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EXPERIMENTAL ARTICLES

Antimicrobial Activity of *Bacillus megaterium* Strains

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Abstract—A bacterial strain with a high level of antimicrobial activity was isolated from soil and identified as *Bacillus megaterium*. Production of antibiotics by nine strains of this species from the collection of the State Research Institute for Genetics and Selection of Industrial Microorganisms was investigated. In submerged cultures, nine out of ten *B. megaterium* strains were found to produce antibacterial antibiotics differing in their spectra of action. Physicochemical characteristics of five compounds were described. Three of them belonged to peptide antibiotics. All five compounds were active against the methicillin-resistant strain *Staphylococcus aureus* INA 00761. Three of them were shown to be the previously undescribed compounds. Antibiotics produced by various *B. megaterium* strains were also active against the *Leuconostoc mesenteroides* VKPM B-4177 strain resistant to glycopeptide antibiotics and against gram-negative bacteria *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922.

Keywords: Bacillus megaterium, antibacterial antibiotics, antibiotic producers, MRSA, resistance to glycopeptide antibiotics.

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Wide occurrence of the pathogenic microorganisms resistant to medical antibiotics necessitates the search for new compounds to overcome microbial resistance to medicines. Soil microorganisms (fungi, actinomycetes, and eubacteria, including *Bacillus* species) are known to be the major antibiotic producers [1, 2]. Among the antibiotics produced by bacilli, gramicidin S from *B. brevis*, the first Soviet antibiotic preparation [3], and polymyxins M and B from *B. polymixa* [4] are presently used in medicine.

In the framework of the program of search for new natural antibiotics active against resistant pathogenic bacteria, we isolated from soil the strain subsequently deposited in the collection of the Gause Institute of New Antibiotics, no. INA 01083. This strain was of interest because it exhibited antimicrobial activity against bacterial test cultures resistant to β-lactam and glycopeptide antibiotics.

The goal of the present work was to determine the species position of strain INA 01083, broaden the range of investigated members of this species using collection strains, and describe the antibiotics produced.

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MATERIALS AND METHODS

Experimental subjects. Strain INA 01083 was isolated from a sample of leached chernozem soil (Krasnodar krai) [5]. After determination of its species position, other strains were obtained from the All-Russian Collection of Industrial Microorganisms: VKPM B-600, VKPM B-602, VKPM B-603, VKPM B-608, VKPM B-607, VKPM B-604, VKPM B-605, VKPM B-606, VKPM B-4801, VKPM B-44-2, and VKPM B-402. The test cultures used for determination of antimicrobial activity were the following: gram-positive bacteria *Bacillus subtilis* ATCC 6633, *B. pumulis* NCTC 8241, *B. mycoides* 537, *Micrococcus luteus* NCTC 8340, *Leuconostoc mesenteroides* VKPM B-4177, *Staphylococcus aureus* FDA 209P (MSSA), and *S. aureus* INA 00761 (MRSA); gram-negative bacteria *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853; and fungi *Aspergillus niger* INA 00760 and *Saccharomyces cerevisiae* RIA 259.

Cultivation conditions. For surface cultivation of all the above strains, modified Gause no. 2 agar medium was used containing the following (%): glucose,1; peptone, 0.5; tryptone, 0.3; NaCl, 0.5; agar, 2; pH 7.2–7.4. For submerged cultivation, the following media were used (%): (1) potato, 2.0; (2) malt extract (Maltax, Finland), 2.0; (3) complete Gause no. 2 medium:

glucose, 1; NaCl, 0.5; peptone, 0.5; tryptone, 0.3; (4) medium no. 802: peptone, 0.5; tryptone, 0.3; yeast extract, 0.2; $MgSO_4 \cdot 7H_2O$, 0.1; (5) glucose, 1; pea flour, 5; peptone, 0.5; NaCl, 0.5; (6) mannitol, 1; NaCl, 0.5; peptone, 0.5; tryptone, 0.3; (7) $(NH_4)_2SO_4$, 0.4; NaCl, 1; chalk, 0.6; wheat flour, 5; (8) soy flour, 5; starch, 2; $(NH_4)_2SO_4$, 0.2; chalk, 0.2; (9) glucose, 1; glycerol, 0.25; peptone, 2.5; soy flour, 1; NaCl, 0.3.

Submerged cultivation of *B. megaterium* strains was carried out in 750-mL flasks with 100 mL of the medium on a shaker (220 rpm). *B. megaterium* strains and test cultures of *L. mesenteroides, A. niger*, and *Sac. cerevisiae* were grown at 28°C; other test cultures, at 37°C.

Determination of antimicrobial activity. Antimicrobial activity was determined by the agar diffusion method on Gause no. 2 medium. The culture liquid was applied to 9-mm wells and the isolated compounds or their fractions were applied to 6-mm paper disks, which were dried and placed on the agar medium inoculated with a test culture. The level of antimicrobial activity was determined from the diameter of growth inhibition zones around the wells or disks.

Microscopy. The microscopic structure was investigated under an Olympus BX41TF light microscope (Japan). Strains *B. subtilis* ATCC 6633 and *E. coli* ATCC 25922 were used as positive and negative controls, respectively, for Gram staining.

DNA isolation. DNA was isolated from 24-h agar culture of *B. megaterium* INA 01083. The isolation was carried out according to Boulygina et al. [6]. PCR of the 16S rRNA gene and sequencing of the PCR products were carried out using the system of universal primers [7]. The PCR reaction mixture contained the following: primers, 25 pmol each; 10× buffer, 2.5 μL; 2 mM dNTP, 2.5 μL; 5 U/μL BioTaq polymerase (Dialat, Russia), 0.2 μL; template DNA, 50 ng; water, to 25 μL. PCR was carried out according to the following scheme: 30 cycles at 94°C for 0.5 min, 45°C for 1 min, 72°C for 1 min; and final polymerization for 7 min.

Analysis of PCR products. Analysis was carried out by electrophoresis in 2% agarose gel at 6 V/cm. The gels were photographed using a BioDocII video documentation system (Biometra, Germany). Isolation and purification of the PCR products corresponding to different gene sites was carried out from low-melt agarose using the Wizard PCR Preps reagent kit (Promega, United States) according to the manufacturer's recommendations. Sequencing of the products was carried out using the Silver Sequencing reagent kit (Promega, United States) according to the manufacturer's recommendations. Both internal and external primers were used for the sequencing, and the reading was bi-directional.

The 16S rRNA gene sequences were analyzed using the data and software of the RDP (Ribosome Database Project, http://rdp.cme.msu.edu). The sequences were edited using the BioEdit software package (http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html). The sequences were aligned with the relevant sequences of the most closely related species using the CLUSTALW v. 1.75 software package. The unrooted phylogenetic tree was constructed using the methods implemented in the TREECONW software package (http://bioc-www.uia.ac.be/u/yudp/treeconv.html).

Isolation, purification, and physicochemical characterization of the antibiotics. Antibiotics were isolated from the culture liquid by sorption on Amberlite ZAD-2 and desorption with the *n*-butanol–acetone–water mixture (1 : 1 : 1). Chromatographic purification was carried out on Kieselgel 60 columns using the following solvent systems: chloroform–methanol (95 : 5 and 9 : 1) and toluene–methanol (9 : 1). The biological activity of the fractions was tested against *B. subtilis* ATCC 6633 and *S. aureus* INA 00761 (MRSA).

Acid hydrolysis of the isolated antibiotics in 6 N HCl for 18 h at 105°C was followed by paper chromatography in the systems *n*-butanol–acetic acid–water (4 : 1 : 5) (upper layer) and *n*-butanol–acetic acid–water (4 : 1 : 1).

Analytical HPLC was carried out on a Shimadzu LC10 chromatograph with the Diaspher C-18 columns, 4.0 × 250 mm with 7 μm grain (BioKhimMak, Russia). The injector loop volume was 10 μL; detection was carried out at 254 nm. The elution system contained 0.01 M H_3PO_4 (pH 2.6) and acetonitrile. Elution was carried out in a gradient mode with acetonitrile concentration varying from 30 to 70% and flow rate of 1 mL/min during 25 min. Prior to injection into the column, the sample was diluted with the mobile phase.

UV-VIS spectra of the antibiotics were determined on an UV-1601 PC spectrophotometer (Shimadzu, Japan).

Mass spectra of the antibiotics were determined on an Ultraflex II MALDI TOF/TOF mass spectrometer (Bruker Daltonics, Germany) equipped with an UV laser (335 nm, Nd) in the mode of positive ion formation using a reflectron; the accuracy of mass measurement was 0.001%. For analysis, 1 μL of the sample solution was mixed with 0.3 μL of 2,5-dihydroxybenzoic acid (10 mg/mL) in 20% acetonitrile (in water) with 1 μL of 0.5% trifluoroacetic acid on the target, and the mixture was air-dried.

IR spectra were obtained using a Nicolet-iS10 IR Fourier spectrometer (DTGS detector and KBr light splitter) with a Smart Performer appliance (illumination with a ZnSe crystal). The measurement was carried out at 4 cm^{-1} resolution within the 3000–650 cm^{-1} range. The spectra were analyzed using the OMNIC-7.0 software package.

RESULTS AND DISCUSSION

From the sample of leached chernozem soil (Krasnodar krai), a bacterium was isolated and designated as strain INA 01083. Under submerged cultivation conditions, it exhibited antimicrobial activity against the test cultures of gram-negative organisms, including methicillin-resistant *Staphylococcus aureus* MRSA INA 00761, at a significantly high level compared to other isolates obtained from the same sample.

The cultural and morphological characteristics of the isolate were determined. On complete no. 2 Gause medium, strain INA 01083 grew as large nonmotile rods, reaching the size of 1 × 6.5–6.9 μm after 24 h. On the second day of growth, endospore formation occurred in almost all cells, with the spores releasing from the sporangia on the 4th–5th day. The strain grew both aerobically and microaerobically (under a layer of paraffin oil). The temperature range for growth (by biomass) was 18–40°C with the optimum at 25–35°C. The strain hydrolyzed casein and gelatin, possessed oxidase and catalase activity, and did not exhibit nitrogenase activity. The carbohydrates utilized by strain INA 01083 were arabinose, glucose, lactose, xylose, galactose, sucrose, melezitose, and trehalose. On Gause no. 2 agar medium, growth was possible at NaCl concentrations up to 10%. At 8–10% NaCl, the strain formed multicellular filaments. Thus, it was moderately halotolerant.

Gram staining of 1–4-day cultures grown on Gause no. 2 medium revealed gram-negative reaction in contradiction to the listed morphological properties of the strain. Electron microscopy, however, established the cell wall structure typical of gram-positive bacteria: a thick peptidoglycan layer above the cytoplasmic membrane and the absence of an outer membrane (data not shown).

The morphological and physiological characteristics of strain INA 01083 suggested its classification as a gram-positive spore-forming bacterium. Species identification of the isolate was carried out based on its 16S rRNA gene sequence. The DNA concentration in the preparations obtained as described in [6] was 30–50 μg/mL. RNA was present in trace amounts (less than 1% according to the results of electrophoretic analysis). A significant part of the 16S rRNA gene was determined (1401 nucleotide), corresponding to *E. coli* positions from 59 to 1449. Screening of the GenBank database demonstrated that the strain belonged to the low G+C line of gram-positive bacteria and was close to the species of bacilli of the largest phylogenetic group 1 [8]; its phylogenetic position is shown on Fig. 1.

With the highest bootstrap value (100), strain INA 01083 fell into the phylogenetic cluster formed by *B. megaterium* species, including the type strain of this species, and exhibited very high homology with their 16S rRNA gene sequences (99.6–99.9%), typical of the intraspecific level of similarity between *B. megaterium* strains (99.3–99.9%) [9]. The similarity between 16S rRNA gene sequences of strain INA 01083 and other species of the phylogenetic group 1 of bacilli [8] was significantly lower, not exceeding 94.1%. Thus, 16S rRNA gene sequencing made it possible to identify the isolate as a *B. megaterium* strain.

Since strain INA 01083 identified as *B. megaterium* possessed high antibiotic activity, comparative investigation of the antimicrobial properties of this isolate and nine *B. megaterium* strains from the All-Russian collection of industrial microorganisms (VKPM) was carried out. Antimicrobial activity against 11 test organisms was determined daily during 7-day submerged cultivation in the Gause no. 2 medium.

Under these conditions, only one of the tested ten *B. megaterium* strains (VKPM B-402) did not exhibit antimicrobial activity.

Two strains (INA 01083 and VKPM B-605) were efficient against all seven gram-positive test strains, but not against any of the gram-negative strains. Strain VKPM B-604 efficiently suppressed growth of only four gram-positive strains (*S. aureus* FDA 209P (MSSA), *S. aureus* INA 00761 (MRSA), *B. subtilis* ATCC 6633, and *B. mycoides* 537).

Strain *B. megaterium* VKPM B-4801 was efficient against *B. subtilis* ATCC 6633 and *M. luteus* NCTC 8340.

Four strains (VKPM B-600, VKPM B-602, VKPM B-607, and VKPM B-608) were active only against *B. subtilis* ATCC 6633.

Strain VKPM B-44-2 was efficient against gram-positive test organisms (three species of bacilli and *M. luteus* NCTC 8340) and gram-negative test bacteria (*P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922). None of the *B. megaterium* strains studied was active against fungal test organisms (*A. niger* INA 00760 and *Sac. cerevisiae* RIA 259). For investigation of the biosynthetic capacities of antibiotics-producing *B. megaterium*, apart from strain INA 01083, collection strains with high levels of antimicrobial activity, VKPM B-605 (against all seven gram-positive test organism) and VKPM B-603 (against four gram-positive and two gram-negative test bacteria) were selected. Antimicrobial spectra, activity levels of antibiotic compounds, and dynamics of their change in the course of cultivation were determined for these strains. Based on the results obtained, it was suggested that all three strains produced different antibiotics (Fig. 2).

Determination of the antimicrobial activity of the cultures grown in the media listed above (media 1–9) yielded additional information concerning the differences in antibiotic biosynthesis. All three strains exhibited the highest antimicrobial activity at the stationary growth phase on the fourth day of cultivation. Strain VKPM B-605 was exceptional, with the highest antimicrobial activity of the cultures grown in

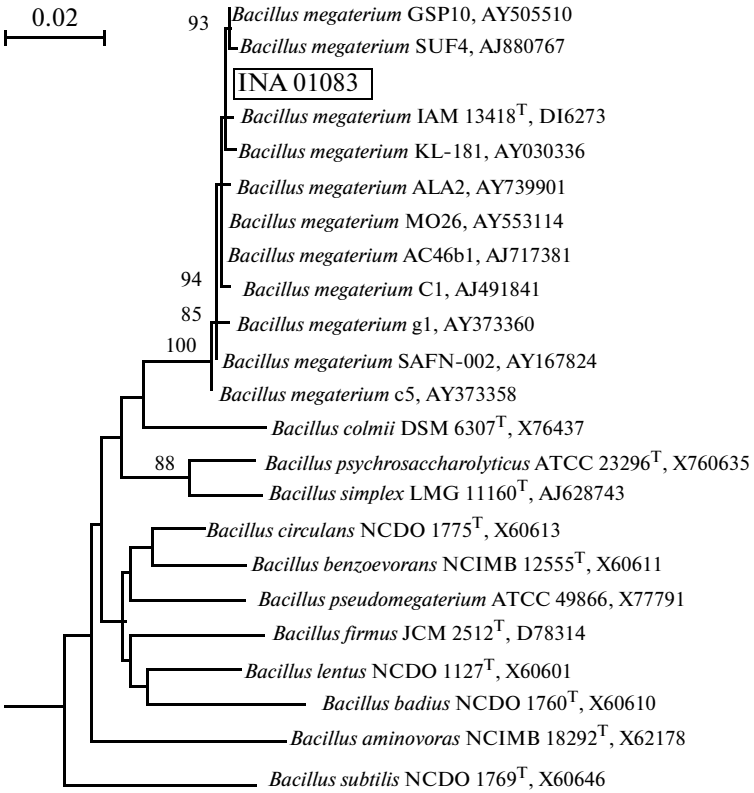

Fig. 1. Phylogenetic position of strain INA 01083. Scale bar corresponds to 2 nucleotide replacements per 100 nucleotides. The numerals indicate the branching order determined by bootstrap analysis of 100 alternative trees (values above 85 were considered reliable).

media 3, 5–9 against *L. mesenteroides* VKPM B-4177 on the 7th day of cultivation.

Strain INA 01083 grown in medium 8, exhibited antimicrobial activity on the second day of cultivation against the only gram-negative test organism, *E. coli* ATCC 25922. Strain VKPM B-603 was active against both *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 when grown on medium 1 (on the fourth day of cultivation) and, to a lesser extent, in media 2, 5, and 6. Strain VKPM B-605 exhibited no activity against gram-negative bacteria. Importantly, strain VKPM B-605 on days 2–7 of cultivation was highly active the test bacterium *L. mesenteroides* VKPM B-4177, which is highly resistant to the glycopeptide antibiotics of the vancomycin group. All three *B. megaterium* strains were active against *S. aureus* INA 00761 resistant to β-lactam antibiotics (MRSA, MPC 32 μg/mL). These results confirm the diversity of antibiotics synthesized by the three most productive *B. megaterium* strains.

For investigation of the chemical nature of antibiotics, strains INA 01083, VKPM B-603, and VKPM B-605 were grown for 4 days in media 5, 1, and 2, respectively. The antibiotics were isolated as described in Materials and Methods; their spectra of molecular decomposition, UV and IR spectra, solubility, chromatographic mobility, and amino acid composition were determined. The results of the physicochemical investigation are presented in the table.

All five antibiotics differed in UV absorption, mobility in different solvent systems (TLC), and the presence or absence of amino acids in acid hydrolysates. Three out of five antibiotics (01083, 603-2, and 605-1) were polypeptide antibiotics, probably cyclic or containing acylated amino groups since they exhibited no reaction with ninhydrin. These three antibiotics differed in molecular mass and amino acid composition.

Analysis of our results (table) and their comparison with the database of the natural biologically active compounds [10] suggest that these antimicrobial compounds (01083, 603-2, and 605-1) belong to the previously undescribed antibiotics. A number of antimicrobial compounds produced by *B. megaterium* strains in presently known. Inducible and constitutive synthesis of a number of bacteriocins (megacins) with molecular masses from 7.5 to 30 kDa has been described [11–13]. The authors suggested their similarity to bacitracins, antibiotics originally reported in the 1940s, which have been found in other *Bacillus* species (*B. licheniformis* and *B. subtilis*). These antibiotics are characterized by broad spectra of antibacterial activity [14]. Bacimethrin (2-methoxy-4-amino-5-hydroxymethylpyrimidine) [15] was among the first described

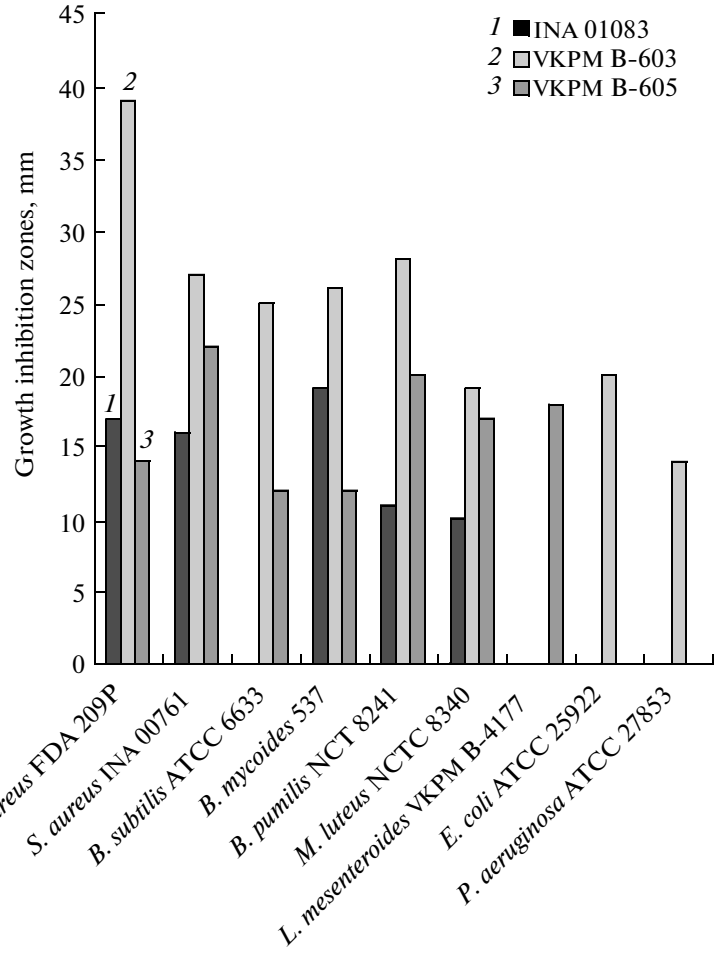

Fig. 2. Antimicrobial activity of the culture liquid of *B. megaterium* strains INA 01083 (*1*), VKPM B-603 (*2*), and VKPM B-605 (*3*) on the fourth day of growth against nine test organisms.

biologically active compounds produced by *B. megaterium*. Bacimethrin possesses antibacterial activity and is also efficient against yeasts, although at MPC exceeding 100 μg/mL [16]. The subsequently described fungitoxin produced by *B. megaterium* B-23 is efficient against fungal pathogens of jute (*Colletotrichum corchori, C. gloesporioides, Myrothecium roridum*, and *Alternaria citri*). This toxin is thermolabile and is not dialyzed. It mass spectrum (*m/z*) is 154, 167, 169, 199. The authors compared it to bacimethrin [17]. Three antibiotics of nucleotide nature (cytidines), also isolated from *B. megaterium*, possess three-amino acid radicals (glutamyl–glutamyl–seryl, glutamyl–glutamyl–glutamyl, and glutamyl–α-glutamyl–seryl), These antibiotics exhibited activity against *Helicobacter pylori* [18]. Oxetanocin, 9-[(2R,3R.4S)-3,4-bis(hydroxymethyl)-2-oxethanyl]adenine, a new compound of nucleoside nature isolated from *B. megaterium* NK 84-0218, exhibited pronounced activity against gram-positive bacteria [19]. Oxetanocin derivatives, 2-amino-OXT-A, OXT-G, OXT-H, and OXT-X, which contain 2-aminoadenine, guanine, hypoxanthine, and xanthine, respectively, as a base fragment, were also described. Apart from antibacterial activity, oxetanocin A was active against the viruses of herpes and human immunodeficiency [20, 21]. Semisynthetic oxetanocin derivatives with pronounced activity against HIV were obtained, which were more resistant to decomposition by human adenosine deaminases in the organism [22].

Thus, *B. megaterium* strains are characterized by high diversity of antimicrobial compounds, indicating the promise for new antibiotics produced by bacilli. The new antimicrobial compounds described in the present article differ from the previously described ones and are of interest primarily due to their activity against the test strains resistant to medical antibiotics (methycillin-resistant staphylococcus and glycopeptide-resistant leuconostoc).

Physicochemical properties of the antibiotics produced by *B. megaterium* strains INA 01083, VKPM B-603, and VKPM B-605 (with *B. subtilis* ATCC 6633 as a test organism)

Characteristics	Antibiotic of strain INA 01083	Antibiotic of strain VKPM B-603		Antibiotic of strain VKPM B-605	
	01083	603-1	603-2	605-1	605-2
MALDI-MS, (*m/z*) ($M+H^+$)	501.3 575.1 579.1 787.0 791.0 962.6 996.9	–	627	1601 1615 1629 1651	–
IR spectra, ν, cm^{-1}	–	–	3446 2974 2932 1728 1567 1456 1409 1381 1301 1254 1182 1135 1056 974 946 910 862 824	–	–
UV-VIS spectra (in ethanol), λ_{max}, nm	225 274 282 290	204.2 260.7	205 276	205.2 280 347.8	205.2 274.8 345.8
TLC (SiO_2)					
System 1: chloroform–methanol (9 : 1)	0.55	0.66	0.4–0.46	0.3	0.4
System 2: toluene–methanol (5 : 1)	0.4–0.5	–	0.34	0.23	–
System 3: hexane–acetone (1 : 1)	–	0.8–0.9	0.5	–	–
Qualitative reactions					
1. Ninhydrin	–	–	–	–	–
2. $KMnO_4$	+	+	+	+	+
Solubility					
Good solubility	Water, methanol, ethanol, acetone	Methanol, ethanol, acetone	Methanol, ethanol, acetone	Methanol, ethanol	Methanol, ethanol
Poor solubility	Hexane	Hexane	Hexane	Hexane, acetone	Hexane, acetone

Table. (Contd.)

Characteristics	Antibiotic of strain INA 01083	Antibiotic of strain VKPM B-603		Antibiotic of strain VKPM B-605	
	01083	603-1	603-2	605-1	605-2
Amino acid composition after acid hydrolysis of the antibiotics:					
Phenylalanine	+	–	–	+	–
Serine	+	–	–	+	–
Proline	+	–	–	+	–
Leucine or isoleucine	+	–	–	–	–
Leucin		–	–	+	–
Isoleucine		–	+	–	–
Tryptophan	+	–	–	–	–
Threonine	–	–	–	+	–
Valine	–	–	–	+	–
Alanine	–	–	+	+	–
Unidentified amino acid	–	–	+	+	–

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