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EXPERIMENTAL ARTICLES

Natural Polymorphism of the Plasmid Double-Stranded RNA of the *Saccharomyces* Yeasts

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Abstract—The distribution and peculiarities of viral double-stranded RNA in natural *Saccharomyces* strains were studied. It is for the first time that the presence of the L and M fractions in the species *S. kudriavzevii* and *S. mikatae* has been documented. *S. kudriavzevii* has two types of M-dsRNA: M_1 and M_4, whereas the yeast *S. mikatae* is characterized by three types of plasmids: M_2–M_4. Plasmid dsRNAs are absent in *S. cariocanus* strains. A total of eleven types of M-dsRNA were identified; some of them were specific to particular species. Plasmids M_5–M_7 were revealed only in *S. paradoxus* strains and the yeast *S. bayanus* is characterized by M_8–M_{11} double-stranded RNA. According to the results of phenotypic analysis, all the M-dsRNAs revealed were cryptic.

Key words: Saccharomyces, L-dsRNA, M-dsRNA, cryptic plasmids, phylogenetic analysis.

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Killer strains occur among different yeast genera, including *Saccharomyces.* This genus includes six biological species: *S. cerevisiae, S. bayanus, S. cariocanus, S. kudriavzevii, S. mikatae*, and *S. paradoxus* [1, 2]. The yeasts *S. cerevisiae* and *S. bayanus* are associated with different fermentation processes and are very rarely isolated from natural sources. The biological species *S. cariocanus, S. kudriavzevii, S. mikatae*, and *S. paradoxus* occur only in nature and are not associated with human activity. The latter is a cosmopolite; its strains have been isolated from the exudation sap of broad-leaved trees, insects, uncultivated soils, plant leaves, and other sources. Recently, seasonal dominance of the yeast *S. paradoxus* was revealed on living and decomposing leaves of certain plant species in the Moscow oblast [3].

The biological species of *Saccharomyces* may have habitats in common. Thus, the yeasts *S. cerevisiae* and *S. paradoxus* form sympatric populations in the exudation sap of broad-leaved trees, whereas *S. cerevisiae* and *S. bayanus* occur in mixed populations under conditions of wine-making [4, 5]; therefore, their killer antagonism cannot be ruled out. The phenomenon of killing sensitive cells by species-specific toxins is widespread among cultured yeasts, primarily the wine yeast *S. cerevisiae* [6, 7]. The killer strains contain two types of viral double-stranded RNA (dsRNA): M and L [8]. M-dsRNA is responsible for synthesis of the toxin and resistance to it; L-dsRNA is responsible for the formation of protein capsules in which the dsRNA M and L fractions exist separately. In the yeast species *S. cerevisiae*, several types of killer strains (K1, K2, K3, etc.) were found; they differ in the size of M fractions, which determine the formation of different toxins and resistance to them [9, 10]. Most isolates of the biological species *S. bayanus* do not possess viral dsRNA; only several wine strains, primarily of French origin, harbor different types of M-dsRNA: M_1–M_3 and M_8–M_{11} [11, 12]. Viral dsRNAs (L and M fractions) were also found in several *S. paradoxus* strains; at least some of them, e.g., M_6, determine toxin synthesis [13, 14]. Nothing is known about the distribution of killer plasmids in the recently described species *S. kudriavzevii*, *S. mikatae* and *S. cariocanus*, which occur in Japan and Brazil, respectively.

In this work, we studied the distribution and specific features of plasmid dsRNA in *S. cariocanus, S. kudriavzevii*, and *S. mikatae,* as well as in the near-Moscow population of *S. paradoxus.*

MATERIALS AND METHODS

The strains studied and their origins are shown in the table. The origin of the strains of the near-Moscow population of *S. paradoxus* (the "MGU" strains) is described in [3].

Plasmid dsRNA was isolated according to the method described in [15] and modified by us [14]. The plasmid dsRNA was fractionated by electrophoresis in a 1% agarose gel at 50–55 V in 0.5× TBE buffer for 3 h.

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Saccharomyces yeasts studied in this work

Strain	Region of isolation	Source of isolation	dsRNA type
S. bayanus			
Sμ-1	France	Grapes	L + M_8
DDI 4.95	France	Fermenting grape pulp	L + M_{10}
SCU 397	France	Muscatel	L + M_{11}
S. cariocanus			
UFRJ 50791	Brazil	*Drosophila* sp.	0
UFRJ 50816 (T)	Brazil	*Drosophila* sp.	0
S. kudriavzevii			
NBRC 1802 (T)	Japan	Leaf litter	0
NBRC 1803	Japan	Leaf litter	0
NBRC 10990	Japan	Soil	L + M_1
NBRC 10991	Japan	Leaf litter	L + M_4
S. mikatae			
NBRC 1815 (T)	Japan	Soil	0
NBRC 1816	Japan	Leaf litter	0
NBRC 10992	Japan	Leaf litter	L + M_3
NBRC 10993	Japan	Leaf litter	L + M_4
NBRC 10994	Japan	Leaf litter	L + M_3
NBRC 10995	Japan	Leaf litter	L + M_3
NBRC 10996	Japan	Soil	0
NBRC 10997	Japan	Leaf litter	0
NBRC 10998	Japan	Leaf litter	0
NBRC 10999	Japan	Soil	L + M_3
NBRC 11000	Japan	Exudation sap of the oak *Quercus crispula*	0
NBRC 11001	Japan	Exudation sap of the camellia *Camellia japonica*	L + M_2
S. paradoxus			
№ 8	Moscow oblast	Exudation sap of the oak *Q. robur*	L + M_5
№ 9	Uzbekistan	Exudation sap of the oak *Quercus* sp.	0
№ 12	Azerbaijan	Exudation sap of the oak *Quercus* sp.	L + M_4
№ 17	Tatarstan	Exudation sap of the oak *Q. robur*	L + M_5
№ 15	Moscow	Exudation sap of the oak *Q. robur*	L + M_5
№ 16	Moscow	Exudation sap of the oak *Q. robur*	L + M_4
VKM Y-2472	Moscow oblast	Lowland willow bog peat	L + M_6
DBVPG 1373	The Netherlands	Soil	L + M_7
CBS 432 (T)	Not known	Not known	0
CECT 10178	Spain	Cutworm *Noctua pronubula*	0

Notes: Abbreviated collection names: VKM, the All-Russian Collection of Microorganisms, Moscow; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CECT, Coleccíon Española de Cultivos Tipo, Valencia, Spain; DBVPG, the Industrial Collection of the of Plant Biology of the University of Perugia, Italy; NBRC, National Institute of Technology and Evaluation, Japan; SCU, Institut Technique de la Vigne et du Vin, Centre d'Expérimentation de Nantes, France; UFRJ, Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Brazil. The strains designated as "№ ..." are from G.I. Naumov's collection. T letters mark type cultures.

The dsRNA preparations of the reference strains *S. cerevisiae* K7 and M437, which contain plasmids [KIL-k_1] and [KIL-k_2], respectively, were used as controls. The gel was stained with ethidium bromide and photographed in ultraviolet light in a Vilber Lourmat transilluminator (France). The size of the dsRNA fractions was determined by the 1 kb DNA Ladder molecular weight marker (Fermentas, Lithuania).

Killer-sensitive reactions. Killer toxin formation by the strains was determined on the lawn of the sensitive strain *S. cerevisiae* SBY 2576, and the resistant phenotype was determined by the yeast tolerance of strain *S. cereveisiae* M437 (M_2-dsRNA) [9, 16].

PCR and product analysis. DNA isolation from the yeast cells was carried out according to the method described earlier [3]. PCR with the microsatellite primer $(GTG)_5$ was performed in 30 μl of a reaction mixture containing PCR buffer, 20 mM m mM $MgCl_2$, 0.25 mM concentration of each dNTP, 1.0 μl of the primer, 0.5 units of *Taq*-polymerase (Syntol, Russia), and 20 ng of the genomic DNA analyzed. A Tertsik DNA amplifier (DNK-Tekhnologiya, Russia) was used in the following regime: initial DNA denaturing at 94°C for 5 min; 40 cycles of DNA denaturation at 94°C for 1 min, annealing of the primers at 52°C for 2 min, DNA synthesis at 74°C for 3 min; and final extension at 74°C for 10 min. The amplification products were subjected to electrophoresis in 1.7% agarose gel at 60 V in 0.5× TBE (45 mM Tris, 45 mM boric acid, 10 mM EDTA, pH 8.0) for 3 h. The gel was stained with ethidium bromide and photographed in ultraviolet light in a transilluminator.

Phylogenetic analysis. The relatedness between the strains was determined by comparing the profiles of the PCR products obtained with the microsatellite primer $(GTG)_5$. The dendrogram was constructed using the neighbor-joining method implemented in the TREECON software package. The PCR profile of *S. cerevisiae* VKM Y-502 (= CBS 5287) was used as the outgroup. The bootstrap values corresponding to the statistical significance of group identification were determined for 100 pseudoreplicates.

RESULTS AND DISCUSSION

The intraspecies polymorphism of *S. paradoxus*. The distribution of plasmid dsRNAs in *S. paradoxus* yeasts was studied using 28 strains isolated in different seasons from the leaves of various kinds of plants in the Moose Island forest reserve and in the vicinity of the village of Burtsevo, Shakhovskoi raion. Hereafter, this population is referred to as the near-Moscow population. Plasmid dsRNA was absent in all the strains studied, whereas among the *S. paradoxus* yeasts isolated within the city of Moscow and Moscow oblast, we earlier revealed strains possessing different types of M-dsRNA: M_3, M_4, M_5, and M_6 [14]. As distinct from the yeasts of the near-Moscow population, the strains studied earlier were isolated from the exudation sap of oaks, a natural substrate with high sugar content.

A certain correlation, exemplified by more than 50 *S. paradoxus* strains of European origin, was earlier found between the $(GTG)_5$ patterns of the strains and the site of their isolation [17]. Thus, *S. paradoxus* strains from the central parts of European Russia and Ukraine, northern Europe, and regions with a warm climate (Spain, southern coastline of the Crimea, Uzbekistan, and Azerbaijan) exhibited different PCR profiles. The molecular marker $(GTG)_5$ with its multiple localization in the genome of *Saccharomyces* [18] allows the identification of *S. cerevisiae*, *S. bayanus*, and *S. paradoxus*, as well as the differentiation between individual strains of each of these species [17, 19]. We compared the $(GTG)_5$ patterns of the yeasts of the near-Moscow population and of six *S. paradoxus* strains (CBS 432, CECT 10178, N8, 9, 12, and 17) isolated in various regions of Europe and differing in dsRNA and in PCR profiles. dsRNA was absent in the type culture CBS 432 and in strains CECT 10178 and N9, isolated in Spain and Uzbekistan, respectively. The remaining three strains N8 (Moscow oblast), N17 (Tatarstan), and N12 (Azerbaijan) had M_5- and M_4-dsRNA, respectively. All the *S. paradoxus* strains studied differed in the $(GTG)_5$ patterns from the test strain *S. cerevisiae* VKM Y-502, characterized by the presence of the major band approximately 2300 bp in size (not shown). Most of the *S. paradoxus* strains exhibited $(GTG)_5$ patterns with major fragments approximately 2500, 1300, 1000, 700, and 280 bp in size. Strains of the near-Moscow population had virtually identical $(GTG)_5$ profiles. The polymorphism of the PCR products mainly manifested itself in the presence or absence of individual minor bands whose size varied between 280 and 700 bp (not shown).

The dendrogram shown in Fig. 1 was constructed on the basis of similarity of the patterns obtained with the microsatellite primer $(GTG)_5$. The strains of *S. paradoxus* formed a separate cluster in relation to the test strain *S. cerevisiae* VKM Y-502. Inside this cluster, several groups combining strains with similar PCR profiles can be identified. Strains of the near-Moscow population form a separate group in which, in turn, three subgroups can be identified. The division into these subgroups does not correlate with the strain isolation source and time. For example, strains MGU20 and MGU41, isolated from spruce needles in July, fall into different subgroups (Fig. 1). The *S. paradoxus* strains isolated in different regions of Europe are, on the whole, more heterogeneous by the PCR profiles. The phylogenetic analysis gives evidence of genetic homogeneity of the near-Moscow population of *S. paradoxus* yeasts.

Plasmid dsRNA of the yeasts *S. cariocanus*, *S. kudriavzevii*, and *S. mikatae*. We have studied all the so far known strains of the species listed in the table. The *S. cariocanus* yeasts lacked plasmid dsRNA, whereas some of *S. kudriavzevii* and *S. mikatae* strains

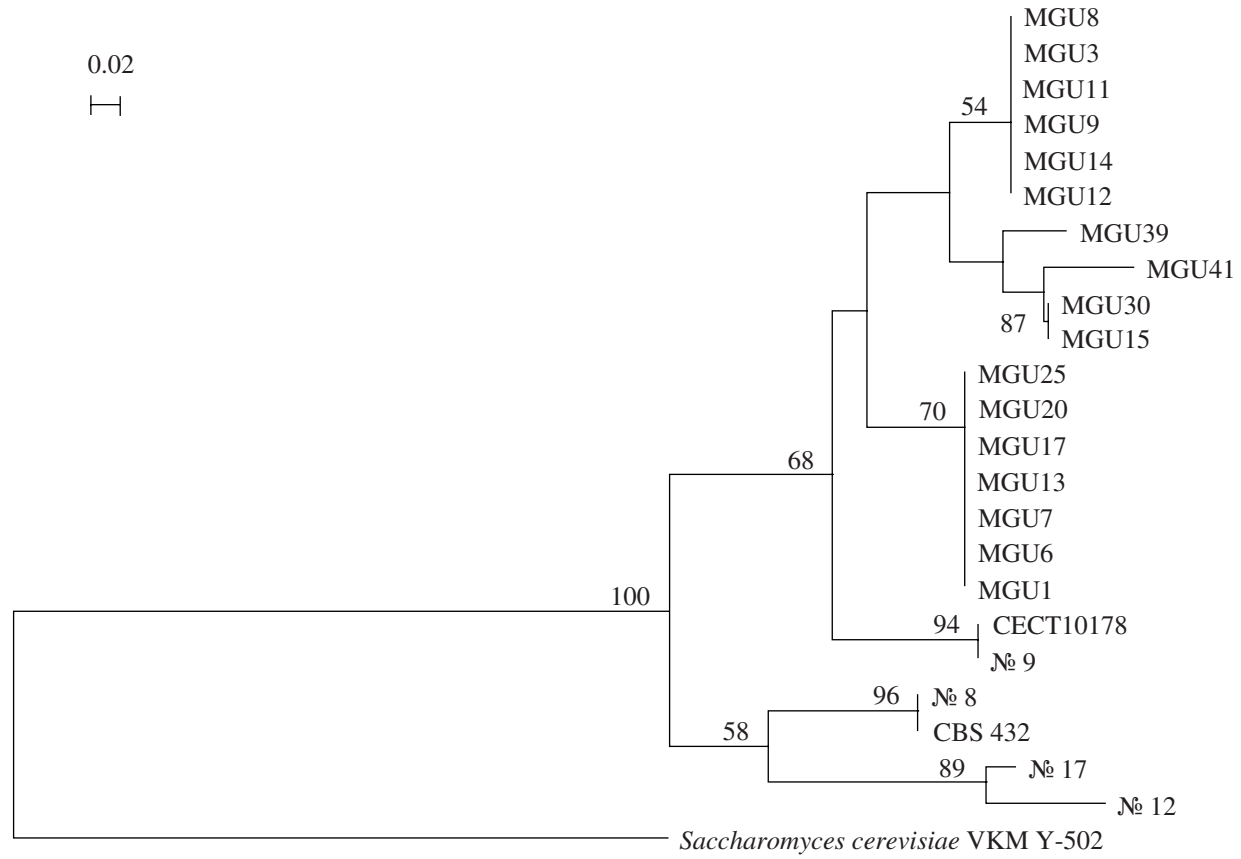

Fig. 1. Dendrogram of relatedness between *S. paradoxus* strains based on the distance matrix of PCR profiles with the microsatellite primer $(GTG)_5$. Strain *S. cerevisiae* VKM Y-502 was used as the outgroup. The data were processed using the neighbor-joining algorithm implemented in the TREECON software package.

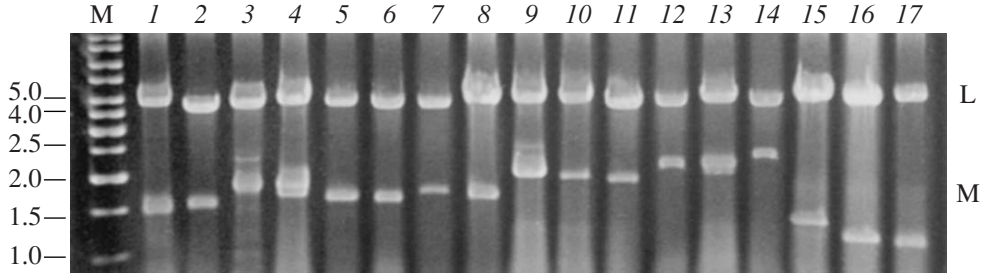

Fig. 2. Plasmid dsRNA of *Saccharomyces* yeasts. *S. cerevisiae* strains: *1*, M 437 (M_2); *3*, K7 (M_1). *S. bayanus* strains: *8*, IFI 373 (M_3); *15*, Sμ1 (M_8); *16*, DDI4.95 (M_{10}); *17*, SCU 397 (M_{11}). *S. kudriavzevii* strains: *4*, NBRC 10990 (M_1); *9*, NBRC 10991 (M_4). *S. mikatae* strains: *2*, NBRC 11001 (M_2); *5*, NBRC 10999 (M_3); *6*, NBRC 10995 (M_3); *10*, NBRC 10993 (M_4). *S. paradoxus* strains: *7*, № 22 (M_3); *11*, № 16 (M_4); *12*, № 15 (M_5); *13*, VKM Y-2472 (M_6); *14*, DBVPG 1373 (M_7). In parentheses, the M-dsRNA type is shown. M is the 1 kb DNA Ladder molecular mass (kb) marker (Fermentas, Lithuania).

exhibited two electrophoretic fractions: L- and M-dsRNA. The size of the M-dsRNA of *S. kudriavzevii* and *S. mikatae* was compared not only with the M fractions of the reference strains *S. cerevisiae* K7 (M_1) and M437 (M_2) but also with different types of M-dsRNA of *S. paradoxus* and *S. bayanus*. The results of dsRNA electrophoresis in agarose gel are shown in the table and, in part, in Fig. 2. In most strains, the size of the main L fraction was about 4.5 kb. In the strains *S. mikatae* NBRC 10995, NBRC 10999, and NBRC 11001 and in the European *S. paradoxus* strains, the L-dsRNA size was somewhat less, about 4.3 kb (Fig. 1, lanes *2*, *5–7*, *11*, *12*, *14*). In strain *S. kudriavzevii* NBRC 10990, the size of the M_1 fraction was similar to that of the reference strain *S. cerevisiae* K7 (about 1.8 kb) (Fig. 1, lanes *4* and *3*, respectively). Another strain of

this species, NBRC 10991, and *S. mikatae* NBRC 10993 had an M-dsRNA whose size (about 1.9 kb) was similar to that of M_4-dsRNA of 16 *S. paradoxus* strain № 16 (Fig. 1, lanes *9*, *10* and *11*, respectively). In strain *S. mikatae* NBRC 11001, the size of M_2-dsRNA(about 1.6 kb) was similar to that of another reference killer strain, *S. cerevisiae* M437 (Fig. 1, lanes *2* and *1*, respectively). The strains *S. mikatae* NBRC 10992, NBRC 10994, NBRC 10995, and NBRC 10999 were characterized by the presence of the M fraction whose size (about 1.7 kb) was similar to that of the M_3-dsRNA of *S. paradoxus* № 22 and *S. bayanus* IFI 373 (Fig. 1, lanes *5*–*8*, respectively).

In the *Saccharomyces* strains studied, irrespective of the presence or absence of dsRNA in them, the production of the killer toxin and resistance/sensitivity to it were determined. According to the phenotypic analysis, none of the *S. paradoxus*, *S. cariocanus*, *S. kudriavzevii*, and *S. mikatae* strains produced a killer toxin under standard conditions. It seems that all plasmid M-dsRNAs that we found are cryptic. The strains of *S. cariocanus* and strain *S. paradoxus* MGU8 from the near-Moscow population were resistant to the toxin of the killer strain M437. Since none of the above-mentioned strains possess plasmid dsRNA, their resistance to the toxin seems to be determined by nuclear genes. Three *S. mikatae* strains (NBRC 10992, NBRC10995, and NBRC 10999) possessing M_3-dsRNA produced an extremely weak zone of methylene blue-stained cells of the sensitive tester strain SBY 2576. In this connection, we have great doubts about the production of a killer toxin by these three stains.

The results of this study and the data obtained by us previously [12, 14] give evidence of significant polymorphism of plasmid dsRNA in the yeasts of the genus *Saccharomyces*. Plasmid dsRNA was absent only in *S. cariocanus*. In *S. kudriavzevii*, two types of M-dsRNA were revealed: M_1 and M_4, whereas the *S. mikatae* yeasts possess three types of plasmids: M_2–M_4. We revealed no new M fractions in either of these species. This may be due to the small size of the strain sample, although, as it was already mentioned, we studied all the known strains of these species. At the same time, *S. paradoxus* and *S. bayanus* are each known to have, along with the standard L fraction, seven different M-dsRNA: M_1–M_7 [14] and M_1–M_3 plus M_8–M_{11} [11, 12], respectively. Thus, a total of eleven different M-dsRNAs have been identified in *Saccharomyces* yeasts. M_1-dsRNA occurs in four of the six *Saccharomyces* species known: *S. cerevisiae, S. bayanus, S. paradoxus, S. skudriavzevii*, and *S. mikatae*. The M_2 fraction was not found only in *S. cariocanus* and *S. kudriavzevii*. The M_1 fraction is typical of the laboratory *S. cerevisiae* strains, and M_2-dsRNA often occurs among wine yeasts, including commercial strains [6, 16]. It is known that, in *S. cerevisiae* strains, the M_1 and M_2 fractions, which differ in dsRNA size, determine the formation of different toxins and different resistance to them [20]. Some of the M-dsRNA fractions were revealed by us only in certain species. Thus, the M_5–M_7 plasmids occur in *S. paradoxus*, and the M_8 –M_{11} fractions are characteristic of the yeast *S. bayanus*.

Despite the fact that a number of *S. paradoxus* strains of different geographic origins do not possess any dsRNA (e.g., the near-Moscow population) or possess only the L fraction, most strains of this species do have both L- and M-dsRNA. At least some of the M fractions, e.g., M_6, determine the synthesis of toxins [13, 14]. Half of the *S. kudriavzevii* and *S. mikatae* strains studied had no dsRNA, whereas all the rest possessed both the L and M fractions. Interestingly, all of the plasmid M-dsRNAs found were not functional and are likely to be the mutant forms of killer plasmids. A well known fact, exemplified by *S. cerevisiae*, is that various physical and chemical factors (increased temperature, UV irradiation, acridine dyes and cycloheximide) may cause elimination of the killer plasmids [21]. Evidently, mutations in plasmid dsRNAs or their complete elimination also occur in natural populations of *Saccharomyces* yeasts under the influence of various environmental factors.

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