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EXPERIMENTAL ARTICLES

New Antibiotics Produced by *Bacillus subtilis* Strains

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Abstract—Two *Bacillus subtilis* strains isolated from the fruiting body of a basidiomycete fungus *Pholiota squarrosa* exhibited a broad range of antibacterial activity, including those against methicillin-resistant *Staphylococcus aureus* INA 00761 (MRSA) and *Leuconostoc mesenteroides* VKPM B-4177 resistant to glycopeptide antibiotics, as well as antifungal activity. The strains were identified as belonging to the "*B. subtilis*" complex based on their morphological and physiological characteristics, as well as by sequencing the 16S rRNA gene fragments. Both strains (INA 01085 and INA 01086) produced insignificant amounts of polyene antibiotics (hexaene and pentaene, respectively). Strain INA 01086 also produced a cyclic polypeptide antibiotic containing Asp, Gly, Leu, Pro, Tyr, Thr, Trp, and Phe, while the antibiotic of strain INA 01085 contained, apart from these, two unidentified nonproteinaceous amino acids. Both polypeptide antibiotics were new compounds efficient against gram-positive bacteria and able to override the natural bacterial antibiotic resistance.

Keywords: *Bacillus subtilis*, basidiomycetes, polypeptides, polyenes, antibiotic resistance in bacteria, MRSA, resistance to glycopeptide antibiotics

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Inefficiency of antibiotics due to drug resistance of the pathogenic microorganisms is presently becoming more and more acute. One of the solutions for this problem is the search for new antibiotics that overcome the drug resistance of pathogenic microorganisms.

For decades soil, which contains a complex community of organisms belonging to various taxonomic groups, has been a major source in the search for producers of new antibiotics. Nowadays, an increasing number of works in this field is carried out using the organisms isolated from other habitats, such as benthic deposits, plants, marine invertebrates, intestines of insects, etc. [1]. In the course of our research aimed at isolation of new natural antibiotics efficient against methicillin-resistant bacteria, as well as against bacteria resistant to the glycopeptide antibiotics of the vancomycin group, basidial fungi and the microorganisms inhabiting their fruiting bodies were the subjects of the studies. It was noticed that upon cultivation of higher fungi, the accompanying microorganisms, which often formed a stable and difficult-to-separate contamination, exhibited pronounced antimicrobial activity. In the course of the present work, two bacterial strains exhibiting antibiotic activity, including activity against the above-mentioned antibiotic-resistant test cultures, were isolated.

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The goal of the present work was to determine the taxonomic position of the isolated bacteria and to characterize the antibiotics they produce.

MATERIALS AND METHODS

Subjects of the study. Bacterial strains INA 01085 and INA 01086 were obtained as stable contaminants in the course of cultivation of the basidiomycete *Pholiota squarrosa* by tissue method from the fruiting body found on an *Acer negundo* maple in Moscow.

The following microorganisms were used as test cultures for determination of antimicrobial activity: gram-positive bacteria *Bacillus subtilis* ATCC 6622, *B. pumilus* NCTC 8241, *B. mycoides* 537, *Micrococcus luteus* NCTC 8340, *Leuconostoc mesenteroides* VKPM V-4177 (glycopeptide antibiotics-resistant strain), *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, modified Gause agar medium no. 2 of the following composition was used (%): glucose, 1; peptone, 0.5; tryptone, 0.3; NaCl, 0.5; and agar, 2. The pH ranged from 7.2 to 7.4. For submerged cultivation, the same medium without agar was used. Submerged cultiva-

tion was performed on a shaker (200 rpm) in 750-mL Erlenmeyer flasks containing 100 mL medium.

Fungal test cultures and *L. mesenteroides* were grown at 28°C; all other strains were grown at 37°C. Submerged cultivation of *B. subtilis* was performed at 28°C.

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

Antimicrobial activity was determined by the agar diffusion method. The level of activity was determined from the diameter of test culture growth inhibition zones around the wells or discs.

Analysis of the nucleotide sequences of the 16S rRNA gene fragments. Bacteria were plated onto agar medium and grown for one day; individual clones were selected for DNA isolation according to the standard technique [2]. The following universal conservative primers were used for amplification of DNA of the 16S rRNA gene by polymerase chain reaction (PCR): 27f, 341f, 785f, 519r, 907r, and 1492r. The following PCR protocol was applied: (1) 94°C for 5 min, (2) 30 cycles of 1 min at 94°C, 1 min at 51°C, and 2 min at 72°C, and (3) 72° for 7 min. PCR products were analyzed by electrophoresis in 1% agarose gel at a voltage gradient of 5 V/cm. The gels were imaged using a BioDoc II (Biometra, Germany) documentation system. Isolation and purification of PCR products were performed by re-precipitation of DNA with ethanol under mild conditions (0.125 M ammonium acetate in 70% ethanol). Purified DNA fragments were sequenced on a Genetic Analyzer 3500 automated sequencer (Applied Biosystems, United States). The GenBank (www.ncbi.nlm.nih.gov) and Ribosomal Database Project (http://www.cme.msu.edu) databases were used to analyze the sequences and to construct the phylogenetic tree.

Isolation and physicochemical characterization of the antibiotics. Primary sorption of antibiotics was performed using the Amberlyte XAD-2 sorbent and subsequent elution with a mixture of *n*-butanol–acetone–water (1 : 1 : 1) at neutral pH. The eluates were evaporated to dryness under vacuum at 37°C and the dry residue was dissolved in 60% aqueous ethanol. Further purification of the antibiotics was performed on a column (14 × 180 mm) filled with Kieselgel 60 (Merck). The antibiotics were eluted in a step gradient: ethyl acetate followed by the mixture of ethyl acetate–ethanol–water (6 : 3 : 3). Each fraction obtained upon column chromatography was analyzed by UV–VIS spectrophotometry and diffusion in agar with *S. aureus* INA 0076 (MRSA) as a test organism. Biologically active fractions were combined, evaporated to dryness under a vacuum at 37°C, dissolved in 60% aqueous ethanol, and precipitated with acetone. Further purification of the antibiotics was performed by thin-layer chromatography (TLC) on DC-Alufolien Kieselgel 60 (Merck) (10 × 10 cm) in the mixture of ethyl acetate–methanol–water (40 : 15 : 15), followed by bioautography. To study the amino acid composition, acidic hydrolysis of the isolated antibiotics was performed in 6 N HCl at 105°C for 18 h, followed by TLC of the hydrolysate on 20 × 10 cm DC-Alufolien Cellulose F (Merck) plates in the system *n*-butanol–CH_3COOH–H_2O (4 : 1 : 1). Mass spectra were registered on Autoflex Bruker (Germany) equipment in the (+) ions mode using the 2,5-dihydroxybenzoic acid as a matrix. UV–Vis spectra of the antibiotics were registered on a UV-1601 PC spectrophotometer (Shimadzu, Japan).

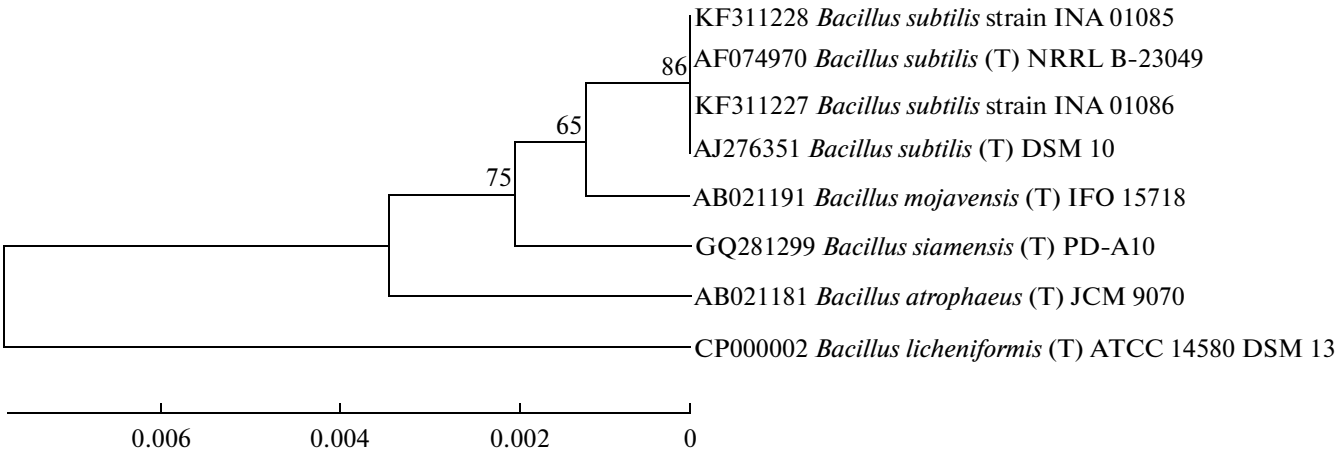
Analysis of the isolated polypeptide antibiotics of the strains INA 01085 and INA 01086 was performed by comparison of their physicochemical and biological properties with those of the antibiotics described in the literature, as well as those registered in the computer database on natural biologically active compounds (BNPD, J. Berdy, Hungary), which was initiated in 1977 [3] and is constantly updated by its authors.

RESULTS AND DISCUSSION

Both bacterial strains, INA 01085 and INA 01086, were stable contaminants of the fungal culture. Upon isolation into pure culture, they grew well on agar medium, forming beige colonies after one day of cultivation and excreting pink pigment into the medium. Both bacteria were shown to be rods, 3 × 0.5 μm in size, and exhibited positive Gram reaction. Starting from day one and until day three the bacteria formed endospores; by day four, the content of the endospores was close to 100%. In contrast to strain INA 01086 forming flat smooth colonies, strain INA 01085 grew as leathery rugose colonies.

For further study of the strains, DNA of the 16S rRNA gene was amplified by PCR and sequenced. Sequences of variable regions of strains INA 01085 and INA 01086 (1091 and 958 nucleotides long, respectively) were obtained and deposited in GenBank under respective accession numbers KF11228 and KF11227. The primary screening in the GenBank database revealed that the analyzed DNA sequences matched those of *B. subtilis* strains by 100%. Then, the sequences were aligned with appropriate sequences of the type strains of the most closely related bacterial species in the RPD database. Using the Mega 5.2.2 software, a phylogenetic tree was constructed basing on the type strains (figure).

Production of antibiotics was studied under conditions of submerged cultivation. Since the leathery growth of strain INA 01085 prevented submerged cultivation of the two strains under uniform conditions, two-stage submerged cultivation was used. For this purpose, bacteria grown on agar medium were inoculated into flasks and grown for two days. In the inocula obtained at the first stage, cell titer was determined



Phylogenetic tree showing the position of strains INA 01085 and INA 01086 constructed based on the 16S rRNA gene sequences. The scale bar indicates evolutionary distance corresponding to 2 nucleotide replacements per 1000 nucleotides. The numerals indicate the branching reliability determined by bootstrap analysis of 100 alternative trees.

and the inocula (10^5 cells/mL) were introduced into flasks with fresh medium for the second stage of cultivation. In the course of further cultivation, for nine days samples of the culture liquid were collected daily for analysis of the antibiotic activity. Culture liquids of both strains were found to contain antimicrobial compounds efficient against all the studied gram-positive test bacteria, as well as against *E. coli* ATCC 25922, with the highest level of activity against gram-positive test bacteria observed on days 2–4 of cultivation, followed by a decrease in activity and its disappearance on days 7 and 8. Antimicrobial activity of the culture liquid against *E. coli* ATCC 25922 was observed during the first three days of cultivation, reaching its peak on days 2–3 and disappearing on day 4. Strain INA 01085 also demonstrated a low-level antifungal activity against *A. niger* INA 00760 and *S. cerevisiae* RIA 259, and strain INA 01086 exhibited only traces of antifungal activity against *A. niger* INA 00760. Both strains were inactive against the strain *P. aeruginosa* ATCC 27853.

In both strains, two antibiotics of differing chemical nature were found. The first fractions eluted with ethyl acetate in the course of isolation and purification on a Kieselgel 60 column contained polyene antibiotics. Based on the UV–Vis spectrophotometric analysis, antibiotics of the following nature were established: strain INA 01085 formed a hexaene absorbing at λ_{max} (60% ethanol) 340, 362, and 385 nm, and strain INA 01086 formed a pentaene absorbing at λ_{max} 319, 336, and 355 nm. Purified raw antibiotics exhibited activity against gram-positive bacteria *B. subtilis* ATCC 6633 and *S. aureus* INA 0076 (MRSA), as well as weak activity against *A. niger* INA 00760, but were inactive against the strain *E. coli* ATCC 25922. Since both strains formed trace amounts of polyenes under the described conditions of biosynthesis, polyenes were not studied further.

Two antibiotics of polypeptide nature were further eluted from the column with the mixture of ethyl acetate–ethanol–water (6 : 3 : 3); their physicochemical properties are presented in the table. Polypeptide antibiotics of both strains were close by their amino acid compositions, but the antibiotics produced by strain INA 01085, in contrast to the one produced by INA 01086, contained two unidentified nonproteinaceous amino acids. Since negative ninhydrin reaction indicated the absence of free amino groups, it could be assumed that both antibiotics were of cyclic structure or contained acylated amino groups. The absence of reaction with potassium permanganate indicated the absence of unsaturated bonds. Positive reaction with the Pauli reagent indicated the presence of phenolic groups in the molecules; in particular, it confirmed the presence of tyrosine amino acid residue(s). Positive reaction with the Ehrlich's reagent confirmed the presence of an indole group comprising tryptophan residue. The R_f value (>5.0) evidenced the hydrophobicity of both compounds. Average yields of the polypeptide antibiotics obtained according to the adopted scheme of cultivation, isolation, and purification were 12.9 and 7.9 mg per one liter of culture liquid of strains INA 01085 and INA 01086, respectively. Both antibiotics were efficient against all of the gram-positive test bacteria studied, but inactive against fungi and gram-negative bacteria.

Since the culture liquid collected at early stages of cultivation was found efficient against the gram-negative strain *E. coli* ATCC 25922, while the isolated antibiotics did not exhibit such activity, it was assumed that both strains additionally produced an antibiotic that either was formed in insignificant amounts, or was easily degraded, or did not exhibit its activity in the purification scheme used, which was based on the control of biological activity against *S. aureus* INA 0076 (MRSA), and thus was not isolated.

Physicochemical properties of the polypeptide antibiotics produced by *B. subtilis* strains INA 01085 and INA 01086

Characteristics	Antibiotics of strain INA 01085	Antibiotics of strain INA 01086
Molecular mass, Da	2059	1505
UV–Vis spectrum (60% ethanol), λ_{max}, nm	203; 270–280	202; 224; 278–282
TLC (SiO_2, Merck, F_{254}), R_f in the systems:		
(1) methanol	0.70	0.74
(2) ethyl acetate–ethanol–water (40 : 15 : 15)	0.9	0.76
Identification test:		
$KMnO_4$	–	–
Pauli reagent	+	+
Ehrlich's ragent	+	+
ninhydrin, 0.5% in ethnol	–	–
Solubility:		
(1) good	Methanol, 60% ethanol	Methanol, 60% ethanol
(2) poor	Water, acetone, hexane	Water, acetone, hexane
Amino acid composition	Asp, Gly, Leu, Pro, Tyr, Thr, Trp, Phe, 2 unidentified nonproteinaceous amino acids	Asp, Gly, Leu, Pro, Tyr, Thr, Trp, Phe
Susceptible microorganisms	Gram-positive bacteria	Gram-positive bacteria

Among bacilli, the species *B. subtilis* is known to have the highest number of reported antibiotics of varying chemical nature, including peptides, differing by their chemical properties, mechanism of biosynthesis, and the antimicrobial action spectrum. Among the known *B. subtilis* peptides, there are comparatively large peptide antibiotics bacteriocins with molecular masses in the range of thousands of Daltons, which are characterized by a narrow antibiotic spectrum, and are known to exert their effect against other *B. subtillis* strains, that is, functioning as intercellular communication factors regulating the *B. subtilis* population [4–7]. In contrast to bacteriocins, lantibiotics are characterized by a broad range of antimicrobial activity; they also belong to the group of peptide antibiotics [8]. A large group of lipopeptide antibiotics with a wide range of activity has been described; these are oligopeptides synthesized in multienzyme complexes to which fatty acids are subsequently conjugated [9]. Rhizocticins, which were isolated as antifungal phosphono-oligopeptides from the culture liquid of *B. subtilis* ATCC 6633 [10], belong to oligopeptides of the lowest molecular mass containing two to three amino acids.

From the practical point of view, *B. subtilis* antibiotics may find varied applications, including medical ones. A peptide antibiotic bacitracin, described among the first ones as early as 1945 and produced by a *B. subtilis* strain isolated from a human wound [11], is still used in medicine. A number of closely related antibiotics forming a bacitracin complex is presently known [12]. Due to its low toxicity, bacitracin has been approved in 2010 for treatment of staphylococcus infections in newborns in the United States [13]. Combination of bacitracin with neomycin was efficient against most clinical isolates of resistant staphylococci (MRSA), which is important for modern antimicrobial therapy [14].

Since *B. subtilis* is a cosmopolitan species occurring in various substrates, it is characterized by high adaptability to various environmental conditions, which is particularly manifested in the wide range of antibiotics produced. The described antibiotics of the strains INA 01085 and INA 01086 differ from the known peptide antibiotics and thus belong to the previously unknown natural compounds. Important is the efficiency of the newly isolated antibiotics against bacteria resistant to antibiotics of the beta-lactam and glycopeptide groups. Both producer strains were isolated from a single source, a fruiting body of a basidial fungus, and their antibiotics are similar in amino acid composition, which is an additional indication of the high biosynthetic potential of the species and its variability. Discovery of new antibiotics produced by *B. subtilis* strains and by closely related species of the "*B. subtilis*" complex may thus be expected.

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