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EXPERIMENTAL ARTICLES

Polygenic Control for Fermentation of β-Fructosides in the Yeast *Saccharomyces cerevisiae*: New Genes *SUC9* and *SUC10*

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Abstract—Using molecular karyotyping and genetic hybridization analysis, two new polymeric β-fructosidase genes, *SUC9* and *SUC10,* were identified in the yeast *Saccharomyces cerevisiae*, which are located on chromosome XIV and on the chromosome XVI/XIII doublet, respectively. The genes are responsible for fermentation of sucrose and raffinose. The *SUC* gene genotypes of strains VKM Y-1831 and DBVPG 1340 are *SUC2 SUC9* and $suc2^0$ *SUC10,* respectively. $suc2^0$ is a silent sequence. The scientific and applied significance of *SUC* genes is discussed.

Key words: yeast, *Saccharomyces cerevisiae*, β-fructosidases, *SUC* genes, fermentation of sugars, sucrose, raffinose.

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The study of β-fructosidase (invertase) *SUC* genes in the yeast *Saccharomyces cerevisiae* is of both scientific and applied significance. The presence of several cumulative polymeric genes *SUC1–SUC5*, *SUC7* and *SUC8* in various sets [1–4] allows enzyme evolution to be studied in the same organism. Actually, the formation of β-fructosidase isozymes takes place. The enzyme isozymes subjected to functional and ecological specialization are known to be of adaptive significance. Many cultured yeast strains are grown on the substrates containing β-fructosides sucrose and raffinose, primarily on molasses. The introduction and selection of cultured yeasts should therefore consider the utility designation of the polymeric *SUC* genes.

The goal of this work was to saturate the known genome of *S. cerevisiae* [5–7] with new *SUC* genes represented in natural populations of this yeast.

MATERIALS AND METHODS

The studied and reference *S. cerevisiae* strains are shown in Table 1. The generally accepted international symbols of the yeast genes are used for genotype designation. The origin of the strains analyzed is as follows: (1) VKM Y-1831, rotten apples, the town of Michurinsk [8], and (2) DBVPG 1340, soil, Netherlands [9]. The strains were isolated by A.A. Imshenetskii (Soviet Union) and A. Capriotti (Italy), respectively. The following abbreviations of the collection names are used: VKM, the All-Russian Collection of Microorganisms, Moscow; DBVPG, Industrial Yeast Collection of the Dipartimento di Biologia Vegetale of the University of Perugia, Italy; and CLIB, Collection de Levures d'Intérêt Biotechnologique, Centre de Grignon, France.

The yeasts were cultivated on YPD complete medium containing the following (g/l): bacto agar (Difco, United States), 20; glucose (Reakhim, Soviet Union), 20; Difco yeast extract, 10; and Difco peptone, 20. Spore formation was induced on the standard acetate medium containing the following (g/l): bacto agar, 20; CH_3COONa, 10; and KCl, 5. The capacity for fermenting the β-fructosides sucrose and raffinose was determined by two methods: (1) by the evolution of carbon dioxide in liquid YP medium in test tubes with floats and (2) by changes in the color of the yeast growing on agarized medium with a pH-indicator. Suc^+ strains usually ferment sucrose and raffinose after 24 h; in rare cases, sucrose is fermented after 48–72 h and raffinose after 72–120 h. Suc^- yeasts do not ferment sucrose and raffinose after 10 days. Originally, the capacity of segregants to ferment sucrose and raffinose was determined on the pH-indicator medium after 24 h. The yeasts that did not ferment sucrose or raffinose under these conditions were additionally studied in test tubes with floats. The auxotrophic mutations and/or maltose fermentation served as the control markers. Monogenic segregation of the latter characteristic was recorded on the pH-indicator medium. The composition of the pH-indicator medium (g/l) was as follows: bacto agar, 20; peptone, 3.15; yeast extract, 2.7; KH_2PO_4, 1.8; $MgSO_4$ ·

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Table 1. Genetic strains of *S. cerevisiae* used

Strain	Origin	Genotype	Reference
1831-51	VKM Y-1831	*HO SUC2 SUC9 MAL GAL mel*	[8], Tw*
1340-1D	DBVPG 1340	*HO suc2^0 SUC10 MAL GAL mel*	[9], Tw
S288C	Genetic line	*MAT*α *SUC2 mal gal2 mel*	[10, 11]
X2180-1A	Ditto	*MAT*a *SUC2 mal gal2 mel*	[10, 11]
YNN 295	"	*MAT*α *ura3 ade1 ade2 lys2 lys7 trp1*Δ	[12, 13]
SH 4.108.-2D	"	*MAT*α *SUC1 suc2*Δ::*URA3 mal GAL leu2 trp1 ura3 ade*	[14, 15]
SH 4.107.-1A	"	*MAT*α *SUC3 suc2*Δ::*URA3 mal gal leu2 ura3*	[14, 15]
SH 1.82.-2B	"	*MAT*α *SUC4 suc2*Δ::*URA3 mal GAL leu2 ura3*	[14, 15]
SH 1.103.-5D	"	*MAT*α *SUC5 SUC2 mal GAL leu2 trp1 ura3*	[4, 14, 15]
FL200**	CLIB 335	*MAT*α *SUC2 SUC7 MAL*	[4, 16]
XII_7-2	XII_7	*MAT*α *SUC2 SUC5 SUC8 MAL*	[4, 17]
SCO-39B	SH 4.108.-2D	*MAT*a *suc2*Δ::*URA3 mal gal leu2 trp1 ade*	[4]
S0-2A	XII_7-2	*MAT*a *SUC8 suc2*Δ::*URA3 MAL gal leu2*	[4]
S8-4B	1340-1D	*MAT*a *SUC2 SUC10 mal GAL*	Tw
S20-6A	1831-51	*HO suc2*Δ::*URA3 SUC9 MAL gal leu2 trp1*	Tw
S20-6C	1831-51	*MAT*a *SUC2 mal gal ade*	Tw
S22-6A	FL200	*MAT*α *SUC7 suc2*Δ::*URA3 MAL gal trp1*	[4]
SUO-10B	SH 4.108.-2D	*MAT*α *suc2*Δ::*URA3 mal gal ade*	[4]
SUO-15B	SH 4.108.-2D	*MAT*α *suc2*Δ::*URA3 mal gal trp1*	[4]
SUO-19D	SH 4.108.-2D	*MAT*α *suc2*Δ::*URA3 mal gal trp1*	[4]
S26-32B	XII_7-2	*MAT*a *SUC5 suc2*Δ::*URA3 mal gal leu2 trp1*	[4]
S29-1A	1340-1D	*MAT*α *suc2 SUC10 mal gal trp1*	Tw
S29-5C	1340-1D	*MAT*a *suc2 SUC10 mal GAL leu2*	Tw
S51-6D	SH 1.103.-5D	*MAT*α *SUC5 suc2*Δ::*URA3 mal gal leu2 trp1*	[4]
S66-6A	XII_7-2	*MAT*a *SUC8 suc2*Δ::*URA3 mal gal leu2*	[4]
S77-3A	1831-51	*MAT*a *suc2*Δ::*URA3 SUC9 mal gal trp1 ade*	Tw
S77-5C	1831-51	*MAT*α *suc2*Δ::*URA3 SUC9 mal gal*	Tw
S78-5B	FL200	*MAT*α *SUC7 suc2*Δ::*URA3 mal gal leu2 ade*	[4]
SV-10B	1340-1D	*MAT*a *suc2 SUC10 mal GAL leu2 ade*	Tw
S101-4A	1340-1D	*MAT*α *suc2*Δ::*URA3 SUC10 mal gal*	Tw
S101-4B	X2180-1A	*MAT*a *SUC2 mal GAL trp1*	Tw
S101-4C	1340-1D	*MAT*α *suc2*Δ::*URA3 SUC10 mal GAL*	Tw
S101-4D	X2180-1A	*MAT*a *SUC2 mal gal trp1*	Tw

Notes: * Tw stands for this work.

** According to F. Lacroute, strains FL200 and FL100 [2] are isogenic.

$7H_2O$, 0.9; $(NH_4)_2SO_4$, 0.9; sucrose or maltose (Sigma, Germany), 20; raffinose (Fluka, Switzerland), 30; potassium eosin (Veb Laborchimie Apolda, German Democratic Republic), 0.4; methylene blue (Reakhim), 0.025. Eosin as 2% solution in 50% ethanol, 0.5% aqueous solution of methylene blue, and 10% solution of sucrose or maltose (15% of raffinose) were added before pouring the medium. The composition of YP fermentation medium was the same as that of YPD medium, but without agar; sucrose or raffinose were used instead of glucose. On all the media, the yeast was cultivated at 28°C. The spores were isolated with the glass needle of a Carl Zeiss micromanipulator (Jena, German Democratic Republic) after destroying the ascus walls with the enzyme preparation we isolated from the stomach of the garden snail *Helix pomatia*. The hybrids of heterothallic strains were obtained by mass hybridization of cells of the opposite mating types on complete medium with the subsequent isolation of the zygotes with the micromanipu-

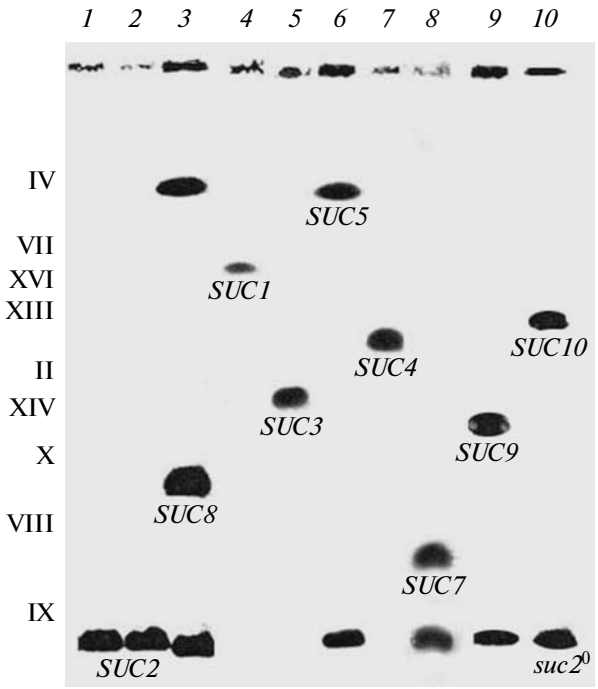

Fig. 1. Comparative Southern analysis with probe *SUC2* of the chromosomal localization of the *SUC* genes in *S. cerevisiae* (VKM Y-1831 and DBVPG 1340) and testers *SUC1–SUC5, SUC7*. Lanes: YNN 295 (*1*); X2180-1A (*2*); XII_7-2 (*3*); SH 4.108.-2D (*4*); SH 4.107.-1A (*5*); SH 1.103.-5D (*6*); SH 1.82.-2B (*7*); FL200 (*8*); VKM Y-1831 (*9*); DBVPG 1340 (*10*). The chromosomes are numbered according to the standard strain YNN 295.

lator. The hybrids of homo- and heterothallic strains were obtained using the spore per haploid cell method by means of the micromanipulator [18].

The chromosomal DNA preparations were prepared according to G.F. Carle and M.V. Olson [19]. The electrophoretic separation of chromosomal DNA was carried out on a Chef-DR II apparatus (Bio-Rad, United States) at 200 V for 24 h: for 15 h with a field-switch time of 60 s and for 9 h with a field-switch time of 90 s. The buffer used was 0.5× TBE (45 mM of Tris, 45 mM of boric acid, and 10 mM of EDTA, pH 8.2) cooled to 14°C. After electrophoresis, the gel was stained with ethidium bromide, washed with distilled water, and photographed. The chromosomal DNAs were transferred to the nitrocellulose membrane using Southern blotting. The DNA was fixed on the membrane by annealing at 80°C for 2 h. The following cloned genes were used as probes: the *Bam*HI-*Hin*dIII fragment (0.9 kb) isolated from plasmid pRB117 [2] and the *Xba*I fragment (2.2 kb) of the *TRK1* gene isolated from plasmid pRG295-1 [20]. The fragment (1 kb) of the plasmid pUC9 kindly provided to us by E.J. Louis (United Kingdom) served as the probe *CEN14*. The probes were prepared according to [21]. The label was introduced by means of the nonradioactive method according to the Roche Applied Science (Germany) instructions using dihydroxygenine Dig-II-dUTP. Hybridization and the hybridization signal events were also carried out according to the Roche Applied Science instructions.

RESULTS

Karyotypic Analysis

The Southern hybridization of the *SUC2* probe with the chromosomal DNA of the two strains studied allowed the preliminary identification of their *SUC* genes [22]. Both strains, VKM Y-1831 and DBVPG 1340, have two *SUC* genes, one of which is probably *SUC2*, whereas the second genes are different (Fig. 1, lanes *9* and *10*). We arbitrarily designated them as *SUC9* and *SUC10*. The use of the control probes allowed us to localize the genes *SUC2, SUC9*, and *SUC10* in the chromosomes IX, XIV, and the chromosome XVI/XIII doublet, respectively (the data are not given).

Genetic Analysis

Mono- and digenic segregation of the hybrids of strains DBVPG 1340, VKM Y-1831, and their heterothallic derivatives. Hybridization with the tester SCO-39B that does not contain *SUC* genes (genotype *suc2*Δ was used to determine the number of active *SUC* genes in the strains analyzed. In the hybrid S20, digenic segregation was observed in the ability to ferment β-fructosides, whereas in the hybrid SV, despite the presence of two genes revealed by Southern hybridization in parental DBVPG 1340, monogenic segregation was noted in a random spore sample (Table 2). Moreover, in the surviving triads, the following segregation was observed: $2Suc^+ : 1Suc^-$ (4), $1Suc^+ : 2Suc^-$ (6). (Tetrad analysis of the hybrid SV was impossible due to low survival of its spores). Hence, the parent VKM Y-1831 contains two active *SUC* genes and DBVPG 1340 contains only one active *SUC* gene. The putative genes *SUC2* and *SUC9* of strain VKM Y-1831 were isolated in hybrid S20 segregants in the tetrads of the nonparental ditype $4Suc^+ : 0Suc^-$ (Figs. 2, 3). The isolation of *SUC2* and *SUC9* genes was confirmed by monogenic segregations of hybrids between the corresponding segregants and testers having the *suc2*Δ genotype (Table 2, hybrids S45, S71, and S77). Hybrid S77 was obtained for selecting the heterothallic *suc2*Δ *SUC9* segregants of two mating types (Fig. 2, Table 2). Hybrid S29 was obtained for selecting the *suc2 SUC10* segregants of two mating types (Fig. 2, Table 2).

Identification of the *SUC2* and *SUC9* genes of strain VKM Y-1831. Hybrid between the segregant S20-6C, inheriting the putative *SUC2* gene from strain VKM Y-1831 (Fig. 1), and tester S288C of the genotype *SUC2* showed it to be *SUC2*-homozygous, because all its segregants fermented β-fructosides (Table 2, hybrid S37). Therefore, segregant S20-6C and, hence, its parent VKM Y-1831 contain the gene *SUC2*. The homothal-

Table 2. Identification of the polymeric *SUC* genes of *S. cerevisiae* VKM Y-1831 and DBVPG 1340

Hybrid	Origin of Suc^+ hybrids	Number of tetrads with the $Suc^+ : Suc^-$ segregation			Genotype
		4 : 0	3 : 1	2 : 2	
SV	1340-lD × SCO-39B	22Suc^+ : 21Suc^-			*suc2*^{0} *SUC10/suc2*Δ
S6	SV-10B × SH l.82.-2B	20	12	0	*suc2 SUC10/suc2*Δ *SUC4*
S8	1340-1D × X2180-1A	5	8	3	*suc2*^{0} *SUC10/SUC2*
S19	SV-10B × S288C	10	22	4	*suc2 SUC10/SUC2*
S20	1831-51 × SCO-39B	3	7	5	*SUC2 SUC9/suc2*Δ
S28	SV-10B × SH4.107.-lA	7	14	2	*suc2 SUC10/suc2*Δ *SUC3*
S29	SV-10B × SUO15B	0	0	18	*suc2 SUC10/suc2*Δ
S37	S20-6C × S288C	28	0	0	*SUC2/SUC2*
S45	S20-6A × SCO-39B	0	0	11	*suc2*Δ *SUC9/suc2*Δ
S46	S20-6A × SH4.107.-lA	9	18	2	*suc2*Δ *SUC9/suc2*Δ *SUC3*
S59	S29-1A × S0-2A	9	9	0	*suc2 SUC10/suc2*Δ *SUC8*
S60	S29-5C × S22-6A	9	3	0	*suc2 SUC10/suc2*Δ *SUC7*
S61	S29-5C × SH4.108.-2D	7	16	6	*suc2 SUC10/suc2*Δ *SUC1*
S71	S20-6C × SUO-19D	0	0	10	*SUC2/suc2*Δ
S77	S20-6A × SUO-10B	0	0	9	*suc2*Δ *SUC9/suc2*Δ
S83	S77-3A × SH4.108.-2D	10	24	7	*suc2*Δ *SUC9/suc2*Δ *SUC1*
S84	S77-5C × 66-6A	11	26	9	*suc2*Δ *SUC9/suc2*Δ *SUC8*
S87	S77-3A × SH4.107.-lA	8	28	3	*suc2*Δ *SUC9/suc2*Δ *SUC3*
S88	S77-3A × SHl.82.-2A	13	5	0	*suc2*Δ *SUC9/suc2*Δ *SUC4*
S89	S77-3A × S78-5B	5	20	3	*suc2*Δ *SUC9/suc2*Δ *SUC7*
S91	S77-3A × S26-32B	11	16	1	*suc2*Δ *SUC9/suc2*Δ *SUC5*
S94	S29-5C × S77-5C	5	16	1	*suc2 SUC10/suc2*Δ *SUC9*
S95	S29-5C × S51-6D	3	11	5	*suc2 SUC10/suc2*Δ *SUC5*
S99	S77-5C × X2180-lA	3	14	5	*suc2 SUC9/SUC2*
S101	S8-4B × SUO-19D	4	3	1	*SUC2 SUC10/suc2*Δ
S102	S101-4A × X2180-1A	2	14	5	*suc2*Δ *SUC10/SUC2*
S103	S101-4B × S288C	25	0	0	*SUC2/SUC2*
S104	S101-4C × X2180-lA	6	16	3	*suc2*Δ *SUC10/SUC2*
S105	S101-4D × S288C	21	0	0	*SUC2/SUC2*
S106	S101-4B × S29-1A	2	18	4	*SUC2/suc2 SUC10*
S107	S101-4D × S29-1A	5	11	4	*SUC2/suc2 SUC10*
S108	S101-4A × S29-5C	27	0	0	*suc2*Δ *SUC10/suc2 SUC10*
S109	S101-4C × S29-5C	25	0	0	*suc2*Δ *SUC10/suc2 SUC10*

lic segregant S20-6A (or its heterothallic derivatives S77-3A and S77-5C of the genotype *suc2*Δ *SUC9*) were hybridized with all the testers containing the known genes *SUC1*–*SUC4*, *SUC5*, *SUC7*, and *SUC8* (Table 2, hybrids S46, S83, S84, S88, S89, S91, and S99). The digenic segregations of all these hybrids indicated that we actually dealt with the novel gene *SUC9*. Thus, that strain VKM Y-1831 has the genotype *SUC2 SUC9* should be considered proven.

Identification of the *suc2*^{0} and *SUC10* genes of strain DBVPG 1340. In order to reveal which of the two β-fructosidase genes is active in strain DBVPG 1340, we first hybridized it with tester X2180-1A of the genotype *SUC2*. The corresponding hybrid S8 yielded digenic segregation (Table 2). Hence, strain DBVPG 1340 possesses an active *SUC10* and an inactive *suc2*^{0} gene. The digenic segregations of the hybrids of this strain (or its heterothallic derivatives SV-10B and S29-5C of the genotype *suc2 SUC10*) with the remaining *SUC* testers give evidence of the fact that its active *SUC* gene is not allelic to any of the already known nine *SUC* genes, i.e., in fact, it is a novel *SUC10* gene

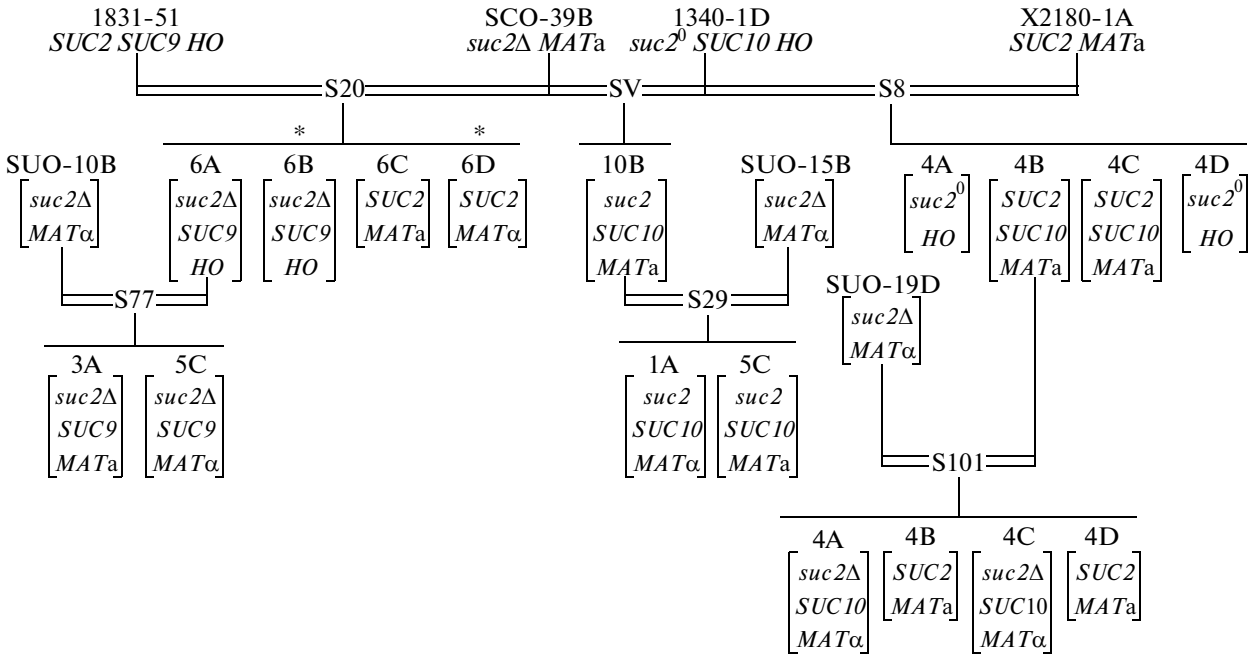

Fig. 2. Genealogy of the meiotic segregants of hybrids and genetic isolation of the genes *SUC2, SUC9* and *suc2*^{0}, *SUC10* of *S. cerevisiae* VKM Y-1831 and DBVPG 1340. The asterisk marks the segregants the genotypes of which were determined only by molecular methods.

(Table 2; hybrids S6, S8, S19, S28, S59–S61, S94, and S95). Owing to the fact that it remains unknown which allele (*suc2*^{0} or *suc2*Δ) the segregants SV-10B, S29-1A, and S29-5C possess, we used the designation *suc2*.

Isolation of the *suc2*^{0} and *SUC10* genes of strain DBVPG 1340. In our further studies of *SUC* genes, their sequencing and intraspecific phylogenetic analysis are planned. Therefore, it was necessary to isolate DBVPG 1340 *suc2*^{0} and *SUC10* genes and to obtain the recombinant meiotic segregants of the genotypes *suc2*^{0} and *suc2*Δ *SUC10*. At first, the nonparental ditype tetrad S8-4 with the phenotypic segregation 2Suc$^+$: 2Suc$^-$ was chosen (Fig. 2). In such a tetrad, the gene *suc2*^{0} was isolated in two Suc$^-$ segregants (S8-4A and S8-4D), whereas hybridization of two other segregants (S8-4B and S8-4C) with strain SUO-19D of the genotype *suc2*Δ allowed gene *SUC10* to be isolated in the nonparental ditype tetrad (4Suc$^+$: 0Suc$^-$). For this purpose, the segregant S8-4B was chosen. The hybrid S8-4B × SUO-19D yielded digenic segregation (Table 2, hybrid S101). The tetrad S101-4 was chosen for the isolation of the gene *SUC10* (Fig. 2). By means of hybridization with testers *SUC2* and *SUC10* (Table 2, hybrids S102–S109), we identified the genotypes of all the four segregants of this tetrad: (1) S101-4A–*suc2*Δ *SUC10*, (2) S101-4B–*SUC2*, (3) S101-4C–*suc2*Δ *SUC10*, and (4) S101-4D–*SUC2*. Note that, at first, the substitution of *SUC2* for the allele *suc2*^{0} in segregant S8-4B and then of *suc2*Δ for *SUC2* in segregants S101-4A and S101-4C unambiguously creates the genotype of the two latter segregants as *suc2*Δ *SUC10* (Fig. 2). Thus, in segregants S8-4A, S101-4D and S101-4A, and S101-4C the genes *suc2*^{0} and *SUC10*, respectively, were isolated.

DISCUSSION

The literature and our data [4, 22–25] give evidence of significant chromosomal polymorphism of the *SUC* genes in *S. cerevisiae* cultured strains. The polymeric *SUC* genes (*SUC1–SUC3* R_1–R_3 according to the original classification) were initially revealed [1] in strain CBS 400 isolated from young wine from the juice of the African palm *Elacis guieensis* (Western Africa/Cote d'Ivoire). Six polymeric *SUC* genes are known on the basis of the material of the genetic lines of intraspecific hybrid origin, namely: *SUC1–SUC5* and *SUC7* [2, 26]. All the *SUC* genes, except *SUC2*, are located in highly mobile telomere regions of different chromosomes: *SUC1*, in chromosome VII; *SUC3*, in chromosome II; *SUC4*, in chromosome XIII; *SUC5*, in chromosome IV; and *SUC7*, in chromosome VIII [2, 3]. The gene *SUC2* is localized at the end of chromosome IX, but not in the telomere region. The distiller's race XII forming the basis of the Russian Peterhof and Gatchina genetic lines of *S. cerevisiae* was the second cultured *SUC*-analyzed strain. As we have established [4], this strain has three polymeric β-fructoside genes: *SUC2, SUC5*, and *SUC8*. Our previous new *SUC8* gene is located in chromosome X [4]. Large-scale screening of the *SUC* genes of *S. cerevisiae* strains of different origin (91 strains were analyzed) by

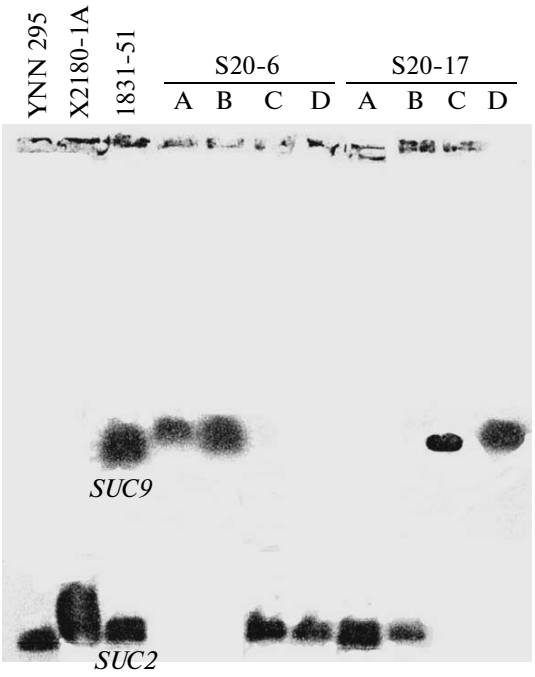

Fig. 3. Identification of the VKM Y-1831 *SUC2* and *SUC9* genes according to molecular karyotyping and hybridization of probe *SUC2* with the chromosomes of segregants from two nonparental ditype tetrads of hybrid S20. The first three lanes are represented by the control strains.

means of Southern hybridization of probe *SUC2* allowed us to tentatively identify the corresponding genotypes [22]. All the strains contained gene *SUC2* located in chromosome IX. Not only 80 Suc^+ strains, but also 11 Suc^- strains, had at least a significant part of the *SUC2* sequence, the silent $suc2^0$ gene. Four strains had three *SUC* genes. Seven strains, including VKM Y-1831 and DBVPG 1340, had two *SUC* genes each. This investigation allowed us to determine unambiguously the genotypes of the latter two strains: *SUC2 SUC9* and $suc2^0$ *SUC10*, respectively. It is the first time we have identified genes *SUC9* and *SUC10*. Note that strain DBVPG 1340 has the $suc2^0$ *SUC10*, genotype, suggesting that, in some populations, stabilization of the Suc^+ phenotype at the expense of *SUC* polymery may occur, despite the loss of the active *SUC2* gene. For comparison, recall the presence of silent $suc2^0$ copies in 11 natural Suc^- strains from certain populations [22].

Thus, our comparative study of *SUC* genes and, earlier [27–30], of the melibiose fermentation *MEL* genes indicates that natural strains may possess a pool of new genes compared to the standard laboratory genetic lines studied in the framework of the international genome sequencing project [5, 6]. The known genome of the yeast *S. cerevisiae* cannot reflect the genetic diversity of natural populations, thus restricting the evolutionary and selection studies. Speaking figuratively, the sequenced and annotated genome of *S. cerevisiae* is only the skeleton of the species, whereas the gene pool of natural and cultured strains is its body.

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