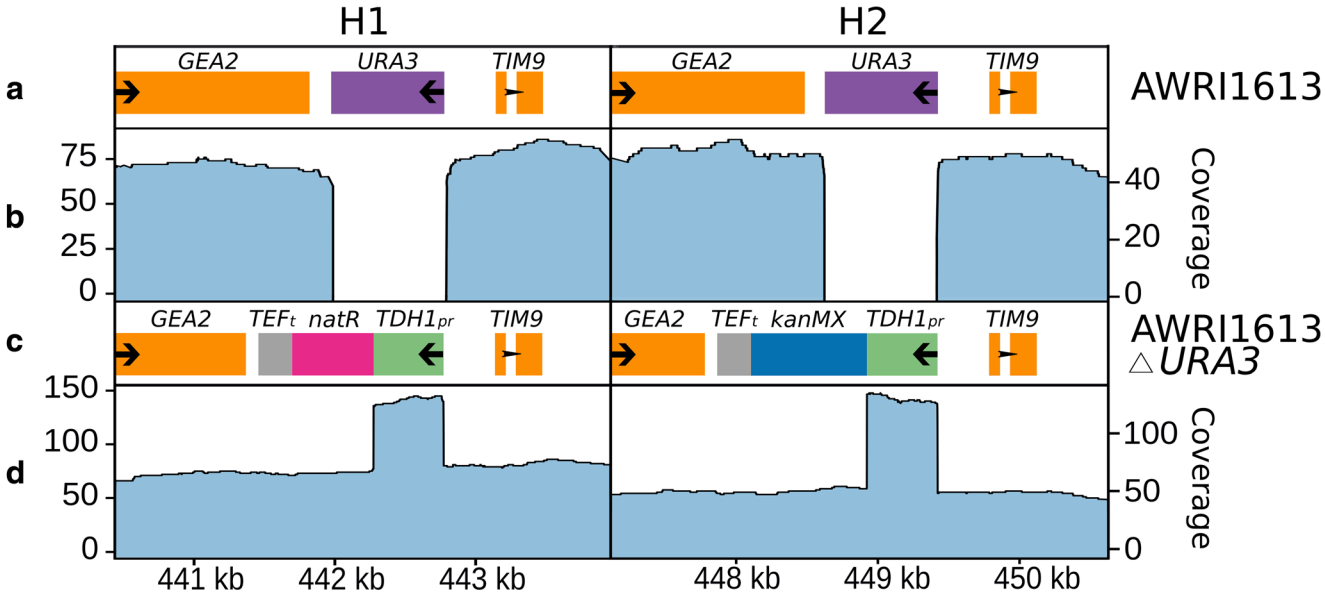


Fig. 5 Genome sequence for AWRI1613 and AWRI1613 Δ*URA3* double deletion transformants. **a** Genes near the *URA3* locus in AWRI1613 for both haplotypes (H1 and H2). **b** Coverage of AWRI1613 Δ*URA3* genome sequencing reads mapped to regions in **a**. **c** Location of the *natMX* and *kanMX* cassettes in the *URA3* locus for AWRI1613 Δ*URA3*. **d** Coverage of AWRI1613 Δ*URA3* genome sequencing reads mapped to region in **c**

RNPs. From a practical and economical perspective, this strategy is very attractive, especially when only a limited number of transformants are required.

In *S. cerevisiae*, the high efficiency of the HR repair mechanism makes the generation of heterozygous alleles by standard CRISPR/Cas9 techniques impossible, and therefore, alternative approaches have been developed (EauClaire and Webb 2019). Similarly, HR is responsible for large-scale loss of heterozygosity in *S. cerevisiae* after the Cas9-mediated introduction of a double-strand break (de Vries et al. 2019). However, in this study, only heterozygous gene deletions were observed following a single round of transformation in a diploid strain and multiple rounds, and selectable markers were required to produce homozygous deletions.

Although time-consuming for studying loss of gene function in polyploid strains, the ability to generate heterozygous deletions in *B. bruxellensis* has its advantages. The generation of heterozygous alleles can be key in understanding gene structure and function, and at the same time it should be possible to create different allelic copy numbers in polyploid strains. Recently, several *SSU1* haplotypes have been described in *B. bruxellensis* which confer different sulfite tolerance when expressed in a *S. cerevisiae SSU1* null mutant (Varela et al. 2019) and the *SSU1* deletion in AWRI2804 performed in this study has now confirmed the role of *SSU1* in conferring sulphite tolerance in *B. bruxellensis*. Deleting each *SSU1* haplotype separately in a range of strains would therefore enable the relative efficiency and the effect of allele copy number to be assessed with respect to the different sulfite tolerances observed between *B. bruxellensis* strains.

While the deletion of multiple alleles in a diploid strain was shown to require step-wise transformations and multiple selectable markers, this should be readily applicable for the study of triploid isolates as three selectable markers have been previously shown to be applicable in this system (Varela et al. 2018). It also may be possible to extend the number of genes that can be addressed in any one strain through various marker recycling strategies that have been established in other yeast systems.

In summary, a combination of an expression-free CRISPR-Cas9 system with DNA cassettes tailored for *B. bruxellensis* have enabled to successfully perform targeted gene deletion in this species. This approach was employed to delete genes in haploid and diploid *B. bruxellensis* strains. Given the highly complex genetic structure of this species, as some strains are diploid while others are allotriploid, resulting from independent hybridization events between different diploid parent strains and unknown donors of the additional haploid genome (Borneman et al. 2014), we expect that the methodology described here will be a valuable tool to study the differences between *B. bruxellensis* strain genetic clusters.

Authors' contributions CV and AB conceived and designed the research. CB and CV conducted the transformation experiments. CO performed the genome sequencing, assembly and annotation. CV and AB prepared the manuscript. All authors read and approved the submitted manuscript.

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Data availability All strains and plasmids used in this study are available from the Australian Wine Research Institute Wine Microorganism Culture Collection. Sequencing reads included in this study and the reference genome of AWRI1613 are available in NCBI under Bioproject PRJNA622385.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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