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Short Communication

Codon optimization enables the Zeocin resistance marker's use in the ascomycete yeast *Debaryomyces occidentalis*

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Debaryomyces occidentalis (*Schwanniomyces occidentalis*) is a non-conventional yeast species with a large enzymatic potential because it secretes several kinds of extracellular enzymes, such as α-amylase (Dohmen et al., 1989), invertase (Klein et al., 1989), and glucoamylase (Dohmen et al., 1990). The α-amylase and glucoamylase genes are strongly induced in the presence of starch. Thus, two strong inducible promoters are available for heterologous enzyme expression (Piontek et al., 1998). Several selectable markers have been reported, including *LEU2* (Iserentant and Verachtert, 1995), *TRP5* (Dohmen et al., 1989), *URA3* (Ávaro-Benito et al., 2013; Klein and Roof, 1988) and *ADE2* (Ávaro-Benito et al., 2013; Klein and Favreau, 1988), that are capable of being used in *D. occidentalis*. Drug-resistance markers have also been used in *D. occidentalis* transformations. The phleomycin resistance-conferring gene is a suitable marker because of its DNA composition and codon usage, which influence expression. The DNA of *D. occidentalis* genes is very AT-rich (36% GC content), and a high percentage of GC-rich (47–62%) genes are not expressed (Janatova et al., 2003). The *Staphylococcus aureus* bleomycin (*Sa ble*) gene having an AT-rich composition conferred to the phleomycin resistance could, therefore, be expressed in *D. occidentalis* (Janatova et al., 2003).

Zeocin is a copper-chelated glycopeptide antibiotic related to bleomycin that causes cell death by intercalating into DNA and cleaving it. It is very toxic to a range of organisms, including mammalian and insect cells, as well as yeast, bacteria and plants. Resistance to Zeocin is conferred by the *Streptoalloteichus hindustanus* bleomycin gene (*Sh ble*), which binds and prevents Zeocin from interacting with DNA (Leiting and Noegel, 1991). The broad applicability of Zeocin resistance means that a single selectable marker can be used for several different cell types. Native and commercial *Sh ble* genes (Zeocin resistance genes) have high G+C compositions (69.9%), but our study showed that the native *Sh ble* gene was not functionally expressed in *D. occidentalis*. We speculated that the codon usage in the Zeocin resistance gene was not appropriate for *D. occidentalis*. Codon optimization is known to improve the expression of heterologous proteins (Gustafsson et al., 2004). A gene's sequence can influence protein expression in a number of ways, including affecting messenger RNA (mRNA) levels, translational velocities, initiation efficiencies, and consumption rates of charged tRNA (Gustafsson et al., 2012). In this study, we optimized the codon use in the Zeocin marker for expression in *D. occidentalis* as part of a useful transformation system.

A codon-optimized Zeocin resistance gene (*Zeo*/opt) was synthesized and subcloned in pUC57 using GenScript (GenScript, Piscataway, NJ, USA). The resulting plasmid was pUC57-*Zeo*/opt. The codons were designed based on the *D. occidentalis* codon usage database (http://www.kazusa.or.jp/codon/). The GC content of the coding region of the native Zeocin was 69.9% GC, while the GC content of *D. occidentalis* was 35.8%. Using codons optimized for *D. occidentalis*, the GC content of the modified Zeocin resistance gene (accession number LC164249) was 41.9%. The alignments of nucleotide sequences and codon usage rates of amino acids are shown in Fig. S1 and Table S1, respectively. The CTG codon was not found in the modified Zeocin resistance gene for *D. occidentalis*. However, some CTG codons were found in the native Zeocin resistance gene (Table S1). *Debaryomyces*

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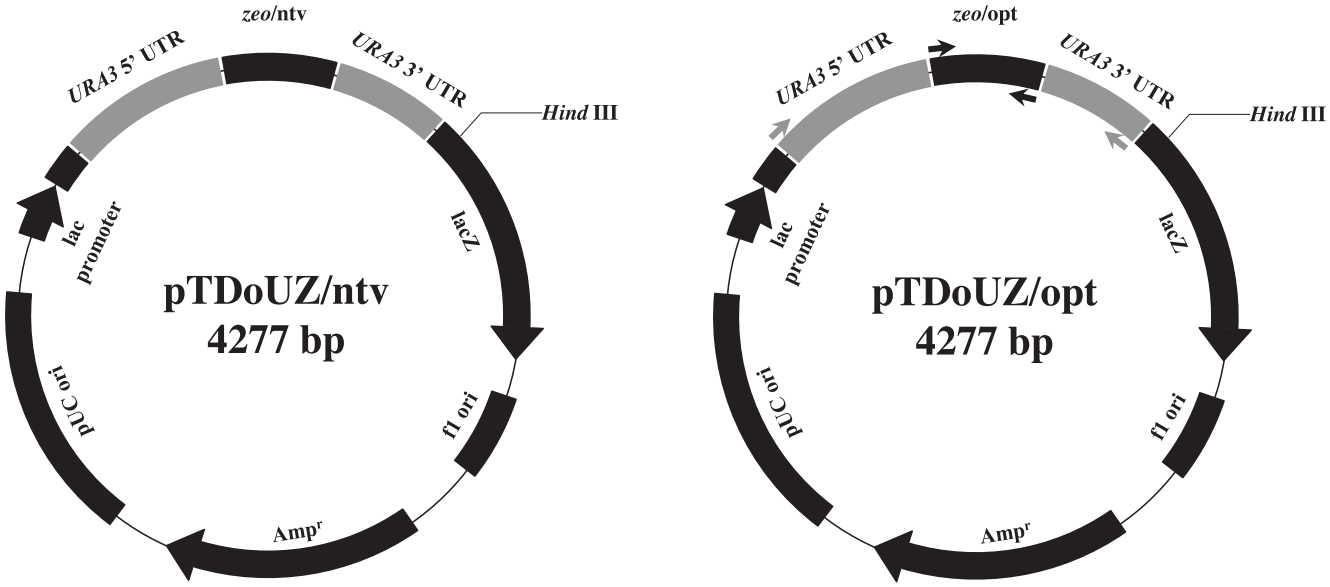

Fig. 1. The pTDoUZ/ntv and pTDoUZ/opt plasmids. The Zeocin resistance gene was cloned into the pTA2 (Toyobo).

*Hind*III was used to linearize the plasmids for transformation. The black and gray arrows in the pTDoUZ/opt plasmid indicate the primers (ZO-F/ZO-R and DoU3P-F/DoU3T-R, respectively) used to confirm the transformations.

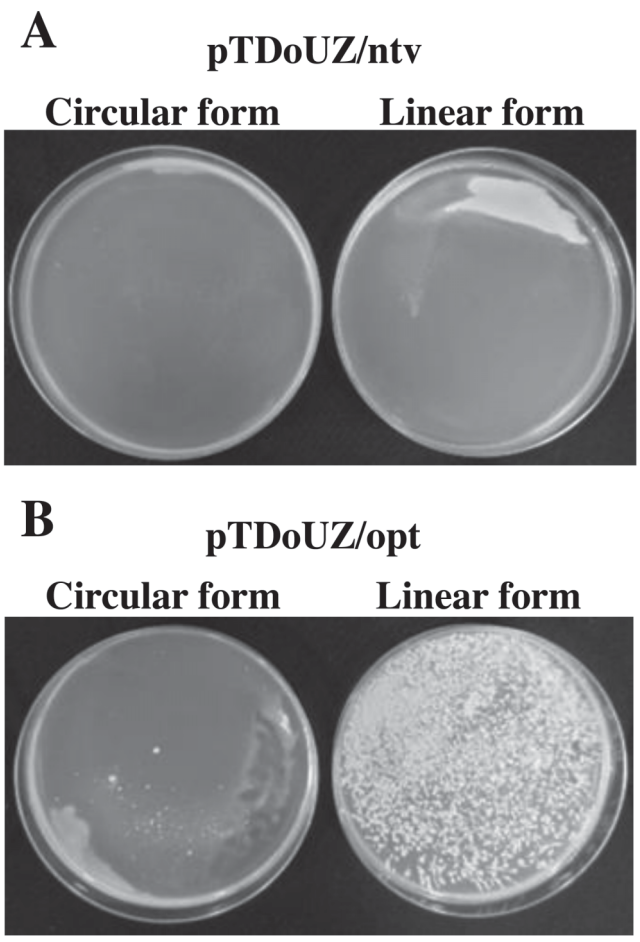

Fig. 2. Transformation of *D. occidentalis* 'NBRC 1840' with plasmids containing the Zeocin resistance gene.

Results of transformation with pTDoUZ/ntv containing the native Zeocin resistance gene (A), and pTDoUZ/opt containing the codon-optimized Zeocin resistance gene (B), on YM medium supplemented with 1 mg/ml Zeocin.

Table 1. Transformation efficiencies of the *Debaryomyces occidentalis* 'NBRC 1840' strain containing plasmids carrrying Zeocin resistance genes.

Plasmid	Formation	Transformation efficiency (colonies/microgram DNA)
pTDoUZ/ntv	Circular	0
	Linearized	0
pTDoUZ/opt	Circular	9.9
	Linearized	110.3

Experiments were carried out in at least triplicate. Results summarized in the table correspond to mean values. In all cases, the mean deviations were less than 10%.

hansenii, a species related to *D. occidentalis*, was classified as a "CTG clade yeast" (Fitzpatrick et al., 2006). The exclusion of the CTG codon in the codon-optimized Zeocin resistance gene might be a key factor for functional expression in *D. occidentalis*.

The wild-type *D. occidentalis* strain '1840' was deposited in the Biological Resource Center, NITE (NBRC) as 'NBRC 1840'. An approximately 400-bp fragment of *URA3* encoding orotidine-5′-phosphate decarboxylase of *D. occidentalis* 'NBRC 1840' was amplified by degenerate PCR using Ura3-F/Ura3-R primers (Table S2), which were designed based on highly conserved regions of *URA3* from other yeasts. Using this fragment of the *D. occidentalis* 'NBRC 1840' *URA3* gene (accession number LC171378), four primers, DoUra3-F1, DoUra3-F2, DoUra3-R1 and DoUra3-R2 (Table S2), were designed to amplify the 5′ and 3′ flanking regions, which were obtained using the LA PCR *in vitro* Cloning Kit (Takara, Otsu, Japan). The flanking regions of the *URA3* gene were used to determine the functional expression of the Zeocin resistance gene.

The plasmids, pPICZ A (Invitrogen, Carlsbad, CA, USA) and pUC57-*Zeo*/opt, were used as the template DNA for the amplification of the Zeocin resistance gene (375 bp).

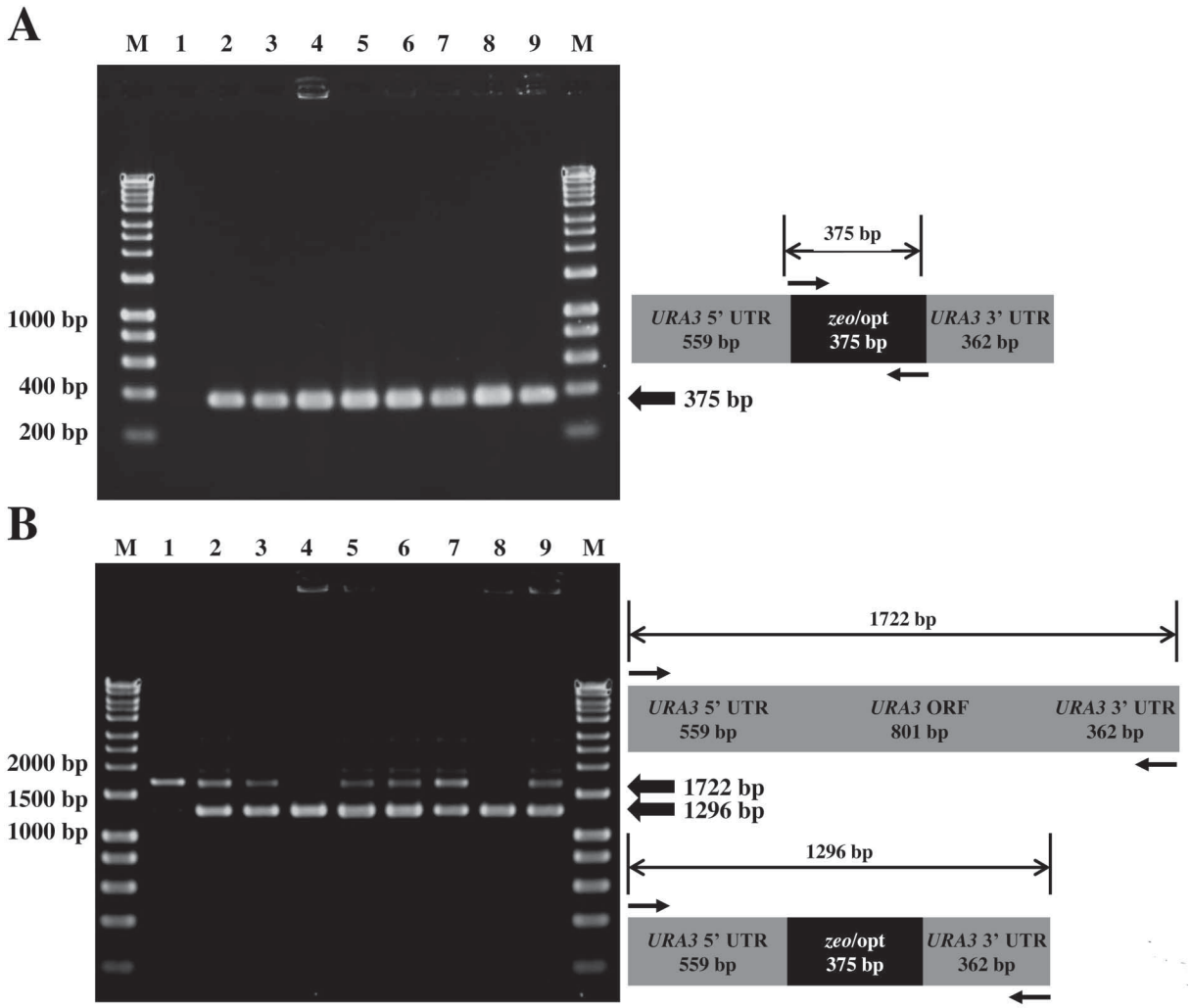

Fig. 3. PCR confirmation of transformations. Transformations were confirmed by PCR using ZO-F/ZO-R primers (A), and DoU3P-F/DoU3T-R primers (B).

Lane 1 is the *D. occidentalis* 'NBRC 1840' recipient strain. Lanes 2–9 are transformants containing the linearized pTDoUZ/opt with the codon-optimized Zeocin resistance gene. M denotes the Loading Quick DNA Mass Ladder (Toyobo).

The purified genomic DNA of the *D. occidentalis* 'NBRC 1840' strain was used as the template DNA for the amplification of the 5′ (559 bp) and 3′ (362 bp) flanking regions of the *URA3* gene. The scheme and primers for plasmid construction are summarized in Fig. S2 and Table S2, respectively. Zeocin-resistance cassettes, consisting of the three DNA fragments, were fused using the In-Fusion HD Cloning Kit (Takara) and subcloned into pMD20 or pMD19 (Takara). The resulting plasmids were named pMDoUZ/ntv or pMDoUZ/opt. The subcloned Zeocin-resistance cassettes were amplified by DoU3P-F/DoU3T-R (Table S2). The amplified fragments were inserted into pTA2 (Toyobo, Osaka, Japan) following the *lac* promoter, resulting in pTDoUZ/ntv or pTDoUZ/opt (Fig. 1).

The transformation of *D. occidentalis* 'NBRC 1840' was carried out by electroporation, essentially following the method used for *Cryptococcus* sp. strain S-2 with slight modifications (Masaki et al., 2012). Briefly, cells were cultured overnight in 60 ml of YM medium (1.0% D-glucose, 0.5% peptone, 0.3% yeast extract and 0.3% malt extract) in a 200-ml baffled Erlenmeyer flask at 25°C with vigorous aeration in a shaker at 180 rpm. The culture was harvested by centrifugation at 2,300 × *g* at 4°C for 3 min. The harvested cells were washed twice in a wash solution, and resuspended in an electroporation buffer to an optical density of 50 at 660 nm. The cell suspension (50 μl) in the electroporation buffer was transferred to 0.2 cm-gap disposable electroporation cuvettes (Bio-Rad, Hercules, CA, USA). After the addition of circular or linearized plasmid (5.0 μg at 1 μg/μl), the cells were subjected to an electrical pulse from a Gene Pulser (Bio-Rad). The pulse setting was 470 V, 25 μF and $\Omega = \infty$. Under these conditions of the electroporation, circular and linear plasmids were used independently in the transformations. For the latter, the *Hind*III restriction enzyme was used to digest the multicloning site located outside of the 3′ flanking region of the Zeocin resistance marker (Fig. 1). After electroporation, 1 ml YM medium was added, and cells were incubated for 24 h. Then, the cells were plated on YM (Zeo) (YM medium + 1 mg/ml Zeocin (InvivoGen, San Diego, CA, USA)) and incubated at 25°C for 3 days. The colonies on YM (Zeo) plates were counted to estimate the transformation efficiencies. Growing colonies were picked and suspended to 0.18 ml 50 mM NaOH,

and then treated at 98°C for 10 min. Alkaline and heat treated colonies were neutralized by adding 0.02 ml 1 M Tris-HCl, pH 8.0. The supernatant was used for PCR with ZO-F/ZO-R and DoU3P-F/DoU3T-R (Fig. 1 and Table S2), respectively. The PCR was performed using KOD FX Neo DNA polymerase (Toyobo) under the following reaction conditions: 94°C for 2 min pre-denaturation, followed by 30 cycles of denaturation at 98°C for 5 s, annealing at 50°C for 5 s, and extension at 68°C for 30 s/kb.

Two forms were prepared: a circular plasmid and a linear plasmid digested by *Hind*III outside the *URA3* 3′ flanking region. No transformants were acquired using pTDoUZ/ntv, in either the circular or linear form. When using pTDoUZ/opt, both the circular and linear (*Hind*III digested) plasmids resulted in transformants (Fig. 2 and Table 1). The transformation efficiency was ~11 times higher for the linear plasmid compared with the circular plasmid under our conditions. Thus, the transformation efficiency with the linearized pTDoUZ/opt is sufficient for library construction. Although the methods described by Costaglioli et al. (1994) were applied to the transformation of *D. occidentalis* 'NBRC 1840', no colonies were obtained on YM (Zeo) plates. Zeocin (2 mg/ml) causes spontaneous mutations in *Candida guilliermondii* (Papon et al., 2013). The complete inhibition of *D. occidentalis* 'NBRC 1840' growth was observed on YM plates containing 1 mg/ml Zeocin, which allowed the selection of Zeocin-resistant transformants in the transformation procedure.

To confirm the insertion of the Zeocin marker, alkaline and heat treated colonies were subjected to a PCR analysis (Fig. 3). The codon-optimized Zeocin gene fragment (375 bp) was amplified by ZO-F/ZO-R in all eight randomly selected transformants. The amplification of the Zeocin-resistance cassette (1,296 bp) by DoU3P-F/DoU3T-R was confirmed in all of the tested transformants. Moreover, the specific amplification of the internal *URA3* gene and its flanking regions was also observed by a band at 1,722 bp. This fragment was not seen in two of the eight transformants, suggesting that the *URA3* gene might be disrupted by homologous recombination in two of the transformants. The rate of homologous recombination was similar to that reported in a putative xylose reductase gene of *D. hansenii* (Minhas et al., 2009). The low efficiency of homologous recombination suggested that transforming DNA was predominantly integrated into the *D. occidentalis* genome by random events. The results indicated that we successfully constructed a useful and efficient transformation marker for *D. occidentalis*.

D. occidentalis is a microorganism that has been studied for over 30 years. Although some auxotrophic markers for this species have been constructed, the only drug-resistance marker for transformations that has been reported is the phleomycin resistance-conferring gene (Janatova et al., 2003). In this research, codon optimization enabled the use of the Zeocin marker for *D. occidentalis* transformations. Thus, the already developed Zeocin marker is usable in this yeast. The acquisition of auxotrophic mutants requires skill and time, but drug-resistance markers are advantageous because they can be used to directly transform wild type strains. *D. occidentalis* may be useful as a heterologous protein-production host, and we hope this codon optimization technique will be used in further studies of *D. occidentalis*.

Supplementary Materials

Supplementary figures and tables are available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

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