High-Temperature Ethanol Fermentation and Transformation with Linear DNA in the Thermotolerant Yeast *Kluyveromyces marxianus* DMKU3-1042[▽]

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We demonstrate herein the ability of *Kluyveromyces marxianus* to be an efficient ethanol producer and host for expressing heterologous proteins as an alternative to *Saccharomyces cerevisiae*. Growth and ethanol production by strains of *K. marxianus* and *S. cerevisiae* were compared under the same conditions. *K. marxianus* DMKU3-1042 was found to be the most suitable strain for high-temperature growth and ethanol production at 45°C. This strain, but not *S. cerevisiae*, utilized cellobiose, xylose, xylitol, arabinose, glycerol, and lactose. To develop a *K. marxianus* DMKU3-1042 derivative strain suitable for genetic engineering, a uracil auxotroph was isolated and transformed with a linear DNA of the *S. cerevisiae* Sc*URA3* gene. Surprisingly, Ura+ transformants were easily obtained. By Southern blot hybridization, the linear Sc*URA3* DNA was found to have inserted randomly into the *K. marxianus* genome. Sequencing of one Lys− transformant confirmed the disruption of the Km*LYS1* gene by the Sc*URA3* insertion. A PCR-amplified linear DNA lacking *K. marxianus* sequences but containing an *Aspergillus* α-amylase gene under the control of the Sc*TDH3* promoter together with an Sc*URA3* marker was subsequently used to transform *K. marxianus* DMKU3-1042 in order to obtain transformants expressing *Aspergillus* α-amylase. Our results demonstrate that *K. marxianus* DMKU3-1042 can be an alternative cost-effective bioethanol producer and a host for transformation with linear DNA by use of *S. cerevisiae*-based molecular genetic tools.

Ethanol production at high temperature has received much attention because fermentation processes conducted at elevated temperatures will significantly reduce cooling costs (14). Other advantages of elevated temperatures include more-efficient simultaneous saccharification and fermentation, a continuous shift from fermentation to distillation, reduced risk of contamination, and suitability for use in tropical countries (3, 5, 27). However, the temperatures suitable for conventional strains of *Saccharomyces cerevisiae* are relatively low (25 to 30°C). While screens for *S. cerevisiae* mutants able to produce ethanol efficiently at high temperature have been performed, only a modest increase in temperature has been obtained, 40°C maximum (35, 40). Alternatively, attention has also focused on thermotolerant yeast species capable of producing ethanol at elevated temperatures. Isolates of *Kluyveromyces marxianus* appear to be particularly promising (3, 5, 20, 27). This species has been reported to grow at 47°C (3), 49°C (20), and even 52°C (6) and to produce ethanol at temperatures above 40°C (14). Moreover, *K. marxianus* offers additional benefits (36) including a high growth rate (34) and the ability to utilize a wide variety of industrially relevant substrates such as sugar cane, corn silage juice, molasses, and whey powder (17, 27, 32, 42, 49). Because of these advantages, *K. marxianus* is currently being promoted as a viable alternative to *S. cerevisiae* as an ethanol producer. However, systematic comparison of the ethanol productivity of *K. marxianus* with that of *S. cerevisiae*, or of those among *K. marxianus* strains isolated from different areas under the same conditions, has not been reported, making it difficult to determine which yeast strain is the best for specified conditions. Therefore, the present study was undertaken to assess growth and ethanol productivity at high temperature and the carbon source utilization of *K. marxianus* strains (11, 25–27, 45) and two *S. cerevisiae* strains, of which NCYC3233 is an isolate from a Brazilian ethanol plant. Furthermore, because *K. marxianus* is relatively undeveloped as a host for heterologous gene expression, in contrast to *S. cerevisiae* and *K. lactis* (15, 17), we demonstrate that *K. marxianus* DMKU3-1042 can be efficiently transformed with linear DNA to yield random insertions, which promises to facilitate both strain improvements and efforts to learn more about the biology of this important thermotolerant species.

MATERIALS AND METHODS

Strains and culture media. Yeast strains used are listed in Table 1. YPD medium (1% yeast extract, 2% peptone, and 2% glucose) and minimal medium (MM; 0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 2% glucose) were prepared as described previously (23). YP medium (1% yeast extract, 2% peptone) or MM without glucose was supplemented with 5% glucose or with 2% glucose, galactose, fructose, mannose, sucrose, raffinose, inulin, arabinose, cellobiose, glycerol, lactose, xylose, or xylitol. Starch medium (YPD supplemented with 1% soluble starch) and MM supplemented individually with uracil, adenine, lysine, histidine, methionine, tryptophan, or leucine were also used. 5-Fluoroorotic acid (5-FOA) and uracil dropout

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TABLE 1. Yeast strains used in this study

Strain[a]	Description	Reference(s) and remarks
K. marxianus strains		
DMKU3-1042	Isolated in Thailand	27
NCYC587	ATCC 36907	11, 31, 33
NCYC1429	ATCC 12424	25, 48
NCYC2791	CBS712; type strain	30
RAK3605	*ura3*	DMKU3-1042 FOA[+]
RAK3626	*ura3* Ade[−] [Sc*URA3*]	RAK3605 transformant
RAK3627	*ura3 lys1*::Sc*URA3*	RAK3605 transformant
RAK4687	*ura3* Sc*TDH3p-TAA*-Sc*URA3*	RAK3605 transformant
S. cerevisiae strains		
BY4704	*MAT**a** ade2*::*hisG his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63*	10
BY4700	*MAT**a** ura3Δ0*	10
BY4713	*MAT**a** leu2Δ0*	10
RAK3599	*MAT**a**/α ura3Δ0/+ +/leu2Δ0*	BY4700 × BY4713
RAK3603	*MAT**a** ura3Δ0*::Sc*TDH3p-TAA-PGKter*-Sc*URA3 his3Δ1 leu2Δ0 met15Δ0*	This study
NCYC3233	Isolated from fermented ethanol broth in Brazil	

[a] NCYC, National Center for Yeast Collection, United Kingdom.

TABLE 2. Primers used in this study

Primer name	Sequence (5′–3′)
URA3-40	ATCAAAGAAGGTTAATGTGGCTGTGG
URA3-40c	TTCGTCATTATAGAAATCATTACGAC
URA3-300	GAAGAGTATTGAGAAGGGCAAC
URA3-300c	TGTTGTGAAGTCATTGACACAG
URA3+720	GGGAAGGGATGCTAAGGTAGAGGGT
URA3+25Down	ACCCGGGAATCTCGGTCGTAATGAT
URA3+1054	GACAGGACTGTAAAGATGGACGCAT
URA3+61c	GCTTGGCAGCAACAGGACTAGGATG
URA3-35c	TGCAGTTGGGTTAAGAATACTGGGC
URA3-400c	TCTACATCAGATAACTTCGGTTTG
15C+9A	CCCCCCCCCCCCCCCAAAAAAAAA
TDH3-572	GCTGTAACCCGTACATGCCCAA
LYS1-551	AAGGCAAGTGAAAGCCATTGCC
LYS1+1642	GTATTGTGGAGCAGTCAGCATC
KmLYS1-225Up	CTTCCTGAAGAATAATTTATGCCGACTTACTGCTTGAAAGTG
KmLYS1-606Down	CGGACACTTTCCCTCTAGTTCATGAGCATATTCAATTTGCCC

An Sc*TDH3p-TAA*-Sc*URA3* DNA was amplified using genomic DNA from RAK3623 as the template and primers TDH3-572 and URA3-300c for the transformation of *K. marxianus*.

Chromosomal DNA isolation. Cells from a 1.5-ml YPD culture were collected by centrifugation and washed once with sterile water. The cells were suspended in 300 μl of SET buffer (1.2 M sorbitol in 20 mM Tris-HCl, pH 7.5, and 10 mM EDTA) to which 20 μl of zymolyase solution (3 mg/ml zymolyase 100T [Seikagaku Corp., Tokyo, Japan] in 0.9 ml SET, mixed with 0.1 ml β-mercaptoethanol) was added. The mixture was incubated at 37°C for 30 min, after which 50 μl of 10% sodium dodecyl sulfate (SDS) and 200 μl of phenol-chloroform-isoamylalcohol (Sigma-Aldrich, MO) were added. After centrifugation, the upper layer containing chromosomal DNA was transferred to a microtube, and DNA was precipitated with ethanol. The precipitated chromosomal DNA was then washed once with 70% ethanol and dissolved in 100 μl of sterile water.

Transformation. Yeast transformation was performed by a lithium acetate method (15). Yeast cells were grown overnight in YPD, diluted 1:10 in 10 ml of fresh YPD, and allowed to grow 5 h at 28°C with shaking. The cells were then collected by centrifugation, washed once with sterile water, and suspended in 100 μl of sterile water. Fifty microliters of the cell suspension was then mixed with 115 μl of 60% polyethylene glycol 3350, 5 μl of 4 M lithium acetate, 15 μl of sterile water, 10 μl of 10 mg/ml carrier DNA, and 5 μl of PCR product. The mixture was vortexed for 30 s, incubated at 42°C for 40 min, and spread on uracil dropout plates.

S. cerevisiae centromere/autonomously replicating sequence (ARS) plasmid pRS316 (41) containing Sc*URA3* as a marker was used for *K. marxianus* transformations. pRS316 (4.8 kb) was linearized by digestion with SmaI (New England Biolabs, MA).

Southern blot hybridization. Chromosomal DNA from BY4704, *K. marxianus* DMKU3-1042, a *K. marxianus ura3* mutant (RAK3605), and Sc*URA3* transformants of the *ura3* mutant was isolated and digested with BamHI (Roche Diagnostics GmbH, Mannheim, Germany). The digests were separated on a 0.8% agarose gel and transferred to a positively charged nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany) as described previously (38). Sc*URA3* amplified from BY4704 chromosomal DNA with primers URA3-40 and URA3-40c was used as a hybridization probe. The probe was labeled using the digoxigenin high prime DNA labeling and detection kit II (Roche Applied Science, IN). Hybridization was performed overnight at 42°C with gentle shaking. The blot was washed twice for 5 min at 25°C in a wash solution (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] containing 0.1% SDS) as described previously (38). Further washes were carried out twice for 15 min at 65°C in a preheated solution of 0.5× SSC containing 0.1% SDS. Blocking of the blots, antibody reaction, removal of the unbound conjugate, and signal generation were performed according to the manufacturer's instructions. The membrane was exposed to an LAS-1000 imaging system (Fuji Photo Film Co., Ltd., Tokyo, Japan) in order to capture the signal.

Isolation and sequencing of Sc*URA3* flanking sequences. For screening of auxotrophic mutants generated by Sc*URA3* integrative transformation, transformants were replica plated to MM and uracil dropout medium. Colonies that

media were prepared as described previously (1, 2). For the preparation of solid plates, 2% agar was added. Anaerobic cultures were grown in an AnaeroPouch (Mitsubishi Gas Chemical, Tokyo, Japan).

Ethanol fermentation and analytical methods. Forty milliliters of YP plus 5% glucose in 250-ml flasks was inoculated with an overnight YPD culture to an initial optical density at 600 nm of 0.2. Cells were incubated at 30°C or 45°C with shaking at 150 rpm. Supernatant samples were collected by centrifugation and were analyzed directly by high-performance liquid chromatography (Hitachi, Tokyo, Japan) using a Shim-pack SPR-Pb column (Shimadzu, Kyoto, Japan) to determine sugar and ethanol concentrations. Deionized water was used as the mobile phase with a flow rate of 0.6 ml/min; the column temperature was set at 80°C.

Cell growth was analyzed using a TVS062CA biophotorecorder (Toyo Seisakusho, Ltd., Chiba, Japan). To detect α-amylase activity, transformed cells were spotted on starch medium plates and incubated at 28°C for 1 day. The starch plates were then exposed to iodine vapor to detect the halo produced by starch degradation.

Ura⁻ mutant isolation. *K. marxianus* DMKU3-1042 was grown overnight in 2 ml of YPD, centrifuged, washed once with sterile water, and resuspended in 1 ml of sterile water. Aliquots (200 μl) of cells were then spread on 5-FOA plates and exposed to UV for 30 s. Colonies were picked after 2 days at 28°C and tested for a uracil requirement by assessing growth on MM with and without uracil.

PCR amplification and primers. DNA fragments used for transformation were amplified by PCR using a KOD Plus kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The PCR was initiated at 94°C for 1 min, followed by 30 cycles of the following program: 94°C for 20 s, 55°C for 30 s, and 68°C for 3 to 4 min. The size of PCR products was analyzed by agarose gel electrophoresis. Primers used are listed in Table 2.

The *S. cerevisiae* Sc*URA3* (1.7-kb) gene was amplified using chromosomal DNA from BY4704 as the template and primers URA3-300 and URA3-300c. The Sc*LYS1* gene was amplified using chromosomal DNA from BY4743 as the template with primers LYS1-551 and LYS1+1642. Integration of the Sc*URA3* at the *K. marxianus* Km*LYS1* locus was confirmed by PCR using KmLYS1-225Up and KmLYS1-606 primers. The Sc*TDH3* promoter-driven *Aspergillus oryzae* α-amylase gene, *TAA*, was integrated at the *ura3Δ0* locus in BY4741 to generate strain RAK3623 (K. Cha-aim, H. Hoshida, and R. Akada, unpublished data).
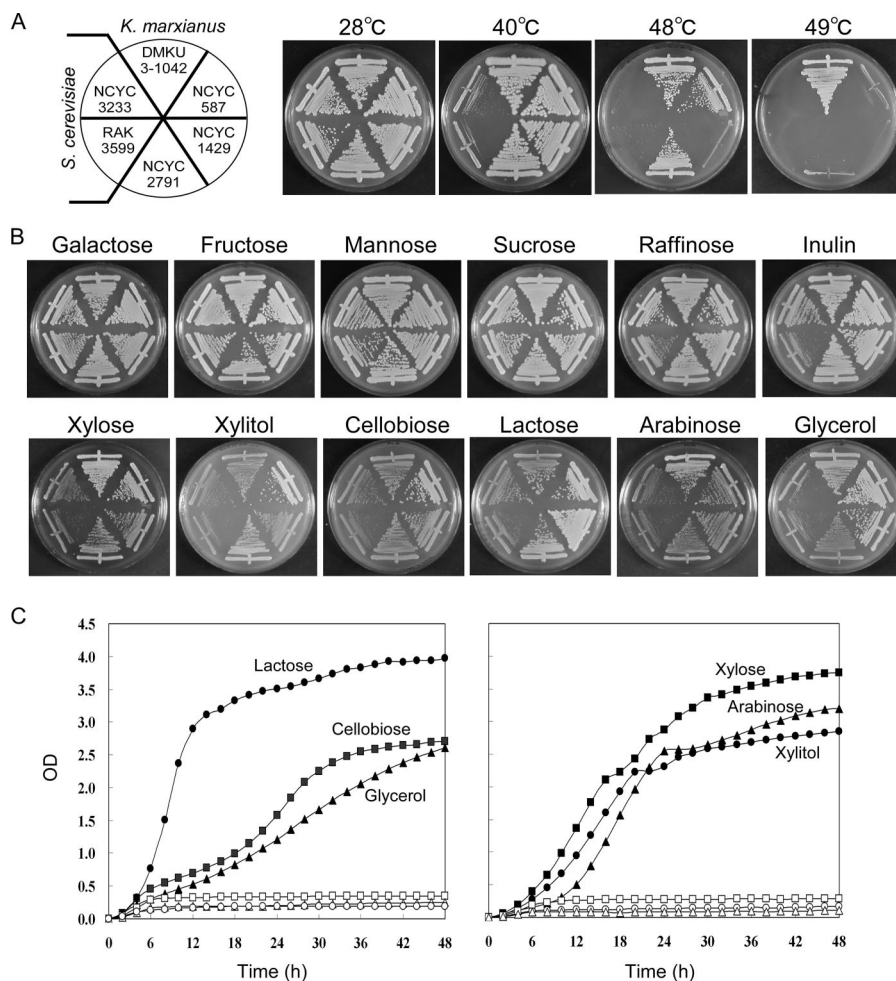
FIG. 1. Growth properties of *K. marxianus* and *S. cerevisiae* strains. (A) Strains of *K. marxianus* and *S. cerevisiae* were streaked on YPD plates and incubated at the indicated temperatures for 2 days. (B) Strains were streaked as described for panel A and grown at 28°C on MM supplemented with 2% of the indicated sugars. (C) *K. marxianus* DMKU3-1042 (closed symbols) and *S. cerevisiae* NCYC3233 (open symbols) were grown in liquid MM supplemented with 2% lactose (circles), cellobiose (squares), or glycerol (triangles) for the graph on the left and with xylitol (circles), xylose (squares), or arabinose (triangles) for the graph on the right. OD, optical density.

failed to grow on MM were picked and their growth was tested on MM supplemented with adenine, histidine, leucine, methionine, tryptophan, or lysine to identify the nutrient requirement.

To amplify the region flanking Sc*URA3* in RAK3627, a thermal asymmetric interlaced PCR (TAIL-PCR) method (28) was used with the following modifications. A universal primer, 15C+9A, was used instead of the arbitrary degenerate primer. The primers URA3+61c, URA3-35c, and URA3-400c were used for amplification upstream of Sc*URA3*, and primers URA3+720, URA3+25Down, and URA3+1054 were used for the downstream sequence. The PCR mixture (10 μl) contained 200 pmol of each primer, 1× KOD Plus buffer, 200 μM of each deoxynucleoside triphosphate, 0.2 μM MgSO₄, and 0.1 U KOD Plus DNA polymerase. Thermal settings for the TAIL-PCR were annealing and extension temperatures of 60°C and 68°C, respectively. The extension time was changed to 2 min in the primary and secondary PCRs. In the tertiary PCR, the reaction was initiated at 94°C for 1 min followed by 30 cycles of the following program: 94°C for 20 s, 50°C for 1 min, and 68°C for 2 min. In the primary TAIL-PCR, 4 ng of RAK3627 genomic DNA template and 15C+9A and URA3+720 or URA3+61c primers were used. One microliter of a 100-fold dilution of the primary PCR product in a total 10-μl mixture was used as a template for the secondary TAIL-PCR with primers 15C+9A and URA3+25Down or URA3-35c. The secondary PCR product was loaded on a 0.7% (wt/vol) agarose gel, the band was excised, and the PCR product was isolated. The PCR product was then used as a template with primers 15C+9A and URA3+1054 or URA3-400c for the tertiary TAIL-PCR. The PCR product

of the tertiary PCR was purified using a purification cartridge with a 30,000-molecular-weight cutoff (Takara Bio Inc., Shiga, Japan). The DNA samples were sequenced using a BigDye Terminator cycle sequencing kit (Applied Biosystems, CA) according to the manufacturer's instructions.

RESULTS

Growth and ethanol production by *K. marxianus* and *S. cerevisiae* strains. We compared thermotolerant growth levels, utilizable carbon sources, and ethanol production levels of *K. marxianus* strains DMKU3-1042 (27), NCYC587 (ATCC 36907) (31, 33), NCYC1429 (ATCC 12424) (48), and NCYC2791 (CBS712, *K. marxianus* type strain) (30) and *S. cerevisiae* strains NCYC3233 and RAK3599 (diploid laboratory strain with no special nutritional requirements). The growth levels of these six strains were examined at 28°C, 40°C, 48°C, and 49°C (Fig. 1A). All *K. marxianus* strains except NCYC1429 grew at temperatures of less than 48°C but only the DMKU3-1042 strain grew at 49°C. We also examined the growth of 15 *K. marxianus* strains obtained from NBRC (NITE Biological Resource Center, Japan) at 48°C.
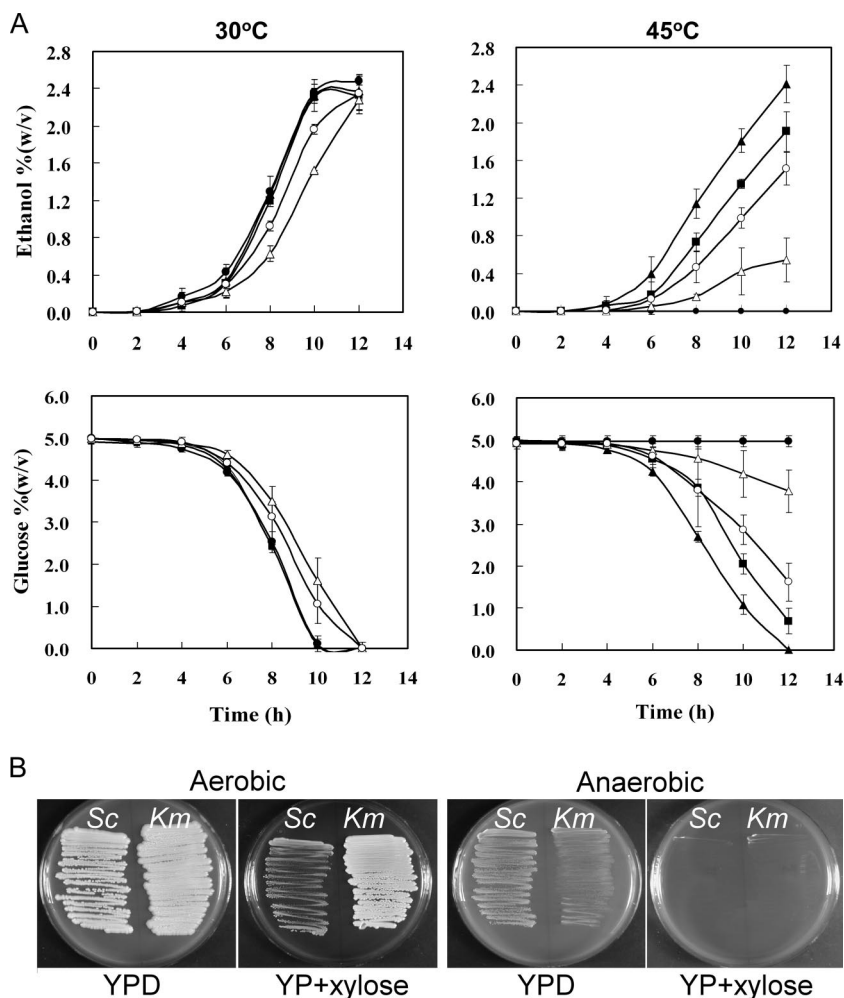
FIG. 2. Ethanol fermentation. (A) Ethanol production (top) and glucose consumption (bottom) by *S. cerevisiae* NCYC3233 (closed circles) and *K. marxianus* strains DMKU3-1042 (closed triangles), NCYC587 (closed squares), NCYC1429 (open triangles), and NCYC2791 (open circles) in YP medium supplemented with 5% glucose at 30°C (left) and 45°C (right). (B) Growth of *K. marxianus* DMKU3-1042 (*Km*) and *S. cerevisiae* NCYC3233 (*Sc*) on YPD and YP supplemented with 2% xylose (YP+xylose) under aerobic or anaerobic conditions (3 or 5 days, respectively).

The DMKU3-1042 strain showed the best growth property (data not shown). In contrast, neither *S. cerevisiae* strain grew at ≥40°C.

Next, the utilization of a wide variety of carbon sources was tested (Fig. 1B and C). All *K. marxianus* and *S. cerevisiae* strains utilized galactose, fructose, mannose, sucrose, raffinose, and inulin for growth (Fig. 1B, top). However, in contrast to *S. cerevisiae*, *K. marxianus* utilized xylose, xylitol, cellobiose, lactose, arabinose, and glycerol (Fig. 1B, bottom). This growth phenotype in liquid medium was also confirmed (Fig. 1C). The growth test for *K. marxianus* DMKU3-1042 and *S. cerevisiae* NCYC3233 in liquid MM supplemented with 2% of each carbon source indicated that *K. marxianus* DMKU3-1042 was able to utilize xylose, xylitol, cellobiose, lactose, arabinose, and glycerol for growth (Fig. 1C).

Ethanol production and glucose consumption were also examined at 30°C and 45°C (Fig. 2A). All *K. marxianus* and *S. cerevisiae* strains exhibited similar levels of ethanol production and glucose consumption at 30°C (Fig. 2A, left). However, when the ethanol fermentation was carried out at 45°C, *S. cerevisiae* did not grow and thus did not produce ethanol, but

the *K. marxianus* strains produced ethanol (Fig. 2A, right). Among the four strains of *K. marxianus*, DMKU3-1042 converted glucose to ethanol faster than the others at 45°C. From these results, we conclude that DMKU3-1042 had the fastest ethanol productivity at high temperatures among the six strains examined.

Ethanol production by *K. marxianus* strains grown on xylose as a sole carbon source was also tested, but very little ethanol was observed. Similar results were obtained for DMKU3-1042 when cellobiose, arabinose, and lactose were used as sole carbon sources (data not shown). We next tested the anaerobic growth of *K. marxianus* when xylose was the sole carbon source (Fig. 2B). *K. marxianus* DMKU3-1042 was found to be unable to grow on YP-plus-xylose medium under anaerobic conditions, indicating that the anaerobic fermentation of xylose is not a native trait of *K. marxianus*.

Development of a *K. marxianus* transformation system by use of linear DNA. In order to develop a transformation system for *K. marxianus*, spontaneous uracil auxotrophs were sought by 5-FOA selection (9). Colonies that arose on 5-FOA plates
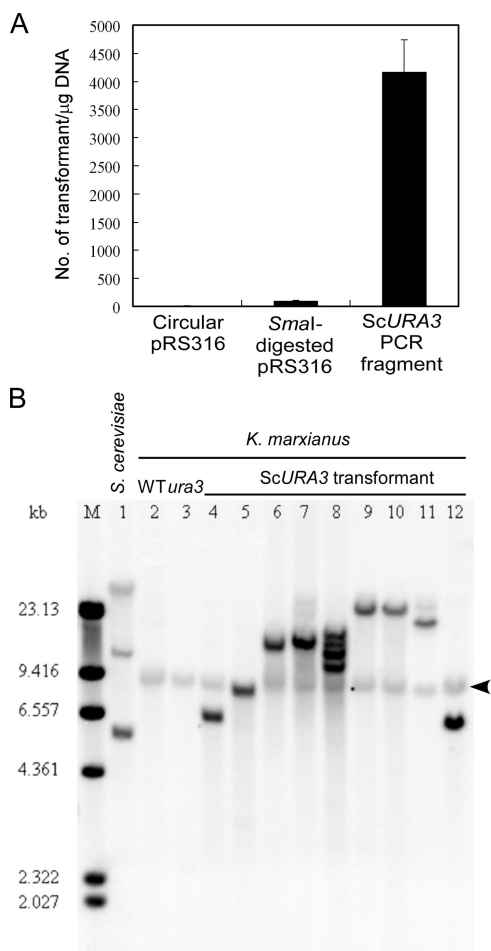
FIG. 3. Transformation of a *K. marxianus ura3* mutant with a linear DNA of *S. cerevisiae URA3* (Sc*URA3*). (A) The *K. marxianus ura3* mutant was not transformed with the intact Sc*URA3* plasmid (pRS316) but with SmaI-digested pRS316 (4.8 kb) and the Sc*URA3* PCR fragment (1.7 kb). (B) Southern blot hybridization of chromosomal DNA of *S. cerevisiae* BY4704 (lane 1), *K. marxianus* wild-type (WT) DMKU3-1042 (lane 2), *ura3* mutant RAK3605 (lane 3), and nine Sc*URA3* transformants from strain RAK3605 (lanes 4 to 12). Chromosomal DNA was digested with BamHI, run on a 0.8% agarose gel, transferred to a nylon membrane, and hybridized with a digoxigenin-labeled Sc*URA3* probe. The bands indicated by an arrowhead are likely the authentic *K. marxianus URA3* that cross-hybridizes with Sc*URA3*. M, DNA size marker.

were streaked onto MM and MM supplemented with uracil (MM-plus-uracil) plates. One mutant that grew on MM plus uracil but not on MM was designated RAK3605 and used as a transformation host.

To confirm that the mutation in RAK3605 was actually in the *ura3* gene, we attempted to complement the strain with *S. cerevisiae URA3*, because Sc*URA3* has been shown to complement a *K. marxianus ura3* mutant (21). Initially, *S. cerevisiae* replicative plasmid pRS316, carrying a Sc*URA3* marker, was used directly, but no transformants were obtained even at a DNA amount as high as 1 μg, suggesting that the *S. cerevisiae* ARS cannot function in *K. marxianus*. Plasmid pRS316 was then linearized with SmaI prior to the transformation, which yielded 90 colonies/μg DNA (Fig. 3A). Transformation with

the PCR-amplified Sc*URA3* from *S. cerevisiae* genomic DNA was tested next. Surprisingly, transformants were obtained at a frequency of 4.2×10^3 colonies/μg DNA (Fig. 3A).

Because the Sc*URA3* transformants were stable even after growth in YPD medium (data not shown), we anticipated that the Sc*URA3* had integrated into a *K. marxianus* chromosome. Integration was subsequently demonstrated by Southern hybridization using Sc*URA3* as a probe (Fig. 3B). Hybridization with Sc*URA3* revealed one faint ~9-kb band in all *K. marxianus* strains tested, suggesting that the band represents the authentic cross-hybridizing Km*URA3* gene. In addition to this faint band, most Sc*URA3* transformants had single additional bands of various sizes, indicating single and random integration events. In one transformant (Fig. 3B, lane 8), multiple integrations at different loci were observed.

Insertional mutagenesis by linear Sc*URA3* transformation. If Sc*URA3* DNA could insert randomly into *K. marxianus* chromosomes during transformation, we reasoned that the procedure could be used for insertional mutagenesis, much like transposon mutagenesis (24). Therefore, a screen for auxotrophic mutants was undertaken following transformation with Sc*URA3*. Among several thousand Sc*URA3* transformants, two were found to be unable to grow on MM. One was subsequently found to be Ade⁻ (RAK3626) and the other Lys⁻ (RAK3627). To identify the integration site of Sc*URA3* in RAK3627, upstream and downstream regions flanking the insertion were isolated by a modified TAIL-PCR method. The sequence of the flanking regions was compared to a *K. lactis* database (13). The nucleotide sequence of the flanking 240 bp was 81.7% identical to that of Kl*LYS1*. Sc*URA3* was found to have inserted into the *LYS1* open reading frame without generating any additional nucleotide changes (Fig. 4A).

For further confirmation, primers KmLYS1-225Up and KmLYS1-606Down were designed according to the obtained sequence to amplify a PCR fragment containing 225 bp upstream and 606 bp downstream of the insertion site. As shown in Fig. 4B, a band of 831 bp corresponding to the wild-type *LYS1* region and a single band of 2.6 kbp corresponding to the Sc*URA3* insert are apparent. This result also suggested that *K. marxianus* DMKU3-1042 is haploid, because there was no 0.8-kb band corresponding to the wild-type allele in the *lys1*::Sc*URA3* strain. If it is diploid, two bands, one containing the insert and other the wild type, should have appeared (18). Further, complementation of the *lys1* mutant with Sc*LYS1* was tested. The Sc*LYS1* gene was amplified from *S. cerevisiae* by PCR and used directly to transform the *K. marxianus lys1* strain. Lys⁺ transformants were obtained, indicating that functional complementation by the *S. cerevisiae* gene was feasible in *K. marxianus*.

Expression of *Aspergillus* α-amylase gene under the control of the Sc*TDH3* promoter in *K. marxianus*. Based on the complementation results, we expected that *S. cerevisiae* promoters would also be functional in *K. marxianus*. Therefore, an expression cassette consisting of the *Aspergillus oryzae* α-amylase (*TAA*) gene under the control of the *S. cerevisiae TDH3* promoter (Sc*TDH3p*) was constructed at the *ura3Δ0* locus in *S. cerevisiae*. Amylase expression in *S. cerevisiae* was confirmed (data not shown). A PCR fragment containing Sc*TDH3p-TAA-*Sc*URA3* was amplified from the *S. cerevisiae* chromosome and directly used to transform the *K. marxianus* RAK3605 *ura3*
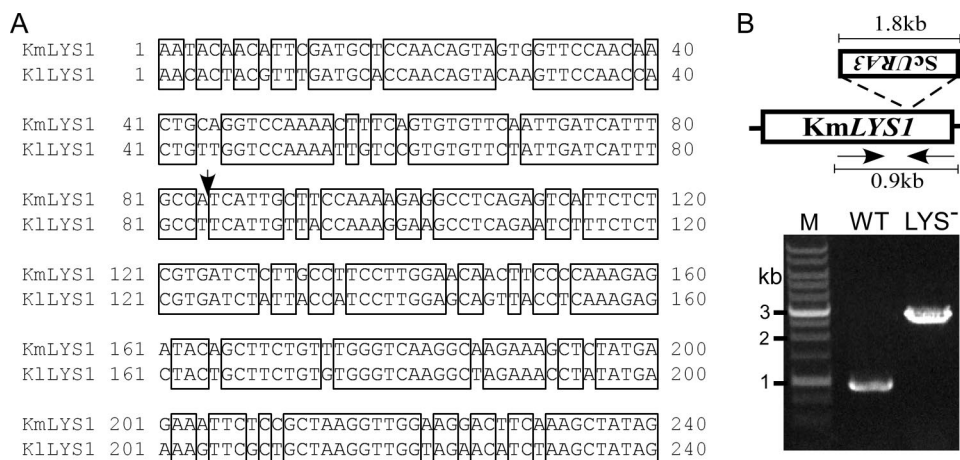
FIG. 4. Screening for auxotrophic mutants following transformation with Sc*URA3*. (A) Sequence flanking the Sc*URA3* insertion was obtained by TAIL-PCR (Km*LYS1*) and was found to be highly homologous to that of *K. lactis* (Kl*LYS1*). An arrow indicates the insertion site. (B) PCR amplification using genomic DNA from DMKU3-1042 (wild type [WT]) and the Lys⁻ mutant RAK3627 (LYS⁻) as templates confirmed the disruption of Km*LYS1* by Sc*URA3*. Sc*URA3* was inserted in an inverse direction relative to Km*LYS1*. The absence of a wild-type band in the *lys1* disruptant suggests a haploid chromosomal constitution.

strain (Fig. 5). The appearance of clear zones around colonies of Ura⁺ transformants after exposure to iodine vapor indicated functional α-amylase expression in *K. marxianus* (Fig. 5). When a promoterless *TAA*-Sc*URA3* cassette was used for transformation, only 3% (38/1,235) of the colonies had halos (data not shown), suggesting fortuitous insertion downstream of authentic promoters. These results indicate that *K. marxianus* strains expressing foreign genes can be constructed by simple transformation using *S. cerevisiae* promoters and open reading frames.

DISCUSSION

K. marxianus is a well-known thermotolerant yeast species. Its ability to produce ethanol by fermentation at high temperature and its use of a wide range of carbon sources make this species attractive for industrial applications (14). While high-
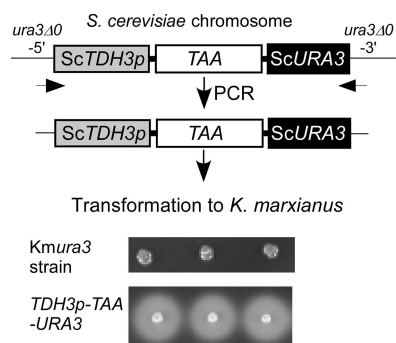

FIG. 5. Construction of an α-amylase expression strain in *K. marxianus* by transformation with a linear DNA. An Sc*TDH3* promoter-driven *TAA* (*Aspergillus oryzae* α-amylase gene) was constructed at the *URA3* locus of *S. cerevisiae* and the PCR-amplified construct was then used without additional manipulation to transform a *K. marxianus ura3* mutant. The appearance of clear zones around colonies indicates that the α-amylase gene was expressed under the control of the *S. cerevisiae TDH3* promoter in *K. marxianus*.

temperature growth and fermentation by *K. marxianus* strains have been previously reported (3, 6, 20, 27, 42), with maximum growth temperatures varying between 47°C and 52°C (14), to our knowledge, no comparative studies performed under the same experimental conditions have been published. Although a maximum growth temperature of 52°C was reported for *K. marxianus* IBM (5), this value is likely to be dependent on the experimental conditions and growth medium used. The results of the present study clearly show that NCYC587 and NCYC2791 grew at 48°C but that only DMKU3-1042 grew at 49°C on a YPD plate (Fig. 1A). All *K. marxianus* strains produced ethanol at 45°C, but the highest productivity was that of DMKU3-1042 (Fig. 2A). All *K. marxianus* strains tested shared similar abilities to use nonglucose carbon sources. Strain DMKU3-1042 utilized lactose, cellobiose, glycerol, xylose, arabinose, and xylitol similarly to the other *K. marxianus* strains and unlike *S. cerevisiae*. These parallel comparisons, undertaken under identical conditions, indicate that DMKU3-1042 is the most suitable strain for high-temperature growth and fermentation among the *K. marxianus* strains tested.

Recently, bioethanol production from lignocellulosic biomass has become an increasingly attractive process, because cellulose is economical, abundant, and renewable. However, because of the high pentose content of lignocellulosic biomass, (i.e., xylose and arabinose), the use of conventional non-pentose-fermenting *S. cerevisiae* strains alone will not be sufficient to produce all the ethanol that potentially can be obtained. This recognition has stimulated extensive studies on recombinant *S. cerevisiae* strains capable of utilizing xylose and arabinose (8, 16, 39, 44, 47). The natural ability of *K. marxianus* to ferment xylose and arabinose is one important advantage it has over *S. cerevisiae*. Indeed, direct ethanol production from the fermentation of D-xylose by *K. marxianus* strains has been reported (29, 46). However, under the conditions used in the present study, we were unable to confirm efficient ethanol production from D-xylose by all tested strains. We also tested whether the strains could grow on YP-plus-xylose medium

under anaerobic conditions. While the strains were able to grow anaerobically on YPD, none could grow on YP plus xylose. These results suggest that the production of ethanol from xylose by *K. marxianus* may not be possible or may require unknown conditions. Nevertheless, the natural ability of *K. marxianus* to use cellobiose, xylose, and arabinose for growth is certainly an attractive trait. Molecular genetic analysis of the native xylose utilization pathway in *K. marxianus*, based in part on tools developed in the present study, should help to explain the physiological impediments to the efficient bioconversion of xylose to ethanol.

With respect to a genetic engineering approach, appropriate host strains and vectors are generally required for transformations and gene manipulation. Vectors used for transformation of eukaryotic microorganisms are typically constructed as *Escherichia coli* shuttle plasmids containing a species- or genus-specific ARS, even in *K. marxianus* (4, 7, 21, 22). For example, plasmids containing a *K. lactis* ARS (8, 12, 24) or a 1.6-μm circular plasmid from *K. drosophilarum* (16) have been used to transform *K. marxianus* (17). In the present study, *K. marxianus* could not be transformed with the intact *S. cerevisiae* plasmid pRS316, containing an *S. cerevisiae* CEN6-ARS4 sequence, indicating that this *S. cerevisiae* ARS cannot function in *K. marxianus*. However, transformation with the linearized pRS316 plasmid and PCR-amplified Sc*URA3* yielded transformants. Southern blot hybridization with the Sc*URA3* probe indicated that Sc*URA3* inserted randomly into the *K. marxianus* genome. These results demonstrate that *K. marxianus* DMKU3-1042 is an excellent host for efficient random integration of nonhomologous DNA sequences.

This finding was verified by mutagenesis using a linear, PCR-generated Sc*URA3* to obtain insertion mutations via transformation. Two auxotrophic mutants (Ade⁻ and Lys⁻ strains) were identified from among several thousand transformants by screening on MM. The Lys⁻ strain was analyzed further. The DNA sequence flanking Sc*URA3* confirmed that the insertion had occurred in the *K. marxianus LYS1* gene. This kind of random integration is typical of transposons, some of which have been modified and used extensively in *S. cerevisiae* for large-scale insertion mutagenesis (24, 37). This suggests the possibility that PCR-amplified linear DNA instead of a functional transposon may be used for efficient insertional mutagenesis in *K. marxianus*.

Moreover, an insertion of Sc*URA3* at the *lys1* locus in *K. marxianus* provided a means for determining the haploid status of *K. marxianus* DMKU3-1042. The ploidy of other *K. marxianus* strains has not been determined. Steensma et al. (43) indicated that some *K. marxianus* strains, including CBS6556, were haploid because they mated with one another. Subsequently, Ribeiro et al. (36) suggested a diploid status based on results of an experiment involving gene disruption and subsequent PCR. Our interpretation of the *LYS1* integration results presented in the present study is that *K. marxianus* DMKU3-1042 is haploid. In *K. marxianus* NBRC1777, the disruption of *URA3*, *LEU2*, and *TRP1* led to the detection of the disrupted alleles but no detection of the wild-type alleles, indicating that this strain is also haploid (19). Haploidy may predominate in native *K. marxianus* strains.

The expression of foreign genes in *K. marxianus*, e.g., the lactate dehydrogenase gene from *Bacillus megaterium* (34), the endo-β-1,4-glucanase gene from *Aspergillus niger*, the cellobiohydrolase and β-glucosidase genes from *Thermoascus aurantiacus* (19), and the β-glucuronidase gene from *E. coli* (4), has been reported. All of these genes were initially manipulated in *E. coli* plasmids prior to their introduction into *K. marxianus* by transformation. In the present study, we used a PCR-amplified DNA for transformation and expression without additional manipulation (Fig. 5). The use of linear DNA for transformation eliminates time-consuming plasmid construction, including restriction digestions, ligations, cloning in *E. coli*, plasmid isolation, and purification. The possibility of critical genes being disrupted following integrative transformation can be minimized by analyzing a number of independent transformants.

In the present study, we used *S. cerevisiae* genes, namely, Sc*URA3* and Sc*LYS1*, as selectable markers and an *S. cerevisiae* promoter, Sc*TDH3p*, for expressing a foreign gene in *K. marxianus*. The results suggest that gene function is conserved between *K. marxianus* and *S. cerevisiae*. Native selectable markers and promoters have also been cloned and used for gene manipulation in *K. marxianus* (4, 19). We speculate here that many *S. cerevisiae* genes (12) and probably many in *K. lactis* (13) as well will be functional in *K. marxianus*. We anticipate that this evolutionary conservation and transformability using linear DNA will facilitate the development of genetic engineering and molecular biological analyses of *K. marxianus*.

In conclusion, with respect to bioethanol production, the nonconventional yeast *K. marxianus* offers several advantages lacking for *S. cerevisiae*. Notably, *K. marxianus* is able to grow and ferment at high temperature, has the natural ability to use xylose, arabinose, and cellobiose, and can be transformed efficiently via nonhomologous manner with linear DNA. For these reasons, we anticipate that the yeast *K. marxianus* will be a promising choice for cost-effective ethanol production and an alternative host for molecular genetic studies.

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