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Note

Homothallism in Sugar-Tolerant *Saccharomyces rouxii*†

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Salt-tolerance and mating type of *Saccharomyces rouxii* isolated from marzipan and honey were examined; the most sugar-tolerant strains were tolerant of high NaCl concentrations such as 2.5 M, while one isolated from honey showed lower-tolerance and could not grow on medium containing more than 0.5 M NaCl. This suggests that the salt-tolerance is independent of the sugar-tolerance. However, the optimum concentration of NaCl for copulation and sporulation of the sugar-tolerant strains was 0.09–0.15 M. We constructed hybrids between sugar-tolerant and salt-tolerant strains, but genetic analysis of them was unsuccessful due to the sterility of the ascospores. Mating response and ploidy estimation of the sugar-tolerant strains indicated that they are essentially homothallic and one of them is diploid. This is the first evidence of the existence of diploid *S. rouxii* cells in natural habitats.

Salt-tolerant *Saccharomyces rouxii*, an industrially important yeast for *miso* and *shoyu* making,[1,2] is a typical heterothallic haploid yeast, which has a stable haploid vegetative phase and its diplophase is limited in zygotes.[3] It is also known that some strains of the same species are harmful for various foods of high sugar content such as marzipan and honey.[4,5] Wickerham and Burton found heterothallism in salt-tolerant *S. rouxii* isolated from Japanese *miso* pastes.[6] To obtain more useful strains for *shoyu* and *miso* fermentation than the natural isolates, we constructed diploid and triploid strains.[7–9] The diploid strains showed stable growth in liquid nutrient, high salt liquid or solid nutrient medium without sporulation.[8] During the course of the above study, we suggested the existence of diploid vegetative cells in natural habitats.[9] This paper concerns the following aspects of the sugar-tolerant *S. rouxii* strains: their salt-tolerance, mating potency and the existence of diploid vegetative cells in natural habitats. We also describe the successful construction of hybrids between sugar-tolerant and salt-tolerant strains, but the hybrids did not produce meiotic progenies.

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Materials and Methods

Organisms Five sugar-tolerant strains of *Saccharomyces rouxii* listed in Table 1 (G4103, G4106, G4110, G4116, and G4118) were isolated from marzipan by one of the authors (S. W.),[4] and the remaining one sugar-tolerant and salt-sensitive strain (0.19–5) isolated from honey was kindly supplied by Dr. A. Rodriguez-Navarro of Cátedra de Microbiologia, E.T.S. Ingenieros Agrónomos, Madrid.[5] Auxotrophic mutants were isolated from free-ascospores of strain 0.19–5 by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.[10] Salt-tolerant auxotrophic strains, Arg-M1 (*a*, *arg1*) (ATCC 26390) and Lys-M5 (*a*, *lys1*) (ATCC 26394), isolated in a previous study[9] were used as the standard haploid strains for mating type determination. In this study, we designated a yeast strain isolated from foods of high sugar content as a sugar-tolerant strain and a strain which could not grow in the presence of 1.0 M NaCl as a salt-sensitive strain.

Media Nutrient medium composed of 10 g of yeast extract, 10 g of Polypepton, and 20 g of glucose

per liter of tap water, and the minimal medium described in the previous paper[10] were used for general cultivation and characterization of auxotrophic traits, respectively. Diluted *shoyu-koji* extract agar[7] containing 0.15 M or 0.85 M NaCl with or without the addition of 0.28 M glucose was used as the copulation or the sporulation medium, respectively.

Genetic methods Vegetative diploid and triploid hybrids were constructed according to the procedure described in the previous paper.[7] Hybrid cells between two haploid strains carrying different auxotrophic traits were selected by the prototroph recovery method.

Azygotic and zygotic asci were directly dissected with a micro-glass needle, since the viability of spores decreased markedly on treatment of asci with yeast cell lytic enzymes such as snail juice, Helicase and Zymolyase.

Mating type was determined from the response to the standard strains on the sporulation medium and confirmed by the complementation of auxotrophic traits on a test plate, if the two haploid clones to be crossed had auxotrophic genetic markers.

Ploidy was judged from the cell size, cell shape, dry cell weight, DNA content, mating potency and ability of sporulation, as described in the previous paper.[9]

Results and Discussion

Salt-tolerance Sugar-tolerant strains could grow in medium containing high amounts of sugar such as 2 M sucrose, and most of them showed lower salt-tolerance as they could not grow on medium containing 3.5 M NaCl, while most of the salt-tolerant strains isolated from *shoyu* mashes and *miso* pastes showed tolerance of more than 3.5 M NaCl. Especially, cellular growth of strain 0.19–5 was almost completely inhibited by 1.0 M NaCl in the medium.[5] This indicates that the salt-tolerance is independent of the sugar-tolerance and is specific for the strain.

Optimum conditions for copulation and sporulation Optimum conditions for copulation and sporulation of the sugar-tolerant strains were examined. They showed a rare mating response with the standard haploid mating types on a diluted *shoyu-koji* extract agar containing 0.85 M NaCl, which is suitable for salt-tolerant strains.[7] However, we found that the sugar-tolerant strains could not copulate nor sporulate on the same sporulation medium as for the salt-tolerant strains, while some sugar-tolerant ones, 0.19–5, G4103 and G4118, could form many zygotes and zygotic asci on the sporulation medium when the NaCl concentration of the above medium was reduced to 0.15 M.

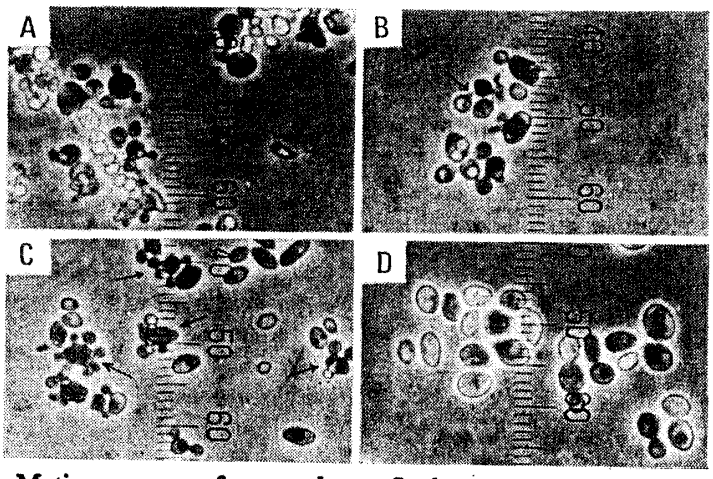

Fig. 1. Mating response of sugar-tolerant *Saccharomyces rouxii*.
(A) Zygotic asci and cells with copulation tube (arrow) of strain 0.19–5.
(B) Azygotic ascus and zygote (arrow) of strain G4103.
(C) Durable cells (arrow) of strain G4106.
(D) Large cells of strain G4116.
Cells were inoculated from the nutrient medium onto the sporulation agar and incubated for 3 days at 25°C.
(Each small division of the scale corresponds to 2.5 μm.)

Mating potency and ploidy On the sporulation medium, the sugar-tolerant strains, 0.19–5, G4103 and G4118, showed a mating response in each culture without mixing with any other strains (Fig. 1). However, the other strains, G4106, G4110 and G4116, never showed a mating response in a single strain culture or even in mixed cultures with the standard strains either of the *a* or *α* mating type. The ploidy estimation showed that the sugar-tolerant strains are all haploid except for strain G4116 (Table 1). Cells of G4116 were larger in cell size, dry weight and DNA content than those of the other strains, suggesting that they are non-mating diploids. This is the first observation of diploid cells of *S. rouxii* in a natural habitat and it confirmed the previous suggestion[7,9] of the existence of a stable diploid phase of this yeast.

To investigate the mating potency more critically, single cell cultures of strains 0.19–5, G4103 and G4118 were performed by isolating cells with the aid of a micromanipulator in a droplet of the nutrient medium which were then incubated at 30°C. The mating type of the clones was examined; all clones of two strains, 12 clones of G4103 and 20 of G4118 showed a bisexual reaction so far as examined. Nine of 15 clones from strain 0.19–5 showed a bisexual reaction, while the remaining 6 clones showed the *α* mating type. The results indicate that strain 0.19–5 has a population consisting of cells of bisexual mating potency and the *α* mating type. On the other hand, we isolated two auxotrophic strains, Lys-S9 (lys$^-$) and Arg-S1 (arg$^-$), from the same strain, 0.19–5. It was interesting that the lysine auxotrophic Lys-S9 showed a bisexual reaction, whereas the arginine auxotrophic Arg-S1 behaved as an *α* mating type clone. A hybrid was constructed by mating between Lys-S9 and Arg-S1 and it was subjected to tetrad analysis. Though the viability of spores was low (about 13%), the hybrid showed 2 bisexual: 2*α* segregation for the mating types and 2+:2− segregation for both the Lys and Arg phenotypes so far as the 48 asci tested were concerned. These results indicate that the bisexual clone of strain 0.19–5 is composed of haploid cells and the Lys and Arg markers are controlled by each single pair of alleles, respectively.

The bisexuality of *S. rouxii* cells observed in strains 0.19–5, G4103 and G4118 indicates that they are homothallic, while strain 0.19–5 may have a life cycle of Hp homothallism which gives rise to *α* mating type cells and homothallic diploid cells when it sporulates and copulates similarly to as described for the life cycle of *Saccharomyces* yeasts of *sensu stricto*.[11,12]

Salt-tolerance is dominant over the salt-sensitivity The optimum concentrations of NaCl for copulation and sporulation of the salt-tolerant and the sugar-tolerant strains are 0.85 M and 0.15 M, respectively, as mentioned above. However, the heterothallic salt-tolerant strains, Arg-M1 (*a*) and Lys-M5 (*α*), can mate more or less by mixing of both cells even in the presence of 0.15 M NaCl. Therefore, construction

Table 1. Ploidy estimation of sugar-tolerant *Saccharomyces rouxii*.

Strain no.	Isolated from	Cell shape*	Cell size (major axis, μm)	Dry cell wt ($\times 10^{-8}$ mg/cell)	DNA content (μg/10^{10} cells)	Mating potency***	Expected ploidy
G4103	Marzipan	R–O	6 - 8	1.99	197	+	Haploid
G4106	Marzipan	E	6 - 8	1.82	207	−	Haploid
G4110	Marzipan	E	6 - 8	—**	—**	−	(Haploid)
G4116	Marzipan	O–E	8 - 12	4.25	422	−	Diploid
G4118	Marzipan	O–E	6 - 8	2.13	201	+	Haploid
0.19–5	Honey	R–O	4 - 7	2.78	211	+	Haploid

* R: round, O: ovoid, E: ellipsoid. ** —: not estimated.
*** Mating potency with the standard strains of both mating types, respectively.

Table 2. Ploidy estimation of hybrids and their parents.

Strain no.	Combination $a \times a$	Cell shape*	Cell size (major axis, μm)	Dry cell wt ($\times 10^{-8}$ mg/cell)	DNA content ($\mu g/10^{10}$ cells)	Auxotrophic trait	Mating type	Expected ploidy
Lys-S**2	(salt-sensitive)	R-O	5 - 7	2.23	185.22	Lysine	*a*	Haploid
Lys-M**5	(salt-tolerant)	R-O	5 - 7	2.52	296.16	Lysine	*a*	Haploid
Arg-M1	(salt-tolerant)	R-O	5 - 7	2.19	228.73	Arginine	*a*	Haploid
SM**-66	Arg-S1 × Lys-M5	O-E	7 - 10	4.65	498.82	Wild	—	Diploid
SM-115	Arg-S1 × Lys-M5	O-E	7 - 10	6.02	698.35	Wild	—	Triploid
SM-710	Arg-S6 × Lys-M5	O-E	7 - 10	4.07	436.49	Wild	—	Diploid
SM-214	Arg-S6 × Lys-M5	O-E	7 - 13	6.31	612.11	Wild	—	Triploid
SM-514	Lys-S2 × Arg-M1	O-E	7 - 10	3.67	421.60	Wild	—	Diploid

* R: round, O: ovoid, E: ellipsoid.
** S and M of strain numbers represent salt-sensitive and salt-tolerant characters, respectively.

of hybrids between a salt-tolerant and a salt-sensitive auxotrophic strain was possible in the copulation medium containing 0.15 M NaCl. Some hybrids obtained by this method are listed on Table 2. They were judged as diploid or triploid from the data for cell shape, cell size, cell weight and cellular DNA content. Although it is obscure why the triploid cells were formed, they might be constructed by copulation between a haploid *a* cell and a diploid *a*/*a* cell as these diploid cells are expected to occur in cultures of the homothallic strain. Salt-tolerance of the hybrids was examined using the nutrient medium containing various concentrations of NaCl (Fig. 2). All the hybrids between salt-tolerant and salt-sensitive strains showed the same phenotype as that of the salt-tolerant strain. Thus, the salt-tolerance is dominant over the salt-sensitive character. To study the segregation of the salt-tolerance and sugar-tolerance phenotypes in the meiotic segregants of these hybrids, we dissected 212 four-spored asci produced in 5 hybrids of 3 combinations listed in Table 2. Unfortunately, none of the 845 spores could germinate and only three of the spore cultures survived. This may due to the genetic incompatibilities between the parental strains, but we do not know the exact reasons.

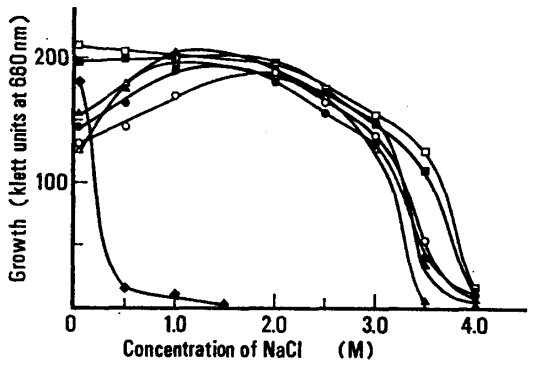

Fig. 2. Effect of NaCl on growth of the hybrids constructed between salt-tolerant and salt-sensitive *Saccharomyces rouxii*.
—◆—: Lys-S*2 (haploid, salt-sensitive strain),
—○—: Lys-M*5 (haploid, salt-tolerant strain),
—●—: SM*-66 (diploid, Arg-S1 × Lys-M5),
—□—: Arg-M1 (haploid, salt-tolerant strain),
—■—: SM-514 (diploid, Lys-S2 × Arg-M1),
—△—: SM-214 (triploid, Arg-S6 × Lys-M5),
—▲—: SM-710 (diploid, Arg-S6 × Lys-M5).
* S and M of strain numbers represent salt-sensitive and salt-tolerant characters, respectively.

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