ISSN 0026-2617, Microbiology, 2014, Vol. 83, No. 3, pp. 195–204. © Pleiades Publishing, Ltd., 2014.
Original Russian Text © N.G. Loiko, M.A. Krasnova, T.V. Pichugina, A.I. Grinevich, V.I. Ganina, A.N. Kozlova, Yu.A. Nikolaev, V.F. Gal'chenko, G.I. El'-Registan, 2014, published in Mikrobiologiya, 2014, Vol. 83, No. 3, pp. 284–294.

EXPERIMENTAL ARTICLES

Changes in the Phase Variant Spectra in the Populations of Lactic Acid Bacteria under Antibiotic Treatment

N. G. Loiko[a, 1], M. A. Krasnova[b], T. V. Pichugina[b], A. I. Grinevich[b], V. I. Ganina[b], A. N. Kozlova[a], Yu. A. Nikolaev[a], V. F. Gal'chenko[a], and G. I. El'-Registan[a]

[a] *Winogradsky Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117312 Russia*

[b] *Moscow State University of Food Production, Moscow, Russia*

Received October 10, 2013

Abstract—Effect of the antibiotics kanamycin and ampicillin on the growth and phase variation of the populations of four strains of lactic acid bacteria (*Lactobacillus* sp. M76AT, *L. casei* MB, *Enterococcus faecium* M, and *E. faecium* M3185) was studied. The presence of antibiotics in the medium resulted in a dose-dependent decrease in viable cell numbers and in partial or complete substitution of the dominant S variant with the minor Sm and Sb variants. The variants differed in colony morphology, as well as in some physiological, biochemical, biotechnological, and probiotic characteristics. The Sm type variants of all strains exhibited the highest resistance to antibiotics. High production of exopolysaccharides was found in Sb variants of lactobacilli and in S variants of enterococci. The highest antibacterial activity was found in Sm variants of lactobacilli, especially in *Lactobacillus* sp. M76AT. The latter is biotechnologically the most promising strain, since all variants fermented milk yielding the products with uniformly pronounced functional and organoleptic properties. These patterns are of importance for the understanding of the mechanisms of antibiotic resistance and for selection of the variants with desired properties, as well as for quality control of the lactic acid bacteria starter cultures.

Keywords: lactic acid bacteria, *Lactobacillus, Enterococcus*, antibiotics, kanamycin, ampicillin, intrapopulation variability, phase variants

DOI: 10.1134/S0026261714030114

Survival and development of an organism depends on its adaptation to a constantly changing environment. In microorganisms, potency for adaptation is provided by their intrapopulation variability, which is the result of reversible intragenomic rearrangements and is observed as phase variation and replacement of the dominant genotype with the minor ones [1–3]. This mechanism of adaptation, by means of alteration of the population phase variation spectrum, providing for survival of a species under unfavorable growth conditions and selective development of its most adapted variant, was discovered and studied by us in a number of microorganisms [4–7], including lactic acid bacteria (LAB) [7]. Importance of the studies on population variability of LABs is motivated by their wide application as probiotics in the food industry and animal farming [8, 9]. Stable demand for high-quality fermented milk products heightens the requirements for starter cultures of LABs [10, 11]. They should possess a number of useful biotechnological characteristics, and also maintain viability for long periods, exhibit resistance to stress conditions, and rapidly adapt to the environment of the gastrointestinal tract of humans and animals [12, 13]. However, due to wide application of antibiotics in animal farming, they may be present in raw milk, which invariably leads to a decrease in quality and safety of dairy products for humans and animals [14]. Moreover, unjustified application of antibiotics in therapy is dangerous, because it affects unfavorably the lactic acid bacterial population in the human gastrointestinal tract and causes dysbiosis [15]. These reasons motivated stricter requirements for antibiotic resistance (AR) as one of the selection criterion of industrial LAB strains [16]. Application of antibiotic-resistant LAB strains, while promoting stabilization of normal intestinal flora upon antibiotics therapy, may lead, however, to transfer of the antibiotic resistance genes from lactic acid bacteria to pathogenic microorganisms and to rapid spread of drug resistance among the clinical strains, which, in turn, may increase the inefficiency of antibiotics application [17–19]. Therefore, elucidation of basic patterns of LAB adaptive resistance to stress effects, including resistance due to emergence of antibiotic-resistant variants, is needed.

The goal of the present work was to study the changes in LAB population spectrum under effect of antibiotics, to isolate the variants, and to compare

[1] Corresponding author; e-mail: loikonat@mail.ru

their major biotechnological and probiotic characteristics.

MATERIALS AND METHODS

Subjects of the study were four strains of lactic acid bacteria (LAB): *Lactobacillus* sp. M76AT, *L. casei* MB, *Enterococcus faecium* M, and *E. faecium* M3185 from the culture collection of cultures of the Department of Sugar Technology, Fermenting Manufacturing, and Wine Production (Moscow State University of Food Production, Russia).

Bacteria were grown on glucose–yeast medium (GYM) of the following composition (g/L): yeast autolysate, 20.0; glucose, 20.0; pH 7.0. Cultivation was carried out in 20-mL tubes containing 10 mL medium under static conditions (without stirring) at 28°C during 24–120 h. Early stationary growth phase cultures were used as inocula at the initial optical density of 0.1 ($\lambda = 540$ nm, $l = 10$ mm; Specord UV Vis, Carl Zeiss Jena, Germany).

Cell viability was determined by the number of colony-forming units (CFU) upon plating the relevant dilutions of cell suspensions onto agarized GYM (1.5% agar-agar).

Antibiotic resistance was determined by the number of CFU upon plating of the early stationary phase cultures (24 h) onto the agarized glucose–yeast media with kanamycin (10, 50, 100, and 150 μg/mL) or ampicillin (0.5, 1, 2, 2.5, and 3 μg/mL), if compared with growth on the same medium without antibiotics. In the same experiments, population phase variation index of antibiotic-resistant LABs was determined as the percent of colonies of certain morphology to the total number of colonies. The AR clones of certain morphotypes were isolated.

Stability of the isolated antibiotic-resistant colony-morphological variants was determined as their ability to retain the characteristic morphotype after 5 sequential transfers of the variants onto GYM agar with antibiotics.

Microscopic observations were performed using a Reichert (Austria) phase-contrast microscope.

Determination of antagonistic activity was performed by the method of development of mixed cultures of LABs and the *Staphylococcus aureus* 209-P reference strain by comparing their growth with that of the reference strain growth in monoculture [7].

Synthesis of exopolysaccharides (EPS) by LABs was evaluated qualitatively, by coloration of the colonies obtained on agarized GYM with ruthenium red dye (80 mg/L). Intense red color evidenced cell inability to produced EPS, and white coloration indicated synthesis of large amounts of EPS [20].

To determine the functional activity of LABs (time required to ferment milk), 3 mL of 16-h culture of a variant under study were added to 100 mL sterile (autoclaved for 7 min at 0.5 atm) milk (10%, low fat), thoroughly mixed, and incubated at 28°C to curd formation. Time of curd formation (h) was considered a measure of fermentation activity.

Moisture retaining (syneresis) capacity of the curd was determined by centrifugation. Six milliliters of an LAB culture were added to 200 mL sterile milk and incubated at 37°C until a dense curd was formed; it was then cooled to 5°C. After 24 h, 10 mL of the curd heated to 20°C were stirred to form a homogeneous pulp and centrifuged for 5 min at 3000 rpm. Moisture retaining capacity was expressed as the volume of the whey (mL) isolated from 10 mL curd [21].

Determination of the energy and threshold of acid formation. A tube with 10 mL sterile fat-free milk was inoculated with one loop of a strain under study and incubated at optimal temperature (37°C) for 24 h (to determine acid formation energy) and 7 days (to determine acid formation threshold), then acidity was determined by titration [21].

Acidity titration. Measurements were performed according to the State Standard R 54669-2011. For this purpose, 20 mL distilled water and two drops of 1% phenolphthalein were added to the sample (10 mL). Then, the sample was titrated with 0.1 N NaOH until pale-pink coloration appeared. The volume (mL) of NaOH used for titration was multiplied by 10 and used as an indicator of acidity expressed in Turner's degrees (°T) [21].

Organoleptic characteristics (color, texture, flavor, and taste) were determined according to the standard sensor testing techniques [21].

Statistical analysis was performed using the standard mathematical approaches (Student's *t*-test and standard deviation) implemented in the Microsoft Excel XP software package. A data group was considered homogeneous if the standard deviation σ did not exceed 10%. The differences between data groups were considered reliable at $P > 0.95$.

RESULTS

Effect of antibiotics on growth and phenotypic heterogeneity of LAB populations. To study the effect of antibiotics on growth and heterogeneity of LAB populations, cells of four strains, *Lactobacillus* sp. M76AT, *L. casei* MB, *Enterococcus faecium* M, and *E. faecium* M3185, grown in rich glucose–yeast medium to the stationary phase (28 h), were plated onto agarized media with antibiotics. The inocula were incubated for 10 days at 28°C. After 10-day incubation, the colonies were counted and colony morphological types recorded (Table 1). Heterogeneity among antibiotic-resistant LAB populations was thus assayed using the morphological characteristics of the colonies. Two broad-spectrum antibiotics often used in medical applications (kanamycin and ampicillin), differing by chemical structure and mechanism of action, were used in the work. Kanamycin is an aminoglycoside which binds to specific protein receptors on the 30S

Table 1. Viability and phase variation spectrum of AR clones in populations of LAB grown on antibiotic-containing medium

LAB	Antibiotic in the medium*		CFU/mL (% to CFU value in the absence of antibiotics)	Phase variation index, %		
	Variant	Concentration, μg/mL		S	Sm	Sb
Lactobacillus sp. M76AT	Without antibiotic	0	4.5×10^8 (100)	100	0	0
	Kanamycin	10	4.2×10^8 (93.3)	97.4	2.6	0
		50	3.6×10^5 (0.08)	69.8	30.2	0
		100	1.5×10^5 (0.03)	22.6	69.5	7.9
	Ampicillin	0.5	3.8×10^8 (84.4)	98.5	1.5	0
		1.0	2.1×10^8 (46.7)	86.9	13.1	0
		2.0	9.3×10^7 (20.7)	65.5	34.5	0
		2.5	1.2×10^3 (2.7×10^{-4})	0	100	0
L. casei MB	Without antibiotic	0	4.2×10^8 (100)	100	0	0
	Kanamycin	100	2.2×10^5 (0.05)	12.3	82.5	5.2
	Ampicillin	2.0	9.1×10^7 (21.7)	64.7	35.3	0
Enterococcus faecium M	Without antibiotic	0	5.6×10^8 (100)	100	0	0
	Kanamycin	10	5.4×10^8 (96.4)	98.7	1.3	0
		50	5.3×10^5 (0.09)	58.6	41.4	0
		100	2.6×10^5 (0.05)	14.7	79.7	5.6
	Ampicillin	0.5	5.3×10^8 (94.6)	99.5	0.5	0
		1.0	4.5×10^8 (80.3)	75.5	24.5	0
		2.0	1.3×10^8 (23.2)	69.7	30.3	0
		2.5	2.6×10^3 (4.6×10^{-4})	0	100	0
E. faecium M3185	Without antibiotic	0	6.5×10^8 (100)	100	0	0
	Kanamycin	100	9.0×10^6 (1.4)	62.4	31.8	5.8
	Ampicillin	2.0	1.9×10^7 (29.2)	78.5	21.5	0

* No growth was observed (CFU = 0) at concentrations of kanamycin of ≥150 μg/mL and ampicillin, ≥3.0 μg/mL.

ribosome subunit and prevents formation of the complex between tRNA and mRNA, thus inhibiting protein synthesis [22]. Ampicillin is a derivative of penicillin inhibiting transpeptidase activity and thus synthesis of peptidoglycan, preventing the formation of peptide cross-links in the process of cell wall synthesis in bacteria [22].

With increasing concentrations of antibiotics in the agar medium, the number of colony-forming cells of LABs decreased (Table 1). No significant difference in resistance to the antibiotics tested was observed between lactobacilli and enterococci.

Under experimental conditions, three types of variants differing by colony morphology were found for all strains studied: S, Sm, and Sb (Fig. 1). The S variant was the dominant one, with round, convex, white, shiny colonies approximately 1 mm in diameter in *Lactobacillus* sp. M76AT and *L. casei* MB and 1.5 mm, in *E. faecium* M and *E. faecium* M3185 (colony size was measured after 10 days of growth). The Sm variant was characterized by round, convex, semitransparent, shiny colonies less than 1 mm in diameter. The Sb type formed white, flat, opaque colonies with uneven edge, over 1.5 mm in diameter.

Antibiotics exhibited a dose-dependent effect on the phase variation spectrum of LAB populations. On the antibiotic-free medium, all strains grew in the form of S type (the dominant one). Colonies of the minor subtypes Sm and Sb were detected in the phase variation spectrum upon introduction of kanamycin or ampicillin into the medium (Fig. 1, Table 1). In all strains, the Sb-type variant was revealed only on the medium with high kanamycin content (100 μg/mL) and its share did not exceed 7.9% (Table 1). The share

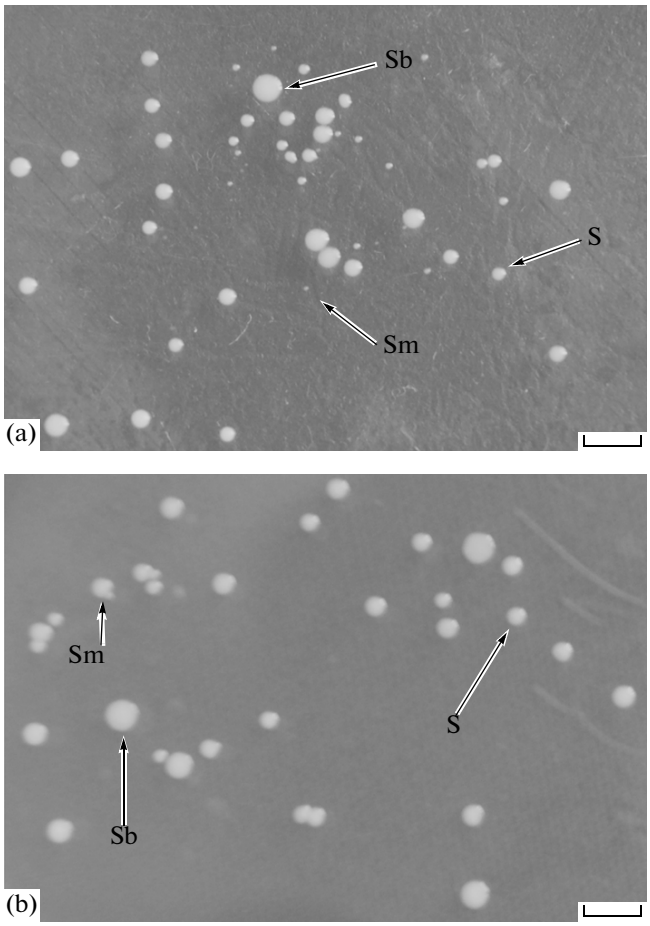

Fig. 1. Images of the colonies of the S, Sb, and Sm variants grown on GYM with 100 μg/mL kanamycin: *Lactobacillus* sp. M76AT (a) and *E. faecium* M (b). Scale bar, 5 mm.

of the colonies of the small Sm variant increased proportionally with increasing antibiotics content in the medium, and at ampicillin concentration of 2.5 μg/mL it completely replaced the dominant S variant in strains *Lactobacillus* sp. M76AT, *L. casei* MB, and *E. faecium* M. The strain *E. faecium* M3185 exhibited higher population stability than the other ones: only 37.6% cells developed the minor variants and 62.4% cells were of the dominating S-type on the medium with kanamycin concentration of 100 μg/mL, and 21.5 and 78.5%, respectively, were formed on the medium with 2 μg/mL ampicillin. Under these conditions, the amount of Sm variant colonies reached 82.5% in *L. casei* MB and 69.5%, in *Lactobacillus* sp. M76AT. This population stability of the strain *E. faecium* M3185 under stress conditions is an important biotechnological feature and also an important criterion for LAB strain selection for practical applications. On the other hand, high resistance of *E. faecium* M3185 to antibiotics is explained by the presence of AR determinants in its genome, which may be dangerous in case of transmittance [23].

Earlier, we demonstrated that the physiological age of bacterial populations (in submerged cultures) affects their phase variation spectrum [24]. Upon plating of LABs onto media with antibiotics, the effect of inoculum age on the phase variation spectrum of the populations derived was also noted (Table 2). With increasing age of the inoculum (slow growth phase, 19 h; stationary phase, 24 h; and post-stationary phase, 48 h), the numbers of cells yielding the Sm variant colonies decreased, although they remained predominant, and the number of the S and Sb type colonies increased.

It should be noted that short-term effect of subinhibitory concentrations of antibiotics had little effect on the phase variation spectrum of LAB populations: upon plating onto the medium without antibiotics of the inoculum (24 h) preincubated with kanamycin (100 μg/mL) or ampicillin (2 μg/mL) for 1 h, only segregation of the minor Sm variants (not more than 11%) was observed (Table 3). The share of the Sm variant was lower in enterococci, which turned out to be more stable to short-term stress effects than lactobacilli.

Thus, the presence of antibiotics in the medium affected not only the viability of LAB cells, but also the heterogeneity of AR clone populations in terms of colony morphology, with the dominant S variant partially or completely replaced with minor colony morphol-

Table 2. Effect of inoculum age on viability and phase variation spectrum of AR clones in populations of *Lactobacillu* sp. M76AT and *E. faecium* M grown on kanamycin-containing medium (100 μg/mL)

Inoculum age, h (growth phase)	*Lactobacillus* sp. M76AT				*E. faecium* M			
	CFU/mL (% to CFU value in the absence of antibiotics)	Phase variation index, %			CFU/mL (% to CFU value in the absence of antibiotics)	Phase variation index, %		
		S	Sm	Sb		S	Sm	Sb
19 (slow growth)	0.7×10^5 (0.02)	12.3	82.8	4.9	1.5×10^5 (0.04)	8.9	87.2	3.9
24 (stationary)	1.5×10^5 (0.03)	22.6	69.5	7.9	2.6×10^5 (0.05)	14.7	79.7	5.6
48 (post-stationary)	2.4×10^5 (0.05)	24.6	67.3	8.1	3.9×10^5 (0.07)	13.3	81.3	5.4

Table 3. Change in the number of cells and phase variation spectrum of LAB populations grown from the inoculum preincubated with antibiotics for 1 h

LAB	Control (without antibiotics), CFU/mL	Antibiotics effect					
		Kanamycin, 100 μg/mL			Ampicillin, 2 μg/mL		
		CFU/mL (% to CFU in the control)	Phase variation index, %		CFU/mL (% to CFU in the control)	Phase variation index, %	
			S	Sm		S	Sm
Lactobacillus sp. M76AT	4.5×10^8 (100)	1.2×10^8 (26.7)	90.5	9.5	1.3×10^8 (28.9)	88.9	11.1
L. casei MB	4.2×10^8 (100)	1.4×10^8 (33.3)	93.7	6.3	1.3×10^8 (31.0)	89.2	10.8
E. faecium M	5.6×10^8 (100)	1.4×10^8 (25.0)	90.8	6.7	1.6×10^8 (28.6)	93.7	6.3
E. faecium M3185	6.5×10^8 (100)	1.9×10^8 (29.2)	94.7	5.3	2.0×10^8 (30.8)	94.2	5.8

ogy variants. Differences between the variants by morphological, physiological, and biotechnological characteristics and their probiotic properties were studied in the next part of the work.

Properties of Colony Morphological Variants of LABs

Morphological properties of the variants. Microscopy revealed that the cells of the Sm variants were smaller (*Lactobacillus* sp. M76AT, (0.4–0.5) × (2.0–2.5) μm; *L. casei* MB, (0.6–0.7) × (1.0–1.5) μm; *E. faecium* M and *E. faecium* M3185, (0.8–0.9) μm in diameter) than the cells of the dominant phenotypes (*Lactobacillus* sp. M76AT, (0.5–0.7) (2.5–3.5) μm; *L. casei* MB, (0.7–0.9) × (1.5–2.0) μm); *E. faecium* M and *E. faecium* M3185 cocci (0.9–1.0) μm in diameter), which was probably to some degree the cause of the small size of the colonies that are formed by the Sm variants. Sizes of the Sb variant bacteria were comparable to those of the S type cells, and an increase in the diameter of colonies they formed was probably associated with the type of cell arrangement. However, decreased biosynthesis of density autoregulators or higher cell resistance to their effects are more probable reasons.

Stability of the variants was determined by repeated transfer of the clones (colonies of a certain morphotype) onto media with or without antibiotics. Upon inoculation onto media without antibiotics, Sm and Sb type variants of all four strains reverted to the dominant S type by 90% at the second or third transfer. After multiple transfers (over 5) onto media with antibiotics, the Sm variant retained its morphotype, and in the population grown upon plating of the Sb variant, splitting into S, Sb, and, mainly, Sm variants was observed in the first transfer. In the following transfer, S and Sb types gradually accumulated (Fig. 2).

Biotechnological properties. The isolated variants differed in their colony morphology characteristics, as well as in a number of other properties that are important for production of fermented milk products and drinks (Table 4). All variants of the strains under study grew better on milk than on GYM; the strain *E. faecium* M accumulated more cells than the others, and in each strain most cells represented the Sb and Sm variants. The dominant variants of all strains exhibited higher milk-fermenting activity. The *Lactobacillus* sp. M76AT and *E. faecium* M3185 were the leading ones in this respect. The dominant variant of the strain *E. faecium* M fermented milk within 16 h, while its minor Sm and Sb variants fermented it weakly, without curd formation. The energy and threshold of acid formation in milk by all minor variants were also much lower than in the dominant ones. On the other hand, moisture retaining ability of the curd in the dominant variant was lower than in the minor ones in all strains under study.

Organoleptic properties of the fermented milk products obtained with the variants were also considerably different (Fig. 3). The products obtained by fermenting of milk with the minor variants turned out to be inferior in their organoleptic properties to the drinks obtained with the dominant variants. Only in one of the four strains under study, that is *Lactobacillus* sp. M76AT, the differences were minimal; the drinks were of homogeneous texture and creamy white color and possessed a pleasant smell and pure fermented milk taste.

Probiotic features of the LAB variants. Antagonistic activity of LAB is an important functional feature affecting the medical and prophylactic properties of fermented milk products and probiotic preparations. The data shown on Fig. 4 demonstrate that all studied LAB variants exhibited antagonistic effect against the test organism (*Staphylococcus aureus* 209-P). Strain *Lactobacillus* sp. M76A, and especially its minor variants had the highest antagonistic activity.

Another important adaptive characteristic of LABs is their ability to synthesize exopolysaccharides (EPS), possessing the functions of adhesion factors and promoting better adsorption of LAB cells on colonocytes of intestinal epithelium for biofilm formation [25]. All

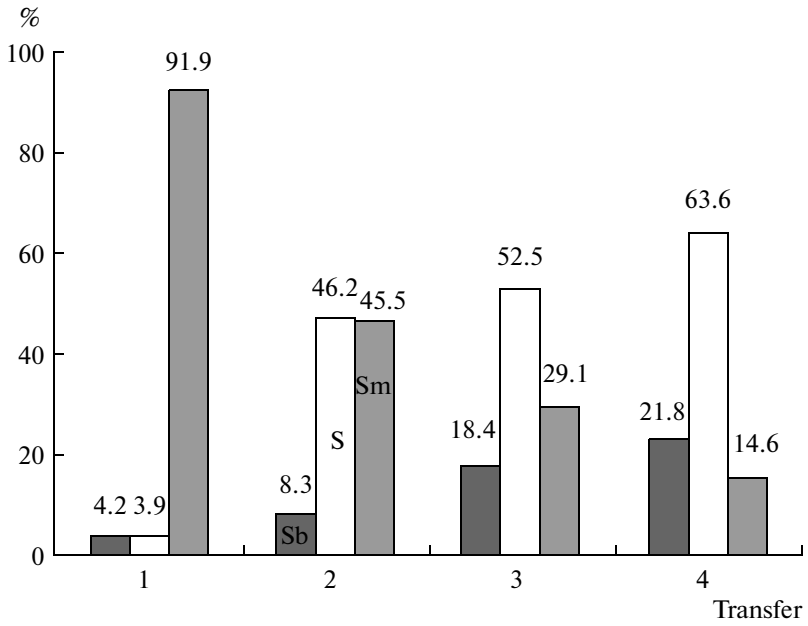

Fig. 2. Phase variation of the Sb variant of *E. faecium* M upon sequential transfers on antibiotic-containing media.

isolated colony morphology variants of the four LAB strains under study grew as heterogeneous populations in terms of EPS synthesis, which could be observed as different (from white to red) coloration of their colonies on ruthenium red supplemented medium (Table 5). Such heterogeneity in EPS production by the populations (colonies developed from a single cell) was noted previously for other LAB strains [11], where it was demonstrated that the level of heterogeneity depended on the strain and cultivation conditions. Among the strains under study, strains *Lactobacillus* sp. M76AT and *E. faecium* M3185 (all three variants) were the most productive in terms of EPS synthesis, with up to 90% of colonies producing EPS (Table 5). Sb and Sm variants of *E. faecium* M turned out to be less productive in EPS synthesis (Table 4).

DISCUSSION

Rapid spread of microbial resistance to antimicrobial preparations [26] and emergence of new pathogenic strains of the previously nonpathogenic bacteria

Table 4. Biotechnological properties of LAB variants

LAB		Cell titer, CFU/cm^3 (GYM)	Cell titer, CFU/cm^3 (milk)	Energy of acid formation (milk)	Acid formation threshold (in milk)	Curd moisture retaining ability, cm^3	Milk fermenting activity, h
				after 24 h, °T	after 7 days, °T		
Lactobacillus sp. M76AT	S	4.5×10^8	8.6×10^8	90	120	6.0	18
	Sm	4.2×10^8	8.4×10^8	77	88	5.0	21
	Sb	2.4×10^8	9.1×10^8	80	91	5.0	20
L. casei MB	S	4.2×10^8	7.5×10^8	80	110	6.0	23
	Sm	3.3×10^8	6.8×10^8	74	86	5.5	26
	Sb	4.4×10^8	9.4×10^8	68	85	5.2	24
E. faecium M	S	5.6×10^8	12.0×10^8	90	115	6.4	16
	Sm	6.7×10^8	17.9×10^8	68	81	No curd	–
	Sb	11.4×10^8	15.7×10^8	68	72	No curd	–
E. faecium M3185	S	6.5×10^8	6.4×10^8	100	120	6.0	18
	Sm	5.5×10^8	6.8×10^8	64	80	5.4	23
	Sb	3.2×10^8	6.8×10^8	62	74	5.0	22

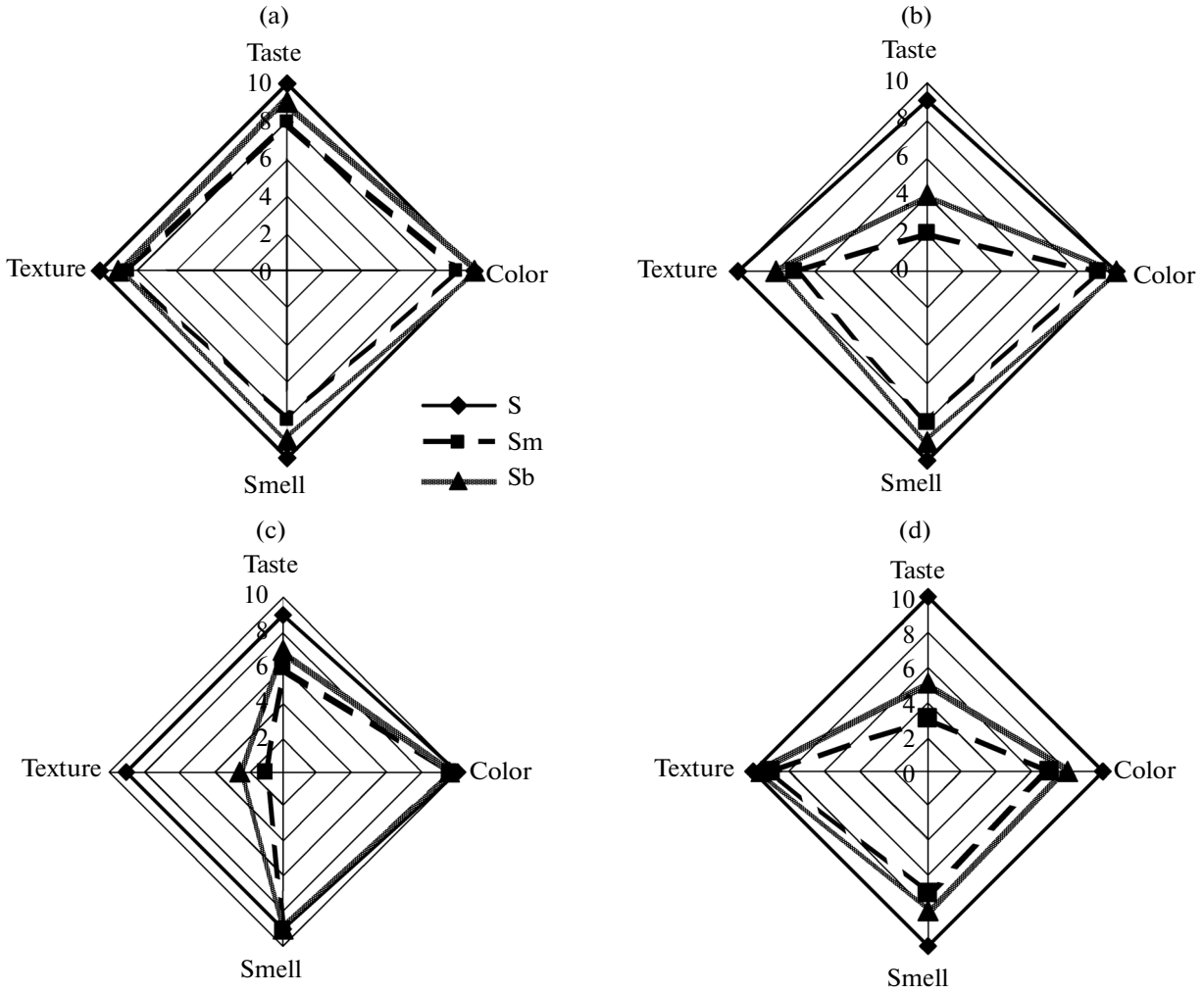

Fig. 3. Organoleptic properties of fermented milk products obtained using the variants of *Lactobacillus* sp. M76AT (a); *L. casei* MB (b); *E. faecium* M (c); and *E. faecium* M3185 (d), evaluated according to a 10-unit scale [21].

[19] has been a serious problem of the last few decades. This caused deeper studies on molecular genetic mechanisms of cell development, elucidation of AR determinants, and of the pathways for their horizontal transfer [18]. Treating microbial cultures as multicellular self-regulating organisms comprising phenotypically and genotypically different subpopulations [2, 27, 28] provides more possibilities for analysis of the reasons for antibiotics resistance and for development of new approaches to its prevention. It was established that in a heterogeneous population, different groups of resistant cells are formed: (a) cells escaping the cell cycle, quiescent in terms of proliferation, preadapted to stress, which ensures their survival in case of antibiotics attack (persister cells) [29]; (b) cells capable of attaining anabiosis, or a metabolically quiescent state, providing for their stress resistance and prolonged (up to hundred thousand and million years) survival under unfavorable growth conditions (cystlike quiescent and nonculturable cells) [30]; and (c) cells having obtained genes responsible for their resistance to antibiotics and developing into clones (variants) with an antibiotic-resistant genotype [2].

The studies reported in the present work demonstrated that the effect of antibiotics on LAB cells in growing cultures not only led to a decrease in their number (or even death), but also caused changes in phase variation spectrum of their populations with the development of variants with different colony morphology possessing an AR genotype in a dose-dependent manner; in other words, the subpopulation of AR cells is heterogeneous in terms of cell morphotypes. Heterogeneity of AR subpopulations by the level of cell resistance at which the share of the AR morphotype with the dominating colony morphology decreases and its partial or complete replacement with the minor phenotypes occurs should be noted. In case of both ampicillin (0.5–2.5 μg/mL), producing stronger growth inhibiting effect, which is in agreement with the literature data [31], and kanamycin

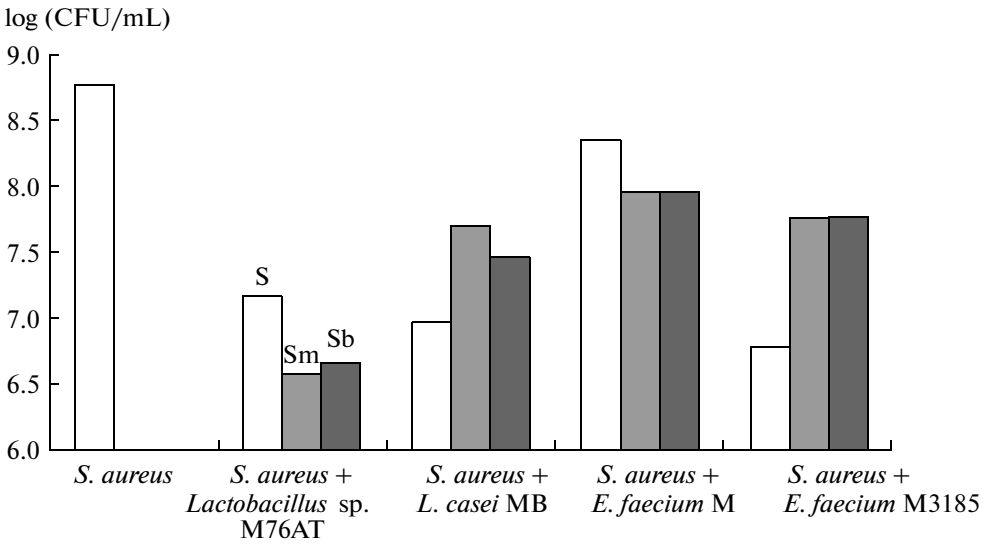

Fig. 4. Viability of the cells of the reference strain *Staphylococcus aureus* 209-P upon co-cultivation with the LAB variants.

(10–100 μg/mL), the antibiotics-resistant Sm variant characterized by small colony size (Fig. 1) and altered characteristics, compared to the dominant S type (Tables 4 and 5 and Fig. 4), was revealed in all strains. Since the Sm variant dominated in the populations growing at high concentrations of antibiotics in the medium, it may be assumed that it develops in the course of germination of persister cells, which does not contradict the literature data [32]. Investigation of the small-colony morphological variants in connection with bacterial AR and pathogenicity attracted much attention lately [32, 33]. Formation of rough small colony variants (RSCV) germinating as small R-type colonies and possessing increased resistance to a wide spectrum of antibiotics was detected upon plating of *Pseudomonas aeruginosa* onto solid medium with kanamycin [34]. In *Enterococcus faecium*, the Sm colony morphotype expressing atypical growth and forming colonies of decreased size was found, with the cells with the Sm genotype characterized by dysfunction of spontaneous autolysis, compared to the normal genotype [35].

In our work, apart from the Sm variant, a small fraction (up to 8%) of colonies of the Sb phenotype differing from the dominant one was discovered on the medium with high kanamycin content (100 mg/mL),

Table 5. Capacity for EPS synthesis in LAB phase variants

LAB		Number of colonies with different capacity for EPS synthesis, %		
		EPS+	EPS±*	EPS–
Lactobacillus sp. M76AT	S	45	43	12
	Sm	47	45	8
	Sb	55	36	9
L. casei MB	S	17	68	15
	Sm	14	75	11
	Sb	27	15	58
E. faecium M	S	32	43	25
	Sm	3	11	86
	Sb	5	12	83
E. faecium M3185	S	51	39	10
	Sm	67	25	8
	Sb	65	27	8

* Pink colonies with intermediate position between EPS-producing (white) and non-producing (red) colonies.

which is another confirmation of the heterogeneity of antibiotics-resistant subpopulations. In addition to the increased size of the colonies (compared to the dominant variant), this variant differed by a number of physiological and biotechnological characteristics. Both minor AR variants (Sm and Sb types) were inferior to the dominant S type by their biotechnological characteristics. Thus, replacement of the dominant variants of LAB with the minor ones will, in most cases, worsen the characteristics of fermented milk products, altering their organoleptic properties. Changing population spectra of LABs in the gastrointestinal tract of humans and animals upon the effect of antibiotics will support LAB species at the expense of highly resistant AR clones of Sm and Sb types, thus realizing the adaptive potential of bacterial populations resulting from their genotype heterogeneity. Therefore, directed production of intrapopulation AR variants of LABs with useful biotechnological and probiotic properties seems promising. The data on the effect of the physiological age of the cells on induction of AR resistance are also of interest (Table 2). It has been proven that genotypic variability increases upon aging of microbial cultures [2, 24] and is the most pronounced in anabiotic cystlike cells [4–7], germination of which yields a population with up to 60% of cells represented by minor genotypes. However, in the present work, with LAB age the number of AR clones of the dominant S type in AR subpopulations was shown to increase at the expense of the number of minor AR clones of the Sm type (Table 2). Since the share of AR cells at high concentration of kanamycin (100 μg/mL) did not exceed 0.07%, one may suppose that the colonies that developed under such conditions originated from persister cells of a multitolerant phenotype, the number of which increases by the stationary growth phase [29]. It is reasonable to suggest that double protection of a bacterial population from the effect of antibiotics—that is, formation of persister phenotype in a genotypically AR subpopulation—is possible. It should be noted that short-term incubation (1 h) of initial culture (inoculum) with antibiotics at lethal concentrations caused a decrease in the titer of viable cells (by 70–75%) and had little effect on the changes in its phase variation spectrum (segregation of the minor variants) (Table 3). Therefore, the antibiotic is a selection factor, rather than an inducer of phase variation transitions.

Another conclusion is related to the conjugation between the morphological features of the cells (colony morphology) and their AR properties (Fig. 2). Determination of stability of individual antibiotic-resistant colony morphology variants demonstrated that variants of the Sb type grew as Sm variants in the first transfer on the medium with antibiotics, but in further transfers reverted to the dominant S type (over 60%) and their own Sb type (up to 22%) with a decrease in the share of Sm variant (from 45 to 14.6%). Therefore, prolonged (several generations) development of LAB in the presence of antibiotics is not necessarily associated with cardinal replacement of the dominant genotype with minor ones; here, we observed the opposite process. Its mechanism is probably related to the functioning of factors of intercellular communication, which requires further studies.

Thus, the study demonstrated that heterogeneity of bacterial populations (by the example of LAB) is manifested at different levels: formation of AR cell populations with their further ranging by the level of resistance to antibiotics; intrapopulational colony morphology variability of AR cells; and variability of AR clones by their antimicrobial activity, probiotic properties, and physiological and biochemical characteristics.

ACKNOWLEDGMENTS

The work was supported by the Russian Foundation for Basic Research (projects nos. 13-04-01145 A and 13-04-40231-N).

REFERENCES

1. Woude, M.W., Phase variation: how to create and coordinate population diversity, *Curr. Opin. Microbiol.*, 2011, vol. 14, no. 2, pp. 205–211.
2. Magdanova, L.A. and Golyasnaya, N.V., Heterogeneity as an adaptive trait of microbial populations, *Microbiology* (Moscow), 2013, vol. 82, no. 1, pp. 1–10.
3. Zgur-bertok, D., Phenotypic heterogeneity in bacterial populations, *Acta Agric. Slovenica*, 2007, vol. 90, pp. 17–24.
4. Mulyukin, A.L., Kozlova, A.N., and El'-Registan, G.I., Properties of the phenotypic variants of *Pseudomonas aurantiaca* and *P. fluorescens, Microbiology* (Moscow), 2008, vol. 77, no. 6, pp. 681–690.
5. Pogorelova, A.Yu., Mulyukin, A.L., Antonyuk, L.P., Gal'chenko, V.F., and El'-Registan, G.I., Phenotypic variability in *Azospirillum brasilense* strains Sp7 and Sp245: association with dormancy and characteristics of the variants, *Microbiology* (Moscow), 2009, vol. 78, no. 5, pp. 559–568.
6. Kryazhevskikh, N.A., Demkina, E.V., Loiko, N.G., Baslerov, R.V., Kolganova, T.V., Soina, V.S., Manucharova, N.A., Gal'chenko, V.F., and El'-Registan, G.I., Comparison of the adaptive potential of the *Arthrobacter oxydans* and *Acinetobacter lwoffii* isolates from permafrost sedimentary rock and the analogous collection strains, *Microbiology* (Moscow), 2013, vol. 82, no. 1, pp. 29–42.
7. Golod, N.A., Loiko, N.G., Mulyukin, A.L., Neimatov, A.L., Vorob'eva, L.I., Suzina, N.E., Shanenko, E.F., Gal'chenko, V.F., and El'-Registan, G.I., Adaptation of lactic acid bacteria to unfavorable growth conditions, *Microbiology* (Moscow), 2009, vol. 78, no. 3, pp. 280–289.
8. Weichselbaum, E., Probiotics and health: a review of the evidence, *Nutr. Bull.*, 2009, vol. 34, pp. 340–373.

9. De Vrese, M. and Schrezenmeir, J., Probiotics, prebiotics, and synbiotics, *Adv. Biochem. Eng. Biotechnol.*, 2008, vol. 111, pp. 1–66.

10. Balakrishnan, M. and Floch, M.H., Prebiotics, probiotics and digestive health, *Curr. Opin. Clin. Nutr. Metab. Care*, 2012, vol. 15, no. 6, pp. 580–585.

11. Ganina, V.I., Anan'eva, N.V., Borisova, L.A., and Zharkova, E.Yu., Investigation of stability of the properties of lactic acid bacteria, *Molochnaya Promyshlennost'*, 2006, no. 10, pp. 39–40.

12. Górska, S., Jarzab, A., and Gamian, A., Probiotic bacteria in the human gastrointestinal tract as a factor stimulating the immune system, *Postepy Hig. Med. Dosw. (Online)*, 2009, vol. 63, pp. 653–667.

13. Lebeer, S., Vanderleyden, J., and De Keersmaecker, S., Adaptation factors of the probiotic *Lactobacillus rhamnosus* GG, *Beneficial Microbes*, 2010, vol. 1, no. 4, pp. 335–342.

14. Samanidou, V. and Nisyriou, S., Multi-residue methods for confirmatory determination of antibiotics in milk, *J. Sep. Sci.*, 2008, vol. 31, no. 11, pp. 2068–2090.

15. Quévrain, E. and Seksik, P., Intestinal microbiota: from antibiotic-associated diarrhea to inflammatory bowel diseases, *Presse Med.*, 2013, vol. 42, no. 1, pp. 45–51.

16. Zhou, N., Zhang, J.X., Fan, M.T., Wang, J., Guo, G., and Wei, X.Y., Antibiotic resistance of lactic acid bacteria isolated from Chinese yogurts, *J. Dairy Sci.*, 2012, vol. 95, no. 9, pp. 4775–4783.

17. Muñoz-Atienza, E., Gómez-Sala, B., Araújo, C., Campanero, C., del Campo, R., Hernández, P.E., Herranz, C., and Cintas, L.M., Antimicrobial activity, antibiotic susceptibility and virulence factors of lactic acid bacteria of aquatic origin intended for use as probiotics in aquaculture, *BMC Microbiol.*, 2013, vol. 13, p. 15.

18. Gillings, M.R. and Stokes, H.W., Are humans increasing bacterial evolvability?, *Trends Ecol. Evol.*, 2012, vol. 27, no. 6, pp. 346–352.

19. Millar, M., Constraining the use of antibiotics: applying Scanlon's contractualism, *J. Med. Ethics*, 2012, vol. 38, no. 8, pp. 465–469.

20. Figueroa, L.A. and Silverstein, J.A., Ruthenium red adsorption method for measurement of extracellular polysaccharides in sludge flocs, *Biotechnol. Bioeng.*, 1989, vol. 33, no. 8, pp. 941–947.

21. Ermolaeva, G.A. and Kolcheva, R.A., *Tekhnologiya proizvodstva piva i bezalkogol'nykh napitkov* (Technologies for Production of Beer and Nonalcoholic Beverages), Moscow: Akademiya, 2000.

22. Egorov, N.S., *Osnovy ucheniya ob antibiotikakh* (Basics of Antibiotic Science), Moscow: Nauka, 2004.

23. Gueimonde, M., Sánchez, B., de los Reyes-Gavilán, C., and Margolles, A., Antibiotic resistance in probiotic bacteria, *Front Microbiol.*, 2013, vol. 4, p. 202.

24. Doroshenko, E.V., Loiko, N.G., Il'inskaya, O.N., Kolpakov, A.N., Gornova, I.B., Klimanova, E.V., and El'-Registan, G.I., Characterization of *Bacillus cereus* dissociants, *Microbiology* (Moscow), 2001, vol. 70, no. 6, pp. 698–705.

25. Botina, S.G., Rozhkova, I.V., and Semenikhina, V.F., Use of exopolysaccharide-synthesizing lactic acid bacterial strains in production of cultured milk foods, *Khranenie i pererab. sel'khozsyr'ya,* 2010, no. 1, pp. 38–40.

26. Lew, W., Pai, M., Oxlade, O., Martin, D., and Menzies, D., Initial drug resistance and tuberculosis treatment outcomes: systematic review and meta-analysis, *Ann. Intern. Med.*, 2008, vol. 2, no. 149, pp. 123–134.

27. *Bacteria as Multicellular Organisms*, Shapiro, J.A. and Dworkin, M., Eds., New York: Oxford Univ. Press, 1997.

28. Stewart, P.S. and Franklin, M.J., Physiological heterogeneity in biofilms, *Nat. Rev. Microbiol.*, 2008, vol. 6, pp. 199–210.

29. Lewis, K., Persister cells, *Annu. Rev. Microbiol.*, 2010, vol. 64, pp. 357–372.

30. Bukharin, O.V., Gintsburg, A.L., Romanova, Yu.M., and El'-Registan, G.I., *Mekhanizmy vyzhivaniya bakterii* (Mechanisms of Bacterial Survival), Moscow: Meditsina, 2005.

31. D'Aimmo, M.R., Modesto, M., and Biavati, B., Antibiotic resistance of lactic acid bacteria and *Bifidobacterium* spp. isolated from dairy and pharmaceutical products, *Int. J. Food Microbiol.*, 2007, vol. 115, no. 1, pp. 35–42.

32. Proctor, R.A., von Eiff, C., Kahl, B.C., Becker, K., McNamara, P., Herrmann, M., and Peters, G., Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections, *Nat. Rev. Microbiol.*, 2006, vol. 4, pp. 295–305.

33. Bald, D. and Koul, A., Advances and strategies in discovery of new antibacterials for combating metabolically resting bacteria, *Drug Discov. Today*, 2013, vols. 5–6, pp. 250– 255.

34. Drenkard, E. and Ausubel, F.M., *Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation, *Nature*, 2002, vol. 416, pp. 740–743.

35. Gröbner, S., Beck, J., Schaller, M., Autenrieth, I.B., and Schulte, B., Characterization of an *Enterococcus faecium* small-colony variant isolated from blood culture, *Int. J. Med. Microbiol.,* 2012, vol. 302, no. 1, pp. 40–44.

Translated by N. Kuznetsova