KU80 deletion does not improve homologous recombination in *Brettanomyces bruxellensis*

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Running title: *KU80* deletion in *B. bruxellensis*

Key words: *Brettanomyces*, wine, gene deletion, yeast

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ABSTRACT

Integration of heterologous DNA in yeast can proceed either by non-homologous end joining (NHEJ), with the foreign DNA inserted into the genome at a random locus, or by homologous recombination (HR), with the foreign DNA targeted to a specific site in the genome by homology to the site of interest. Gene targeting is key for studying gene function and investigating cell physiology, and usually relies on HR. Although HR is the dominant mechanism in *S. cerevisiae*, NHEJ is the dominant repairing mechanism in *Brettanomyces bruxellensis*, a microorganism with broad biotechnological potential and responsible for significant economic losses during the production of fermented beverages. One strategy to increase HR in NHEJ-proficient strains is to delete the genes responsible for NHEJ. Thus, we attempted to produce a homologous-recombining *B. bruxellensis* strain by deleting the *KU80* gene. Stimulation of homologous recombination was then tested by targeting two different genomic loci for deletion via HR in the Δ*KU80* strain. While it was possible to obtain large numbers of transformants, homologous recombination was no observed. These results indicate that *KU80* deletion does not improve homologous recombination in *B. bruxellensis*.

INTRODUCTION

A common approach to stable gene modification in yeast is the integration of heterologous DNA cassettes into the genome. This integration can be mediated by either non-homologous end joining (NHEJ) or homologous recombination (HR), two independent mechanisms that compete with each other (Shrivastav, et al. 2008). While NHEJ mediates the integration of a DNA fragment into essentially random sites within the host genome, HR targets the fragment to a specific site in the genome via stretches of DNA homology between the heterologous fragment and the site of interest (Löbs, et al. 2017). Targeted gene deletion (targeted gene knockout), which involves the precise removal/substitution of specific genomic loci is an essential tool for studying gene function and investigating cell physiology, and is completely dependent on efficient HR. While HR is the dominant mechanism in *S. cerevisiae* (Fraczek, et al. 2018), providing the means for rapid genomic engineering, NHEJ is the dominant repairing

mechanism in several non-conventional yeast species (Klinner and Schafer 2004, Wang, et al. 2001).

One strategy to increase HR in NHEJ-dominant strains is to mutate the genes responsible for NHEJ. This is often performed using targeted gene deletion, however given the dominance of NHEJ in these species, very long homologous flanking sequences are required to increase HR and large numbers of transformants must be screened to find the rare, targeted events from within the background of random integrants. Accordingly, deletion of genes encoding the NHEJ components such as *KU70* or *KU80* resulted in enhanced gene targeting efficiency in several non-conventional yeast and fungal species of biotechnological interest, including *Candida glabrata* (Ueno, et al. 2007), *Cryptococcus neoformans* (Goins, et al. 2006), *Hansenula polymorpha* (Saraya, et al. 2012), *Kluyveromyces lactis* (Kooistra, et al. 2004), *Kluyveromyces marxianus* (Choo, et al. 2014), *Penicillium chrysogenum* (Snoek, et al. 2009), *Pichia pastoris* (Näätsaari, et al. 2012), *Pichia stipitis* (Maassen, et al. 2008), *Rhodosporidium toruloides* (Koh, et al. 2014) and *Yarrowia lipolytica* (Kretzschmar, et al. 2013).

Although *Brettanomyces bruxellensis* is well known for the spoilage of fermented beverages including, wine, beer and cider (Curtin, et al. 2015, Varela and Borneman 2017), this yeast species has several significant attributes for industrial applications (Conterno, et al. 2006, Curtin, et al. 2015, de Barros Pita, et al. 2013, Reis, et al. 2014). In addition, *B. bruxellensis* has a positive role during the production of Belgian lambic and gueze 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). Despite the biotechnological potential and the significant economic impact of this species, targeted genome editing tools in *B. bruxellensis* have only been reported recently (Varela, et al. 2020). Thus, targeting gene deletion in this species was possible using an expression-free CRISPR-Cas9 system (Varela, et al. 2020).

In this work, we describe the development of a *B. bruxellensis* strain lacking the *KU80* gene with the aim of enhancing homologous recombination in this species.

MATERIALS AND METHODS

Strain and media

The haploid *B. bruxellensis* AWRI2804 strain (UC Davis collection UCD2041) was obtained from the Australian Wine Research Institute (AWRI) Wine Microorganism Culture Collection (WMCC). 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; 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 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 target specific genes. Cassettes targeting the genes *KU80* and *SSU1* contained long flanking regions (1.0-1.5 kb) and were constructed as follows. The up- and down-stream regions of the targeted ORF were cloned in plasmid pMA-TDH1pr-natMX and pMK-T-TDH1pr-kanMX, respectively. Up- and down-stream regions to *KU80* and *SSU1* were synthesised by Invitrogen GeneArt (ThermoFisher Scientific, Massachusetts, USA) and cloned using BamHI/XbaI sites for upstream regions and SpeI for downstream regions. A cassette targeting *URA3* contained short flanking regions (60 bp) and was obtained by amplifying the kanMX cassette from the plasmid pMK-T-TDH1pr-kanMX (Varela, et al. 2018). Primers containing 60 bp flanking regions corresponding to up- and down-stream regions outside *URA3* were used to amplify

this cassette. All plasmids used in this study are listed in Table 1, while all primers used in the construction of the cassettes are listed in Table 2. For transformation, DNA cassettes were amplified from the respective vectors using the primers M13-F and M13-R. PCR products obtained from all cassettes were purified using the Wizard® SV Gel and PCR clean-up system (Promega, Madison, USA) and used for transformation as indicated below.

Transformation and confirmation of positive transformants

B. bruxellensis strains were transformed by electroporation following the protocol described by Miklenic et al. (2015). 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. (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.

Genome sequencing and bioinformatic processing

Chromosomal DNA was isolated using the Gentra Puregene Yeast/Bact. kit (Qiagen, Hilden, Germany) as described previously (Varela, et al. 2020). Whole genome sequencing was performed using the MinION platform (Oxford Nanopore Technologies, Oxford, UK) as described previously (Varela, et al. 2020). Genome assembly was performed using Canu v.1.7.1 (Koren, et al. 2017) and polished with Nanopolish v. 0.11.2. Sequencing reads included in this study are available in NCBI under Bioproject PRJNA827945.

RESULTS AND 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 only been reported recently (Varela, et al. 2020). *B. bruxellensis*, similar to other non-conventional yeast (Klinner and Schafer 2004, Wang, et al. 2001), favours NHEJ

for DNA repair, making targeted gene deletion, which relies on HR, very challenging in these species. While very short homology (50 bp) is sufficient for HR in *S. cerevisiae* (Cai, et al. 2019), often very long homology sequences (over 1 kb) are needed in other species (Klinner and Schafer 2004, Löbs, et al. 2017, Wang, et al. 2001). One strategy to increase HR in non-conventional yeast is the deletion of some of the genes involved in NHEJ (Dudasova, et al. 2004, Koh, et al. 2014, Verbeke, et al. 2013).

To attempt to engineer a HR-dominant *B. bruxellensis* strain, the *B. bruxellensis* ortholog of the *KU80* gene was targeted for gene deletion using a low-efficiency, long-flanking targeting DNA cassette. From PCR screening of 110 transformants, only one putative deletant strain was obtained. Whole genome sequencing of this transformant indicated that a copy of the *nat*MX cassette had integrated into the *KU80* locus with no other copies of the cassette detectable in the genome (Figure 1). This strain was named AWRI5224.

To evaluate the suitability of the Δ*KU80* strain as a platform for HR-dominant engineering, this strain was transformed with DNA cassettes which targeted either the gene *URA3* or *SSU1*. While *URA3* was targeted with a cassette containing short flanking regions, *SSU1* was targeted with a cassette containing long flanking regions. Despite targeting these two different genomic loci and screening over 100 colonies for each locus, it was not possible to detect homologous recombination at either of the two targeted loci.

Although deletion of *KU80* has been shown to improve gene deletion frequency in other yeast species (Choo, et al. 2014, Colombo, et al. 2014, Kretzschmar, et al. 2013, Ueno, et al. 2007), this was not the case in *B. bruxellensis*, indicating that the DNA repair machinery in this species is not easy to manipulate. It is possible that targeting other genes involved in NHEJ, such as *YKU70*, *NEJ1* or *DNL4* (Abdel-Banat, et al. 2010, Nambu-Nishida, et al. 2017, Valencia, et al. 2001) may increase HR in *B. bruxellensis*.

FUNDING

The AWRI, a member of the Wine Innovation Cluster in Adelaide, is supported by Australia's grape growers and winemakers through their investment body Wine Australia with matching funds from the Australian Government.

CONFLICT OF INTEREST

The Authors declare no conflict of interest.

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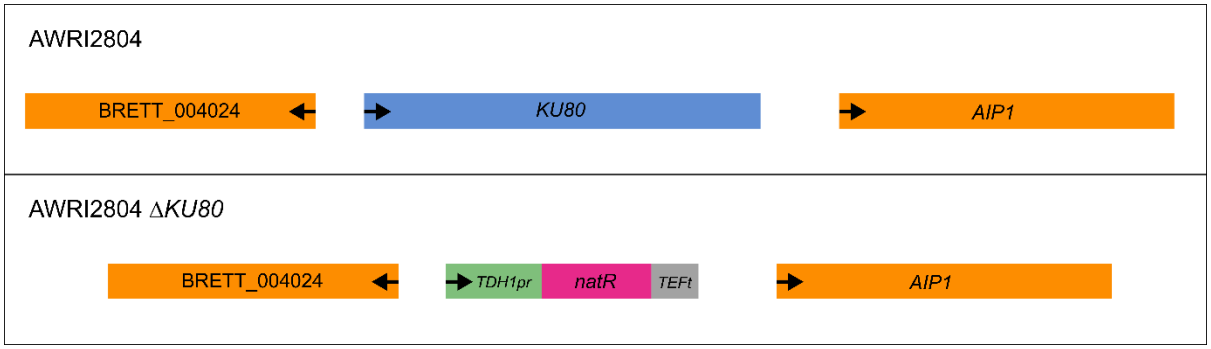

Figure 1. *B. bruxellensis KU80* locus in AWRI2804 and AWRI2804 Δ*KU80*.

Table 1. Plasmids used in this study.

Plasmid	Description/genotype	Source/reference
pMA-TDH1pr-natMX	natR flanked by the *B. bruxellensis TDH1* promoter and the *TEF2* terminator, ampicillin^R	(Varela, et al. 2018)
pMK-T-TDH1pr-kanMX	kanR flanked by the *B. bruxellensis TDH1* promoter and the *TEF2* terminator, kanamycin^R	(Varela, et al. 2018)
pMK-T-TDH1pr-kanMX KU80 up/down	kanR cassette flanked by 1.2 kb flanking regions up- and down-stream of *KU80*	This study
pMA-TDH1pr-natMX SSU1 up/down	natR cassette flanked by 1.5 kb flanking regions up- and down-stream of *SSU1*	This study

267 **Table 2.** Primers used in this study.

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Primer	Sequence (5’ → 3’)	Aim
KU80up_BamHI-F	catg ggatcc atctgctctttttcctcctgtttat	Amplify upstream region of *KU80* for cloning
KU80up_XbaI-R	catg tctaga tttcggttgcttatgttagttaatg	Amplify upstream region of *KU80* for cloning
KU80down_SpeI-F	catg actagt aaagcaaggagaaatcggcacagga	Amplify downstream region of *KU80* for cloning
KU80down_SpeI-R	catg actagt gggagtcaacggaccaaagacggac	Amplify downstream region of *KU80* for cloning
SSU1up_BamHI-F	catg ggatcc taaatgcaagcgtcacctgc	Amplify upstream region of *SSU1* for cloning
SSU1up_XbaI-R	catg tctaga tgtttgcttgctctgctcg	Amplify upstream region of *SSU1* for cloning
SSU1down_SpeI-F	catg actagt atttgcaatgactaacccgc	Amplify downstream region of *SSU1* for cloning
SSU1down_SpeI-R	catg actagt aggcggaaatgttaaccacg	Amplify downstream region of *SSU1* for cloning
M13-F	gtaaaacgacggccagtg	Amplify DNA cassettes for transformation
M13-R	ggaaacagctatgaccatg	Amplify DNA cassettes for transformation
TDH1pr-R	accgtcatactgatttgagcc	Anneals on the *TDH1* promoter, used to confirm positive transformants
marker-F	ttcgcatctgggcagatgatgtcga	Anneals on the *TEF2* terminator, used to confirm positive transformants
ampKU80-F	ccactcatctctaaagccttc	Used to confirm positive transformants for *KU80* deletion
ampKU80-R	ggttttaccactcattttccagg	Used to confirm positive transformants for *KU80* deletion

ampSSU1-F	taaatgcaagcgtcacctgc	Used to confirm positive transformants for *SSU1* deletion
ampSSU1-R	cgtgtcgttatgggtcagat	Used to confirm positive transformants for *SSU1* deletion
coURA3-F	gacatctgcttctgctcaac	Used to confirm positive transformants for *URA3* deletion
coURA3-R	atgcgttgcgagtgaaaatg	Used to confirm positive transformants for *URA3* deletion

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