J. Microbiol. Biotechnol. (2013), **23**(5), 674–680
http://dx.doi.org/10.4014/jmb.1211.11047
First published online March 13, 2013
pISSN 1017-7825 eISSN 1738-8872

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Genetic Transformation of the Yeast *Dekkera/Brettanomyces bruxellensis* with Non-Homologous DNA

Miklenić, Marina, Anamarija Štafa, Ana Bajić, Bojan Žunar, Berislav Lisnić, and Ivan-Krešimir Svetec*

Laboratory for Biology and Microbial Genetics, Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, 10000 Zagreb, Croatia

Received: November 19, 2012 / Revised: December 29, 2012 / Accepted: December 31, 2012

Yeast *Dekkera/Brettanomyces bruxellensis* is probably the most common contaminant in wineries and ethanol production processes. The considerable economic losses caused by this yeast, but also its ability to produce and tolerate high ethanol concentrations, make it an attractive subject for research with potential for industrial applications. Unfortunately, efforts to understand the biology of *D. bruxellensis* and facilitate its broader use in industry are hampered by the lack of adequate procedures for delivery of exogenous DNA into this organism. Here we describe the development of transformation protocols (spheroplast transformation, LiAc/PEG method, and electroporation) and report the first genetic transformation of yeast *D. bruxellensis*. A linear heterologous DNA fragment carrying the *kanMX4* sequence was used for transformation, which allowed transformants to be selected on plates containing geneticin. We found the spheroplast transformation method using 1 M sorbitol as osmotic stabilizer to be inappropriate because sorbitol strikingly decreases the plating efficiency of both *D. bruxellensis* spheroplast and intact cells. However, we managed to modify the LiAc/PEG transformation method and electroporation to accommodate *D. bruxellensis* transformation, achieving efficiencies of 0.6–16 and 10–20 transformants/μg DNA, respectively. The stability of the transformants ranged from 93.6% to 100%. All putative transformants were analyzed by Southern blot using the *kanMX4* sequence as a hybridization probe, which confirmed that the transforming DNA fragment had integrated into the genome. The results of the molecular analysis were consistent with the expected illegitimate integration of a heterologous transforming fragment.

*Corresponding author
Phone: +385-1-4836013; Fax: +385-1-4836016;
E-mail: iksvetec@pbf.hr

Key words: *Dekkera/Brettanomyces bruxellensis*, transformation, integration of foreign DNA, genetic manipulation, non-*Saccharomyces* yeast, osmotic stabilization

The ability to genetically modify organisms has been one of the major advances in science and technology in the last 30 years. The first eukaryote modified by integration of foreign DNA into the genome was baker's yeast *Saccharomyces cerevisiae* [21]. Besides its wide range of industrial applications, *S. cerevisiae* is one of the most informative model organisms in fundamental research of eukaryote genetics and molecular biology. Many other yeasts have also been successfully transformed with exogenous DNA, even though the efficiency of transformation varies greatly among species [23, 25, 40]. However, a vast majority of microorganisms of industrial and scientific interest have not been genetically transformed. One such microorganism is the yeast *Dekkera/Brettanomyces bruxellensis*.

D. bruxellensis is probably the most common cause of wine spoilage, generating considerable economic losses in the wine industry [12]. It produces volatile phenols (4-ethylphenol and 4-ethylguaiacol), giving the wine a specific Brett aroma [3–5]. Although a very mild Brett aroma is desirable in some types of wines, the presence of *D. bruxellensis* usually results in unpleasant flavors described as the smell of horse sweat, burnt plastic, and mice [26]. *D. bruxellensis* produces and tolerates high ethanol concentrations (up to 14%), grows under anaerobic conditions, and exhibits the Crabtree effect [31]. In industrial ethanol production, it has so far been regarded mostly as a contaminant that can replace *S. cerevisiae* starter cultures [8, 9]. However, *D. bruxellensis* has high potential for industrial use in ethanol production from renewable sources (*e.g.*, lignocellulosic hydrolysate), which requires potential to ferment various sugars (hexoses and pentoses) and the ability to adapt to

toxic compounds in the hydrolysate [2, 13]. Recently, the genome sequencing project of two strains of *D. bruxellensis* has been completed [7, 29], and genetic transformation of this yeast could facilitate in-depth research leading to better contamination control and construction of more suitable strains for large-scale bioethanol and acetic acid production [2, 11] and other applications.

There are several commonly used methods for genetic transformation of yeasts: spheroplasts transformation, LiAc/PEG transformation, and electroporation [14]. We successfully modified the LiAc/PEG method and electroporation to achieve *D. bruxellensis* transformation, with the transformation efficiency ranging from 0.6 to 20 transformants/μg. The transforming DNA was a linear heterologous fragment, which had to be integrated into the genome *via* illegitimate integration in order to establish a stable transformant. The fragment carried the *kanMX4* sequence, allowing transformants to be selected on plates containing geneticin. All putative transformants were confirmed by Southern blot analysis and, as expected, the results suggest a random (illegitimate) integration of the transforming DNA into the genome. Additionally, the attempt to transform spheroplasts revealed a high sensitivity of both spheroplasts and intact cells of *D. bruxellensis* to sorbitol.

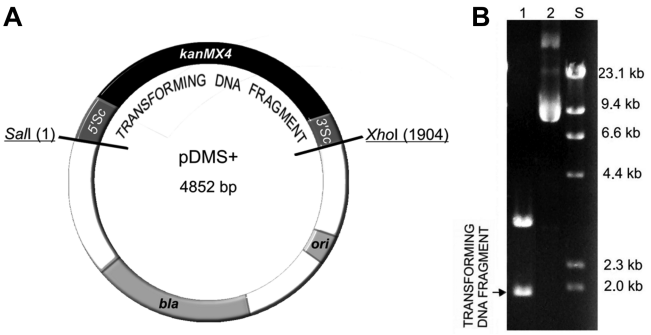

Fig. 1. Plasmid pDMS+ (4,852 bp) carries the *kanMX4* sequence responsible for geneticin resistance, and regions 5'*Sc* and 3'*Sc*, which represent 220 bp of the 5' end and 165 bp of the 3' end of YMR224C ORF from *S. cerevisiae*, respectively.
(**A**) The central 1,694 bp of YMR224C ORF were replaced with the *kanMX4* sequence. The plasmid origin of replication in *E. coli* (*ori*) and ampicilin resistance gene (*bla*) are also indicated. The plasmid was cut with *Sal*I and *Xho*I to create a linear fragment used for transformation (1,903 bp). (**B**) Lane 1, Plasmid pDMS+ cut with *Xho*I and *Sal*I ; Lane 2, uncut plasmid; Lane S, λ/*Hin*dIII ladder.

Materials and Methods

Yeast Strains

Dekkera/Brettanomyces bruxellensis Van der Walt strain CBS2499 was used in this study. The CBS2499 strain originates from France and was isolated from wine. The genome sequence of strain CBS2499 is available at http://genome.jgi.doe.gov/ [19]. The strain was obtained from Centraalbureau voor Schimmelcultures, CBS. Yeast *Saccharomyces cerevisiae* strain BY4741 (*MAT***a** *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0) was used as a control in experiments in which the influence of sorbitol on plating efficiency was investigated. The strain was obtained from the European *Saccharomyces cerevisiae* Archive for Functional Analysis, EUROSCARF.

Media and Growth Conditions

Standard complete complex medium (YPD) and synthetic complete medium (SCM) were used for the cultivation of *S. cerevisiae* [32]. Complex GYP medium (10 g/l bacto-peptone, 5 g/l yeast extract (both supplied by Biolife, Italy), 20 g/l glucose (Fluka, Switzerland)) and synthetic complete medium (identical to SCM for *S. cerevisiae*, but containing 2 g/l instead of 5 g/l of ammonium sulfate (Alkaloid, Macedonia)) were used for *D. bruxellensis* cultivation. Geneticin-resistant transformants were selected on GYP plates containing 200 μg/ml of G418 (Sigma Chemical Co. Ltd, USA). Both yeasts were grown at 28°C.

Preparation of Transforming DNA Fragment

The transforming DNA (1.9 kb) was obtained by digesting the plasmid pDMS+ (Fig. 1A) with restriction endonucleases *Xho*I and *Sal*I (Fig. 1B). The plasmid was isolated from an overnight culture of *Escherichia coli* (DH5α *fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*) using a Qiagen Maxi Prep Kit (Qiagen, Germany). Following thermal inactivation of restriction endonucleases, DNA was used for transformation. When it was prepared for electroporation, DNA was additionally precipitated using ammonium acetate and ethanol and dissolved in sterile deionized water.

LiAc/PEG Transformation Procedure

The LiAc/PEG transformation procedure for *S. cerevisiae* described by Giets and Schiestl [16] was modified for *D. bruxellensis* transformation. The culture was grown up to 2–2.5×10^7 cells/ml (determined by counting in Thoma counting chamber), which corresponded to OD_{600} of 0.60–0.75, washed, and aliquoted as described by Gietz and Schiestel [16]. Three different transformation procedures were tested: (1) Cells were resuspended in 150 μl of 0.1 M lithium acetate (Sigma Chemical Co., Germany) and incubated for 40 min at 28°C. Then the lithium acetate was removed and the cells were resuspended in 360 μl of transformation mixture and incubated for 40 min at 42°C. (2) Cells were resuspended in 360 μl of transformation mixture and incubated for 40 min at 28°C and then for another 40 min on 42°C. (3) The cells were resuspended in 360 μl of transformation mixture and immediately subjected to heat shock for 40 min at 42°C. In all cases, the transformation mixture was prepared as described by Giets and Schiestl [16]. After heat shock, the cells were centrifuged to remove the transformation mixture and resuspended in 2 ml of GYP medium, incubated for 6 h at 28°C/200 rpm on an orbital shaker, and inoculated onto GYP plates containing geneticin. Plates were incubated at 28°C for 5 days. During the course of the procedure, the cells were always centrifuged at ~ 2,500 ×*g*/5 min/22°C and were never vortexed, but mixed gently using a micropipette.

Spheroplast Transformation Procedure

The spheroplast transformation procedure described by Štafa *et al.* [38] was used. Regeneration of spheroplasts was carried out in 5 ml of liquid SCM containing 1 M sorbitol (Kemika, Croatia) at 28°C for

12 and 24 h. After regeneration, the cells were inoculated on GYP plates containing geneticin and incubated at 28°C.

Electroporation Procedure

One colony of yeast *D. bruxellensis* was inoculated into 200 ml of GYP medium and incubated at 28°C and 200 rpm on an orbital shaker. The culture was grown up to 0.8–1.0×10^7 cells/ml (determined by counting in Thoma counting chamber), which corresponded to OD_{600} of 0.25–0.35, and chilled on ice. The culture was centrifuged for 5 min at ~2,500 ×*g* and 4°C and washed twice in 100 ml of ice-cold sterile deionized water. Several variations of competent cell preparation and electroporation procedures were tested, so cells were distributed into three cuvettes and were either (1) washed with 25 ml of ice-cold sterile deionized water, (2) washed with 25 ml of 0.1 M lithium acetate, or (3) resuspended in 25 ml of 0.1 M lithium acetate and incubated for 40 min at room temperature. In each case, cells were subsequently washed twice in 25 ml of ice-cold sterile deionized water, and then in 10 ml of ice-cold osmotic stabilizer (0.5 M sorbitol, 0.75 M sucrose, and 1 M sucrose were tested). Finally, the cells were resuspended in the respective osmotic stabilizer to the final concentration of 10^9 cells/ml. A volume of 40 µl of cell suspension was gently mixed with 2 µl (0.1 µg) of DNA dissolved in deionized water and incubated on ice for 10 min. The mixture was then transferred to a chilled 0.2 cm electroporation cuvette (Bio-Rad) and placed in a Bio-Rad MicroPulser electroporation apparatus (10 µF capacitator, 600 Ω resistor in parallel with the sample cuvette, and 30 Ω resistor in series with the sample cuvette). Voltage was set to either 1.5 or 1.8 kV, whereas the time parameter was kept constant (5 ms). Immediately after the pulse, the cells were gently resuspended in 1 ml of ice-cold GYP medium containing an appropriate osmotic stabilizer (0.5 M sorbitol, 0.75 M sucrose (Gram-mol, Croatia), or 1 M sucrose). The suspension was transferred to 5 ml test tubes and incubated for 6 h at 28°C without shaking. The cells were then inoculated onto GYP plates containing geneticin and were incubated at 28°C for 5 days.

Testing the Influence of Sorbitol on *D. bruxellensis*

D. bruxellensis strain CBS2499 and *S. cerevisiae* strain BY4741 were grown in complete complex medium to stationary phase. A volume of 100 µl of the appropriate 10-fold serial dilutions (10^{-3}, 10^{-4}, 10^{-5}) was mixed with 50 ml of SCM (maintained at 42°C) containing 0, 0.5, or 1 M sorbitol and immediately poured into two Petri dishes. In addition, 100 µl (10^{-5} dilution) of cells was inoculated on a GYP plate, and another 100 µl (10^{-5} dilution) of cells was mixed with GYP agar, also maintained at 42°C, and immediately poured into two Petri dishes. After a 2-day (*S. cerevisiae*) or 5-day (*D. bruxellensis*) incubation the colonies were counted and the percentage of surviving cells was calculated in respect to the cell count on GYP plates.

Testing the Stability of Transformants

Transformants were streaked on GYP plates containing geneticin. Two colonies of each of the 12 transformants that were tested were picked and inoculated into 3 ml of liquid GYP medium. Cultures were grown at 28°C and 200 rpm on an orbital shaker until the stationary growth phase was reached. Serial dilutions were made in GYP medium and cells were spread on GYP plates (approximately 100 cells per plate). After a 5-day incubation period, colonies were counted and replica plated on GYP plates containing geneticin. After 2 days, colonies grown on GYP with geneticin were counted and the stability of transformants was expressed as a percentage of colonies that retained geneticin resistance.

Molecular Analysis of Transformants

Transformants selected on plates containing geneticin were streaked on fresh GYP plates containing geneticin. A single colony was inoculated into 3 ml of liquid GYP medium containing 200 µg/ml of geneticin and incubated for 5 days on an orbital shaker at 28°C/ 200 rpm. Genomic DNA was isolated using the protocol for yeast DNA isolation described by Sambrook and Russel [32]. The entire amount of isolated DNA was cut with *Xho*I. After restriction, DNA was precipitated using ammonium acetate and ethanol and dissolved in TE buffer (pH 8.0). Electrophoresis was run on a 0.8 % agarose gel and 1× TAE buffer. Southern blotting was performed as described by Gjuračić and Zgaga [17]. A DIG-labeled *kanMX4* sequence was synthesized by PCR using primers 5'-aaaaaataggcgtatcacgag-3' and 5'-tcgatgataagctgtcaaac-3'. Genomic DNA of bacteriophage λ, which was used as a size marker, was DIG-labeled by random priming (DIG DNA Labeling and Detection Kit; Roche Applied Science, Germany). The hybridization solution contained both *kanMX4* and bacteriophage λ DNA probe.

RESULTS AND DISCUSSION

The aim of this work was to develop a method for transformation of *Dekkera bruxellensis*. Hence, a transformation system harboring an appropriate selectable marker was required. Since the genetics of *D. bruxellensis* is very poorly investigated, origins of replication and autonomously replicating elements have not been identified yet. Therefore, we used a linear transforming fragment, which had to be integrated into the genome *via* non-homologous (illegitimate) recombination in order to transform the cell. The transforming fragment (Fig. 1) carried a *kanMX4* sequence, which is often used as a selectable marker in the transformation of eukaryotic cells, making them resistant to the aminoglycoside antibiotic geneticin. By inoculating the cells on a series of plates containing 0–50 µg/ml of geneticin, we determined that *D. bruxellensis* showed sensitivity to geneticin in the same concentration range as the reference BY4741 *S. cerevisiae* strain (no growth above 30 µg/ml; data not shown). Since non-homologous recombination was required to produce stable transformants and this process can be associated with partial degradation of DNA ends [6, 24, 28, 39], selectable marker was flanked with DNA originated from yeast *S. cerevisiae* (Fig. 1A). As well as *kanMX4*, these flanking regions were heterologous to the *D. bruxellensis* genome (share less than 60% of homology) and served to protect the selectable marker in case of degradation of DNA ends.

Three most frequently used methods for yeast transformation are the LiAc/PEG method, spheroplast transformation, and electroporation procedure. These methods differ significantly by the treatment that causes the yeast cells to become

Table 1. Transformation efficiencies and numbers of transformants obtained in two independent experiments using the LiAc/PEG transformation procedure.

Experiment	Incubation (40 min/28°C)	Mass of the transforming DNA (μg)	Number of transformants (Transformation efficiency [μg^{-1}])					Total number of transformants
			No carrier	Double-stranded carrier	Single-stranded carrier	Double-stranded sonicated carrier	Single-stranded sonicated carrier	
1	0.1 M LiAc	1.6	0 (0)	1 (0.6)	5 (3.1)	3 (1.9)	0 (0)	9
2	LiAc/PEG/DNA	0.25	0 (0)	1 (4)	3 (12)	4 (16)	0 (0)	8
		1.1	/	/	2 (1.8)	/	/	2

The two experiments differ by the treatment prior to heat shock.

competent and by the manner of DNA uptake. Hence, we decided to test and, if necessary, modify all three methods to find the most appropriate for *D. bruxellensis*.

Transformation of *D. bruxellensis* Using LiAc/PEG Method

The LiAc/PEG transformation method is based on increasing the permeability of the cell wall by treating the cells with 0.1 M lithium acetate and 50% PEG 4000, and exposing the cells to a prolonged heat shock. Commonly, carrier DNA is added in a large quantity (up to 100 μg) into the transformation mixture [15]. However, the carrier DNA is sometimes omitted from the transformation mixture because DNA that has no selectable marker can also enter the cell and integrate into the genome, which can lead to undesirable and uncontrollable genetic events.

We found that the LiAc/PEG transformation procedure can be used to transform *D. bruxellensis* (Table 1). The basis for the procedure described in this paper is a commonly used protocol for *S. cerevisiae* transformation by Gietz and Shiestl [16] to which several modifications were necessary. Most importantly, we found that prior to heat shock, a 40 min incubation at 28°C in 0.1 M LiAc or in the entire transformation mixture (LiAc/PEG/DNA) is needed. Two independent transformation experiments, in which cells resuspended in the transformation mixture were immediately subjected to heat shock, yielded 0 and 1 transformant per experiment (data not shown). In addition, we tested several variations of transformation procedure using ss and ds carrier DNA, both sonicated and non-sonicated, and omitting carrier DNA from the transformation mixture. Even though the number of the obtained transformants is too low to make a definite conclusion, we would suggest using ss or sonicated ds carrier DNA, which are also most effective in *S. cerevisiae* transformation [29, 33]. The highest transformation efficiency achieved during these transformation experiments was 16 transformants/μg. Adding more than 0.25 μg of DNA per sample did not increase the total number of transformants, suggesting that all competent cells were saturated with DNA. Therefore, when more transformants are required, the number of individual samples should be increased.

Spheroplast Transformation: Sorbitol Decreases Plating Efficiency of Spheroplasts and Intact Cells

The spheroplast transformation method is based on partially removing the cell wall by enzymatic digestion (using zymolyase), producing spheroplasts that presumably (in *S. cerevisiae*) take up the DNA by endocytosis [23]. Spheroplasts need to be handled in an osmotic stabilizer to avoid rupturing. For osmotic stabilization of *S. cerevisiae* and many other yeasts, 1 M sorbitol is used. We tested the spheroplast transformation procedure described by Štafa *et al*. [38] with appropriate modifications, but no transformants were obtained. During the course of the transformation procedure, we also performed a control of spheroplast regeneration efficiency, which indicated that *D. bruxellensis* might be sensitive either to 1 M sorbitol or to the temperature of the medium in which the spheroplasts were inoculated (42°C). To examine the influence of sorbitol and brief heat

Table 2. The influence of heat shock and sorbitol concentration on the plating efficiency of intact cells of yeasts *D. bruxellensis* and *S. cerevisiae.*

Plating method – medium	Plating efficiency (%)	
	D. bruxellensis	*S. cerevisiae*
Complex medium	100.0	100.0
Complex medium (42°C)	99.2	57.8
Synthetic complete medium (42°C; 0 M sorbitol)	93.0	67.5
Synthetic complete medium (42°C; 0.5 M sorbitol)	75.0	63.0
Synthetic complete medium (42°C; 1 M sorbitol)	7.4	51.5

shock on *D. bruxellensis* in more detail, *D. bruxellensis* and *S. cerevisiae* cultures were grown to the stationary phase and inoculated on complex medium plates, in complex medium maintained at 42°C, and in synthetic complete medium maintained at 42°C containing 0, 0.5, and 1 M sorbitol. The average plating efficiencies from two independent experiments are shown in Table 2. Data presented in Table 2 clearly demonstrate the negative effect of sorbitol on the plating efficiency of *D. bruxellensis* cells, with the percentage of colony forming units in 1 M sorbitol being less than 10%. It is possible that 1 M sorbitol is hypertonic or has a negative effect on the cells of *D. bruxellensis per se*. Therefore, it would be interesting to determine the cell osmotic pressure and investigate the properties of the cell wall as well as sorbitol metabolism in *D. bruxellensis*. Additionally, the use of other osmotic stabilizers might lead to development of a spheroplast transformation procedure. Moreover, it is possible that sorbitol induces a state in which cells remain viable but become non-culturable, which was recently reported for *D. bruxellensis* exposed to sulfite stress [35]. Altogether, we conclude that the spheroplast transformation procedure using 1 M sorbitol as the osmotic stabilizer is inappropriate for this yeast.

Electroporation of *D. bruxellensis*

The electroporation transformation procedure uses brief high voltage pulses to create pores in the cell surface that allow macromolecules to enter the cell. During the electroporation and subsequent incubation of cells, osmotic stabilization in a low-ionic-strength stabilizer is needed. Usually, appropriate concentrations of sorbitol or sucrose are used. Since 1 M sorbitol decreases the plating efficiency of *D. bruxellensis*, we used 0.5 M sorbitol, 0.75 M sucrose, or 1 M sucrose. Several procedures for preparing electrocompetent cells were tested by varying the amount of time the cells were exposed to 0.1 M lithium acetate (see Materials and Methods) and the electric field strength used for electroporation. The transformants were obtained using the following procedures: 1 M sucrose osmotic stabilization without LiAc treatment, 0.75 M sucrose stabilization with brief LiAc treatment, and 0.5 M sorbitol stabilization with 40 min LiAc treatment. In all cases, transformation was achieved using the electric field strength of 9 kV/cm, whereas 7.5 kV/cm, which is recommended for *S. cerevisiae* in our experimental setup, did not yield any transformants. The efficiency of transformation achieved by electroporation was 10–20 transformants/μg (1–2 transformants per sample), which is comparable to the efficiency achieved by the LiAc/PEG method.

Molecular Analysis and Stability of Transformants

All putative transformants were verified by Southern blot analysis using the labeled *kanMX4* sequence as a hybridization probe. A typical membrane is shown in Fig. 2. Evidently, the genomic DNA of the transformants contains the *kanMX4* sequence, whereas the control sample (genomic DNA of yeast that was not transformed) shows no signal when hybridized with the *kanMX4* probe. As expected, bands hybridized with the *kanMX4* probe appeared in random positions, which is consistent with illegitimate integration of the heterologous transforming DNA.

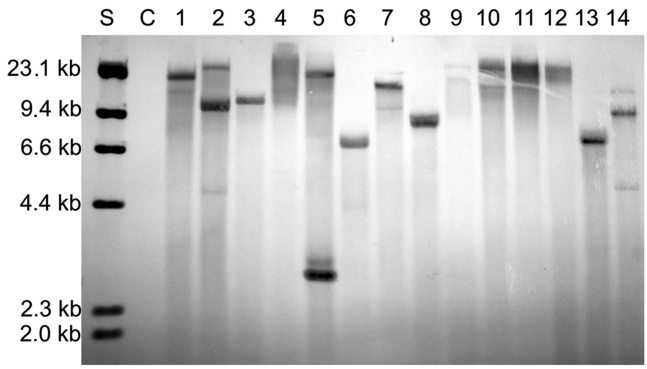

Fig. 2. Molecular analysis of transformants by Southern blotting. Lane S, λ/*Hin*dIII ladder. Fragment sizes are indicated on the left. Lanes 1–14, Genomic DNA of transformants; Lane C, control sample, genomic DNA of non-transformed yeast *D. bruxellensis*. Genomic DNA of *D. bruxellensis* was digested with *Xho*I. The hybridization solution contained DIG-labeled *kanMX4* and DNA of bacteriophage λ.

Since *D. bruxellensis* is known to be genetically unstable, in addition to molecular analysis we determined the genetic stability of 12 randomly chosen transformants (see Materials and Methods). The stability of inheritance of geneticin resistance ranged from 93.6% to 100%, suggesting that neither the transformation procedure nor illegitimate integration of the transforming DNA initiated further genetic instability or formation of highly unstable genetic elements carrying the transforming sequence.

Future Perspectives

In this paper, we described protocols for transformation of *D. bruxellensis* using a linear heterologous transforming DNA fragment. The transformation efficiencies achieved by the LiAc/PEG method and electroporation ranged from 0.6 to 20 transformants per μg of DNA. For comparison, Schiestl and Petes [34] reported the first transformation of *S. cerevisiae* by non-homologous linear DNA with an efficiency of 0.75 transformants/μg. It is expected that future efforts in genetic manipulation of *D. bruxellensis* will be focused on improving the efficiency of the transformation methods *per se* by optimizing individual parameters of transformation, such as the cell growth phase, as well as achieving targeted integration of transforming DNA using a similar approach that is routinely used in *S. cerevisiae* [20, 36]. As in *S. cerevisiae,* the efficiency of gene targeting might be influenced by the length of the

flanking homologies [18]. Nevertheless, since transforming DNA might not undergo homologous recombination, as is often the case in non-*Saccharomyces* yeasts [25, 40], the efficiency of gene targeting in *D. bruxellensis* might be considerably low regardless of the length of the terminal homology. An alternative approach to increase the efficiency of targeted integration might be the targeting of transforming DNA in palindromic and palindrome-like sequences, since such DNA motifs have been shown to be highly recombinogenic [10, 27] and they stimulate plasmid integration in *S. cerevisiae* [37]. Once the targeted modification and the construction of knock-out mutants becomes possible, further increase of transformation efficiency might be achieved by inactivation of *S. cerevisiae* homologs *pde2*, *spf1*, *pmr1* [22], *sgs1*, and *exo1* [38], whereas the fidelity of gene targeting might be increased in *ku70* mutants [1]. Finally, the transformation efficiency might be improved by restriction enzyme mediated integration [34], which was successfully applied in several non-*Saccharomyces* species [25].

To conclude, we believe that the work presented in this article, in hand with the genome sequence, will encourage further improvement and development of procedures and methods that will enable targeted genetic manipulations and a thorough genetic characterization of yeast *Dekkera/Brettanomyces bruxellensis*.

References

1. Abdel-Banat, B. M., S. Nonklang, H. Hoshida, and R. Akada. 2010. Random and targeted gene integrations through the control of non-homologous end joining in the yeast *Kluyveromyces marxianus*. *Yeast* **27:** 29–39.
2. Blomqvist, J., E. South, I. Tiukova, M. H. Momeni, H. Hansson, J. Ståhlberg, *et al*. 2011. Fermentation of lignocellulosic hydrolysate by the alternative industrial ethanol yeast *Dekkera bruxellensis*. *Lett. Appl. Microbiol*. **53:** 73–78.
3. Chatonnet, P., D. Dubourdieu, J. N. Boidron, and M. Pons. 1992. The origin of ethylphenols in wines. *J. Sci. Food Agric.* **60:** 165–178.
4. Chatonnet, P., D. Dubourdieu, and J. N. Boidron. 1995. The influence of *Brettanomyces/Dekkera* spp. yeasts and lactic acid bacteria on the ethylphenol content of red wines. *Am. J. Enol. Vitic.* **46:** 463–468.
5. Chatonnet, P., C. Viala, and D. Dubourdieu. 1997. Influence of polyphenolic components of red wines on the microbial synthesis of volatile phenols. *Am. J. Enol. Vitic.* **48:** 443–448.
6. Chua, G., L. Taricani, W. Stangle, and P. G. Young. 2000. Insertional mutagenesis based on illegitimate recombination in *Schizosaccharomyces pombe*. *Nucleic Acids Res*. **28:** E53.
7. Curtin, C. D., A. R. Borneman, P. J. Chambers, and I. S. Pretorius. 2012. *De-novo* assembly and analysis of the heterozygous triploid genome of the wine spoilage yeast *Dekkera bruxellensis* AWRI1499. *PLoS One* **7:** e33840.
8. de Barros Pita, W., F. C. Leite, A. T. de Souza Liberal, D. A. Simões, and M. A. de Morais Jr. 2011. The ability to use nitrate confers advantage to *Dekkera bruxellensis* over *S. cerevisiae* and can explain its adaptation to industrial fermentation processes. *Antonie Van Leeuwenhoek*. **100:** 99–107.
9. de Souza Liberal, A. T., A. C. Basílio, A. do Monte Resende, B. T. Brasileiro, E. A. da Silva-Filho, J. O. de Morais, *et al*. 2007. Identification of *Dekkera bruxellensis* as a major contaminant yeast in continuous fuel ethanol fermentation. *J. Appl. Microbiol*. **102:** 538–547.
10. Farah, J. A., E. Hartsuiker, K. Mizuno, K. Ohta, and G. R. Smith. 2002. A 160-bp palindrome is Rad50.Rad32-dependent mitotic recombination hotspot in *Schizosaccharomyces pombe*. *Genetics* **161:** 461–468.
11. Freer, S. N., B. Dien, and S. Matsuda. 2003. Production of acetic acid by *Dekkera/Brettanomyces* yeasts under conditions of constant pH. *World J. Microbiol. Biotechnol.* **19:** 101–105.
12. Fugelsang, K. C. 1997. *Wine Microbiology*. Chapman and Hall New York, NY.
13. Galafassi, S., A. Merico, F. Pizza, L. Hellborg, F. Molinari, J. Piškur, and C. Compagno. 2011. *Dekkera/Brettanomyces* yeasts for ethanol production from renewable sources under oxygen-limited and low-pH conditions. *J. Ind. Microbiol. Biotechnol*. **38:** 1079–1088.
14. Gietz, R. D. and R. A. Woods. 2001. Genetic transformation of yeast. *Biotechniques* **30:** 816–820, 822–826, 28 passim.
15. Gietz, R. D., R. H. Schiestl, A. R. Willems, and R. A. Woods. 1995. Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. *Yeast* **11:** 355–360.
16. Gietz, R. D. and R. H. Schiestl. 2007. High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat. Protoc*. **2:** 31–34.
17. Gjuračić, K. and Z. Zgaga. 1996. Illegitimate integration of single stranded DNA in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **253:** 173–181.
18. Gray, M. and S. M. Honigberg. 2001. Effect of chromosomal locus, GC content and length of homology on PCR-mediated targeted gene replacement in *Saccharomyces*. *Nucleic Acids Res.* **29:** 5156–5162.
19. Grigoriev, I. V., H. Nordberg, I. Shabalov, A. Aerts, M. Cantor, D. Goodstein, *et al*. 2012. The genome portal of the Department of Energy Joint Genome Institute. *Nucleic Acids Res*. **40(Database issue):** 26–32.
20. Hastings, P. J., C. McGill, B. Shafer, and J. N. Strathern. 1993. Ends-in vs. ends-out recombination in yeast. *Genetics* **135:** 973–980.
21. Hinnen, A., J. B. Hicks, and G. R. Fink. 1978. Transformation of yeast. *Proc. Natl. Acad. Sci. USA* **75:** 1929–1933.
22. Kawai, S., T. A. Pham, H. T. Nguyen, H. Nankai, T. Utsumi, Y. Fukuda, and K. Murata. 2004. Molecular insights on DNA delivery into *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun*. **317:** 100–107.
23. Kawai, S., W. Hashimoto, and K. Murata. 2010. Transformation of *Saccharomyces cerevisiae* and other fungi: Methods and possible underlying mechanism. *Bioeng. Bugs* **1:** 395–403.
24. Kegel, A., P. Martinez, S. D. Carter, and S. U. Aström. 2006. Genome wide distribution of illegitimate recombination events in *Kluyveromyces lactis. Nucleic Acids Res.* **34:** 1633–1645.

25. Klinner, U. and B. Schäfer. 2004. Genetic aspects of targeted insertion mutagenesis in yeasts. *FEMS Microbiol. Rev.* **28:** 201–223.
26. Licker, J. L., T. E. Acree, and T. Henick-Kling. 1999. What is "Brett" (*Brettanomyces*) flavour? A preliminary investigation, pp. 96–115. *In* A. L. Waterhouse and S. E. Ebeler (eds.). *Chemistry of Wine Flavour*. American Chemical Society, Washington, DC.
27. Lisnić, B., I. K. Svetec, A. Štafa, and Z. Zgaga. 2009. Size-dependant palindrome-induced intrachromosomal recombination in yeast. *DNA Repair* **8:** 383–389.
28. Mezard, C. and A. Nicolas. 1994. Homologous, homeologous, and illegitimate repair of double-strand breaks during transformation of a wild-type strain and a *rad52* mutant strain of *Saccharomyces cerevisiae*. *Mol. Cell. Biol*. **14:** 1278–1292.
29. Piškur, J., Z. Ling, M. Marcet-Houben, O. P. Ishchuk, A. Aerts, K. LaButti, *et al*. 2012. The genome of wine yeast *Dekkera bruxellensis* provides a tool to explore its food-related properties. *Int. J. Food Microbiol*. **157:** 202–209.
30. Rose, M. D. 1987. Isolation of genes by complementation in yeast. *Methods Enzymol.* **152:** 481–504.
31. Rozpędowska, E., L. Hellborg, O. P. Ishchuk, F. Orhan, S. Galafassi, A. Merico, *et al*. 2011. Parallel evolution of the make-accumulate-consume strategy in *Saccharomyces* and *Dekkera* yeasts. *Nat. Commun*. **2:** 302.
32. Sambrook, J. and D. W. Russel. 2001. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbour Laboratory Press, 3th Ed, Cold Spring Harbour, NY.
33. Schiestl, R. H. and R. D. Gietz. 1989. High efficency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr. Genet.* **16:** 339–346.
34. Schiestl, R. H. and T. D. Petes. 1991. Integration of DNA fragments by illegitimate recombination in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **88:** 7585–7589.
35. Serpaggia, V., F. Remize, G. Recorbet, E. Gaudot-Dumas, A. Sequeira-Le Grand, and H. Alexandre. 2012. Characterization of the "viable but nonculturable" (VBNC) state in the wine spoilage yeast *Brettanomyces*. *Food Microbiol.* **30:** 438–447.
36. Svetec, I. K., A. Štafa, and Z. Zgaga. 2007. Genetic side effects accompanying gene targeting in yeast: The influence of short heterologous termini. *Yeast* **24:** 637–652.
37. Svetec, I. K., B. Lisnić, and Z. Zgaga. 2002. A 110 bp palindrome stimulates plasmid integration in yeast. *Period. Biol.* **104:** 421–424.
38. Štafa, A., I. K. Svetec, and Z. Zgaga. 2005. Inactivation of the *SGS1* and *EXO1* genes synergistically stimulates plasmid integration in yeast. *Food Technol. Biotechnol.* **43:** 103–108.
39. Tatebayashi, K., J. Kato, and H. Ikeda. 1994. Structural analyses of DNA fragments integrated by illegitimate recombination in *Schizosaccharomyces pombe*. *Mol. Gen. Genet*. **244:** 111–119.
40. Wang, T. T., Y. L. Choi, and B. H. Lee. 2001. Transformation systems of non-*Saccharomyces* yeasts. *Crit. Rev. Biotechnol*. **21:** 177–218.