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Transformation System for an Asporogenous Methylotrophic Yeast, *Candida boidinii*: Cloning of the Orotidine-5′-Phosphate Decarboxylase Gene (*URA3*), Isolation of Uracil Auxotrophic Mutants, and Use of the Mutants for Integrative Transformation

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An integrative transformation system was established for an asporogenous methylotrophic yeast, *Candida boidinii*. This system uses a uracil auxotrophic mutant of *C. boidinii* as the host strain in combination with its *URA3* gene as the selectable marker. First, the *C. boidinii URA3* gene coding for orotidine-5′-phosphate decarboxylase (ODCase) was cloned by using complementation of the *pyrF* mutation of *Escherichia coli*. Next, the host ODCase-negative mutant strains (*ura3* strains) were isolated by mutagenesis and selection for 5-fluoro-orotic acid (5-FOA) resistance. Five *ura3* host strains that exhibited both a low reversion rate and good methylotrophic growth were obtained. All of these strains could be transformed to Ura$^+$ phenotype with a *C. boidinii URA3*-harboring plasmid linearized within the *Candida* DNA. The transformants had a stable Ura$^+$ phenotype after nonselective growth for 10 generations. These results and extensive Southern analysis indicated that the linearized plasmid was integrated into the host chromosomal DNA by homologous recombination at the *URA3* locus in *C. boidinii*.

Methanol is one of the most promising carbon sources expected in industrial microbiology, where the cost of culturing process is a crucial factor. We have been investigating methylotrophic yeasts extensively for the production of useful chemicals from methanol (28, 31). During these studies, *Candida boidinii* has often been the most efficient organism among our methanol yeast culture collection (which includes all representative genera of methylotrophic yeasts, i.e., the ascosporogenous genera *Hansenula*, *Pichia*, and *Saccharomyces* and the asporogenous genera *Candida* and *Torulopsis*). For example, *C. boidinii* had an especially high ability to accumulate citric acid in a methanol culture broth (29) and to produce ATP from adenine by its biocatalytic function (28). Also, *C. boidinii* was superior to other methylotrophic yeasts in the production of L-methionine, L-iditol, glycerol, NAD, and formaldehyde (24, 28, 31).

Besides these applied aspects, *C. boidinii* has been the most biochemically well characterized methylotrophic yeast, being the first methylotrophic yeast isolated (28). In recent years, it has been used for studying peroxisomal membrane transport or peroxisome proliferation (3, 11, 14).

Despite these interesting features, the breeding of *C. boidinii* has been limited to classical mutagenesis because of its inability to form spores. A transformation system for *C. boidinii* would provide a means to introduce exogenous DNA into the organism and thus enable a molecular-level study of peroxisome proliferation or the expression and regulation of methanol-metabolizing enzymes. Furthermore, heterologous or homologous gene expression using this transformation system would improve the potent high productivity of useful metabolites of this yeast. Although a transformation system in methylotrophic yeasts has been developed with two ascosporogenous methylotrophic yeasts, *Pichia pastoris* and *Hansenula polymorpha* (8, 13, 20), such a system for asporogenous methylotrophic yeasts has not been reported. This report describes an integrative transformation system in an asporogenous methylotrophic yeast, *C. boidinii*, which is based on homologous recombination between chromosomal DNA of a uracil-requiring host and a cloned *C. boidinii URA3* fragment coding for orotidine-5′-phosphate decarboxylase (ODCase; EC 4.1.1.23) on a vector. The integrative transformation system is considered to be advantageous for industrial application of this yeast because of its genetic stability.

MATERIALS AND METHODS

Strains and media. The parent strain for deriving uracil auxotrophic mutants was *C. boidinii* S2 strain AOU-1 (30), preserved in the AKU type culture collection (Department of Agricultural Chemistry, Kyoto University). Complex medium for yeast growth was YEPD medium (1% Bacto-yeast extract, 2% Bacto-tryptone [Difco Laboratories, Detroit, Mich.], 2% glucose). The minimal medium (YNB) contained 0.67% yeast nitrogen base without amino acids (Difco) and 2% glucose. Uracil was supplemented at a concentration of 20 μg/ml. The methanol synthetic medium was described previously (24).

Escherichia coli SA116 (F$^-$ *thi-1 supE44 leuB6 pyrF lacY1 tonA21 mcrA mcrB*$^-$) was a kind gift from S. Asahi (Takeda Pharmaceutical Co. Ltd., Osaka, Japan). *E. coli* cells were cultured on 2 × TY medium (1.6% Bacto-tryptone, 1.0% Bacto-yeast extract [Difco], 0.5% NaCl) or M9 medium (25) supplemented with 0.2% glucose, thiamine-HCl (50 μg/ml), L-threonine (40 μg/ml), and L-leucine (10 μg/ml) or uracil (2.4 μg/ml).

Plasmids. The Yeast-*E. coli* shuttle vector YRp7 (27) was a kind gift from Y. Kaneko (Institute for Fermentation Organization, Osaka, Japan). pBR322 was described previously (25).

DNA isolation. Yeast DNA was isolated by the method of Cryer et al. (9). Plasmid DNAs from *E. coli* were isolated by the method of Birnboim and Doly (4).

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Derivation of uracil auxotrophic yeast strains. Cells of *C. boidinii* were collected at the mid-log phase cultured on YEPD medium, washed twice with physiological saline, and mutagenized by UV light or by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as previously described (23). Mutagenized cells were washed with physiological saline twice, spread on a YNB medium plate containing 0.08% 5-fluoro-orotic acid (5-FOA; Sigma Chemical Co., St. Louis, Mo.), and incubated for 5 days at 28°C. Resistant colonies that appeared were replica plated onto YNB medium plates supplemented or not supplemented with uracil. Uracil auxotrophic mutant strains were thus obtained, rechecked for uracil auxotrophy, and subjected to the ODCase assay.

ODCase assay. Yeast cells were grown on YEPD medium and collected at the late log phase. Cell extracts were prepared by disrupting the cells, using glass beads (0.5-mm diameter) with a mini-beadbeater (model 3110BX; Biospec Products, Bartlesville, Okla.), in 0.1 M potassium phosphate buffer, pH 7.5, containing 5 mM β-mercaptoethanol. ODCase activity was assayed by the method of Yoshimoto et al. (32). Protein was determined by the method of Bradford (6), using bovine serum albumin as the standard.

Cloning of the ODCase gene (*URA3*) from the *C. boidinii* genome. *C. boidinii* total DNA (ca. 30 μg) was completely digested with *Bam*HI or *Sal*I and subjected to electrophoresis on a 0.7% low-melting-point agarose gel (SeaPlaque GTG; FMC Bioproducts, Inc., Rockland, Maine). DNA fragments larger than 1 kb were extracted from the gel and ligated into the *Bam*HI or *Sal*I site of YRp7. The recombinant plasmids were transformed into *E. coli* SA116 by the TFB method of Hanahan (16) at a frequency of 5×10^8 CFU/μg of DNA. Among the recombinant clones, seven independent $pyrF^+$ colonies were isolated from the *Sal*I library. All of the clones contained the *C. boidinii URA3* gene as described in Results.

Transformation of *C. boidinii*. Transformation of yeast cells was performed by the conventional lithium acetate method (18) or by the spheroplast method (17).

Southern analysis. Five micrograms of yeast DNA was digested completely with several restriction enzymes. Digested DNA was subjected to 0.6% agarose gel electrophoresis in TBE buffer (25). The gel was alkaline denatured in 0.1 N NaOH–1.5 M NaCl and neutralized in 0.5 M Tris-HCl (pH 7.0)–1.5 M NaCl. Alkaline-denatured DNA was blotted onto Biodyne nylon membrane (Pall Bio Support, New York) by the capillary action of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0) (26). Blotted DNA was fixed by baking the filter at 80°C for 2 h in vacuo. A gel-purified 3.5-kb *Sal*I fragment harboring the *C. boidinii URA3* gene (*URA3* fragment) or pBR322 DNA was ^{32}P labeled by the random primer method (10). The hybridization solution contained labeled probe (10^5 to 10^6 cpm/ml), 6× SSC, 25 mM potassium phosphate (pH 7.5), 5× Denhardt's solution, 0.5% sodium dodecyl sulfate, and 50% formamide (2). Hybridization was performed at 42°C overnight. The hybridized filters were rinsed in 2× SSC, washed with 0.2× SSC at 65°C for three times, and exposed to X-ray film. The ^{32}P-labeled *URA3* fragment DNA was also detected and quantitated by direct counting of the specific band on filters with the AMBIS radioanalytic imaging systems (AMBIS Systems, Inc., San Diego, Calif.). The copy number of integrated plasmids per *C. boidinii* cell was estimated from the radioactive counts of the hybridizing band.

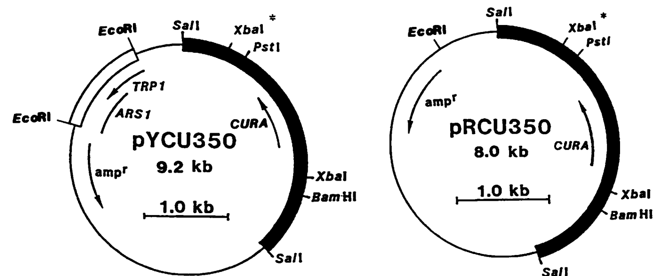

FIG. 1. Restriction maps of *C. boidinii URA3*-harboring plasmids pYCU350 and pRCU350. One of the *Xba*I sites (marked with an asterisk) could not be cut by the enzyme when the plasmid DNA was prepared from an *E. coli dam*$^+$ strain. Positions and directions of transcription of the genes are shown by arrows. The plasmid contains sequences from the following sources: pBR322, thin line; DNA from *C. boidinii*, thick line; DNA from *S. cerevisiae*, open box; *C. boidinii URA3* coding sequence, *CURA*.

RESULTS

Cloning of the *C. boidinii URA3* gene by complementing the *pyrF* mutation of *E. coli*. Among the yeast host-vector systems, the most sophisticated system is considered to be the use of the *ura3* mutation in *S. cerevisiae*, in which a *ura3* strain can be positively selected on 5-FOA plates (5). It seemed most efficient to use this powerful positive selection to isolate *ura3* host strains of *C. boidinii*, whose ploidy was unknown. Furthermore, the *URA3* gene of *S. cerevisiae* is known to complement the *pyrF* mutation of *E. coli*, using its fortuitous promoter activity in *E. coli* (21). Thus, the *E. coli* clone harboring the *C. boidinii URA3* gene was expected to be selected on uracil-deficient medium by using the high competency of *E. coli*. For these reasons, the DNA fragment harboring the *URA3* gene was cloned from the genomic library of *C. boidinii* as the first step in constructing a transformation system.

Total DNA of *C. boidinii* was completely digested by *Bam*HI or *Sal*I, ligated into the corresponding site of YRp7, and transformed into *E. coli* SA116. Each gene library contained ca. 1.0×10^4 independent recombinant clones. When these library clones were spread on uracil-deficient medium, seven independent $pyrF^+$ colonies were isolated from the *Sal*I library. All of these clones had the same 3.5-kb *Sal*I insert DNA on YRp7. The physical map of one of these plasmids, pYCU350, is shown in Fig. 1. The DNA of pYCU350 transformed the cells of *E. coli* SA116 to Ura$^+$ at a high frequency. Verification that the isolated gene was a *C. boidinii URA3* structural gene and not a DNA suppressing the *pyrF* mutation was obtained as follows: (i) pYCU350 complemented the *ura3* mutation in *S. cerevisiae* at a high frequency, (ii) ODCase activity was recovered in *E. coli* SA116, and (iii) the *C. boidinii URA3* gene was similar in sequence to reported ODCase genes from other sources (24b).

Isolation of ODCase-negative (*ura3*) mutant strains capable of growth on methanol. The second step in developing a transformation system was the isolation of *ura3* host mutant strains of *C. boidinii*. *C. boidinii* cells were mutagenized by UV light or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and spread on 5-FOA plates. Among ca. 6.0×10^5 viable cells after mutagenesis, 2.4×10^3 strains showed 5-FOA resistance and 29 strains were uracil auxotrophs. Among these uracil auxotrophs, nine were defective in ODCase activity (Table 1). However, three of them could not grow on

TABLE 1. ODCase activity, reversion rate, and methylotrophic growth of uracil auxotrophic mutant strains of *C. boidinii*

Strain	ODCase activity (U/mg of protein)	Reversion rate[a]	Growth on methanol[b] (h)
Mutant			
TK04	0	3.4×10^{-3}	6.2
TK40	0	4.8×10^{-8}	21
TK51	0	5.9×10^{-7}	15
TK53	0	3.5×10^{-6}	31
TK54	0	1.1×10^{-7}	11
TK62	0	1.0×10^{-9}	6.7
TK38	0	ND[c]	—[d]
TK52	0	ND	—
TK61	0	ND	—
Parent	0.139		4.9

[a] Determined by growing the cells on nonselective YEPD medium for 10 generations and examining them for uracil auxotrophy.
[b] Expressed as the estimated doubling time in synthetic methanol medium (23).
[c] ND, not determined.
[d] —, unable to grow on methanol.

methanol. One mutant, strain TK04, showed a high reversion frequency of 3.4×10^{-3} and could not be used as a host strain. The reversion frequencies of the other five strains were in the range of 3.5×10^{-6} to 1.0×10^{-9}, permissible for use as a host strain for transformation. Each of these five strains (TK40, TK51, TK53, TK54, and TK62) was tested as a host strain for transformation.

Transformation of *C. boidinii* with linearized plasmid pRCU350. To determine which mutant strain is the most suitable host for transformation, the five ODCase-negative strains were treated with lithium acetate, and transformation was examined with a linearized plasmid vector harboring the *C. boidinii URA3* gene. A plasmid vector, pRCU350, was constructed from the isolated plasmid, pYCU350, by eliminating the 1.5-kb *Eco*RI fragment harboring *Saccharomyces cerevisiae TRP1-ARS1* (Fig. 1). Although the *C. boidinii URA3* fragment had two *Xba*I sites, only one of them could be cut by *Xba*I when the plasmid DNA was prepared from *E. coli dam*$^+$ strains. Therefore, pRCU350 plasmid DNA was prepared from *E. coli* JM109 and linearized by *Xba*I digestion. Transformation with linearized pRCU350 resulted in Ura$^+$ transformant colonies after 3 days of incubation. The linearized pRCU350 DNA could transform all of the five putative host strains of *C. boidinii*, giving two colony sizes, large and small (Table 2). The ratio of large colonies to small colonies differed among the host strains. Of the five host strains tested, mutant strain TK62 gave the best transformation frequency (46 CFU/μg of DNA), the lowest reversion rate (1.0×10^{-9}), and good growth on methanol. Therefore, this strain was selected for use as the host strain for transformation in the following experiments.

As shown in Table 2, transformation with the circular pRCU350 vector occurs only at the low frequency of ca. 0.2 CFU/μg of DNA. Although in *H. polymorpha* the *S. cerevisiae ARS1* fragment is known to increase the transformation frequency (20), in this assay no transformant colonies were obtained from a plasmid vector, pYCU350 harboring the *ARS1* fragment. Therefore, *ARS1* from *S. cerevisiae* did not appear to function in *C. boidinii*. Transformation with a circular or linearized vector, YIp5 (harboring the *S. cerevisiae URA3* gene on pBR322), resulted in no transformants.

The spheroplast method is known to yield a higher transformation frequency than the lithium acetate method in *S. cerevisiae* (18). However, in *C. boidinii*, the transformation efficiency by the spheroplast method was almost the same as that by the lithium acetate method (Table 2).

Stability and Southern analysis of the transformants. To characterize the mode of transformation in *C. boidinii* TK62 with a linearized vector, pRCU350, further analysis was made with 12 independent transformants, which consisted of 8 transformants (L-1 to L-8) from large colonies and 4 transformants (S-1 to S-4) from small colonies. First, the phenotypic stability of these transformants was examined after nonselective growth for 10 generations. All of the transformants from small colonies as well as those from large colonies were found to retain a Ura$^+$ phenotype after nonselective growth (Table 3). This result suggested that the vector DNA was integrated into the chromosomal DNA of a host strain by homologous recombination in both large and small colonies.

To confirm this possibility, the genomic DNAs from the transformants were isolated and Southern hybridization was conducted, using either the ^{32}P-labeled DNA of pBR322 or the *C. boidinii URA3* fragment as a probe. Because pRCU350 has one *Eco*RI site in pBR322 DNA, two *Xba*I sites in the *URA3* fragment, and no *Bgl*II sites (Fig. 1), the transformant yeast DNAs were digested with these enzymes and separated by gel electrophoresis in 0.6% agarose. From

TABLE 2. Transformation of *C. boidinii ura3* host strains with *C. boidinii URA3*-harboring plasmids

Host strain	Plasmid	Plasmid form	Transformation frequency[a] (CFU/μg of DNA)	Large-/small-colony ratio
TK62	pRCU350	Linearized by *Xba*I	46 (20)	1:4.4 (1:4.0)
		Circular	0.2 (0)	ND[b]
	pYCU350	Circular	0 (0)	
	pBR322	Circular	0 (0)	
		Linearized by *Sal*I	0 (0)	
	YIp5	Circular	0 (0)	
		Linearized by *Nco*I	0 (0)	
TK40	pRCU350	Linearized by *Xba*I	11	1:4.3
TK51	pRCU350	Linearized by *Xba*I	3	1:0
TK53	pRCU350	Linearized by *Xba*I	14	1:1.2
TK54	pRCU350	Linearized by *Xba*I	9	1:1

[a] Transformation was performed by the lithium acetate method or the protoplast method. Two micrograms of each plasmid DNA was used. After incubation at 28°C for 3 days on YNB selection medium, the number of colonies that appeared was counted. Numbers in parentheses indicate the data obtained with the protoplast method.
[b] ND, not determined.

TABLE 3. Summary of the analyzed transformants of *C. boidinii* TK62

Transformant	Colony size	Stability[a] (%)	Pattern of Southern analysis[b]	Copy no. of *C. boidinii URA3*-hybridizing sequences[c]
L-1	Large	100	A	2
L-2	Large	105	C	1
L-3	Large	109	A	2
L-4	Large	100	A	2
L-5	Large	95	B	3
L-6	Large	102	A	2
L-7	Large	98	B	3
L-8	Large	93	B	4
S-1	Small	116	A	2
S-2	Small	113	B	4
S-3	Small	110	C	1
S-4	Small	101	A	2

[a] Stability of the Ura^+ phenotype was determined by growing the cells on nonselective YEPD medium for 10 generations and examining them for uracil auxotrophy.

[b] A; single integrant; B, multiple integrant; C, gene convertant (see Results).

[c] Estimated from Southern analysis as described in Results.

extensive Southern analysis, the transformants were classified into three groups by hybridizing-band patterns (patterns A to C). The most frequent banding pattern was pattern A (Fig. 2A) and had the following characteristics: (i) pBR322 DNA hybridized with undigested high-molecular-weight chromosomal DNA (Fig. 2A, lanes 1 and 4); (ii) the single 4.8-kb band from *Bgl*II-digested DNA (corresponding to the *URA3* locus in a host strain; Fig. 2D, lane 2) shifted to a position showing a larger DNA fragment of approximately 14 kb with both probes, the *URA3* fragment and pBR322 DNA, in the transformant (Fig. 2A, lanes 2 and 5); and (iii) the single 6.0-kb *Eco*RI band in the host strain (Fig. 2D, lane 3) is split into two bands, 9.2 and 4.8 kb, in the transformant (Fig. 2A, lanes 3 and 6). These banding patterns and their sizes are completely consistent with the results representing a single integration event of pRCU350 at the *URA3* locus of chromosomal DNA in the host strain. The most characteristic features of the second most frequent banding pattern (pattern B; Fig. 2B) are (i) the hybridizing band from *Bgl*II-digested DNA (>23 kb; Fig. 2B, lanes 2 and 5), which was higher than that observed in pattern A; and (ii) the presence of an 8.0-kb band from *Eco*RI-digested DNA (corresponding to the size of linear pRCU350 DNA; Fig. 2B, lanes 3 and 6) in addition to the two bands appearing in pattern A. These banding patterns represent multiple integration of pRCU350 into the *URA3* locus of chromosomal DNA. The copy number of the *C. boidinii URA3*-hybridizing sequence was estimated directly by radioisotopic counting the *Bgl*II band with an AMBIS radioisotopic image analyzing system. As shown in Fig. 2E, quantitative analysis of the radioisotopic counts showed linearity with the presumptive copy number of *C. boidinii URA3*-hyridizing sequences. From this analysis, two (L-5) or three (L-8 and S-2) copies of pRCU350 were estimated to be integrated into the host chromosomal DNA. The last banding pattern (pattern C; Fig. 2C) was the same as for the host strain with a *URA3* fragment as a probe (Fig. 2D); ^{32}P-labeled pBR322 DNA did not hybridize with transformant yeast DNA. This is most likely due to gene conversion or double crossing over within the *URA3* locus. All other observed banding patterns not

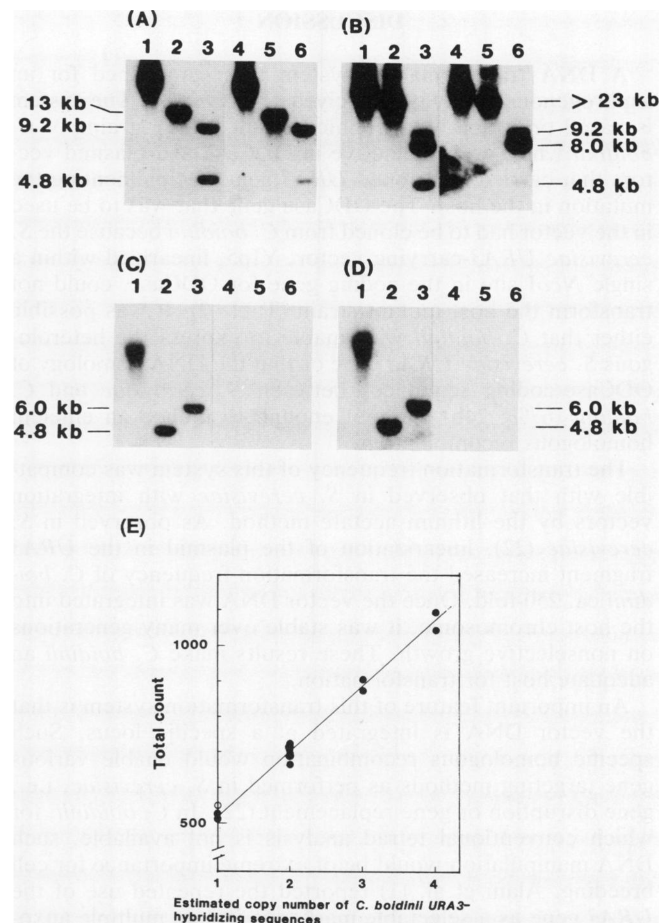

FIG. 2. Southern analysis of genomic DNA from various transformants. (A to D) Autoradiographs; (E) Plot of the radioisotopic count of the *Bgl*II band against the estimated copy number of *C. boidinii URA3*-hybridizing sequence with 12 analyzed transformants (●) and the host strain TK62 (○). The DNA from transformant strain S-1 (single integrant; A), transformant strain L-8 (multiple integrant; B), transformant strain L-2 (gene convertant; C), or the host strain TK62 (D) was used. Five micrograms of undigested DNA (lanes 1 and 4), *Bgl*II-digested DNA (lanes 2 and 5), or *Eco*RI-digested DNA (lanes 3 and 6) was loaded on a 0.6% agarose gel and subjected to Southern analysis as described in Materials and Methods. The hybridization was performed with a ^{32}P-labeled DNA as a probe, the *C. boidinii URA3* fragment (lane 1 to 3) or pBR322 (lane 4 to 6). The sizes of hybridizing bands shown were estimated from mobilities of molecular weight markers, *Hin*dIII- or *Sty*I-digested λ DNA.

mentioned here (e.g., data from *Xba*I-digested DNA) completely correlate with the deduced transformation events in all cases.

Of the 12 transformants analyzed, 6 were single integrations, 4 were multiple integrations, and 2 were gene conversions (Table 3). It is evident from the results obtained that all of the integration events occur at the *URA3* locus and that these events appear to involve homologous recombination with linearized pRCU350. However, the colony size did not reflect the transformation pattern, and all of the patterns were observed with DNA from both large and small colonies. Differences in banding patterns between the large and small colonies were not detected from these experiments.

DISCUSSION

A DNA transformation system was established for an asporogenous methylotrophic yeast, *C. boidinii*. The system is based on uracil auxotrophic mutant (*ura3*) strains of *C. boidinii* which were defective in ODCase and plasmid vectors that carried the cloned *URA3* gene complementing the mutation in the host. The ODCase gene that was to be used in the vector had to be cloned from *C. boidinii* because the *S. cerevisiae URA3*-carrying vector, YIp5, linearized within a single *Nco*I site in the coding gene for ODCase, could not transform the host mutant strain (Table 2). It was possible either that *C. boidinii* was unable to express the heterologous *S. cerevisiae URA3* gene or that the DNA homology of ODCase-coding sequences between *S. cerevisiae* and *C. boidinii* (67%; 24b) was not enough to induce an efficient homologous recombination.

The transformation frequency of this system was compatible with that observed in *S. cerevisiae* with integration vectors by the lithium acetate method. As observed in *S. cerevisiae* (22), linearization of the plasmid in the *URA3* fragment increased the transformation frequency of *C. boidinii* ca. 250-fold. Once the vector DNA was integrated into the host chromosome, it was stable over many generations on nonselective growth. These results make *C. boidinii* an adequate host for transformation.

An important feature of this transformation system is that the vector DNA is integrated at a specific locus. Such specific homologous recombination would enable various gene targeting methods as performed in *S. cerevisiae*, i.e., gene disruption or gene replacement (22). In *C. boidinii*, for which conventional tetrad analysis is not available, such DNA manipulation would be of extreme importance for cell breeding. Alani et al. (1) reported the repeated use of the *URA3* gene as a selectable marker to isolate multiple auxotrophic mutant strains. Similar methods would be applicable for constructing a double-marker strain of *C. boidinii* with the cloned isopropylmalate dehydrogenase gene (*C. boidinii LEU2*) (24b). A double-marker strain (Ura$^-$ Leu$^-$) would provide a more convenient system for subsequent introduction of two different exogenous DNAs into *C. boidinii*.

Candida albicans and *C. maltosa* are considered to be diploid strains (7, 19), and *C. albicans* and *C. tropicalis* are reported to have two loci hybridizable with *URA3* DNA (15, 19). Because the ploidy of *C. boidinii* was not known when we started the investigation, we anticipated difficulties for isolating auxotrophic mutant strains as observed in other *Candida* yeasts by other investigators (7, 12). Fortunately, *ura3* mutant strains arose at a frequency of 1.5×10^{-7} in the mutagenized cell population, and several *ura3* strains were isolated without an enrichment technique such as that by nystatin. From the Southern analysis with *C. boidinii* genomic DNA, only a single *URA3* locus was detected under the lowest-stringency conditions that we tested (37°C, 6× SSC, 30% formamide). Our *C. boidinii* seems to be haploid, at least during its vegetative growth.

Colonies of two sizes, large and small, are observed in the transformants of *C. boidinii*. Haas et al. (15) also observed the two distinct sizes in transformant colonies of *C. tropicalis*. They explained that the presence of plasmids with autonomous elements resulted in the slower growth of the small colonies. To our surprise, *C. boidinii* transformants from small colonies, as well as those from large colonies, showed a stable Ura$^+$ phenotype after nonselective growth. All types of integrative transformants (patterns A to C) were present in both large- and small-colony transformants (Table 3). To date, differences other than colony sizes have not been detected by Southern hybridization experiments. If gene conversion occurred simultaneously with the integration of plasmids into the host chromosome, there is a chance of the defective *ura3* sequence converting to an active *URA3* gene, or vice versa. This would result in transformants with a various number of active *URA3* gene independent of the type of integration event. Although, Southern analysis could not differentiate the mutated *ura3* sequence from the active *URA3* sequence, small-colony transformants may possess only one active *URA3* gene, and large-colony transformants may possess more than one. The difference in large-/small-colony ratio between the isolated *ura3* strains (Table 3) may reflect differences of mutation sites in the host strain that may have affected the recombinant event.

In summary, the established transformation system enables for the first time a fine genetic analysis of *C. boidinii*, which had been hindered by this yeast's lack of the sexual cycle. Previously, we established a method for isolating strains of *C. boidinii* mutant with respect to alcohol oxidase regulation (23). Recently, the alcohol oxidase gene (*AOD*) has been cloned from *C. boidinii* (24a). The established transformation system is necessary to investigate methanol regulation and catabolite repression at the molecular level.

The use of DNA manipulations of *C. boidinii* could be of interest in other areas of study. Goodman et al. (14) established a rapid peroxisome purification technique for *C. boidinii* and analyzed peroxisomal membrane transport. The established transformation system would be extremely useful for investigating peroxisomal proliferation or protein transport (3, 11). Another expected application of this transformation system would be for breeding of industrially useful strains. Heterologous or homologous gene expression under the strong inducible alcohol oxidase promoter or disruption of a specific gene may improve the productivity of useful metabolites that have been investigated with *C. boidinii*.

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