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EXPERIMENTAL ARTICLES

Dedicated to the memory of professor Nadezhda Ivanovna Buriyan

Reidentification of Chromosomal *CUP1* Translocations in the Wine Yeasts *Saccharomyces cerevisiae*

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Abstract—Reciprocal translocations between chromosomes XVI and VIII were revealed in eight *Saccharomyces cerevisiae* strains (mostly wine ones) using pulse-field electrophoresis of native chromosomal DNAs and their hybridizations with the *CUP1* and *GAL4* probes. New and reciprocal translocations of at least the gene *CUP1* occur at the expense of crossing-over in the hybrids of such strains with the genetic lines of normal karyotype during meiosis. Relationship between these reciprocal translocations and the sulfite (Na_2SO_3) resistance gene *SSU1-R* is discussed.

Keywords: Saccharomyces cerevisiae, $CuSO_4$, Na_2SO_3, copper and sulfite resistance, the genes *CUP1* and *SSU1-R*, reciprocal translocations, chromosomes XVI and VIII

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The metallothionine gene *CUP1* regulating copper resistance was discovered in G. Lindegren's laboratory in 1955 [1] and has so far been one of the most actively studied *Saccharomyces cerevisiae* genes. This is favored by both the scientific and applied significance of the pertinent studies. The unique nature of a complex *CUP1* locus, rather than just a gene, is determined by the possibility of amplification of its constituent metallothionine sequences. Tandem iteration of the metallothionine copy number proportionately increases copper resistance of the yeasts [2–6]. Increased copper resistance may probably also occur with a ploidy increase, as well as in the case of cell aneuploidy. Copper resistance, primarily in wine yeasts, is undoubtedly due to the use of copper sulfate (Bordeaux mixture) in viniculture and of copper equipment in wine making.

In our opinion, the standard laboratory isogenic genetic lines S288C, X2180-1A, and X2180-1B widely used in the world [7] (possessing the multicopied gene *CUP1* in chromosome VIII), whose genome has been sequenced and annotated [8, 9], are of wine origin.

Earlier, using molecular karyotyping, we detected among 76 *S. cerevisiae* strains of different origin eight strains with the *CUP1* sequence localized in a much longer chromosome (~1000 kb), rather than in chromosome VIII of the standard size (~580 kb) [10].

Considering the reciprocal translocations between chromosomes VIII and XVI detected recently [11] as well as our experience [12] of the study of chromosomal translocations having the *SUC2* marker, we reidentified the translocation of the gene *CUP1-t* which we previously named *MTH2*.

MATERIALS AMD METHODS

Strains and media. The origin of the analyzed and tester *S. cerevisiae* strains is given in Table 1. The collection name abbreviations are as follows: VKM, All-Russian Collection of Microorganisms, Moscow; VKPM, All-Russian Collection of Industrial Microorganisms, Moscow, Russia; ATCC, American Type Culture Collection, Manassas, VA, United States; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; DVPG, Dipartimento di Biologia Vegetale Universita di Perugia, Italy; MCYC, Departamento de Microbiologia, Escuela Tecnica Superior de Ingenieros Agronomos, Universidad Politecnica de Madrid, Spain. The wine strains designated as L and M were isolated by one of the authors [16] from the Crimean populations; the strains M-427 and M-437 obtained from the collection of the Institute of Grapes and its Processing Products (Magarach), Yalta, Crimea, are an exception.

The yeasts were cultivated on complete YPD medium containing the following (g/L): bacto-agar

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Table 1. List of the *Saccharomyces cerevisiae* strains used in the work

Collection numbers			Source and place of isolation	Resistance to		Genotype	Reference or author
Initial strain	Monosporic culture	VKPM		$CuSO_4$(mM)	Na_2SO_3(mM)		
–	X2180-1A	–	Genetic line	1.0	2.0	*MAT*a *gal2 mal SUC2 CUP1*	[7, 13]
–	S288C	–	Ditto	1.0	2.0	*MAT*α *gal2 mal SUC2 CUP1*	[7, 13]
–	31-1-7B	–	Ditto	0.2	2.0	*MAT*a *trp1 arg4 ade8 ura3 cup1*	S. Fogel
YNN 295	–	–	Ditto	–	–	*MAT*α *ura3 lys2 ade1 ade2 his7 trp1-Δ1*	[14]
L2-43	L2-43-6D	Y-117	Wine-making, Yalta	1.2	3.0	*HO GAL MAL SUC2 CUP1-t*	[15, 16]
L3-44	L3-44-7C	Y-374	Ditto	0.75	3.0	*HO gal4 mal SUC2 CUP1-t*	[15–17]
M3-33	M3-33-6B	Y-124	Ditto	0.3	6.0	*HO gal mal SUC2 cup1-t*	[15–17]
M11-22	M11-22-10B	Y-369	Ditto	0.2	3.0	*HO gal2 MAL SUC2 cup1-t*	[15, 17]
VKM Y-1753	1753-8-2	–	Apple, Michurinsk	0.75	3.0	*MAT*α *GAL MAL SUC CUP1-t*	[10]
CBS 4054	4054-3B	Y-382	Wine-making, Spain	0.2	3.0	*MAT*a *gal4 mal suc2 cup1-t*	[15, 17]
MCYC 2576	2576C	Y-91	Ditto	0.3	2.0	*HO GAL mal suc2 cup1*	[15]
DVPG 1340	1340-1D	–	Soil, The Netherlands	0.7	2.5	*HO GAL MAL SUC10 CUP1-t*	[18]
M-427	427-2A	Y-367	Wine making, Transcarpathia	0.5	2.0	*HO gal2 MAL SUC2 CUP1-t*	[15, 17]
M-437	437-1B	Y-116	Ditto	1.2	2.0	*HO GAL MAL SUC2 CUP1*	[15]

Note: 437-1B = ATCC 48498.

(Difco, United States), 20; glucose (Merck, Germany), 20; yeast extract (Difco), 10; peptone (Difco), 20. Spore formation was induced after 48 h on the standard acetate medium (g/L): bacto-agar, 20; CH_3COONa, 10; KCl, 5. Copper resistance of the yeasts was determined on the minimal agar medium containing different $CuSO_4$ concentrations (0.2–1.2 mM) after 48 h. The yeasts were cultivated on all the media at 28°C. The composition of the minimal medium was as follows (g/L): bacto-agar (Difco), 20; glucose (Merck), 20; the yeast nitrogenous base without amino acids (Difco), 6.7. For auxotrophic yeasts, the necessary amino acids and bases were added to the basal medium. The genes of sucrose, maltose, and galactose fermentation, as well as the auxotrophy genes were used in hybridizations as the control markers (Table 1). The yeast capacity for sugar fermentation was determined on agarized pH-indicator medium with eosin and methylene blue [18]. The spores were isolated with a glass needle using a Carl Zeiss micromanipulator (Jena, GDR) after digestion of the ascus wall with the enzymatic preparation isolated by us from the stomach of the garden snail (*Helix pomatia*). Haploid cells of the opposite mating types were hybridized by the mass method on a complete medium with the subsequent zygote isolation using the micromanipulator. The hybrids of homo- and heterothallic strains were obtained by the "spore for spore" or "spore for a haploid cell" method using the micromanipulator [19]. Resistance to sulfite (Na_2SO_3, Ampresco, United States) was determined according to [20] on the YPD medium in the presence of 75 mM tartaric acid (Loba Chemie, Austria). The technique for the preparation of this medium is given below. Sterilization of agar in water (40 mL) and the tartaric acid solution (60 mL) with the remaining components of the YPD medium, pH 3.5 was carried separately (by autoclaving at 0.8 atm). After sterilization, the solutions were mixed; the media were poured into 25-mL plastic petri dishes, and dried at 25°C for 24 h. The self-sterile 0.5 M Na_2SO_3 solution was then rubbed with a microbiological spatula to the end concentration of 1–8 mM. The petri dishes were allowed to stand for the subsequent 24 h at 25°C for Na_2SO_3 to diffuse. The suspensions of 24-h yeast cultures were then applied onto the dishes containing the medium using a metal replicator. The experimental results were recorded after 24 and 48 h.

Molecular karyotyping and Southern hybridization. The preparations of chromosomal DNA were obtained according to [21]. Electrophoretic separation of the chromosomal DNA was carried out in the CHEF-DF II apparatus (Bio-Rad, United States) at 200 V for 24 h in the following mode: 15 h with a field switching time of 60 s and 9 h with a field switching time of 90 s. The buffer used was 0.5× TBE (45 mM Tris, 45 mM boric acid, 10 mM EDTA, pH 8.2) cooled to 14°C. After electrophoresis, the gel was stained with ethidium bromide, washed in distilled water, and photographed. The chromosomal DNAs were transferred onto the nitrocellulose membrane by Southern blotting. The DNA was fixed on the membrane by annealing at 80°C for 2 h. The 5 kb *Bam*HI-*Hin*dIII fragment of the *CUP1* gene isolated from the plasmid pET13.1 [22, 23] and the 1.0 kb *Sal*I-*Pvu*II fragment isolated from the plasmid pALK79 were used as probes [24]. For amplification of the *CUP1* and *GAL4* genes, the following pairs of primers were used: CUP11/CUP12 (ATGCGTCTTTTCCGCTGAAC and TATTCTTGGGGCGACATATGG) and GAL41/GAL42 (TGGCAGTTGAGGAGAACAAT and ATGGCCTTGTACCACGGTTT). PCR was carried out directly on yeast cells using a Tercyc DNA cycler (DNA Technologies, Russia). The probes were prepared according to [25]. The label was introduced by a nonradioactive method according to the Roche Applied Science (Switzerland) instruction using dioxygenin dig-II-dUTP. Hybridization and development of the hybridization signals were carried out according to the Roche Applied Science instructions.

RESULTS AND DISCUSSION

Monospore cloning. An obligatory step in genetic investigation of yeasts is the creation of their highly fertile homozygous lines by monospore cloning. Natural and, especially, cultured yeast strains may be heterozygous by one or many genes, including the sterility factors. It is possible to obtain the reproducible results of genetic analysis only with homozygous lines. Therefore, monospore clones of the strains analyzed were primarily isolated with a micromanipulator. Table 2 shows the results of monospore cloning. Many initial strains had low spore viability, so it was necessary to additionally determine the spore survival in monospore clones. According to the sporulation of monospore clones, almost all the strains analyzed had the homothallic cycle of development. The heterothallic strains CBS 4054 and VKM Y-1753 were an exception.

Molecular karyotyping of the strains analyzed and Southern hybridization of the *CUP1* and *GAL4* probes with their chromosomes are shown in Fig. 1. Unlike the control strains YNN295 and X2180-1A having the gene *CUP1* in chromosome VIII, all the eight investigated strains had a translocation of the gene *CUP1-t* in the chromosome with the size of chromosome XVI and its specific marker *GAL4*. The data on strain 1753-8-2 are not presented. In Figs. 1a–1c, polymorphism of the size of the translocation chromosome VIII is seen in different yeasts, with the strain M11-22-10B being especially noticeable. Weak *CUP1* hybridization signals in the strains 4054-3B, M3-33-6B, and M11-22-10B, which are sensitive to copper (Table 1) and probably have a small number of copies of the metallothionine sequences, are noteworthy.

Genetic analysis. The presence of only one *CUP1-t* gene in a number of the strains analyzed was con-

Table 2. Ascospore viability of different *S. cerevisiae* strains

Strain	Number of isolated tetrads	Spore viability, %	Strain	Number of isolated tetrads	Spore viability, %
L2-43	13	83	VKM Y-1753	12	25
L2-43-6C	11	100	M-427	13	35
L3-44	25	50	427-2A	5	100
L3-44-1C	12	98	CBS 4054	13	44
M3-33	6	50	4054-3B × 4054-5A	21	71
M3-33-6B	11	80	DVPG 1340	6	100
M11-22	24	50	M-437	10	100
M11-22-10B	14	91			

firmed by their hybridizations with the sensitive strains of the genotype *cup1-t* (Table 3, hybrids 1–5). The monogenic 2 : 2 segregation was observed in the tetrads, with small exceptions. The presence of six irregular tetrads (3 : 1, 1 : 3) among 86 tetrads can easily be explained by the high frequency of gene conversion in the amplified *CUP1* locus discovered earlier [5, 26–28]. The strains L2-43-6D, L3-44-7C, 1340-1D, and 427-2A shared the same gene *CUP1-t*, because the appearance of copper-sensitive meiotic segregants was not observed in their hybrids (Table 3, hybrids 6–8).

Reciprocal translocations between chromosomes XVI and VIII were established when we analyzed the hybrids of strains with a normal localization of the gene *CUP1* (or *cup1*) and with the translocation of *CUP1-t* (Table 4). In the case of polymery of the genes *CUP1-t* and *CUP1*, digenic segregation with emergence of sensitive segregants should be observed in the hybrids of the *CUP1-t/CUP1* genotype. The three relevant hybrids (nos. 11–13) obtained by us had no segregation. Formally, this testifies to allelism of the genes *CUP1-t* and *CUP1* as no recombination occurs between them. This is only possible in the case of reciprocal chromosomal translocations detected earlier [11] with the involvement of chromosomes XVI and VIII. The evidence was also obtained when further analysis of hybrids nos. 9, 10, and 11, respectively, of the genotypes *CUP1-t/cup1* and *CUP1-t/CUP1*, was

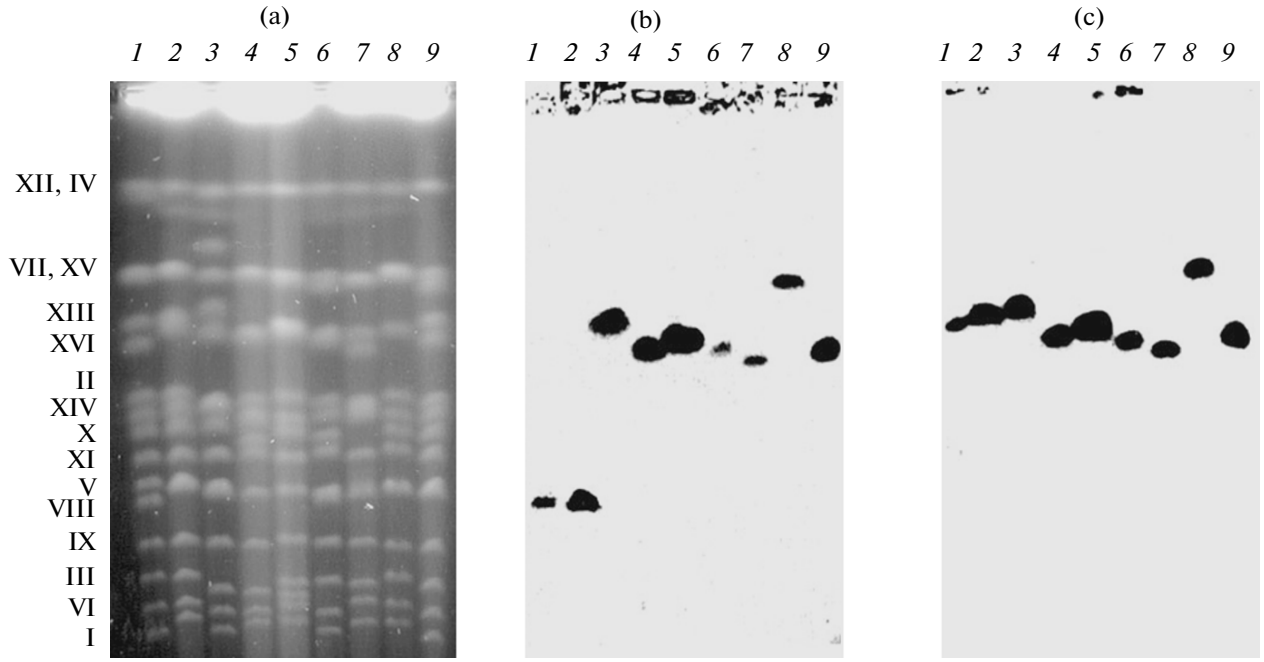

Fig. 1. Detection of reciprocal translocations between chromosomes XVI and VIII. Karyotyping (a) and Southern blotting of the *CUP1* (b) and the *GAL4* (c) localization in different *S. cerevisiae* strains: (*1*) YNN 295; (*2*) X2180-1A; (*3*) L2-43-6D; (*4*) L3-44-7C; (*5*) 427-2A; (*6*) 4054-3B; (*7*) M3-33-6B; (*8*) M11-22-10B; (*9*) 1340-1D.

Table 3. Identification of the *CUP1* gene by genetic analysis

Hybrid	Hybrid origin (parental phenotype)	Ascospore viability, %	Number of tetrads with segregation Cu^R : Cu^S				Hybrid genotype
			2 : 2	3 : 1	4 : 0	1 : 3	
1	L2-43-6D (Cu^R) × 4054-3B (Cu^S)	96	16	3	0	1	*CUP1-t/cup1-t*
2	L2-43-6D (Cu^R) × M11-22-10B (Cu^S)	83	20	0	0	0	*CUP1-t/cup1-t*
3	L3-44-7C (Cu^R) × M3-33-6B (Cu^S)	98	21	2	0	0	*CUP1-t/cup1-t*
4	427-2A (Cu^R) × M3-33-6B (Cu^S)	98	23	0	0	0	*CUP1-t/cup1-t*
5	1340-1D (Cu^R) × M3-33-6B (Cu^S)	90	18	0	0	0	*CUP1-t/cup1-t*
6	L2-43-6D (Cu^R) × L3-44-7C (Cu^R)	78	0	0	9	0	*CUP1-t/CUP1-t*
7	L3-44-7C (Cu^R) × 1340-1D (Cu^R)	93	0	0	23	0	*CUP1-t/CUP1-t*
8	L2-43-6D (Cu^R) × 427-2A (Cu^R)	96	0	0	21	0	*CUP1-t/CUP1-t*

Note: Copper resistance (Cu^R) and copper sensitivity (Cu^S) in the segregants of all hybrids was determined at 0.5 mM $CuSO_4$. Hybrid no. 1 segregants, for which 0.9 mM $CuSO_4$ was used, were an exception.

Table 4. Genetic analysis of the hybrids heterozygous by reciprocal translocations between chromosomes XVI and VIII

Hybrid	Hybrid origin (parental phenotype)	Ascospore viability, %	Number of asci with Cu^R : Cu^S segregation					Cu^R : Cu^S segregation in a random spore sample	Hybrid genotype
			2 : 2	4 : 0	3 : 0	2 : 1	1 : 2		
9	L2-43-6D (Cu^R) × 31-1-7B (Cu^S)	46	1	0	0	3	2	18 : 17	*CUP1-t/cup1*
10	L3-44-7C (Cu^R) × 31-1-7B (Cu^S)	69	2	0	0	11	6	40 : 35	*CUP1-t /cup1*
11	L2-43-6D (Cu^R) × X2180-1A (Cu^R)	70	0	6	29	1	0	113 : 1	*CUP1-t /CUP1*
12	1340-1D (Cu^R) × X2180-1A (Cu^R)	73	0	8	30	1	0	120 : 1	*CUP1-t /CUP1*
13	L2-43-6D (Cu^R) × 437-1B (Cu^R)	66	0	6	25	0	0	99 : 0	*CUP1-t /CUP1*

Note: Copper resistance in all hybrid segregants was determined at 0.5 mM $CuSO_4$. The segregants of the hybrids no. 9 and no. 13, for which 0.9 mM $CuSO_4$ was used are an exception.

performed (Table 4). Although the hybrids nos. 9 and 10 did produce phenotypic monogenic segregation, the Southern analysis of the karyotypes of their meiotic segregants with the *CUP1* probe showed, on the one hand, the absence of the double recombinants *CUP1-t cup1* (the genotype *cup1* is differentiated well by both a low signal and copper sensitivity) and, on the other hand, as a result of meiotic crossing-over, the allele *cup1* could translocate to the chromosome with the size of chromosome XVI; the allele *CUP1*, to chromosome VIII (Figs. 2a, 2b). Evidently, new and reciprocal *CUP1* translocations took place. The segregants of the hybrid no. 11 had no double recombinants *CUP1-t CUP1* and *cup1-t cup1* (Fig. 2c). Note that, along with the letter designations of the segregants within one tetrad, numerical designations are also used; for example, no. 9-8-3 and no. 9-8-4 are two surviving segregants of the same tetrad.

The data on fertility of the hybrids and their parents also agree with the presence of reciprocal translocations between chromosomes XVI and VIII. Higher ascospore viability (Table 3), compared to the heterozygous hybrids (Table 4), 78–98 and 46–73%, respectively, was observed in translocation-homozygous hybrids. The low ascospore viability of the parental strains L3-44, M3-33, M11-22, VKM Y-1753, M-427, and CBS 4054 (Table 2) leads us to suggest that they were heterozygous by the reciprocal translocations analyzed, whereas high ascospore viability of the strains L2-43 and DVPG 1340 gives evidence of the corresponding homozygosity.

Sulfite resistance. Investigation of the natural sulfite resistance gene *SSU1-R* of the wine strains of *S. cerevisiae* played a great role in the understanding of the mechanism of reciprocal translocations between chromosomes XVI and VIII. The sulfite-resistant mutant *RSU1* was originally isolated by Xu et al. [29], together with the sulfite-sensitive *SSU1* mutant. Later, it was shown that the plasma membrane protein was the product of the gene *SSU1* [30]. A natural mutation of the sulfite overresistance *SSU1-R* gene of the wine yeasts was revealed [31]. The encoding region of the

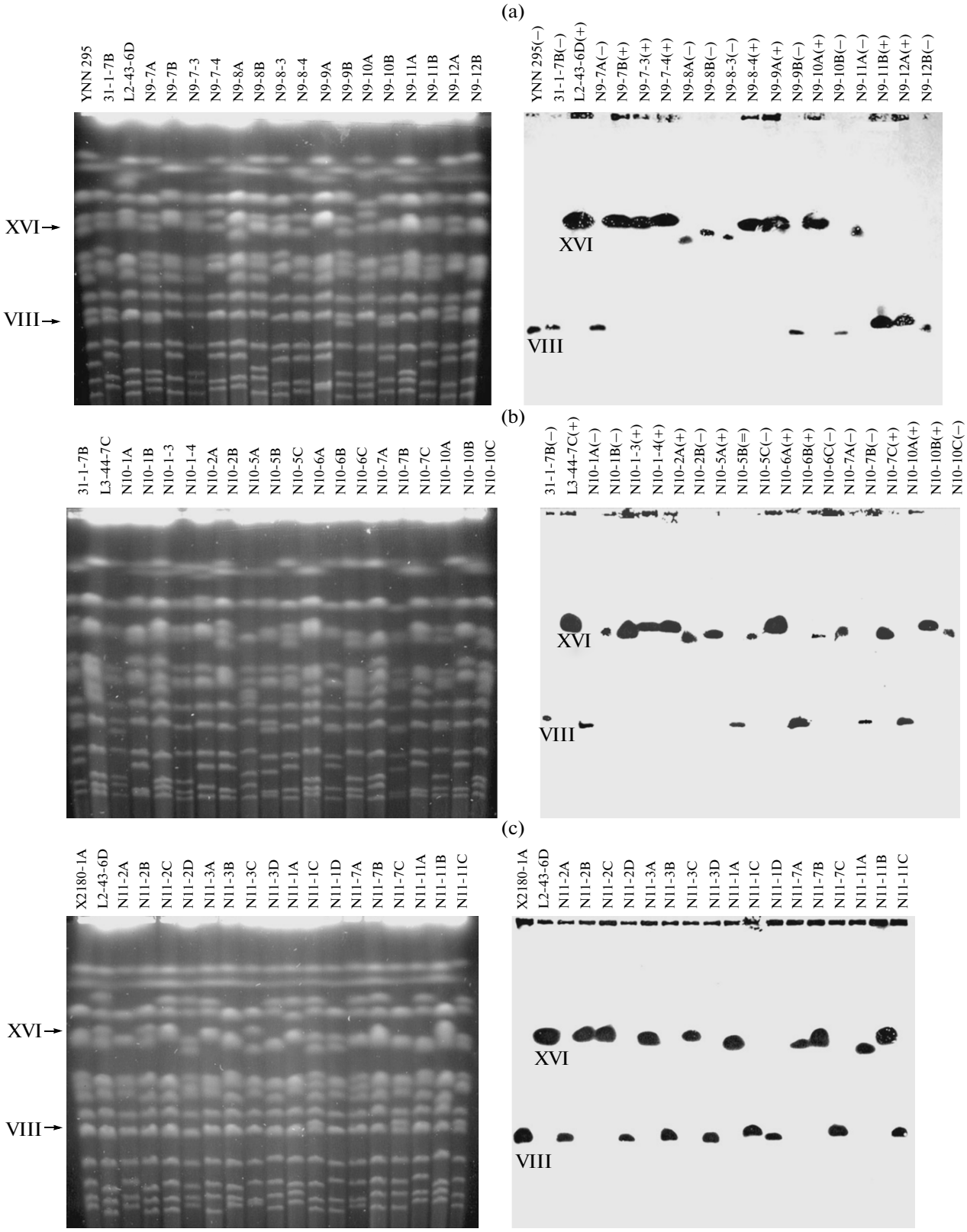

Fig. 2. Karyotypes and Southern analysis of chromosomal localization of the *CUP1* gene in meiotic segregants of the hybrids no. 9 (a), no. 10 (b), and no. 11 (c). Copper resistance and copper sensitivity are designated with the + and – signs, respectively, after the strain number in brackets.

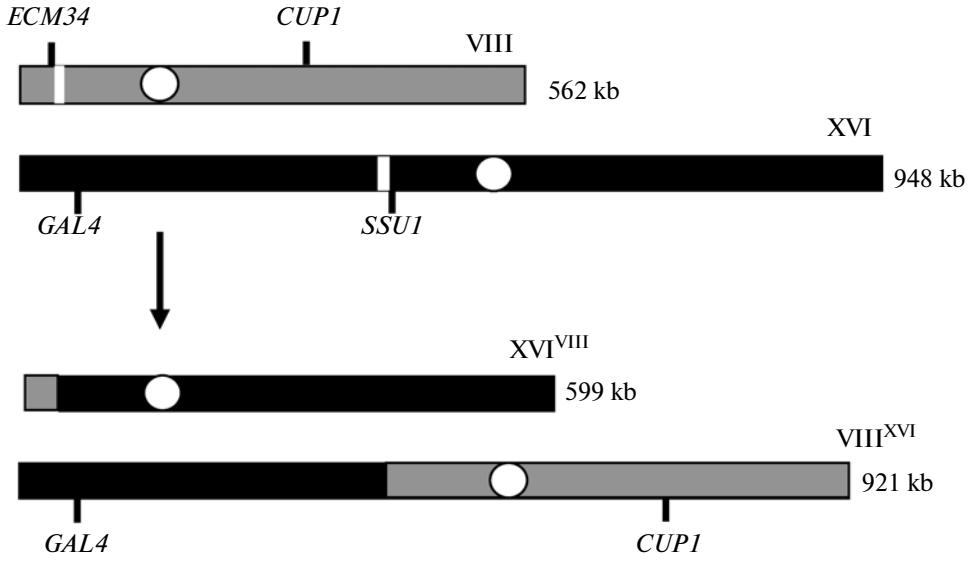

Fig. 3. Scheme of reciprocal translocations between chromosomes VIII and XVI in *S. cerevisiae* according to [11] in our modification. Translocations occur on the basis of crossing-over in the regions of microhomology of the promoters of the genes *ECM34* and *SSU1*, whose localization on chromosomes VIII and XVI, respectively, is designated with white gaps. Localization of the marker genes *CUP1* and *GAL4* is also shown.

SSU1-R sequence is almost identical to that of the *SSU1* located in chromosome XVI but contains the promoter (several copies, each of which is 76 kb) from chromosome VIII. Pérez-Ortín et al. [11] showed by the example of a number of wine strains that the *SSU1-R* allele was the product of the reciprocal translocation between chromosomes VIII and XVI determined by unequal crossing-over in a very short homologous region in the 5'-terminal position of the genes *SSU1* and *ECM34* (Fig. 3). Naturally, the more extended translocation chromosome VIII also carries the gene *CUP1-t* [32]. It was discovered on a large material [11, 31] that emergence of the recombinant promoter resulted in increased transcription of the *SSU1* sequence and, consequently, in overresistance to sulfite, which is an antioxidant and an antimicrobial agent widely used in wine-making. Thus, the yeast selection for sulfite resistance results in the selection of reciprocal translocations between the chromosomes XVI and VIII involving the gene *CUP1*. Sulfite resistance, together with the formation of the type K2 killer toxin (mycocin) [33–35], determines the competitiveness of the strains in wine-making [36].

As it would be expected, our experiments showed that almost all strains with the *CUP1-t* or *cup1-t* translocation (Fig. 1) were sulfite-resistant, although to a different degree, compared to the strains X2180-1A, S288C, 31-1-7B, 437-1B, and 2576C of the normal karyotype (Table 1, Fig. 4). The only exception was the strain 427-2A, which was resistant to only 2.0 mM Na_2SO_3. Earlier, it was revealed that tandem iteration of the number of the *SSU1-R* promoter sequences increased sulfite resistance of the cells [11, 31]. Taking into account the data of Whittaker et al. [6] on increased copper resistance of the yeasts containing an extra chromosome(s) VIII, we can assert that the selection of *CUP1-t* translocation in the yeasts under the wine-making conditions may, apparently, occur due not only to the *SSU1-R* marker, but also to *CUP1*.

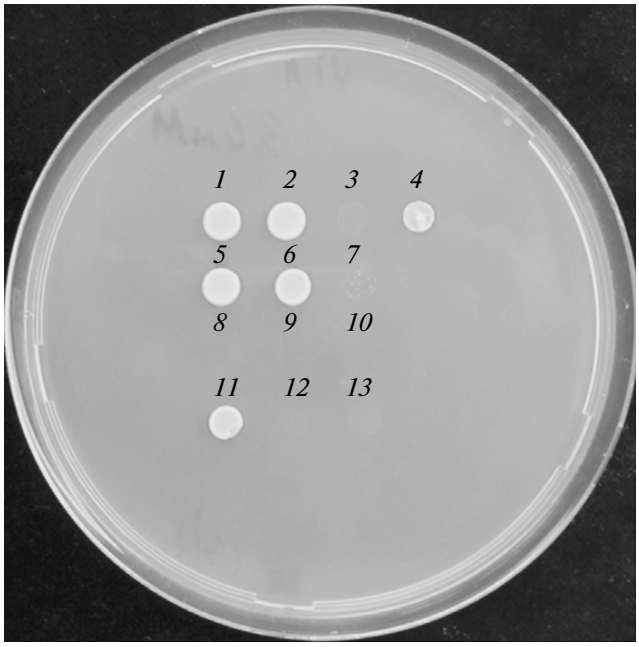

Fig. 4. Growth (24 h) of *S. cerevisiae* yeasts on the YPD medium in the presence of 3.0 mM Na_2SO_3: (*1*) L2-43-6D; (*2*) L3-44-7C; (*3*) 427-2A; (*4*) 4054-3B; (*5*) M3-33-6B; (*6*) M11-22-10B; (*7*) 1340-1D; (*8*) X2180-1A; (*9*) S288C; (*10*) 437-1B; (*11*) 1753-8-2; (*12*) 31-1-7B; (*13*) 257-6C.

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