APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1987, p. 1504–1511
0099-2240/87/071504-08$02.00/0

Vol. 53, No. 7

Induction and Characterization of Artificial Diploids from the Haploid Yeast *Torulaspora delbrueckii*

TAKASHI SASAKI*[1] AND YOSHINOBU OHSHIMA[2]

Sankyo Co., Ltd., Bio-Science Research Laboratories, 1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140,[1] and Sankyo Co., Ltd., Tanashi Plant, Tanashi, Tokyo 188,[2] Japan

Received 4 August 1986/Accepted 17 March 1987

The yeast *Torulaspora delbrueckii*, which propagates as a haploid, was made into a diploid by treatment with dimethyl sulfoxide (DMSO) on the regeneration of protoplasts. The diploid state was stably inherited; the cell volume was three times that of the parent strain and the cellular DNA content was two times that of the parental strain. No essential difference was found between diploids induced by DMSO and those formed through intraspecific protoplast fusion. The diploid strains sporulated fairly well, with their cells converting directly into asci. Random spore analysis revealed that diploids induced through protoplast fusion gave rise to auxotrophic segregants (haploids) with the parental genetic marker or to segregants formed by recombination, while diploids induced by DMSO from a doubly auxotrophic parent gave rise to no recombinant, indicating that it was chromosomally homoallelic in nature. The magnesium level in the protoplast regeneration medium was found to be an important factor for inducing diploid formation. At 0.2 mM magnesium diploids appeared even in the absence of DMSO, while at 2 mM magnesium diploids never appeared unless DMSO was added to the regeneration medium. Evidence is provided that the diploids induced by DMSO or a low magnesium level are due to direct diploidization but not protoplast fusion. UV light irradiation of intact cells (without protoplasts), 10% of which survived, also produced diploids among this surviving population. From these results we conclude that the perturbation of protoplast regeneration or of cell division by the treatments mentioned above somehow induced direct diploidization of *T. delbrueckii*.

Torulaspora delbrueckii (formerly *Saccharomyces rosei* [2, 24]) was developed by Sankyo Co. with its affiliated company (Sankyo Foods Co.) as a bakery yeast which is tolerant to high sugar concentrations (18) and to freeze-thawing in dough (M. Haga and T. Iwata, Japanese patent 1,252,219, February 1985). The former property gives the advantage that this organism can be used to ferment sweet dough, such as buns, and the latter property makes it possible to store dough fermented once with the yeast cells at −20°C and then to ferment it again when it is needed after thawing. This yeast is widely marketed in Japan, but its industrial production has been hampered because of its small cell size. The yeast cell suspension must be dehydrated to make yeast cakes like those on the market after it is harvested and washed. The small cell size gives a particular disadvantage in this dehydration process; filtration of cells for dehydration requires a long time, and even worse, filtration cannot be conducted continuously because the filter for dehydration becomes clogged and must be changed frequently. The small cell size may be ascribed to the fact that *T. delbrueckii* is the organism that propagates vegetatively as a haploid (24).

This study was undertaken to circumvent this problem by obtaining large-sized cell strains, which are easily dehydrated. Successful approaches to this problem were made in the following three ways: (i) induction of artificial diploids by a newly developed method which consisted of perturbing protoplast regeneration; (ii) induction of artificial diploids by a newly established method in which intact yeast cells were irradiated with UV light; and (iii) induction of diploids through intraspecific protoplast fusion, as has been reported with another *T. delbrueckii* strain (Y. Nakatomi, Abstr. Annu. Meet. Agric. Chem. Soc. Japan, 1982, p. 567). Such a diploid state is stably inherited, with the cell volume being three times as large as that of the parent strain. Genetic evidence is also provided that the diploids induced by method 1 are made by direct diploidization, as are those induced by method 2, in contrast with those obtained by method 3, in which diploids are induced by the fusion of two protoplasts.

* Corresponding author.

MATERIALS AND METHODS

Organisms. *T. delbrueckii* SANK 50268 (formerly *S. rosei* SANK 50268) was obtained from our laboratory stock culture. The organism was deposited at the Fermentation Research Institute, Tsukuba, Japan, and is available as *S. rosei* Y-134-5. Auxotrophic mutants were isolated from this strain by treatment with ethyl methanesulfonate (14). Doubly auxotrophic mutants were obtained by stepwise treatments with ethyl methanesulfonate or through recombination-segregation from a diploid strain that was formed through protoplast fusion and that carried two mutagenized genes heterozygously.

Incubation was at 30°C in all experiments and in experiments with diploid strains derived here from SANK 50268.

Media. Complete medium consisted of 1% yeast extract (Difco Laboratories, Detroit, Mich.), 1% polypeptone, and 2% glucose (YPD). Standard minimal medium (MM) was yeast nitrogen base without amino acids (Difco) (6), to which 1% glucose was supplemented. A minimal medium with a low magnesium concentration (LMgMM) was prepared so that it had the same basal composition as MM, except that the $MgSO_4$ concentration was decreased to 1/10, or 0.2 mM. LMgMM contained the following per liter: $(NH_4)_2SO_4$, 5 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 50 mg; NaCl, 100 mg; $CaCl_2 \cdot 2H_2O$, 100 mg; H_3BO_3, 500 μg; $CuSO_4 \cdot 5H_2O$, 40

μg; KI, 100 μg; $FeCl_3 \cdot 6H_2O$, 200 μg; $MnSO_4 \cdot 4H_2O$, 400 μg; $NaMo_2O_4$, 200 μg; $ZnSO_4 \cdot 7H_2O$, 400 μg; biotin, 2 μg; calcium pantothenate, 400 μg; folic acid, 2 μg; inositol, 2,000 μg; nicotinic acid, 400 μg; *p*-aminobenzoic acid, 200 μg; pyridoxine hydrochloride, 400 μg; riboflavin, 200 μg; thiamine hydrochloride, 400 μg. The regeneration medium of protoplasts contained 1% glucose, 0.6 M KCl, and 2% agar in MM or LMgMM. (For conciseness, MM or LMgMM alone indicates those media containing 1% glucose, 0.6 M KCl, and 2% agar whenever protoplast regeneration was involved.) The compositions of other media used only once are described in the appropriate section.

Diploid formation through perturbation of protoplast regeneration (method 1). Cells of wild-type *T. delbrueckii* SANK 50268 grown aerobically in YPD were collected at the exponential growth phase by centrifugation and washed twice with solution A (0.6 M KCl, 20 mM Tris hydrochloride [pH 7.5]). The cells were suspended in solution A, to which 2-mercaptoethanol was added at a final concentration of 200 mM, and incubated with shaking in a water bath at 30°C for 20 min. The cells were collected by centrifugation, washed twice with solution A, and then suspended in 5 ml of the same solution at a concentration of approximately 4×10^8 cells per ml. To the suspension was added 3 mg of Zymolyase 60000 (Kirin Brewery Co., Takasaki, Gummaken, Japan), and the suspension was incubated with gentle shaking at 30°C for 1 h. Protoplast formation was checked by means of microscopy as follows. Two droplets of the suspension were mounted onto a glass slide; included in one of the droplets was 1 μl of 10% sodium *N*-lauroylsarcosinate. Experiments were continued only when protoplast formation was assessed to exceed 99% by comparing the detergent-treated suspension with the untreated control. Protoplasts were collected by centrifugation at $500 \times g$ for 10 min and washed twice with the same solution, from which a 0.2-ml portion was taken and mixed with 8 ml of melted regeneration MM agar (45°C), and poured over 15 ml of solidified regeneration MM agar.

Dimethyl sulfoxide (DMSO) was dispensed in regeneration MM agar for both the over- and underlayer at a concentration of 2.5% (vol/vol). After 3 to 4 days of incubation at 30°C, individual colonies were examined for cell size by microscopy. A total of 1 to 2% of the colonies consisted of large-sized cells, while plates without DMSO never gave large-sized cell clones, except for the case in which the concentration of magnesium in the regeneration medium was decreased to 0.2 mM (LMgMM), as described below. Concentrations of DMSO higher than 2.5% (5%) totally inhibited protoplast regeneration. If LMgMM was employed instead of MM for regeneration medium, large-sized cell colonies appeared in the absence of DMSO. A combination of 2.5% DMSO dispensed in LMgMM was most effective, however. Alternatively, paper disks (for antibiotic assay; Toyo Roshi Co., Tokyo, Japan) containing 50 to 100% (vol/vol) DMSO were placed on MM or LMgMM agar plates. Colonies appearing around the paper disks were examined. (Colonies should not be so close to one another that they mutually inhibit growth.) Large-sized cell clones were purified by repeating single-colony isolation twice. This method is termed perturbed protoplast regeneration.

Diploid formation by irradiation with UV light (method 2). Cells grown in YPD were suspended at an approximate concentration of 1.5×10^5 cells per ml in 67 mM KH_2PO_4 in a petri dish, followed by irradiation with UV light at 2,000 ergs/s per cm^2. A germicidal lamp was used; the intensity of its energy was measured with a Blak-Ray ultraviolet meter (model J225; UVP, Inc., San Gabriel, Calif.). A portion was withdrawn at time intervals and spread onto YPD plates. All manipulations were performed in dim light to avoid photoreactivation. After incubation for 2 days at 30°C in the dark, individual colonies on plates that showed 90 to 99% lethality were examined by microscopy for large-sized cell clones. There were large (normal) and small (petitelike) colonies on such plates. Large-sized cell clones with normal cell morphology without auxotrophic mutations were purified by single-colony isolation as described above. The occurrence of large-sized cell clones with both normal and abnormal cell morphologies comprised about 10% of the colonies examined, and almost all of them were from small colonies.

Diploid formation through protoplast fusion (method 3). Sixty-two mutant strains with singly or doubly auxotrophic requirement(s) were isolated and tested for dough fermentation and tolerance to freeze-thawing (13). Two strains that showed nearly the same activities as the parent (wild type) were selected for protoplast fusion. One required arginine and the other lysine. These strains were each grown aerobically in YPD; and at the exponential growth phase the cells were harvested, washed, and made into protoplasts by treatment with Zymolyase 60000 in 5 ml of solution A, as described above. The cell suspensions were both about 4×10^8 cells per ml. The two protoplast suspensions thus prepared were thoroughly mixed before centrifugation and then were washed twice with 0.4 M $CaCl_2$. The protoplast pellet was suspended in 5 ml of a solution containing 30% polyethylene glycol 4000 and 25 mM $CaCl_2$. The suspension was incubated at 30°C for 20 min, and a 0.2-ml portion was taken into 7 to 8 ml of melted agar (43°C) containing 0.4 M $CaCl_2$ and 2% agar, which was poured over 15 ml of solidified MM regeneration agar. The plates were incubated at 30°C for 3 to 6 days. Fusants (phenotypically prototrophic) were purified by single-colony isolation repeated twice on MM plates. Among a lot of such fusants, strains F26, F31, and F32 were selected through dough fermentation tests, which was done in view of the industrial application. Other combinations of mutants that were less potent in dough fermentation tests were also employed for protoplast fusion, which yielded fusants F6, F7, and F15. The fusion frequency ranged from 3.2×10^{-6} to 1.8×10^{-5}, as calculated from the actual CFU of the same protoplast suspension on MM plates that were supplemented with arginine and lysine, or other nutrients in the case of other parental combinations. These values were far beyond the back mutation rates of the parents ($<10^{-8}$). The fusants had large cell volumes that were compatible with the volumes of the large-sized cell strains that were obtained by perturbed protoplast regeneration or UV light irradiation. They showed no requirement for the nutrients that were essential for their parents, which were used for protoplast fusion and had auxotrophic mutations.

Photomicrographs. Micrographs of cells in Fig. 1 were taken with microscope (model BH-2; Olympus Optics Industry Co., Tokyo, Japan) with Nomarski differential interference equipment.

Measurement of cell sizes. Incubation was performed aerobically for 2 days in medium containing the following ingredients (in grams per liter): molasses, 50 (as glucose equivalent); urea, 2.8; KH_2PO_4, 0.4; $(NH_4)_2SO_4$, 1; $MgSO_4 \cdot 7H_2O$, 0.3 (pH 5.3). This is the medium that our company uses to prepare the seed culture of *T. delbrueckii* for subsequent large-scale cultivation. Cells in the stationary phase of growth were harvested by centrifugation and washed twice with saline, and micrographs were taken with a conventional phase-contrast microscope. The cell lengths

and widths were measured from the enlarged positive images by using a caliper for 50 cells. The cell volumes (V) were determined by using the equation for an ellipsoid, $V = (4/3 \times \pi \times a \times b^2)$, where a is half the length and b is half the width.

Determination of cellular DNA contents. Cells were grown aerobically in YPD for 2 days, collected by centrifugation, and washed twice with distilled water. Cellular DNA contents were analyzed in two ways. For chemical analysis, cellular DNA was extracted by the method described by Bostock (3) and determined by the diphenylamine method described by Burton (4), with calf thymus DNA (type I; Sigma Chemical Co., St. Louis, Mo.) used as the standard. The cell number was calculated by using a hemacytometer (Thoma). Experiments were run in duplicate for obtaining single DNA values. Alternatively, flow microfluorometry was carried out with cells for determining the relative DNA contents by the method described by Slater et al. (17), who used mithramycin for nuclear staining (19). A cell sorter (EPICS; model 753; Coulter Electronics Inc., Hialeah, Fla.) was used for this, with the excitation of mithramycin by a 458-nm line operated at a laser light output of 500 mW. Fluorescence data were collected on 10^5 cells (counted by light scattering) for each sample. Mithramycin was purchased from Sigma.

Measurement of doubling time. The doubling time was measured on an exponentially growing culture in 100 ml of YPD in a shake flask fitted with a stopcock, through which portions were withdrawn at time intervals. The samples were diluted with distilled water when necessary and measured for turbidity with a spectrophotometer at 630 nm with a 0.5-cm path in cuvettes. The final cell yield was determined after growth reached a plateau; the cells were collected by centrifugation, washed twice with distilled water, and then dried on a planchet by using an infrared lamp.

Assimilation of carbon compounds. The carbon compound assimilation test was performed in 5 ml of yeast nitrogen base without amino acids supplemented with 5 mg of a carbon compound per ml. At the early stationary phase YPD-grown cells were harvested by centrifugation, washed with saline, and diluted to 1/50 with the same solution. The test culture was inoculated with 0.1 ml of this cell suspension and incubated. Growth was checked on days 10, 17, and 30, with the same results.

Spore formation and random spore analysis. Cells were grown aerobically in 40 ml of YPD to the stationary phase, harvested by centrifugation, washed twice with saline, and suspended in 5 ml of saline. Portions (0.2 ml) were spread on sporulation agar plates (16), which were incubated at 30°C for 2 days. Cells containing asci were collected from the plates, and micrographs were taken with a phase-contrast microscope. The number of spores in the asci was counted from enlarged positive images for approximately 500 asci. There were cells in which it was not obvious whether they were asci, and there were asci containing spores but it was difficult to count the number of spores. Such cells and asci were classified as unknown when the number of spores in the asci were examined.

Random spore analysis for genetic studies was carried out with spores isolated from asci. The asci were collected from sporulation plates and washed twice with solution A and were made into protoplasts in solution A by treatment with zymolyase as described above. The asci were bursted in 6.7 mM Tris hydrochloride buffer (pH 7.8) after they were washed twice with solution A. The released spores (containing monads, dyads, triads, and tetrads) were washed twice with the same buffer and treated briefly and repeatedly with a sonic oscillator until all the spore complexes were dissociated into single spores.

TABLE 1. Effects of DMSO and magnesium concentration on diploid formation[a]

Mg^{2+} concn (mM)	DMSO concn (%)	No. of colonies examined	No. of large-sized cell colonies (%)
2.0	0	100	0 (0)
	1	15	0 (0)
	2.5	81	1 (1.2)
0.2	0	102	2 (2.0)
	1	60	2 (3.3)
	2.5	30	4 (13.3)

[a] Protoplasts were regenerated on MM or LMgMM containing DMSO at various concentrations. The regenerants were examined by microscopy for the occurrence of large-sized cell colonies, as described in the text. Namely, large-sized cell clones were confirmed by single-colony isolation repeated twice.

RESULTS

Effects of different magnesium and DMSO concentrations on diploid formation. In Table 1 the occurrence of diploid formation (large-sized cell colonies) through perturbed protoplast regeneration by method 1 is shown. When the magnesium level in the protoplast regeneration medium was 2 mM, large-sized cell colonies never appeared, except that DMSO was added at a concentration of 2.5%, although the regeneration frequencies were nearly the same without DMSO. Namely, the regeneration frequencies were 0.41 ± 0.29% for MM, 0.50 ± 0.47% for LMgMM, and 0.81 ± 0.80% for LMgMM containing 2.5% DMSO (mean ± standard deviation of three experiments). Regeneration on other reference hypertonic agar media was evaluated at the same time, as follows: 35.9 ± 14.7% for MM (underlayer)–0.4 M $CaCl_2$ (overlayer), 22.9 ± 10.5% for MM containing 2.5% DMSO (underlayer)–0.4 M $CaCl_2$ containing 2.5% DMSO (overlayer), and 2.0 ± 1.9% for 0.3% yeast extract, 0.3% malt extract, 0.5% polypeptone, 1% glucose, and 0.6 M KCl. Large-sized cell colonies never appeared on MM in the absence of DMSO as confirmed by experiments repeated over 10 times. If the magnesium level was decreased to 0.2 mM in the regeneration medium (LMgMM), large-sized cell colonies appeared in 1 to 2% of the regenerants from protoplasts without DMSO. A combination of a low magnesium concentration with 2.5% DMSO was found to be the most effective for inducing diploid formation. Often, over 10% of regenerants consisted of large-sized cell clones in such media (Table 1).

Intact cells were not converted to large-sized cell clones, even if they were grown in liquid LMgMM with increasing concentrations of DMSO (up to 17.5%; vol/vol), although some morphological changes occurred transiently (physiologically) in such media containing greater than 15% DMSO to inhibit growth totally. In the range of 5 to 15% DMSO, growth was inhibited in response to the concentration, but no morphological change was observed nor was a large-sized cell line produced.

Cell size. Micrographs of cells of the parent strain *T. delbrueckii* SANK 50268 (haploid) and large-sized cell lines that were induced therefrom by the three methods described above are shown in Fig. 1. Strain YL3, which was obtained through the perturbed protoplast regeneration with DMSO by method 1, had a cell volume that was larger than that of the parent strain under the same growth conditions. This also applied to strain LUV1 by method 2 and the fusant F31

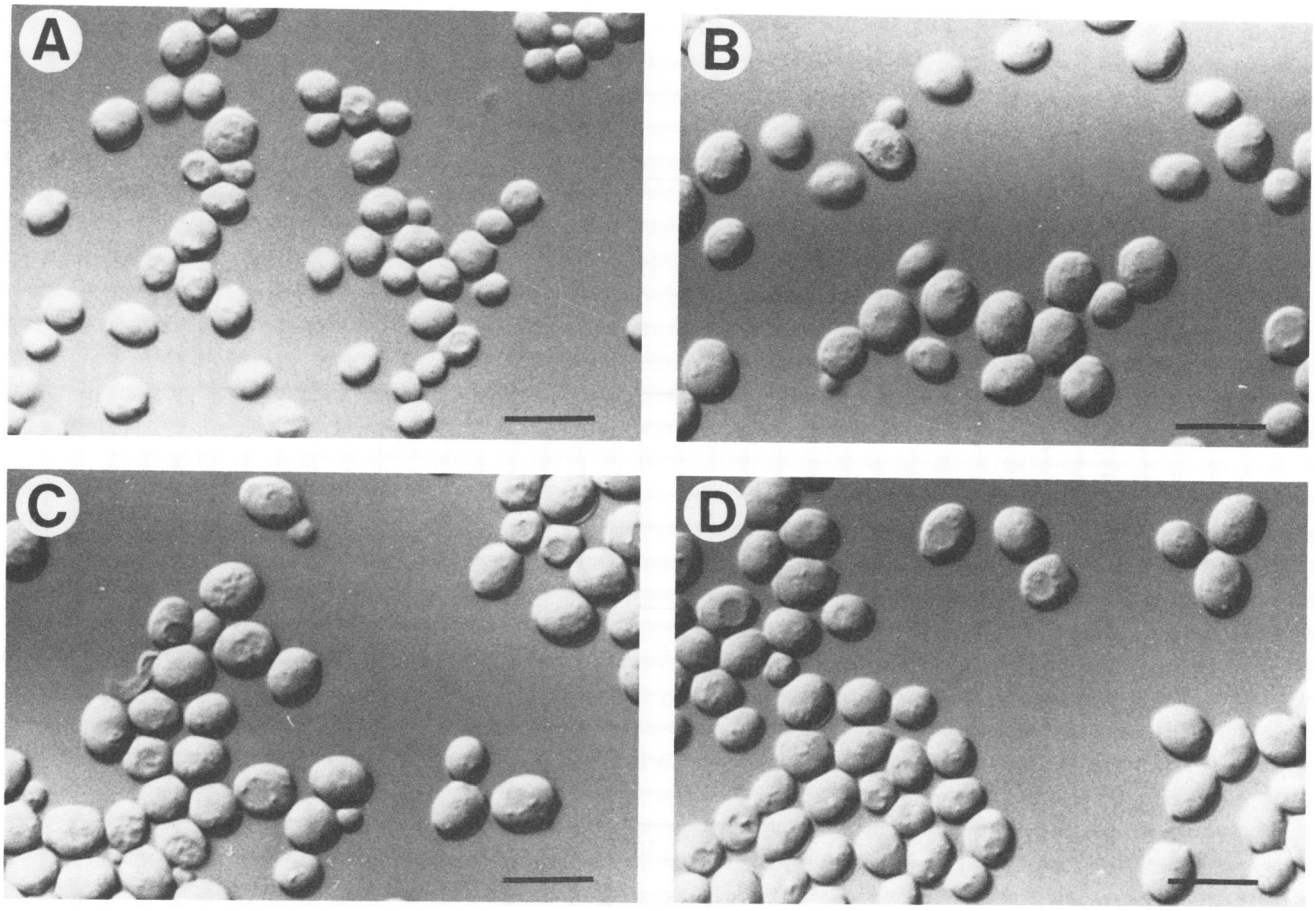

FIG. 1. Micrographs of the parent and diploids induced by three different methods. Overnight standing cultures of the organism in 5 ml of YPD were taken for micrography. (A) SANK 50268; (B) diploid YL3 induced by the perturbed protoplast regeneration in the presence of DMSO on MM; (C) diploid LUV1 induced by UV light irradiation; (D) diploid F31 formed through protoplast fusion. Bars; 10 μm.

by method 3. Values for cell volume (Table 2) suggest that YL1 and YL3 were diploids, as was F31; this suggestion was actually confirmed by later analyzing relative cellular DNA contents (Fig. 2). Strain F31-S12, which was derived from a spore of F31, had a small cell size, like that of SANK 50268. Because F31-S12 required both arginine and lysine, it was concluded that it was haploid. This occurred through recombination and segregation of Arg^- and Lys^-, each of which came from the two parents of protoplast fusion. Then, F31-S12 was made into protoplasts and treated with DMSO by method 1 to give rise to a large-sized cell strain, F31-S12-L1. This strain required arginine and lysine, but its cell volume was about three times as large as that of F31-S12 (Table 2). The large-sized cell strain LMg1 that was obtained by protoplast regeneration in the medium with a low level of magnesium (LMgMM) and LUV1 obtained by UV light irradiation of intact cells had the same range of cell volumes as YL1, YL3, and F31. The distribution of cell sizes in the SANK 50268, YL3, and F31 populations was drawn in histograms. Among the population of SANK 50268 cells, there were cells larger than the small cells of YL3 and F31, but median cell volumes became obviously larger in the last two strains (data not shown).

TABLE 2. Cell sizes of the parent and several derivatives[a]

Expt and strain	Length (μm)	Width (μm)	Vol (μm^3)	Vol ratio
Expt 1				
SANK 50268	3.5 ± 0.5	3.0 ± 0.5	18.3 ± 7.4	1
YL1	5.0 ± 0.7	4.8 ± 0.6	59.1 ± 18.3	3.2
YL3	5.0 ± 0.7	4.9 ± 0.6	60.9 ± 17.6	3.3
F31	4.9 ± 0.7	4.5 ± 0.6	51.4 ± 16.8	2.8
F31-S12	3.5 ± 0.5	3.4 ± 0.5	20.8 ± 8.7	1.1
F31-S12-L1	5.0 ± 0.8	4.9 ± 0.7	61.4 ± 20.2	3.4
Extp 2				
SANK 50268	3.5 ± 0.6	2.8 ± 0.6	15.9 ± 9.2	1
YL3	5.4 ± 0.6	4.4 ± 0.5	55.8 ± 19.5	3.5
YL4	5.1 ± 0.7	4.2 ± 0.5	48.7 ± 16.3	3.1
YL5	5.6 ± 0.7	4.5 ± 0.6	60.5 ± 20.9	3.8
LMg1	5.4 ± 0.6	4.3 ± 0.5	52.6 ± 17.1	3.3
LUV1	5.3 ± 0.7	4.2 ± 0.5	50.6 ± 19.9	3.2

[a] YL1, YL4, and YL5 were obtained as described in the legend to Fig. 1 for YL3. LMg1 was obtained through perturbed protoplast regeneration on LMgMM. For other strain designations, see the legend to Fig. 1 and the text. Cell sizes are expressed as the mean ± standard deviation.

The cell size differed according to cultural conditions, but the large-sized cell lines were always 2 to 3 times larger than the parent SANK 50268 when grown under the same conditions.

DNA contents. YL1, YL3, LMg1, LUV1, and three fusants (F26, F31, F32) were analyzed for relative cellular DNA contents by flow microfluorometry (Fig. 2). Their DNA contents were double that of the parent SANK 50268,

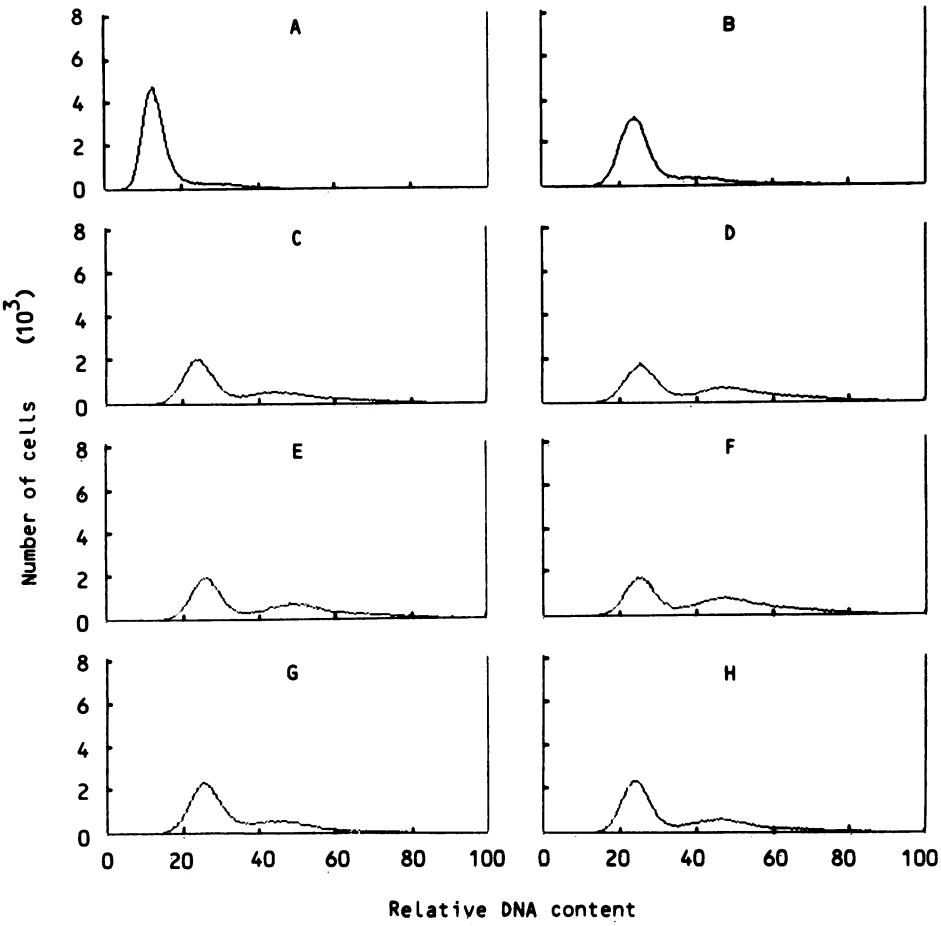

FIG. 2. Flow microfluorometric analysis of population DNA content. (A) SANK 50268; (B) YL1; (C) YL3; (D) F26; (E) F31; (F) F32; (G) LMg1; (H) LUV1. For strain designations see the legend to Fig. 1, Table 2, and the text.

indicating that they were diploids. The chemical analysis by the diphenylamine method (4) revealed that YL3 contained 41.2 ± 4.2 fg of DNA per cell (n = 3), which was nearly double the value of 21.9 ± 1.4 fg of DNA per cell (n = 3) for SANK 50268.

Assimilation of carbon compounds. YL1, YL3, and the fusants that were tested (F6, F7, F31, F32) shared the same spectrum with the parent SANK 50268 in the assimilation of 28 carbon compounds. The assimilated compounds were glucose, inulin, lactic acid, mannitol, raffinose, sorbitol, L-sorbose, sucrose, and trehalose. The unassimilated compounds were D-arabinose, L-arabinose, cellobiose, citric acid, erythritol, galactitol, galactose, lactose, maltose, melezitose, melibiose, α-methyl-D-glucoside, rhamnose, ribitol, ribose, salicin, starch, succinic acid, and xylose. This fact indicates that genomes of artificially induced diploids are expressed perfectly.

Doubling time and final cell yields. SANK 50268, YL1, YL3, F31, and F31 showed the same doubling time of 84 min and almost the same final cell yields (9.7 to 10.3 mg [dry weight]/ml) when grown in YPD, despite the difference in cell sizes (data not shown).

Random spore analysis. Artificially induced diploids sporulated fairly well in comparison with the parent SANK 50268 on the sporulation plate described by Sherman et al. (16). The sporulation rate of diploids ranged from 54.6 to 68.1%, while that of the parent was 21.1% (Table 3). The difference in sporulation rate was remarkable on YPD plates (nonsporulation medium). It was almost impossible to find asci in strain SANK 50268 grown on YPD plates, whereas the diploid strains produced asci well on incubation for over 3 days at 30°C. Diploid cells converted directly into asci without making protuberances, which contained generally one to two and, to a lesser extent, three to four spores. It should be added, however, that they were stable diploids and that sporulation did not seem to lead to haploidization unless spores were isolated out of the asci. In other words, we did not encounter haploids from YPD plates or slants of the diploids once they were established. Actually, after storage for 3 years at 4°C, all the slants retained large-sized cell lines, except for one strain that had been obtained

TABLE 3. Number of spores in asci produced from the parent and diploids[a]

Strain	No. of asci with the following no. of spores:						% Sporulation
	Unknown	0	1	2	3	4	
SANK 50268	53	339	23	42	9	17	21.1
YL3	41	148	164	87	20	45	68.1
LUV1	45	182	140	89	38	65	64.6
F31	47	239	147	92	18	34	54.9
F31-S12-L1	24	206	135	96	8	35	57.1

[a] The method by which the strain was obtained is described in the legend to Fig. 1. For F31-S12-L1, see text. Sporulation was induced on sporulation plates, and asci were inspected as described in the text.

TABLE 4. Random spore analysis with a diploid induced through the perturbed protoplast regeneration and three diploids obtained through protoplast fusion

Strain	Nutritional requirements of meiotic segregants		
F7.....Prototroph (63)[b]	Ade$^-$ (56)	Ura$^-$ (62)	Ade$^-$ Ura$^-$ (50)
F15.....Prototroph (52)	Ade$^-$ (54)	Lys$^-$ (63)	Ade$^-$ Lys$^-$ (47)
F31.....Prototroph (108)	Arg$^-$ (73)	Lys$^-$ (80)	Arg$^-$ Lys$^-$ (105)
F31-S12-L1.....Prototroph (0)	Arg$^-$ (0)	Lys$^-$ (0)	Arg$^-$ Lys$^-$ (162)

[a] F7, F15, and F31 were the fusion products of Ade$^-$ + Ura$^-$, Ade$^-$ + Lys$^-$, and Arg$^-$ + Lys$^-$, respectively. F31-S12-L1 was obtained as described in the text.

[b] Values in parentheses represent the numbers of clones found.

through protoplast fusion. Besides, cultivation of the diploid YL3 on an industrial scale of over 100 kl produced no haploid when individual colonies were examined for cell size by microscopy after transfer and growth on YPD plates. This type of confirmation was made twice for about 60 colonies (data not shown).

Four diploid strains were examined genetically by random spore analysis, as described above (Table 4). The fusants F7 and F15 showed segregation into approximately 1:1:1:1, being tetratypes from diploids. The fusant F31 showed a relative value of 1:0.8:0.8:1, but this deviation was not tested further. On the other hand, the diploid F31-S12-L1 that was obtained by perturbed protoplast regeneration gave rise to Arg$^-$ Lys$^-$ progeny alone, which were the same markers obtained with the parent F31-S12-L1. This fact indicates that this strain is made into a diploid directly and so is homoallelic.

Apart from random spore analysis, the diploid LUV1, which was obtained by UV light irradiation, produced wild-type progeny alone; none of the 147 spores tested was an auxotrophic mutant.

Evidence of direct diploidization. There is no need to present evidence for direct diploidization (fusion of mother and daughter nuclei before cytokinesis) or endomitosis (no nuclear division after chromosome duplication) in diploids that were obtained by UV light irradiation on intact SANK 50268 cells (method 2 above), while such evidence should be provided for method 1 in which perturbation of protoplast regeneration induces diploid formation, because DMSO is known to bring about cell fusion in mammalian cells at a concentration of 35% (1) and also is known to enhance the frequency of polyethylene glycol-mediated protoplast fusion in yeasts at a concentration of 15% (12). The 2.5% DMSO concentration used in this study was much lower than those concentrations used for the fusion experiments described above, but analysis of the fact that method 1 induces no protoplast fusion is still required.

Two doubly auxotrophic mutants FVII-S1 (Ade$^-$ Ura$^-$) and Y-19-15 (Arg$^-$ Met$^-$) were mixed, made into protoplasts, and then regenerated on LMgMM with or without 2.5% DMSO (Table 5). If diploids (large-sized cell lines) were induced through protoplast fusion, the occurrence of prototrophic clones with large cell volumes could be expected because all the markers Ade$^-$ Ura$^-$ Arg$^-$ Met$^-$ should be masked phenotypically by heteroallelism. For the other case in which diploids were induced through direct diploidization or endomitosis, large-sized cell clones could be expected with either of the parental markers, i.e., Ade$^-$, Ura$^-$ or Arg$^-$ Met$^-$. In Table 5 the latter mechanism is shown to be responsible; none of the large-sized cell clones were prototrophic. Essentially the same results were obtained for the plates with and without DMSO in the LMgMM regeneration medium with regard to the occurrence of direct diploidization. The deviations in the occurrence of Ade$^-$ Ura$^-$ over that of Arg$^-$ Met$^-$ among the large-sized cell colonies indicated in Table 5 rather than equal numbers can be ascribed to the fact that regeneration frequencies of the parents differed, as described below. When the same mixed protoplasts were embedded in MM plates of three types, a similar deviation in regeneration was observed. Namely, the regenerant number was 2.5×10^5/ml on the MM plates supplemented completely for both parental requirements (adenine, uracil, arginine, methionine), while the regenerant numbers were 1.4×10^5/ml for the MM plates with adenine and uracil and 0.6×10^5/ml for the MM plates with arginine and methionine.

DISCUSSION

Protoplasts of yeasts are widely used today for intra- and interspecific protoplast fusion and genetic transformation with chimeric plasmids to produce heterologous proteins. We found that regeneration of protoplasts of *T. delbrueckii* SANK 50268 produced large-sized cell clones (diploids) in yeast nitrogen base without amino acids plus glucose, agar, and 0.6 M KCl as a stabilizer if DMSO was dispensed at 2.5%. Although DMSO was essential for inducing diploid formation in that medium, it could be deleted if the magnesium level in the medium was decreased from 2 to 0.2 mM. Combination of the medium with a 0.2 mM magnesium concentration or below with 2.5% DMSO was most effective for such purposes. The exact mechanisms by which DMSO or low magnesium levels in the protoplast regeneration medium induced diploid formation are not known. Difficulty exists for analyzing the phenomena described above because only about 400 colonies at most could be examined by microscopy in 1 day by one person and, furthermore, cell sizes of colonies changed physiologically, becoming large, so that haploids and diploids could not be distinguished even if test plates were stored at 4°C. The optimal period for

TABLE 5. Requirements of large-sized cell clones regenerated from the mixed protoplasts of two mutants[a]

Chemical added	No. of regenerants/ml (10^4)	No. of large-sized/no. examined (%)	No. with the following requirements:		
			Ade$^-$ Ura$^-$	Prototroph	Arg$^-$ Met$^-$
None	4.8	21/98 (21.4)	19	0	2
DMSO	2.4	17/51 (33.3)	14	0	3

[a] The cells of auxotrophic mutants FVII-S1 (Ade$^-$ Ura$^-$) and Y-19-15 (Arg$^-$ Met$^-$) were mixed and made into protoplasts. The protoplasts were regenerated on LMgMM plates with or without 2.5% DMSO. Regenerants were examined by microscopy for cell size. Large-sized cell clones were tested for nutrient requirements after they were purified by single-colony isolation repeated twice.

observation was 2 days; discrimination earlier or later than this was difficult. Only 4 of 400 cell colonies were distinctly large-sized for about 2 days if 1% of the regenerated population became diploids, which was actually the case. This fact made analysis of the mechanism almost impossible. Nevertheless, diploids that were formed under the conditions described above were heritable. On transfer to fresh medium, cells underwent another growth stage, when haploids and diploids were remarkably different in cell size.

Attempts did not succeed in discriminating haploids and diploids by colony color by including dyes in the plates, as has been described in *Saccharomycodes ludwigii* (23). We confirmed this finding with the same strains (one diploid and two haploids) and then tried it with our strains. The dyes tested were eosin yellowish, magdala red, phloxine B, tetrazolium violet, and trypan blue at several concentrations or in several combinations, with *T. delbrueckii* SANK 50268 (haploid), YL3 (directly induced diploid), and F31 (diploid obtained through protoplast fusion) used as standard strains (data not shown). What we know is that 2.5% DMSO produced diploids from the regeneration of protoplasts of the haploid SANK 50268 in yeast nitrogen base without amino acids, and the decrement of the magnesium level in that medium from 2 to 0.2 mM exerted the same effect. We obtained artificial diploids which had no genetic defects in their chromosomes, unlike those induced through intraspecific protoplast fusion in which it was necessary to use mutants as the parents.

Because the treatments described above appeared to perturb protoplast regeneration, resulting in diploid formation by direct diploidization, we tried UV light irradiation on intact cells to perturb cell division and found that diploids were formed. Again, the exact mechanism underlying this induction is not known. Parry et al. (15) have indicated that UV light irradiation causes aneuploidy in *Saccharomyces cerevisiae*. They used diploids, and the frequency of aneuploid occurrence on irradiation was on the order of 10^{-4}, which was two to three magnitudes below that which we obtained with *T. delbrueckii*, which afforded large-sized cell clones on UV light irradiation. Because our yeast was haploid in nature, the monosomic state ($2n - 1$) did not logically exist in one step and the disomic state ($n + 1$) probably did not occur at a frequency of 10^{-2} to 10^{-1}. The same cell size that was obtained between the UV-induced large-sized cell clone and other diploids indicates that the former was actually diploid but not aneuploid. The fact that the UV-induced large-sized cell clone showed good sporulation with the same pattern of spore number in asci as that of other diploids strongly supports this argument (Table 3). Ishitani (10) irradiated conidia of heterokaryons of the fungi *Aspergillus sojae* or *Aspergillus oryzae* with UV light, resulting in diploid formation. Our yeast strain was vegetatively haploid and did not produce a heterokaryon, but the same or similar mechanism should underlie the two phenomena. In fact, a nonsporulating industrial *S. cerevisiae* strain gave rise to stable clones that were 1.9 to 2.5 times as large in cell volume as the parent on UV irradiation. They contained 2.5 times as much DNA as the parent, suggesting that pentaploids are produced from diploids (T. Sasaki and Y. Ohshima, unpublished data).

There are reports (5, 25) in which the effects of DMSO on *S. cerevisiae* cells were examined, but the concentration of DMSO that was used was much higher than that employed here. Results of one report (25) indicated that DMSO induces the formation of respiratory-deficient petite mutants in *S. cerevisiae* at concentrations higher than 8% in the growth medium. The other report (5) dealt with cell lysis at a concentration of 33%. They seemed to have nothing mechanistically in common with the work described here. Most related to our observations is the report by Fulton and Bond (9), who described the occurrence of aneuploidy in the fungus *Sordaria brevicollis* by treatment with DMSO. Differences in *S. brevicollis* and *T. delbrueckii* are as follows. *T. delbrueckii* afforded diploids with DMSO acted on regenerating protoplasts but not on intact cells, whereas *S. brevicollis* produced aneuploids when a 0.55% solution of DMSO was flooded onto fertilized crosses.

The question may arise that our methods to induce diploids through perturbed protoplast regeneration or through UV irradiation of intact cells merely selected spontaneously occurring diploids with a peculiar yeast, *T. delbrueckii*, which undergoes diploidization by autogamy (24). We obtained super-large-sized cell lines with triple amounts of cellular DNA (triploids) by UV irradiation of our diploid YL3 strain at the same frequency, which was comparable to diploid induction from the haploid (T. Sasaki, 127th Meeting of the Society of Yeast Scientists, 1985, Tokyo). They were not stable, unlike the diploids, but the fact that triploidy could be induced by the same treatment excludes the possibility questioned above.

Colchicine was reported to be effective for obtaining polyploids (11) from *Candida scotti* and *Candida tropicalis*, but our *T. delbrueckii* strain did not respond to the drug even when it was used at a concentration of 30 mg/ml (paper disk method) on regenerating protoplasts or added up to 16 mg/ml in a liquid medium with the inoculum of intact cells (data not shown).

DMSO is well known as the agent that exerts versatile effects on cultured mammalian cells and others, but the exact molecular mechanisms are still obscure. Fukui and Katsumaru (8) observed with *Dictyostelium mucoroides* that DMSO induces formation of huge bundles of actin filaments in the nucleus but that magnesium ion inhibits its effect. In this study the decrement of magnesium levels in the protoplast regeneration medium for inducing diploid formation may have some relatedness in biochemical mechanism. Sanui and Rubin have reported (Abstract 3rd International Congress on Cell Biology, Tokyo, 1984, p. 541) that reversion of mouse tumor cells to their normal appearance by treatment with DMSO is correlated with the lowering of cellular levels of magnesium ion. Thus, the effects of DMSO on several organisms, including those used in this study, may be related to the levels of magnesium; high levels of magnesium counteract DMSO action, and low levels exert effects similar to those of DMSO, at least in certain respects. This relationship should be analyzed by using an organism(s) that is more suitable than *T. delbrueckii*.

Enlargement of cell volume in magnesium-limited cultures has been reported with microorganisms such as the cyanobacterium *Anacystis nidulans* (20), the yeast *Schizosaccharomyces pombe* (22), and the alga *Chlorella vulgaris* (7). Such cell enlargement appears to be a physiological rather than a genetic change, however; the enlarged cells became normal in size when they were transferred to the media with normal magnesium levels. In a recent review, Walker (21) has described the relationship between magnesium and physiological cell cycle control that occurs universally, from procaryotes to cultured mammalian cells, including *S. pombe*. Our finding that genetically stable diploids are produced from haploid *T. delbrueckii* protoplasts on regeneration in the medium with low magnesium levels should be considered a consequence of perturbed cell division that first

occurs physiologically but the resulting diploid state was fixed as a genotype during protoplast regeneration.

In the accompanying paper (13) we describe the usefulness of the artificially induced but stable diploids of *T. delbrueckii* SANK 50268 for industrial purposes.

ACKNOWLEDGMENTS

We thank Y. Kishida, former Director of Bio-Science Research Laboratories, and H. Akabori, former Director of the Tanashi Plant, for their interest throughout this study. Thanks are also due to T. Umeda of Japan Science Instrument Co., Ltd., Tokyo, for technical help with the flow microfluorometry.

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