ISSN 0096-3925, Moscow University Biological Sciences Bulletin, 2015, Vol. 70, No. 1, pp. 29–34. © Allerton Press, Inc., 2015.
Original Russian Text © M.A. Trenina, A.S. Yepremyan, L.G. Stoyanova, 2015, published in Vestnik Moskovskogo Universiteta. Biologiya, 2015, No. 1, pp. 31–36.

MICROBIOLOGY

Dependence of Growth Characteristics of Original Strains of *Lactococcus lactis* subsp. *lactis* on the Composition of Agar Media Used for Biomass Growth

M. A. Trenina[a], A. S. Yepremyan[b], and L. G. Stoyanova[c]

[a] *Ivanovsky Institute of Virology, ul. Gamalei 16, Moscow, 123098 Russia*

[b] *Scientific and Production Center "Armbiotechnology," National Academy of Sciences of Republic of Armenia, Yerevan, 0056 Republic of Armenia*

[c] *Department of Biology, Moscow State University, Moscow, 119991 Russia*

e-mail: stoyanovamsu@mail.ru

Received December 22, 2013

Abstract—Data on the dependence of growth characteristics of *Lactococcus lactis* subsp. *lactis* 729 and TB2 extracted from milk and homemade curd on the content of casein peptone (CP) as the main source of nitrogen in the agar medium is presented. The number of colony-forming units (CFUs), cell division rate (CDR), and mean hourly doubling time (HDT) were compared between two time segments of cultivation: from 1 to 2.5 h, and from 2.5 to 3.5 h. In liquid media, logarithmic growth was observed for the 729 strain in all groups for 3.5 h at minimal pH of 6.5. The maximum biomass production rate (2.5×10^8 cells/mL) was observed in the media containing the meat extract; the minimal HDT was in the biomass transferred from the minimal MA4 medium to the enriched M7 medium. In contrast with the TB2 strain, the 729 strain does not form colonies on agar media at CP concentrations below 0.4%. The colonies of the TB2 strain increase in size from 2.85 mm^2 to 5 mm^2 when decreasing CP concentration from 1 to 0.4%. The yeast extract has a stimulating effect in liquid media at said concentration ratios, leading to an increase in the efficiency of cell division at lower rates of lactic acid production, to pH of 5.5 when oligopeptide transport is blocked. The meat extract provides adaptation of bacteria to the conditions of excessive acidity and has positive effect on cell division in the 729 strain at later stages of bacterial colony development. The smaller size of colonies of the strain might be evidence of active acid formation.

Keywords: *Lactococcus lactis* subsp. *lactis,* nutrient media, growth characteristics.

DOI: 10.3103/S0096392515010095

INTRODUCTION

The basic trait of lactic acid bacteria is the capability of fermentation involving formation of lactic acid as the major end product. According to the kind of carbohydrate fermentation products, lactic acid bacteria are divided into homofermentative and heterofermentative. In homofermentation, up to 98% of carbohydrates are converted into lactic acid [1]. Dynamics of development of *Lactococcus lactis* subsp. *lactis* population and their genetic stability are markedly affected by the efficiency of lactic acid production in the medium [2–6]. Some phenotypic differences were observed between fast and slow milk-coagulating isolates of lactococci [7]. Mutations affecting either uptake of β-casein peptides or lactose fermentation were considered to be the reason for these phenotypic differences. Fast coagulating isolates exhibited notable rate of acid formation while slow coagulating isolates needed stimulating effect of the yeast extract to a greater extent. Comparative analysis of phenotype in the colonies of active acid-forming strains *Lactococcus lactis* subsp. *lactis* TB2 and 837 in the presence and absence of the yeast extract, performed by authors before, provided an opportunity to estimate colony size on the agar media with high CP concentration (1%). The average colony size for 837 strain in the presence of the yeast extract was 34% smaller as compared with TB2 strain and 74% smaller in the absence of the yeast extract; this suggests that the bacteria of the 837 strain are more dependent on the stimulating effect of the yeast extract under conditions of CP excess.

The ecological niche of *L. lactis* subsp. *lactis* is represented by plants and the capability of colonizing milk appears to be due to the macroevolutionary selection. The strain specificity of lactococci used in milk processing depends more on the technological environment of cultivation than on the geographical location of the farm. It is suggested that the plasmid DNA needed for the utilization of casein and lactose was received by lactococci from other bacteria, since its GC-content (30–40%) is fairly different from that of

Composition of the nutrient media used for cultivating *L. lactis* subsp. *lactis*

Components, %	Nutrient media							
	MA3	MA4	MA5	MA7	MA6	M4	M7	M6
Casein peptone (CP)	0.3	0.4	0.4	0.8	0.4	0.4	0.8	0.4
Yeast extract	–	–	0.2	0.4	0.4	–	0.4	0.4
Meat extract	–	–	–	–	0.4	–	–	0.4
Lactose	0.3	0.4	0.4	0.5	0.5	0.4	0.5	0.5
Agar	1.5	1.5	1.5	1.5	1.5	–	–	–
Na_6HPO_4	0.85	0.85	0.85	0.85	0.85	–	–	–
KH_2PO_4	0.2	0.2	0.2	0.2	0.2	–	–	–
Water	Rest							

the chromosomal DNA (36–38%) [10]. Controlled isolation of lactococci from the surface of plants and their identification showed that the "wild-type" strains of lactococci exhibit phenotypic traits of *L. lactis* subsp. *lactis*, while phenotypes of *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* bv. *diacetilactis* are typical of the strains obtained from the milk products [11].

The purpose of the work is to determine nutrition needs and time ranges of development in lactococci population in which undesirable lactic acid accumulation does not occur.

MATERIALS AND METHODS

Two original strains of *Lactococcus lactis* subsp. *lactis* were used in the work. The 729 strain is an active acid-former; it was extracted from cow's milk [12], and genotyped using 16S rRNA gene (GenBank EF-100778) [13]. The TB2 strain was obtained from homemade curd and suggested for production of a curd culture [8]; its genome was described by pulse-electrophoresis [4]. Composition of the media in which CP is the main source of nitrogen is presented in the table. The medium components—CP, yeast and meat extracts—used in the study were produced by "HiMedia" (India). The media were prepared in two steps. First, starvation salt agar and nutrient solutions with high concentration were prepared separately. Then, nutrient concentrates were diluted in melted agar with phosphate buffer in Petri dishes. Biomass cultivation on the agar media and washing with normal saline prior to the transfer to the liquid media were carried out according to the procedure described in [4, 15, 16]. The inoculation of diluted bacterial suspensions was carried out with a spatula into 1/4 of the agar medium surface in Petri dishes ($d = 10$ cm). For CFU counting, suspensions were inoculated into MA5 medium. In liquid media, the strains were cultivated in the volume of 5 mL at 30°C without aeration. pH of the media was measured by a pH-meter (Mettler Toledo MP220). The data were recorded in four independent observations and statistically processed.

RESULTS AND DISCUSSION

The investigation was preformed proceeding from the fact that the efficiency of cell division of *L. lactis* subsp. *lactis* on agar media that determines biomass production in colonies is a strain-specific characteristic. The rate and duration of cell division of lactococci in liquid media depend considerably on the size of the energy supply produced and conserved on the agar surface. Therefore, nutritional components were selected not only according to morphological and cultural characteristics of the strains but also considering transfer of bacterial population from agar media to liquid media.

Determination of the minimal CP and lactose concentrations sufficient for colony formation in *Lactococcus lactis* subsp. *lactis* 729 and TB2.

Comparative evaluation of the colony-forming ability of *L. lactis* subsp. *lactis* 729 and TB2 on MA3 and MA4 agar media (table).

The MA4 medium with peptone and lactose concentrations increased by 0.1% was used as a minimal medium for the 729 strain, since the colonies of this strain on MA3 medium are not well defined. The visible difference between colonies of 729 and TB2 strains is more after 24 h (Figs. 1a and 1b) than after 96 h of cultivation (Figs. 1c and 1d). Thus, growth rate advantage of TB2 strain is more prominent at early stages of development of bacterial population. The colony size of 729 strain (diameter ~0.5 mm, area ~0.2 mm^2) after 24 h of cultivation on MA4 medium (Fig. 1a) coincides with the colony size of the slow-coagulating isolates of lactococci (0.5 mm) cultivated at 21°C on GMA medium with glycerophosphate for 2–3 days [7]. The colony diameter of the fast-coagulating isolates on milk medium with glycerophosphate was 1–2.5 mm, which was similar to the colony diameters of TB2 strain cultivated on MA3, MA4, and MA5 media under said conditions (1.5–2.5 mm). On the media

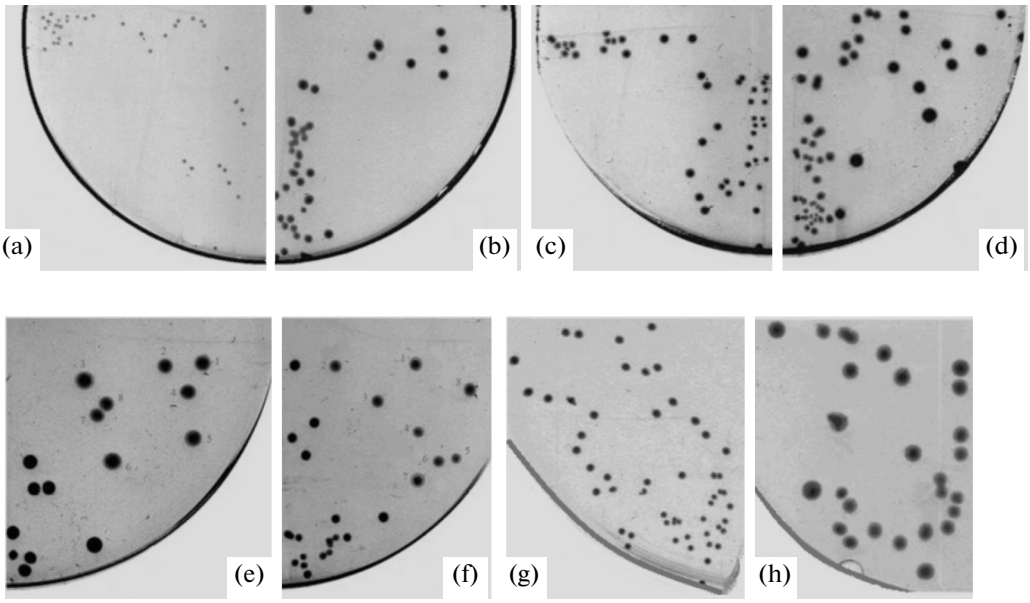

Fig. 1. External view of colonies of *Lactococcus lactis* subsp. *lactis* 729 and TB2 cultivated on CP-minimal agar media: MA3 (0.3% of CP), MA4 and MA5 (0.4% of CP). (a, b) MA4 medium, 729 strain (left, colony diameter is ~0.5 mm), and TB2 strain (right, colony diameter is ~1.5 mm), cultivated at 30°C for 24 h; (c, d) MA4 medium, 729 strain (left, colony diameter is ~1.5 mm), and TB2 strain (right, colony diameter is ~2.5 mm), cultivated at 30°C for 96 h; (e, f) TB2 strain on MA5 medium (left, colony diameter is 2–2.5 mm), and MA3 medium (right, colony diameter is ~1.5 mm), cultivated at 30°C for 48 h; (g, h) MA5 medium, 729 strain (left, colony diameter is ~1 mm), and TB2 strain (right, colony diameter is ~2.5 mm) cultivated at 30°C for 24 h, at room temperature for 48 h.

with decreased (0.4%) CP concentration (MA4 and MA5), an increase in colony size of TB2 strain up to 3–5 mm^2 was observed, as compared with the media with CP concentration of 1% (M02 and M03)—2.85–2.9 mm^2 [8]; this might be evidence that the nitrogen excess has an inhibiting effect on the colony growth. On the other hand, the colony size of this strain on MA3 medium (CP concentration is 0.3%) is lower (1.8 mm^2) than that on M02 and M03 media.

Quantitative estimation of the increase in the colony size of TB2 strain on an enriched medium was carried out using MA5 medium, i.e., after addition of the yeast extract (0.2%) to the minimal MA4 medium (0.4% of CP), and using MA3 medium with CP and lactose concentration of 0.3% (table). The average area of colonies on MA3 medium was 1953 pixels with standard deviation of 292 pixels; that on MA5 medium was 3752 pixels with standard deviation of 518 pixels (Figs. 1e and 1f); i.e., the average colony size increased by 1.9 times. Thus, the limitations of the colony size of TB2 strain cultivated on poor MA3 medium may be accounted for by depletion of the nutrient source.

The morphology of 729 strain colonies on MA4 and MA5 media (Figs. 1c and 1g) is an evidence that MA4 medium enrichment by the yeast extract does not lead to the increase in colony size. This suggests that CP is the main nitrogen source for growth.

The average HDT in *Lactococcus lactis* subsp. *lactis* 729 in liquid media M4, M7, and M6 in two sequential time segments with total duration of 3.5 h. The pairs of media with identical nutrient composition—MA4/M4, MA7/M7, MA6/M6—and a composite pair MA4/M7 were used in order to determine the optimal composition of an agar medium providing the most efficient cell division in nonbuffered liquid media (table). The data on CDR and HDT, pH, and CFU values are presented in Fig. 2 (2a, 2b, 2c, 2d, respectively). The HDT value for the first time segment with total duration of 90 min was estimated by conversion of CDR value for the segment duration of 60 min. Thus, when the number of CFUs in the first time segment in the pair MA4/M4 increased by approximately 1.9 times (Fig. 2a) HDT was 92 min (Fig. 2b). For pairs MA4/M4 and MA4/M7 in which the initial biomass was cultivated on minimal MA4 medium, HDT was 92 and 80 min, respectively; for enriched pairs MA7/M7 and MA6/M6, HDT was 60 and 66.7 min, respectively. Since only agar media MA4 and MA7 had different concentrations of nutritional components in these pairs, it might be suggested that increase in growth rate by 20 min observed on the liquid medium results from MA7 agar medium enrichment by the yeast extract or from longer duration of the adaptation period in bacteria transferred to a liquid medium with different composition.

However, merely the change in HDT occurring in liquid media depending on the composition of the agar medium on which biomass was cultivated provides an opportunity to simulate the physiological activity of

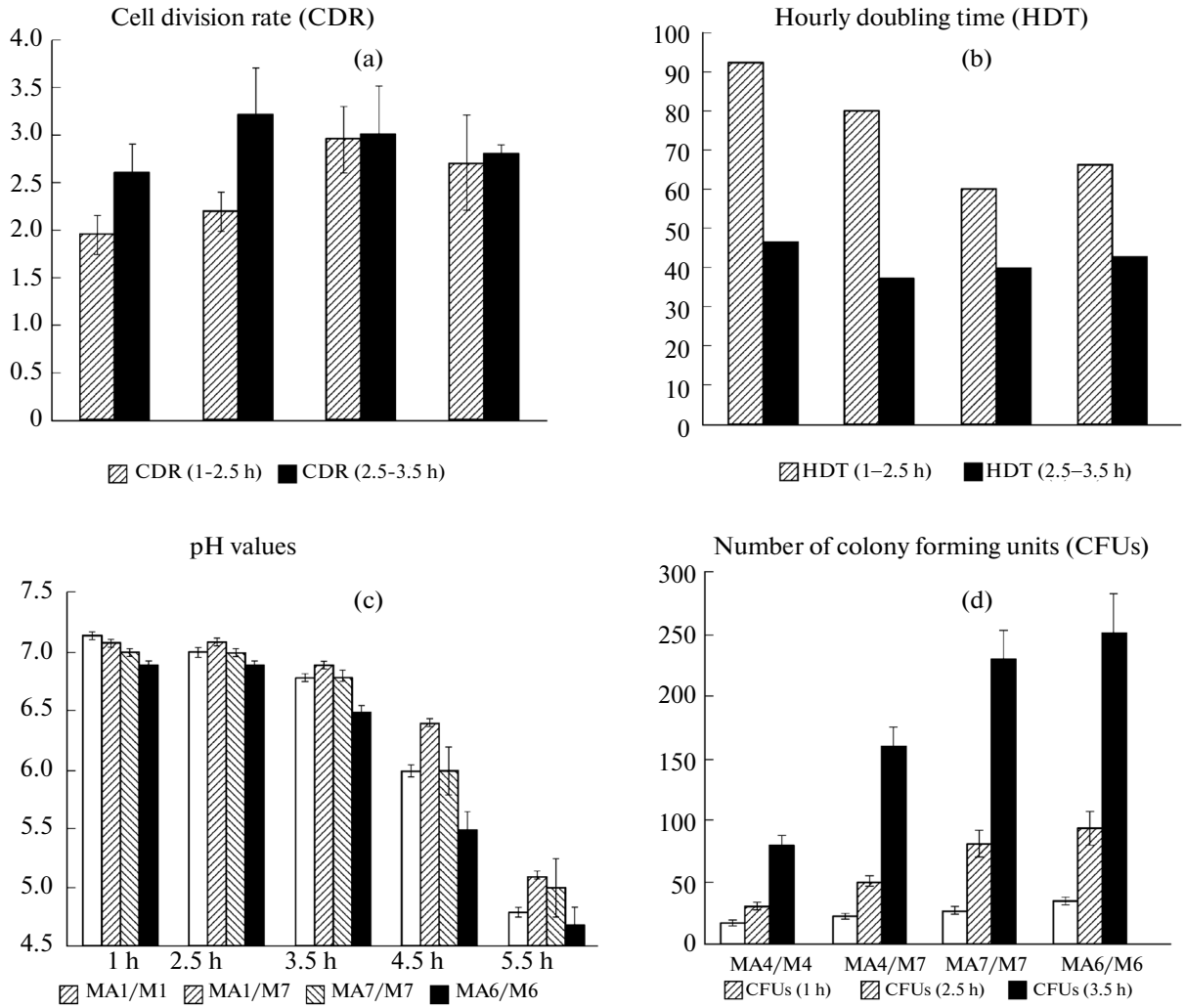

Fig. 2. Growth characteristics of *Lactococcus lactis* subsp. *lactis* 729 in two time segments of cultivation (from 1 to 2.5 h and from 2.5 to 3.5 h) in liquid media: M4, M7, and M6 after the biomass transfer from agar media MA4, MA7, MA6 and in the composite pair MA4/M7. (a) cell division rate (CDR); (b) hourly doubling time (HDT); (c) pH values of liquid media MA4, MA7, and MA6 recorded after 1, 2.5, 3.5, 4.5, and 5.5 h of cultivation after biomass transfer from MA4, MA7, MA6 agar media and in the composite pair MA4/M7; (d) the number of CFUs in MA4, MA7, and MA6 liquid media recorded after 1, 2.5, and 3.5 h of cultivation after biomass transfer from MA4, MA7, MA6 agar media and in composite pair MA4/M7.

produced biomass of lactococci before transferring it to a medium with different composition. In the second time segment (from 2.5 to 3.5 h), HDT for pairs MA4/M4, MA4/M7, MA7/M7, and MA6/M6 was 46.2, 37.5, 40.0, and 42.9, respectively. The closeness of HDT values for the four pairs in the second time segment (from 2.5 to 3.5 h) suggests that the growth of 729 strain is logarithmic.

Comparative evaluation of the acidification rate in liquid media M4, M7, and M6 during the cultivation of *Lactococcus lactis* subsp. *lactis* 729 after the biomass production on agar media. Cell division characteristics—CDR and HDT (Figs. 2a and 2c)—indicated the efficiency of division in this time segment; it is also notable that hardly any changes were observed in pH for the liquid media from pairs MA4/M4, MA4/M7, MA7/M7, and MA6/M6 between 1 and 2.5 h, which corresponds with the results obtained earlier [14]. We determined the time segment of cultivation in which logarithmic growth was observed in liquid media with different levels of enrichment and minimal acidification rate (between 2.5 and 3.5 h). The maximal acidification rate among the liquid media occurred during the cultivation in pair MA6/M6, which contains the meat extract (from 3.5 to 6.5 h); these values do not, however, exceed the optimal range for lactococci [16, 17]. The maximal difference of pH values between MA4/M7 and other pairs was recorded after 4.5 h of cultivation: 6.4 for pair MA4/M7, 6.0 for pair MA4/M4, 6.0 for pair MA7/M7, and 5.5 for pair

MA6/M6. In the composite pair MA4/M7, pH value was close to the optimum (up to 6.5) with allowance for the efficient cell division in the time segment before 4.5 h, while that in pair MA6/M6 pH decreased to the critical value (5.5), which blocked the transport of oligopeptides [18, 19]. Two phases of logarithmic growth were observed in *L. lactis* and *Streptococcus thermophilus* cultivated in milk [19, 20]. Two periods of intensification of the cell division we found earlier during independent measurements of HDT in distinct 729 strain populations with initial biomass produced on a minimal agar medium [14]. Since CP was used as the main nitrogen source in our nutrient media and milk, we suggest that the time of cultivation prior to the decrease in HDT accounts for the nutrition of lactococci by oligopeptides with adequate composition and weight, i.e., before triggering the proteolytic activity [18, 20].

Biomass production rate in *Lactococcus lactis* subsp. *lactis* 729 in M4, M7, and M6 liquid media during the first 3.5 h of cultivation. The initial value of titer of bacterial suspensions in pairs MA4/M4 and MA4/M7 was 7.3×10^6 c/mL; in pair MA7/M7, 1.3×10^7 c/mL; in pair MA6/M6, 1.9×10^7 c/mL (Fig. 2d). The maximal rate of biomass production during these 3.5 h was observed in pair MA6/M6, which contained the meat extract (2.5×10^8 c/mL). Even considering the higher values of initial titers, evaluation of the relative increase in the number of CFUs showed that the meat extract has a positive effect on the biomass production rate; thus, nutritional composition of the M6 liquid medium might be considered complex. The minimal rate of biomass production was recorded in "minimal" pair MA4/M4 (8.0×10^7 c/mL); the nutrient restriction is a limiting factor. In the enriched liquid media, cell division is limited by lactic acid accumulation until a pH value of 5.5 [3, 4, 17] when oligopeptide transport is blocked [18]. Low rate of biomass production in *L. lactis* subsp. *lactis* TB2—to 2.5×10^8 c/mL—in MB medium (0.6% of CP, 0.175% of the yeast extract) corresponds with the absorbance of 0.3 (wave length of 600 nm) after cultivation for 3 h at pH decreased to 5.4 [4]. Similar results were obtained for *L. lactis* cultivated in complex M17 medium containing 1% of glucose [2]. The authors attribute this early inhibition of the logarithmic growth of *L. lactis* at an absorbance of 0.3 (wave length of 600 nm) to the increase in stress resistance of lactococci.

CONCLUSIONS

By cultivation on the agar media prepared with different enrichment levels of the minimal MA4 medium, we determined the period of logarithmic growth (2.5–3.5 h) of *L. lactis* subsp. *lactis* 729 in identical and composite liquid media at slightly decreased pH (6.5). Relatively small size of the colonies might result from the acid-forming properties of the strain.

The efficiency of the colony-forming ability of TB2 strain was investigated as a characteristic of the colony development at minimal CP concentration (0.4%). It was found that colony size of TB2 strain on the minimal MA4 medium reaches 5 mm^2, which is more than that on rich M03 medium [8].

The results of comparative analysis of the bacterial populations developing on agar and liquid media can be used for the population modeling in different strains of *L. lactis* subsp. *lactis*.

The strain-specific characteristics of growth in lactococci are of interest for determining stress conditions of cultivation for a strain. The most wide-spread stress conditions are: lack of nutrients, high acidification rate, raised and reduced temperature range, etc. The changes in the physiological functions of lactococci populations cultivated in milk, accompanied by triggering of the proteolytic activity in the second phase of logarithmic growth, are an example of programmed adaptation to nitrogen starvation.

REFERENCES

1. *Praktikum po mikrobiologii* (A Practical Course in Microbiology), Netrusov, A.I., Ed., Moscow: ACADEMA, 2005, Ch. 44, pp. 467–478.
2. Duwat, P., Cesselin, B., Sourice, S., and Gruss, A., *Lactococcus lactis*, a bacterial model for stress responses and survival, *Int. J. Food Microbiol.,* 2000, vol. 55, pp. 83–86.
3. Hartke, A., Boucheacute, S., Giard, J.C., Benachour, A., Boutibonnes, P., and Auffray, Y., The lactic acid stress response of *Lactococcus lactis* subsp. *lactis*, *Curr. Microbiol.*, 1996, vol. 33, no. 3, pp. 194–199.
4. Trenina, M.A., Lysenko, A.M., Akhverdyan, V.Z., and Mchedlishvili, E.B., Study of intraspecific variations of the bacterium *Lactococcus lactis* in adaptation to high acidity of the medium, *Microbiology* (Moscow), 2006, vol. 75, no. 1, pp. 97–104.
5. Stoyanova, L.G., Sul'timova, T.D., and Netrusov, A.I., Effect of phosphate and carbohydrates on the synthesis of nisin by *Lactococcus lactis* subsp. *lactis* strain 194, *Vestn. Mosk. Univ., Ser. Biol.*., 2003, no. 4, pp. 17–22.
6. Stoyanova, L.G. and Levina, N.A., Components of fermentation medium regulate bacteriocin synthesis by the recombinant strain *Lactococcus lactis* subsp. *lactis* F-116, *Microbiology* (Moscow), 2006, vol. 75, no. 3, pp. 286–291.
7. Huggins, A.R. and Sandine, W.E., Differentiation of fast and slow milk-coagulating isolates in strains of lactic streptococci, *J. Dairy Sci.,* 1984, vol. 67, pp. 1674–1679.
8. Sukhodolets, V.V., Lysenko, A.M., and Trenina, M.A., RF Patent No. 2244002, 2003.
9. Corroler, D., Mangin, I., Desmasures, N., and Gueguen, M., An ecological study of lactococci isolated from raw milk in the camembert cheese registered designation of origin area, *Appl. Environ. Microbiol.*, 1998, vol. 64, no. 12, pp. 4729–4735.
10. Mills, S., McAuliffe, O.E., Coffey, A., Fitzgerald, G.F., and Ross, R.P., Plasmids of lactococci—genetic acces-

sories or genetic necessities?, *FEMS Microbiol. Rev.*, 2006, vol. 30, pp. 243–273.

11. Kelly, W. and Ward, L., Genotypic vs. phenotypic biodiversity in *Lactococcus lactis*, *Microbiology*, 2002, vol. 148, pp. 3332–3333.
12. Stoyanova, L.G. and Egorov, N.S., Derivation of efficient nisin producers by fusing protoplasts of two allied strains of *Lactococcus lactis* subsp. *lactis* with low nisin-synthesizing abilities, *Microbiology* (Moscow), 1998, vol. 67, no. 1, pp. 38–44.
13. Stoyanova, L.G., Sul'timova, T.D., and Netrusov, A.I., Establishment of taxonomic status of new prospective bacteriocin-synthesizing *Lactococci* strains of various origins, *Moscow Univ. Biol. Bull.,* 2008, vol. 62, no. 4, pp. 156–162.
14. Trenina, M.A., Ganina, V.I., Stoyanova, L.G., Rybakov, Yu.A., and Skladnev, D.A., Characteristics of development of lactic acid bacteria *Lactococcus lactis* subs. *lactis* strain 729, *Pererab. Sel'skokhoz. Syr'ya*, 2008, no. 8, pp. 55–57.
15. Trenina, M.A., Skladnev, D.A., Bronnikov, S.V., Ustyugova, E.A., and Stoyanova, L.G., Development of populations of bacteria *Lactococcus lactis* ssp. *lactis* on agar nutrient media simulating the natural substrate, *Ekol. Prom. Ross.*, 2009, no. 5, pp. 42–46.
16. Hutkins, R.W. and Nannen, N.L., pH homeostasis in lactic acid bacteria, *J. Dairy Sci.*, 1993, vol. 76, pp. 2354–2365.
17. Frees, D., Vogensen, F.K., and Ingmer, H., Identification of proteins induced at low pH in *Lactococcus lactis*, *Int. J. Food Microbiol.*, 2003, no. 87, pp. 293–300.
18. Foucaud, C. and Juillard, V., Accumulation of casein-derived peptides during growth of proteinase-positive strains of *Lactococcus lactis* in milk: their contribution to subsequent bacterial growth is impaired by their internal transport, *J. Dairy Res.*, 2000, vol. 67, pp. 233–240.
19. Juillard, V., Le Bars, D., Kunji, E.R.S., Konings, W.N., Gripon, J.-C., Richard, J., Oligopeptides are the main source of nitrogen for *Lactococcus lactis* during growth in milk, *Appl. Environ. Microbiol.*, 1995, vol. 61, pp. 3024–3030.
20. Letort, C., Nardi, M., Garault, P., Monnet, V., and Juillard, V., Casein utilization by *Streptococcus thermophilus* results in a diauxic growth in milk, *Appl. Environ. Microbiol.*, 2002, vol. 68, no. 6, pp. 3162–3165.

Translated by Ya. Atmanskikh