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EXPERIMENTAL ARTICLES

Genetic and Molecular Study of the Inability of the Yeast *Kluyveromyces lactis* var. *drosophilarum* To Ferment Lactose

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Abstract—The fermentation of lactose (Lac^+) in the dairy yeast *Kluyveromyces lactis* var. *lactis* is controlled by the *LAC4* (β-galactosidase) and *LAC12* (lactose permease) genes. The complementation analysis of twelve *Kl. lactis* var. *drosophilarum* natural homothallic Lac^- strains of different origin was carried out using the genetic heterothallic lines of *Kl. lactis* var. *lactis* of the *lac4LAC12* and *LAC4lac12* genotypes. It was shown that the natural Lac^- strains did not possess the *LAC4LAC12* gene cluster. Southern hybridization of chromosomal DNA with *LAC4* and *LAC12* probes, as well as recombination analysis, showed that *Kl. lactis* var. *drosophilarum* yeasts do not have even silent copies of these genes. As distinct from this yeast, natural Lac^- strains of the yeast *Kl. marxianus* are mutants impaired in the lactose permease gene (*lac12* analogue), but possess an active β-galactosidase gene (*LAC4* analogue). The origin of the *LAC4LAC12* gene cluster of the dairy yeasts *Kl. lactis* is discussed.

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Key words: dairy yeast, *Kluyveromyces lactis* var. *lactis, Kluyveromyces lactis* var. *drosophilarum, Kluyveromyces marxianus,* LAC4 and LAC12 genes, b-galactosidase, lactose permease.

According to the latest classification [1–3], the yeasts *Kluyveromyces lactis* are represented by two varieties: *Kl. lactis* var. *lactis* and *Kl. lactis* var. *drosophilarum*. However, the molecular and genetic data [4–6] give evidence that the variety *Kl. lactis* var. *drosophilarum* is heterogeneous and consists of a number of divergent geographical and ecological populations. The populations most studied genetically are "drosophilarum" (var. *drosophilarum* proper) and "phaseolosporus" (North America), "krassilnikovii" (Europe), "vanudenii" (South Africa), "eastern" (Far East), and "aquatic" (North America).

The system of lactose genes in the lactose-fermenting (Lac^+) dairy yeast *Kl. lactis* var. *lactis* is known by virtue of the studies of Dickson et al. [7]. Along with the lactose genes proper, *LAC4* and *LAC12*, which code for β-galactosidase and lactose permease, respectively, the genes responsible for lactose fermentation also include the structural galactose genes *GAL1* (kinase), *GAL7* (transferase), and *GAL10* (epimerase) and the galactose–lactose regulatory genes *LAC9* (positive control) and *LAC10* (negative control).

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No data are available in the literature that could explain why the closest wild relatives of the dairy yeast *Kl. lactis* var. *lactis*, formally assigned to one variety, *Kl. lactis* var. *drosophilarum*, are incapable of utilizing lactose (Lac^-) despite the absence of reproductive isolation between the varieties and the 70–100% reassociation of their nuclear DNAs [4, 5, 8]. A similar situation is observed with the Lac^- strains of the species *Kl. marxianus*. Along with this, in rare natural strains of another yeast, *Saccharomyces cerevisiae*, the inability to ferment maltose and galactose may be determined by mutations in regulatory and structural genes. Thus, Mal^- strains have a defective maltose permease gene and/or a defective regulatory gene, but not a defective α-glucosidase gene [9, 10]. In Gal^- strains, mutations have been identified in one or several of the following genes: *gal2* (permease), *gal4* (regulatory gene), and *gal7* (transferase) [11]. We have already mentioned the relationship between lactose and galactose fermentations. Maltose and galactose fermentations also have some features in common: along with an intracellular hydrolase, a permease is necessary for the disaccharide transport into the cell.

The aim of this work was to reveal which lactose genes are absent in the wild strains of *Kl. lactis* and *Kl. marxianus* that do not utilize lactose.

Table 1. The homothallic strains of *Kl. lactis* var. *drosophilarum* (the populations "drosophilarum," "phaseolosporus", "krassilnikovii", "vanudenii," "aquatic", and "eastern") and *Kl. marxianus* and the results of their hybridization with the *lac* testers

Yeast name	Strain designation[a]	Isolation source	Geographic origin	Reference	Hybridization[b] with testers	
					lac4	*lac12*
"drosophilarum"	VKM Y-1302 (T)	*Drosophila* sp.	California, USA	[4, 5]	+	+
"phaseolosporus"	VKM Y-1296 (T)	*Drosophila* sp.	California, USA	[4–6]	+	+
	UCD 51-272	*Drosophila* sp.	California, USA	[4, 6]	+	+
	UCD 61-200	*Drosophila* sp.	California, USA	[4, 6]	+	+
"krassilnikovii"	VKM Y-831 (T)	Oak sap oozing	Kaluga, Russia	[5, 6]	+	+
	Vor86 (CBS 9058)	Oak exudate	Voronezh, Russia	[5, 6]	+	+
	CBS 2877	Cow caecum	Portugal	[5, 6]	+	+
"vanudenii"	VKM Y-1535 (T)	Winery	South Africa	[4–6]	+	–
"aquatic"	UCD 71-45	Swamp	Louisiana, USA	[4, 6]	+	+
	CBS 6171	Swamp	Louisiana, USA	[6]	+	+
"eastern"	UCD 72-212	Food plant	Thailand	[4, 6]	+	+
	DV30 (CBS 9815)	Oak sap oozing	Far East	[5, 6]	+	+
Kl. marxianus	CBS 5670	Winery	South Africa	[12]	+	+
	ATCC 52486	Sheep caecum	Portugal	–	+	+

[a] Abbreviations: T, the type culture; VKM, All-Russia Collection of Microorganisms, Moscow; ATCC, American Type Culture Collection, Manassas, USA; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; UCD-FST, Herman J. Phaff Yeast Culture Collection, Davis, USA; the rest of the symbols are those of our laboratory collection. The monospore cultures of the strains indicated were used.
[b] The experiments used the following UV-induced auxotrophic mutants: VKM Y-1302, *lys1* and *trp2*; VKM Y-1296, *lys1* and *arg1*; VKM Y-1535, *ade1* and *lys7*; CBS 5670, *cys26* and *ade23*; ATCC 52486, *aux1* and *aux2*. Each of the remaining strains was represented by one *ura* mutant selected on medium with 5-FOA.

MATERIALS AND METHODS

Genetic analysis. The yeasts studied are listed in Table 1 (see also the notes to Table 2). The following heterothallic genetic lines of the yeast *Kl. lactis* var. *lactis* were used for complementation analysis: (1) JD109 (*MAT*α, *lac12-230*, *his2-2*, Ura$^-$) [13]; (2) 11D304 (*MAT*α, *lac12-230*, *his2-2*, Ura$^+$) [13]; (3) JA6/DL4 (*MAT*α, *ade1-600*, *ade2-T*, *ura3-11*, *trp1-11*, *lac4*::*URA3*) from K. Breunig; and (4) RUL 1888/DL4R (*MAT***a**, *ura3*, *lac4*::*ura3*), also from Breunig. In addition, the recombination experiments used I.A. Herman's (United States) auxotrophic lactose-fermenting mutants wm37 (*MAT***a**, *his*, *LAC4*, *LAC12*) and wm27 (*MAT*α, *lys*), derived from strains NRRL Y-1140 and NRRL Y-1118, respectively. The yeasts were cultivated on complete YPD medium of the following composition (g/l): European BactoAgar (Pronadisa, Spain), 20; BactoPeptone (Oxoid, UK), 20; yeast extract (Pronadisa), 10; glucose (Panreac, Spain), 20. Mass hybridizations of the haploid *Kluyveromyces* cells marked with complementary selective markers (auxotrophy and inability to utilize lactose) were performed on starvation medium of the following composition (g/l): maltose (Sigma, USA), 30; BactoAgar, 20. For hybridization, 24-h streaks of the strains hybridized were reprinted with velvet crosswise from complete medium to the maltose medium. After 24 h, the yeasts were again transferred with velvet, this time to minimal selective medium containing (g/l) yeast nitrogen base (Difco, USA), 6.7; BactoAgar (Difco), 20; glucose (Fluka, Switzerland), 20, or lactose (Fluka), 10. Glucose was used when both parents carried auxotrophic markers. Lactose was used when one parent utilized lactose but was an auxotroph, and the second parent did not utilize lactose but was a prototroph. In the prototrophic diploid Lac$^-$ × Lac$^-$ hybrids (Table 1) grown at the streak crossing, the capacity for lactose fermentation in liquid YPL medium was tested (in Durham tubes) as soon as after 24 h (to forestall sporulation). The yeast Lac$^+$ yeasts ferment lactose in 24 h, whereas Lac$^-$ yeasts do not ferment lactose over seven days. The composition of the fermentation YPL medium was the same as that of YPD medium, except that no agar was added to YPL and lactose was used instead of glucose. In all of the media, the yeasts were cultivated at 28°C. The Lac$^+$ × Lac$^-$ hybrids (Table 2) were cloned from single cells on complete YPD medium using a micromanipulator; this was done as soon as after 24–48 h, in order to forestall sporulation. The yeasts from 24- to 48-h colonies were additionally grown for 24 h in complete medium and then transferred to sporulation medium, which contained 3% maltose. Spores were isolated from the asci with a micromanipulator; the ascus walls were preliminarily destroyed with the enzyme preparation obtained from the stomach of the edible snail *Helix pomatia.* The fermentation of lactose

Table 2. Tetrad analysis by the capacity for lactose fermentation of the hybrids of *Kl. lactis* var. *lactis* (NRRL Y-1118, NRRL Y-1140) with the "krassilnikovii" (VKM Y-831, VKM Y-834, Est86, Vor86, CBS 2877, CBS 2896, CECT 1122) and "vanudenii" (VKM Y-1535) populations of *Kl. lactis* var. *drosophilarum*

Origin of the *lac4lac12/LAC4Lac12* hybrids[a]	Tetrad number[b]		
	P	N	T
831 × 1118	21	0	0
834 × 1140	20	0	0
834 × 1118	20	0	0
1535 × 1140	11	0	0
Est86 × 1118	18	0	0
Vor86 × 1118	22	0	0
2877 × 1118	20	0	0
2896 × 1140	23	0	0
1122 × 1118	16	0	0
Control hybridizations [13]			
lac12-230 × *lac4-8*	86	0	3
lac12-230 × *lac4-14*	65	0	0
lac12-230 × *lac4-23*	18	0	2
lac12-230 × *lac4-30*	19	0	0

[a] The origin and identification of strains NRRL Y-1118 = CBS 6513, NRRL Y-1140 = CBS 2359, VKM Y-834 = CBS 9056, Est86 = CBS 9057, CBS 2896, and CECT 1122=CBS 9059 were described in [5, 6, 8]. The experiments used the auxotrophic mutants wm27 and wm37 of strains NRRL Y-1118 and NRRL Y-1140, respectively.
[b] P and N denote the parent and nonparent ditypes; T is the tetrad tetratype.

(2%) by hybrid segregants was determined after 24 h in agarized EMS pH-indicator medium with eosin and methylene blue [14].

Polymerase chain reaction was performed using a Techne Progene DNA amplifier. The primers MR66 (5'-ATGCTTTTGCAAGCTTTC-3'), MR67 (5'-GGTCATGTTCACAGATCC-3') and AC18 (5'-CTTGAGCTCAAAATGGCAGATCATTCGAGC-3'), AC19 (5'-CGGTCTAGAATGGCTTTAAACAGATTCTGC-3') were used for *LAC4* and *LAC12* amplification, respectively [15, 16]. The PCR was performed in 30 μl of $(NH_4)_2SO_4$ buffer containing 2.5 mM $MgCl_2$, dNTPs (0.1 mM each), 50 pmol of each primer, 2.5 units of *Taq* DNA polymerase (Syntol, Russia), and 20–200 ng of DNA. The initial denaturation was carried out at 94°C for 5 min, followed by 36 cycles, each including denaturation at 94°C for 60 s, primer annealing at 52°C for 60 s, and DNA synthesis at 72°C for 120 s; final elongation was allowed to occur at 72°C for 10 min.

Pulsed-field electrophoresis. Chromosomal DNA isolation was described earlier [17]. A CHEF-DRTM III apparatus (Bio-Rad, USA) was used for the separation of chromosomal DNA. Pulsed-field electrophoresis was performed at 200 V in the following mode: (1) at 175 V for 8 h, with the field switching time of 40–120 s; (2) at 130 V for 24 h, with the field switching time of 120–360 s; and(3) at 100 V for 8 h, with the field switching time of 360–1200 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. The chromosomal DNA of the strains *S. cerevisiae* YNM 295 and *Pichia canadensis* (synonym, *Hansenula wingei*) YB-4662-VIA (Bio-Rad, USA) served as the karyotypic standards. After electrophoresis, the gel was stained with ethidium bromide, washed in distilled water, and photographed in ultraviolet light.

Southern hybridization. Chromosomal DNA were transferred onto a nylon membrane using a Pharmacia vacuum apparatus (Sweden). The DNA was fixed on the membrane by UV rays. PCR-amplified fragments of *LAC4* and *LAC12* genes were used as probes. The introduction of the nonradioactive label digoxigenin (DIG-II-dUTP), hybridization, and signal detection were performed according to the Roche Applied Science (Germany) instructions.

RESULTS AND DISCUSSION

Complementation analysis. The ability of the haploid yeasts of the genus *Kluyveromyces* to form both intraspecies and interspecies hybrids allowed complementation analysis to be used to determine the genotypes of natural homothallic Lac⁻ strains. Heterothallic genetic lines of the dairy yeast *Kl. lactis* var. *lactis* that had mutant *lac4* or *lac12* alleles served as testers. In order to avoid accidental errors in the complementation tests, the prototrophic hybrids of auxotrophic strains were selected in minimal selective glucose medium. In the case of using the type cultures, each parent was represented by two different auxotrophic mutants, including the genetic testers. The hybridization results are shown in Table 1. We did not succeed only in obtaining hybrids of strain VKM Y-1535 auxotrophs with the α-type-mating testers and hybrids of 71-45-6C yeasts with the testers JD 109 and RUL 1888/DL4. In the first case, the parents had the same mating type (the haploid cells of strain VKM Y-1535, despite their homothallism, have the α-type of mating [18]). In the second case, the hybrids did not grow on the minimal medium, obviously, due to the fact that they were homozygous with respect to *ura3* auxotrophy. All the 63 hybrids obtained with the involvement of natural Lac⁻ strains of *Kl. lactis* var. *drosophilarum* were incapable of fermenting lactose; i.e., there was no complementation with either the *lac4* or the *lac12* testers. This means that the Lac⁻ strains lacked both the active *LAC4* gene and the active *LAC12* gene. All four Lac⁻ hybrids of the species *Kl. marxianus* with the *lac4* testers were able to ferment lactose, as distinct from the four hybrids with the *lac12* testers. This indicates that the two natural *Kl. marxianus* strains analyzed are mutants by the per-

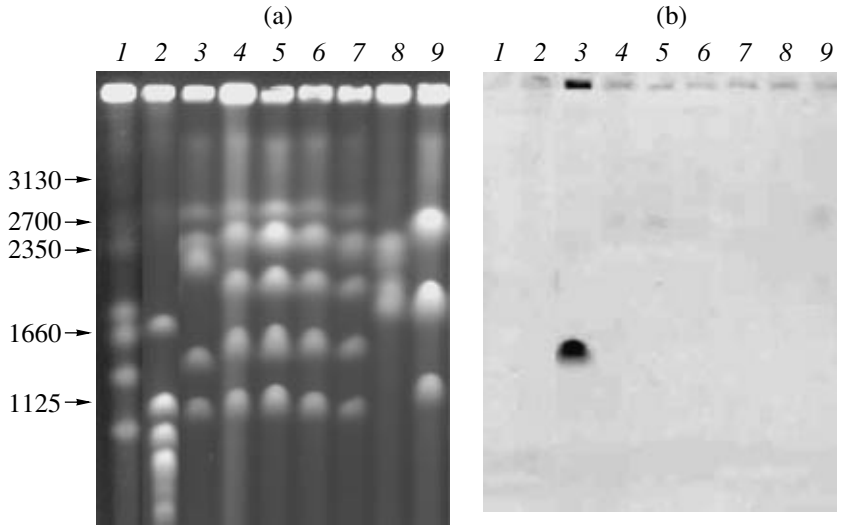

Fig. 1. (a) Pulsed-field electrophoresis and (b) Southern hybridization with the *LAC4* probe of the chromosomal DNA of (*1*) *P. canadensis* YB-4662-VIA (chromosomal standard); (*2*) *S. cerevisiae* YNN 295 (chromosomal standard); (*3*) *Kl. lactis* var. *lactis* wm37; and *Kl. lactis* var. *drosophilarum* strains (*4*) VKM Y-831, (*5*) CBS 2877, (*6*) Vor86, (*7*) Est86 (all from the "krassilnikovii" population), (*8*) VKM Y-1302 (the "drosophilarum" population), and (*9*) VKM Y-1296 (the "phaseolosporus" population).

mease gene, an analogue of *lac12*, but they have an active β-galactosidase gene, an analogue of *LAC4*.

Genetic hybridization analysis. The absence of linked *lac4* and *lac12* genes in natural Lac⁻ strains of the yeast *Kl. lactis* var. *drosophilarum* was confirmed by the monogenic segregation with respect to the lactose fermentation capacity in hybridizations with genetic Lac⁺ lines (Table 2). We analyzed a total of nine *lac4lac12/LAC4LAC12* hybrids and found a 171P : 0N : 0T tetrad cleavage pattern. For comparison, the results of analysis of four similar (*LAC4lac12/lac4LAC12*) hybrids reported in [13] were 188P : 0N : 5T (Table 2). It should be pointed out that, between the *LAC4* and *LAC12* genes, there is a 2.6-kb regulatory sequence [13, 19]. Therefore, in the experiments carried out by Riley et al. [13], the corresponding recombination occurred, although with a low frequency. In our case, *LAC4* and *LAC12* did not recombine. This leads us to conclude that natural Lac⁻ strains of the yeast *Kl. lactis* var. *drosophilarum* do not contain nonfunctional *lac4* and *lac12* genes.

Thus, our study showed that the dairy yeasts *Kl. lactis* var. *lactis* differ from their closest wild relatives *Kl. lactis* var. *drosophilarum*, and in particular, from their immediate progenitor, the European "krassilnikovii" population [5, 6, 8], by the presence of the lactose *LAC4* (β-galactosidase) and *LAC12* (lactose permease) genes.

Molecular analysis. We obtained the additional proof of the absence of silent $lac4^0$ and $lac12^0$ sequences from natural Lac⁻ strains of *Kl. lactis* var. *drosophilarum* by using the PCR-analysis and Southern hybridization with *LAC4* and *LAC12* probes.

Thirteen *Kl. lactis* var. *drosophilarum* strains (those listed in Table 1 and strain Est86) unable to ferment lactose revealed the absence of amplification both with the primers MR66/MR67 and the primers AC18/AC19. The *LAC4* and *LAC12* fragments were amplified only in the control strain *Kl. lactis* var. *lactis* wm37 (data not shown). Figure 1 shows the molecular karyotypes of seven *Kl. lactis* strains and Southern hybridization with the *LAC4* probe. The hybridization signal was revealed only in strain wm37 (lane *3*), whereas in six phenotype Lac⁻ strains, including the strains of the "krassilnikovii," "drosophilarum," and "phaseolosporus" populations, the hybridization signals were completely absent (lanes *4–9*). The same pattern was observed when *LAC12* was used as the probe (data not shown). This probe hybridized only with the DNA of *Kl. lactis* var. *lactis* wm37. Thus, in *Kl. lactis* var. *drosophilarum*, strains incapable of fermenting lactose, even the sequences of the corresponding genes are absent.

Obviously, the domestication of the yeast *Kl. lactis* var. *lactis*, inhabiting dairy products, occurred on the basis of *LAC4* and *LAC12* gene acquisition. If the progenitor (of the "krassilnikovii" population), which produces highly fertile hybrids with *Kl. lactis* var. *lactis* [5, 8], has no such genes, then where were these genes transferred from? The already-known data can give a definite answer to this question. According to K. Huo (see accession number Y09281 in GenBank), strain *Kl. marxianus* CBS 397 (synonym, *Kl. fragilis*) has a β-galactosidase that is highly homologous to *LAC4* β-galactosidase of the yeast *Kl. lactis* var. *lactis.* This fact leads us to believe that at least the *LAC4* gene originates from *Kl. marxianus.* The natural (not dairy) strains of this species are, as a rule, able to utilize lactose, as distinct from *Kl. lactis.* Both species occur in

dairy products. Most probably, the yeast *Kl. marxianus* itself acquired β-galactosidase as a result of horizontal transfer of the corresponding gene from bacteria [20].

At due time, the lactose operon was of great importance in the establishment of bacterial genetics; the role that the yeast *LAC4LAC12* gene cluster may play in evolutionary genetics is by no means less important and intriguing.

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