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EXPERIMENTAL ARTICLES

Investigation of the Antibiotic Complex Produced by *Lactococcus lactis* subsp. *lactis* 194, Variant K

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Abstract—Phase variation in the culture of the environmental strain *Lactococcus lactis* subsp. *lactis* 194 resulted in the formation of two types of colonies differing by 15% in antibiotic activity. The active variant 194-K produced an antibiotic complex with a broad spectrum of antibacterial and antifungal activity. Five components (194-A, B, C, D, and E) were isolated from the complex by solid-phase extraction and thin-layer chromatography. Components 194-A and 194-B were hydrophobic neutral compounds soluble in organic solvents. Component 194-A possessed fungicidal activity, whereas component 194-B exhibited only bactericidal activity. Physicochemical studies of the isolated components 194-A and 194-B revealed that they had no analogs in the Berdy database of biologically active substances (BNPD) and appeared to be novel antibiotics. Component 194-C was a hydrophilic polar compound inhibiting growth of gram-positive and gram-negative bacteria. Component 194-D belonged to peptide antibiotics; it inhibited growth of only gram-positive bacteria and was similar to nisin A in biological properties but differed in electrophoretic mobility and molecular mass.

Keywords: environmental strain *L. lactis* subsp. *lactis* 194, phase variation, antibiotic complex, isolation, physicochemical properties, identification.

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The lactococci *Lactococcus lactis* subsp. *lactis* are known as producers of various bacteriocins, such as nisins (A, B, C, D, E, Z, R, and Q), lacticins 3145 and 481, and lactococcin 140 [1, 2]. The production of bacteriocins is considered to be a strain-specific process. The most-studied bacteriocin, nisin A, is the main active component of the Nisaplin preparation (Aplin & Barrett, Ltd, United Kingdom), which is widely used as a food product preservative. Nisin is the only natural antibiotic that was designated as GRAS (generally recognized as safe). However, nisin is active only against gram-positive bacteria and does not inhibit growth of gram-negative bacteria and micromycetes, which are often responsible for foodstuff spoilage. Populations of the bacteriocin producers contain cells with different biosynthetic capacity. Multiple transfers of the antibiotic producers under unfavorable conditions can provoke phase variation with the prevalence of inactive clones [3]. Strains with a wide spectrum of antimicrobial activity inhibiting growth of gram-negative bacteria and micromycetes rarely occur among *Lactococcus lactis* subsp. *lactis* isolates [4, 5]. In particular a fungicidal peptide was isolated from strain *L. lactis* subsp. *lactis* CHD-28.3 [6]; there is information on the strain producing a phosphoglycolipid of low molecular-mass (below 500 Da) that was able to inhibit growth of *Aspergillus flavus* under mixed cultivation [7].

The goal of the present work was to study the antibiotic complex produced by an active variant 194-K isolated from the phase variants of the heterogeneous population of the environmental strain *Lactococcus lactis* subsp. *lactis* 194.

MATERIALS AND METHODS

Objects, Media, and Cultivation Conditions

The study was carried out with the environmental strain *Lactococcus lactis* subsp. *lactis* 194 isolated from cow milk (Buryatiya) [8].

To prepare the inoculum, lactococci stored in fat-free (skimmed) milk were cultivated under static conditions at 28°C for 20 h in medium containing tap water, yeast extract (20 g/l), and glucose (10 g/l); pH 6.8–7.0. The inoculum (5%) was transferred into the fermentation medium containing the following (g/l): sucrose, 20.0; KH_2PO_4, 10.0; NaCl, 2.0; $MgSO_4$, 0.2; yeast extract, 20.0; pH 6.8–7.0 and cultivated under the same conditions. The biomass was determined nephelometrically on a FEK-56 photom-

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eter (Russia) at 540 nm. Antibiotic activity was determined by the method of diffusion in agar with measuring the zone of growth inhibition of the test culture (mm) [5].

The phase variation of the culture was studied using solid (2% agar) fermentation medium. Bacterial suspension was plated onto the medium, and colonies of different morphology were collected. Individual colonies were plated primarily on the fermentation medium and then transferred onto the medium inoculated with the test culture *Bacillus coagulans* 429. The colonies containing the largest zones of the test culture growth inhibition were selected.

Morphology of the colonies was studied by routine methods using an MBI-15 microscope.

Antibiotic activity of the individual colonies was determined after their incubation, first in the inoculum medium and then in the liquid fermentation medium [8].

To determine the spectrum of antibiotic activity, the active variant *L. lactis* subsp. *lactis* 194-K was cultivated under static conditions in the aforementioned fermentation medium. The following test cultures were used: gram-positive bacteria *Bacillus subtilis* ATTCC 6633, *Micrococcus luteus* NCTC 8340, *Bacillus coagulans* 429, *Staphylococcus aureus* FDA 209P; gram-negative bacteria *Escherichia coli* ATCC 25922 and *Comamonas terrigena* ATCC 8461; micromycetes *Aspergillus niger* INA 00760, *Aspergillus repens* 111, *Penicillium chrysogenum,* and *Candida tropicalis* INA 00763.

Bacilli were grown on a medium based on diluted Hottinger broth (1 : 2) and containing (g/l) glucose, 10.0; NaCl, 2.0; and agar–agar, 20.0; *E. coli* was grown on nutrient agar; fungi were cultivated on the Sabouraud medium containing (g/l) glucose, 40.0; peptone, 10.0; and agar–agar, 20.0.

Cultivation was performed at 37°C (bacilli, staphylococci, and micrococci), 42°C (*E. coli*), 55°C (*B. coagulans*), and 28°C (micromycetes including yeasts). Petri dishes were plated with suspensions of 1-day cultures of bacteria or spore suspensions or 5-day cultures of fungi in phosphate buffer (pH 5.5) in the amount of 1×10^9 cells/ml according to the bacterial density standard (OSO density standard, Tarasevich State Institute for Medical and Biological Standardization and Control, GISK). The antibiotic activity of the strain against various groups of microorganisms was estimated by measuring the growth inhibition zones.

Solutions of 10, 20, 30, 40, and 50 IU/ml of commercial nisin preparation (Nisaplin, Aplin & Burrett, Ltd, United Kingdom) with activity of 1000 IU/mg and nystatin (Sigma, United States) with activity of 4670 IU/mg were used as standards for bacteria and fungi, respectively.

Isolation and Investigation of the Antibiotic Complex from Lactococcus lactis subsp. lactis 194-K

Antibiotic substances were extracted from strain 194-K with a mixture of acetone–acetic acid–culture (4 : 1 : 5) at 55°C for 1.5 h. Acetic acid and acetone were evaporated under vacuum at 40°C. The total antibiotic activity was determined in the aqueous concentrate. Methanol was added to the residual aqueous concentrate in an excessive amount, and the precipitate was collected by filtration through a glass filter to obtain the crude powder. Antibiotics were isolated from the concentrate of the mother solution by successive extraction with ethyl acetate and *n*-butanol. The solvents were evaporated under vacuum on a rotary evaporator; the residue was dissolved in 60% aqueous ethanol to obtain ethanol concentrates (Fig. 1).

The analysis of the antibiotic substances was performed for all fractions (ethanol concentrates, mother solutions, and powder) by both the biological method with the test cultures of *B. subtilis* ATCC 6633 and *A. niger* INA 00760 and the chromatographic method with subsequent bioautography. The TLC was performed on DC Alufoilien Kieselgel-60 plates (Merck, Germany) developed in the chloroform–benzene–methanol (30 : 20 : 7) solvent system. Preparative TLC for the isolation of individual components was carried out under the same conditions.

Electrophoresis was performed on a Filtrak F-14 paper using a Durruma V-shaped device at 550 V for 150 min in electrolytes E1 (30% acetic acid), pH 1.7, and E2 (pyridine–acetic acid–water (2 : 4 : 994)), pH 4.3. The electrophoretic mobility of the antibiotics was determined by measuring a shift of a substance (cm) from the start line to cathode or anode.

The desalting and purification of components 194-A and 194-B were carried out in several stages by using Diapak C-8 and Diapak C-16 cartridges (BioChimMak ST, Moscow, Russia). Solutions with different ratios of acetonitrile (AcCN) and 0.1% trifluoroacetic acid (TFA) were used as desorbents. The minimal inhibitory concentration (MIC) of the antibiotic 194-A was determined by the method of diffusion in agar. The heat stability of the isolated antibiotic was studied after its autoclaving for 15 min (121°C, 0.5 atm) and boiling for 15 min at 100°C.

The UV-VIS spectra were registered on a Shimadzu UV-1601 PC UV-Visible spectrophotometer (Japan).

The mass spectra of the 194-A and 194-B components were recorded on a Bruker Ultraflex device by the MALDI-TOF method in regimes of positive ions and reflectron at accelerating voltage of 25 kV; 2,5hdihydroxybenzoic acid was used as a matrix.

The IR spectra of the isolated antibiotics were registered on a Nicolet-iS10 IR Fourier spectrometer (Thermo Fisher Scientific, United States) equipped with a DTGS detector, a KBr beam divider, and a Smart Performer with a ZnSe crystal. The measure-

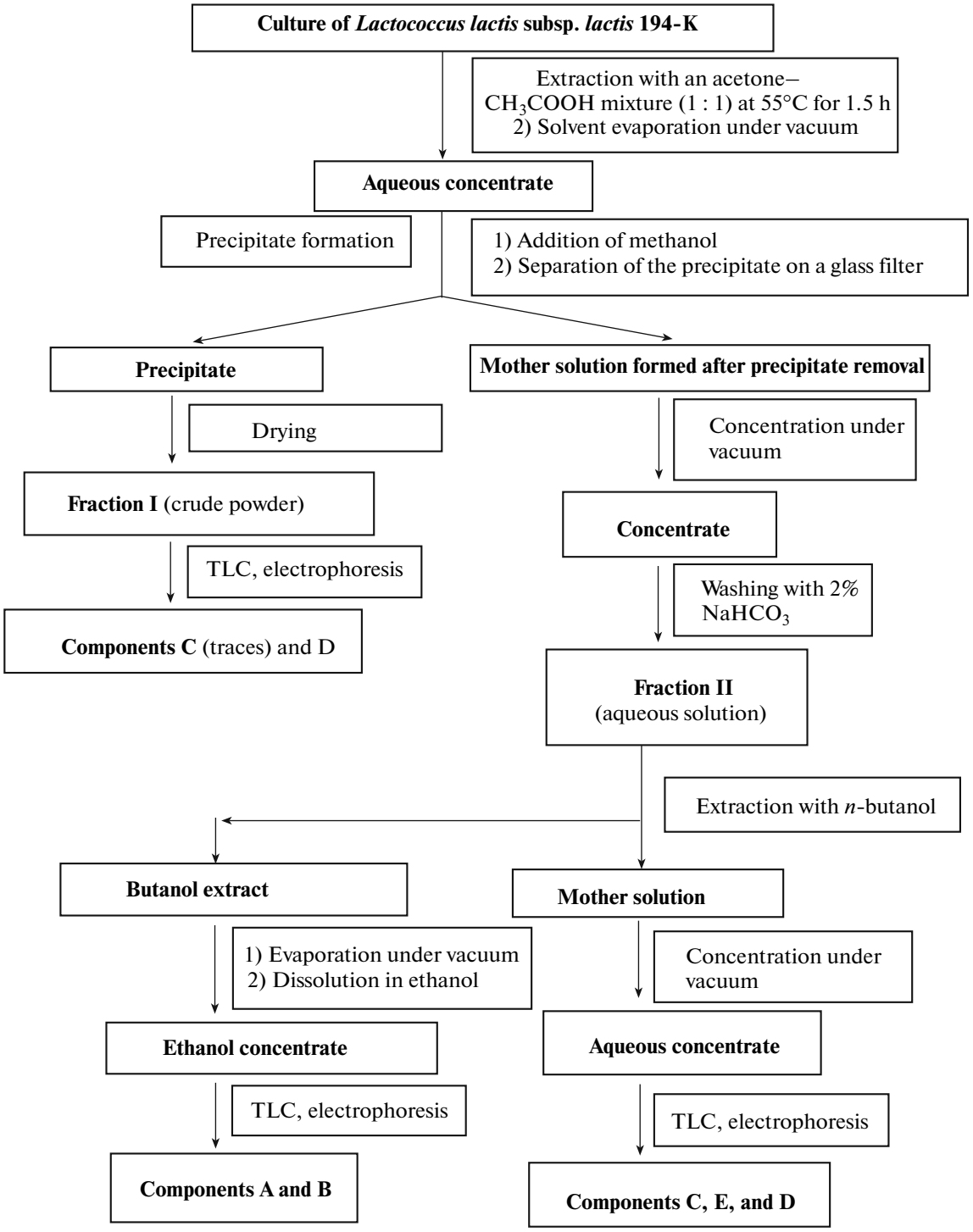


Fig. 1. Scheme of isolation of the components of the antibiotic complex from the culture of strain *Lactococcus lactis* subsp. *lactis* 194-K.

ment was performed at a resolution of 4 cm^{-1} in the spectrum zone of 3000–650 cm^{-1}. The IR spectra were processed using the OMNIC-7.0 software package and interpreted according to [9].

Bioautography of antibiotics was performed with *Bacillus subtilis* ATCC 6633 as a test microorganism [10].

The detection of free amino acid groups and peptide bonds (CONH-) in the antibiotic complex was carried out using specific reagents containing ninhydrin and Cl_2-benzidine, respectively [11].

To determine the distribution of antibiotic substances between the cells and the culture liquid, a 20-h

Table 1. Distribution of antibiotic activity between cells and the cell-free extract of *Lactococcus lactis* subsp. *lactis* 194-K by the 24th hour of incubation

Fractions of the culture broth	Antibiotic activity			
	Relative to nisin		Relative to nystatin	
	IU/ml	%	IU/ml	%
Cell-associated antibiotic activity	2050 ± 50	51 ± 0.01	900 ± 84	47 ± 0.04
Extracellular activity	2000 ± 115	49 ± 0.02	1050 ± 76	53 ± 0.02
Total antibiotic activity*	4050 ± 135	100	1950 ± 60	100

* Antibiotic activity was determined in the culture with $OD_{540} = 0.9$ at pH 4.7.

culture of strain 194-K was centrifuged at 2240 *g* for 30 min; the cells (1 g) were washed with phosphate buffer (pH 7.0), treated with 5 ml of the solution containing 15% AcCN and 0.1% TFA, incubated at room temperature for 1 h, and centrifuged; the supernatant contained the active substances that were washed out from the cells. Then the cells (1 g) were treated with 5 ml of 70% ethanol to obtain the ethanol extract of antibiotics. The native solution of the culture *L. lactis* subsp. *lactis* 194-K was treated with acetone cooled to −10°C (1 : 3, vol/vol) and incubated at that temperature for 24 h; the precipitate formed was separated from the mother solution. In the obtained fractions, antibiotic activity was determined as described above.

RESULTS AND DISCUSSION

Population of *L. lactis* subsp. *lactis* 194 grown on solid fermentation medium with 2% sucrose dissociated into small transparent colonies (G) and large white colonies (S) in a ratio of 82 : 18% exhibiting activities of 4000 and 3400 IU/ml (relative to nisin), respectively (Table 1). The most active phase variant designated as variant 194-K with activity of 4050 IU/ml was selected and used for the isolation and identification of antibiotics.

Table 2. Antimicrobial spectrum of the main fractions of the antibiotic complex produced by *Lactococcus lactis* subsp. *lactis* 194-K

Test organism	Diameter of the growth inhibition zone, mm*	
	Fraction I* (powder)	Fraction II* (aqueous solution)
Bacillus subtilis ATCC 6633	10.0	26.0
B. coagulans 429	12.0	17.5
Comamonas terrigena ATCC 8461	11.0	18.5
Fusarium spp. ИНА G-20	0	21.0
Candida tropicales INA 0073	0	14.0
Aspergillus niger INA 00760	0	15.0

* Fractions I and II were obtained according to the scheme (Fig. 1). Diameter of the paper discs was 7 mm.

In the course of growth, strain *L. lactis* subsp. *lactis* 194-K produced an antibiotic complex with a wide spectrum of biological activities; it inhibited growth of gram-positive bacteria including thermotolerant *B. coagulans*, gram-negative *E. coli* and *C. terrigena*, as well as micromycetes *Aspergillus niger, A. repens*, and *P. chrysogenum*. The total antibiotic activity of a 24-h culture reached 4050 IU/ml (relative to nisin) and 1950 U/ml (relative to nystatin); up to 53% of the antibiotic activity was associated with the cells (Table 1).

For complete isolation of all antibiotic components, the cells were treated with the mixture of acetone–acetic acid–water (4 : 1 : 5) (Fig. 1).

The antibiotic complex isolated from strain 194-K was composed of several biologically active components, which were located in two main fractions (Fig. 1). Fraction I (crude powder) exhibited activity only against gram-positive bacteria, whereas fraction II isolated from the mother solution was characterized by a wide spectrum of antimicrobial action (Table 2). Subsequent chromatographic and electrophoretic studies of these fractions revealed the presence of five biologically active compounds (A, B, C, D, and E).

Components 194-A and 194-B were electrically neutral hydrophobic substances; when divided by TLC on Silica Gel in the chloroform–benzene–methanol (30 : 20 : 7) system, they had R_f of 0.63 and 0.57, respectively (Fig. 2). The compounds were completely extracted with *n*-butanol from the aqueous mother concentrate. Component 194-A showed fungicidal action and weak activity against gram-positive bacteria, whereas component 194-B inhibited growth of both gram-positive microorganisms including

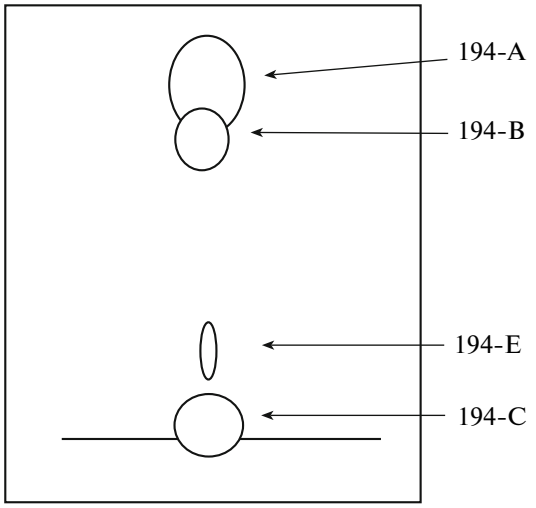


Fig. 2. TLC of the components of the antibiotic complex produced by *L. lactis* subsp. *lactis* 194-K on Kiesegel-60 plates developed in the chloroform–benzene–methanol system (30 : 20 : 7).

motolerant, spore-forming, and acid-resistant bacterium *B. coagulans* and gram-negative microorganisms including *E. coli* and *C. terrigena* (Table 3).

Components 194-B and 194-C were the major compounds of the antibiotic complex, whereas 194-A, D, and E were the minor ones. The fungicidal component 194-A and the bactericidal component 194-B were isolated from the ethanol concentrate of the *n*-butanol extract by the method of solid-phase extraction. The desalting and purification of the components were carried out using the Diapak C-8 and C-16 cartridges by stepwise elution with solvents of different polarity. Component 194-A was a hydrophobic substance well soluble in chloroform and acetonitrile (Table 4). The fungicidal antibiotic 194-A had molecular mass of 290 Da; its IR spectra showed characteristic absorption bands at (ν_{max}, cm^{-1}): 3412 (aldehyde group), 2958 ($-CH_3$), 2923 ($-CH_3$), 2853 ($-CH_2-$), 1738 (saturated aliphatic aldehyde), 1631 (aldehyde group at a double bond), 1466 (alkyl radical in alkyl benzene) 1378 (methyl group in an aliphatic hydrocarbon), 1266 (aldehyde), 1166 ($-C(CH_3)_2$), 1100 (–CH–OH–), 1073 (–C–OH), 949 (C–O–C), and 721 ($CHR_1{=}CHR_2$). The fungicidal activity of component 194-A decreased after both autoclaving at 0.5 atm (by 37–40%) and heating at 100°C for 15 min (by 20%). The minimal inhibitory concentration of compound 194-A against such microorganisms as *A. niger, A. repens, P. chrysogenum*, and *C. tropicalis* was 0.25 mg/ml.

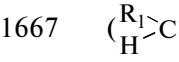
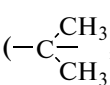
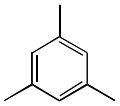
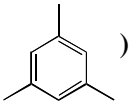
The antibacterial component 194-B was a hydrophobic substance with higher molecular mass (879 Da) (Table 4). The IR spectra showed the following characteristic absorption bands at (ν_{max}, cm^{-1}): 1667 (), 1454 (–C(CH$_3$)–, –CH$_2$–), 1143 (), 847 (), 801 (), 726 (–(CH$_2$)$_4$, ), 669 (C–H)), which indicated the aromatic nature of the substance with alkyl substituents. Component 194-B was well soluble in such organic solvents as ethyl acetate, *n*-butanol, ethanol, acetonitrile, and aqueous acetonitrile.

Table 3. Chromatographic and biological properties of active components from the antibiotic complex produced by *Lactococcus lactis* subsp. *lactis* 194-K

Physicochemical characteristics		Components			
		194-A	194-B	194-C	194-D
TLC, Kieselgel-60	R_f in the system chloroform–benzene–methanol (30 : 20 : 7)	0.63	0.57	0	0
Paper electrophoresis	R_f in 30% acetic acid, pH 1.7 at 550 V, 3 h (migration zone, cm)	0	0	2.0 (toward anode)	4.7 (toward cathode)
Spectrum of antimicrobial action		*B. subtilis*, *A.niger*, *P. chrysogenum*, *C. tropicales*	*B. subtilis*, *B. coagulans*, *M. luteus*, *S. aureus*, *C. terrigena*	*B. subtilis*, *B. coagulans*, *C. terrigena*	*B. subtilis*, *B. coagulans*, *M. luteus*

* The distance from the start line to cathode or anode (cm): "0 cm" means "at the start line" (electrically neutral compound), "4.7 cm" means "migration toward the cathode" (alkaline compound), and "2.0 cm" means "migration toward the anode" (acid compound).

Table 4. Physicochemical properties of components 194-A and 194-B produced by *Lactococcus lactis* subsp. *lactis* 194-K

Physicochemical properties	Component 194-A	Component 194-B
UV spectrum (EtOH), λ_{max}, nm	Not obtained	271
MALDI-MS (*m/z*) $(M + H)^+$	290	879
TLC (Kieselgel-60), R_f in the system chloroform–benzene–methanol (30 : 20 : 7)	0.63	0.57
Reaction with $KMnO_4$	+	+
IR spectrum (ν_{max}, cm^{-1})	3412weak band, 2958, 2923, 2853, 1738, 1631weak band, 1466, 1378, 1266weak band, 1166, 1100, 1073, 949weak band, 721weak band	1667weak band, 1454, 1143, 847weak band, 801, 726, 669 weak band.
Antibacterial spectrum of activity	Gram-positive bacteria: *B. subtilis* Micromycetes: *A. niger*, *A. repens*, *P. chrysogenum*, *C. tropicales*	Gram-positive bacteria: *B. subtilis*, *B. coagulans*, *M. luteus*, *S. aureus* Gram-negative bacteria: *E. coli*, *C. terrigena*
Solubility	Soluble in chloroform, acetonitrile, and ethyl acetate insoluble in water and aqueous ethanol	Soluble in *n*-butanol, ethyl acetate, and aqueous acetonitrile insoluble in ethanol and water

Fraction I (precipitate) obtained after addition of methanol to the culture 194-K contained two components: 194-D and 194-C (Fig. 3). Positive qualitative reactions with ninhydrin and Cl_2-benzidine were indicative of the peptide nature of component 194-D [8]. This component differed from nisin A by its lower electrophoretic mobility toward the cathode in electrolyte E1 (30% CH_3COOH) at pH 1.7. The electrophoresis of component 194-C in electrolyte E2 (pyridine–acetic acid–water) (2 : 4 : 994) at pH 4.3 revealed that it migrated toward the anode indicating that it was a hydrophilic substance with acidic properties. Since component 194-E occurred in the culture of strain 194-K in trace amounts and was unstable, its biological and physicochemical properties could not be studied.

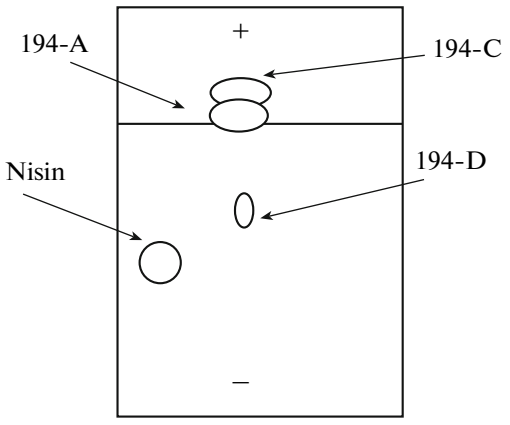


Fig. 3. Scheme of paper electrophoresis in 30% acetic acid of nisin A and the antibiotic complex produced by *L. lactis* subsp. *lactis* 194-K.

Thus, the bacterial isolate *L. lactis* subsp. *lactis* 194-K produced a multicomponent antibiotic complex composed of hydrophobic antibiotic substances 194-A and 194-B, peptide antibiotic 194-D, and hydrophilic components 194-C and 194-E. All components of the antibiotic complex were excreted into the culture liquid. Components 194-D and 194-E were partly associated with the cells. The hydrophobic components 194-A and 194-B were also extracted from the cells with ethanol.

Thus, the active phase variant 194-K obtained from an individual colony of strain *L. lactis* subsp. *lactis* 194 originally isolated from cow milk (Buryatiya). Strain 194-K produced an antibiotic complex containing five biologically active compounds (A, B, C, D, and E), which were characterized by different chemical and biological properties, such as the chromatographic and electrophoretic mobility, solubility, and spectra of antibiotic activities. Component 194-A was a hydrophobic substance exhibiting fungicidal activity; its MIC toward micromycetes and yeasts was 0.25 mg/ml. Component 194-B was a hydrophobic high-molecular-weight substance containing aromatic and alkyl groups; it was active against gram-positive and -negative bacteria. Component 194-D was a peptide differing in electrophoretic mobility from nisin A, which is produced by many strains of *L. lactis* subsp. *lactis*. To identify the isolated antibiotics, information retrieval was performed using the database for the natural biologically active substances (BNPD), the principle of which was developed by J. Berdy [12]. It was concluded that components 194-A and 194-B had

no analogues and represented novel natural biologically active compounds; the presence of fungicidal activity is a rare and poorly studied physiological property of lactococci belonging to the subspecies *L. lactis* subsp. *lactis*.

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