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EXPERIMENTAL ARTICLES

New Metabolites of *Azotobacter vinelandii* Exhibiting Antifungal Activity

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Abstract—New metabolites exhibiting antifungal activity were isolated from the culture liquid of *Azotobacter vinelandii* strain IB 4. The metabolites were characterized by IR and ^{13}C-NMR spectroscopy and defined as sucrose polythiophosphates of tetraamine (α-D-2,3-diaminoglucopyranosyl-β-D-3,4-diaminofructofuranose).

Key words: antifungal activity, *Azotobacter vinelandii*, antibiotics.

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Since bacteria of the genus *Azotobacter* conform to most of the requirements for bacteria promoting plant growth, they possess significant potential for development of microbiological products. The major issue in production of efficient biofertilizers on the basis of azotobacter is the search for strains possessing a complex of advantageous characteristics, namely a high rate of dinitrogen fixation, wide range of antagonistic activity towards phytopathogens, and the ability to produce vitamins and growth factors [1].

Production of antibiotics by bacteria is a major factor affecting phytopathogenic organisms; it is, however, a poorly investigated aspect of azotobacter antagonism. Literature data are available on a single antifungal agent produced by *Azotobacter chroococcum* 92 composed of a methyl ester of a tetraenoic aliphatic acid. The antibiotic was shown to suppress growth of such phytopathogenic fungi as *Bipolaris sorokiniana, Botrytis cinerea, Pythium debarianum, Verticillum dahliae*, and *Fusarium* sp. [2, 3].

The aim of the present work was to study the chemical structure of new metabolites of *Azotobacter vinelandii* possessing antifungal activity and to characterize their effect on phytopathogenic micromycetes.

MATERIALS AND METHODS

Azotobacter vinelandii IB 4 obtained from the Collection of Microorganisms of the Ufa Scientific Center, Russian Academy of Sciences was the subject of the work. The strain is known to possess antagonistic activity against a number of plant pests and a complex of other characteristics mentioned above [4, 5].

Bacteria were cultivated for 3 days at 28°C on a shaker (170 rpm) in 250-ml flasks with 100 ml of the medium containing the following (g/l): sucrose, 20; yeast extract, 0.5; K_2HPO_4, 0.8; KH_2PO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.2; $FeCl_3$, 0.0016; and Na_2MoO_4, 0.001 [6].

Active metabolites were isolated from the culture liquid by ultrafiltration on Amicon 3P-10 fiber modules (United States) followed by a 20-fold concentration by vacuum evaporation at 40°C. Then, the metabolites of interest were precipitated from the concentrate by an equal volume of methanol.

Purity of the isolated metabolites was controlled by HPLC on an apparatus consisting of a 572P high-pressure pump (Gasukuro Kogyo, Japan) and a refractive index detector (Du Pont, United States). A stainless steel column of HP-NH_2 adsorbent (5 μm; 250 × 4.6 mm) was used for separation. A mixture of acetonitrile–water (75 : 25) was used as an eluent at a flow rate of 1 ml/min.

Elemental composition of the metabolites was determined on an HP Model 185 B (United States) C, H, N-analyzer.

IR spectra were recorded on a Specord M80 (Carl Zeiss, Germany) spectrophotometer in the range of 400–4000 cm^{-1} (in mineral oil).

^{13}C-NMR spectra were registered on a Bruker AMX-III-300 (Germany) apparatus at a frequency of 300.13 MHz using tetramethylsilane as an internal standard and D_2O as a solvent.

The following organisms were used to test antifungal activity: *Fusarium gibbosum* VKM 848, *F. culmorum* VKM 844, *F. graminearum* VKM 1668, *F. nivale* VKM 3106, *F. avenaceum* VKM 132, *F. semitectum* VKM 1938, *F. solani* VKM 142, *Alternaria alternata* (Fr.) Keissl. IB-1, and *Bipolaris sorokiniana* (Sacc.) Shoemaker (= *Helminthosporium sativum* Pam., King et Bakke).

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Antagonistic effect against phytopathogenic fungi was initially determined by co-cultivation of bacteria and phytopathogens in petri dishes on a potato–glucose agar [6], which is an optimal medium for growth of both fungal and *Azotobacter* cultures. Suspensions of the spores of the test fungus were inoculated onto an agarized medium and then the culture under study was stab-inoculated. The plates were incubated at 28°C. Antagonism was revealed visually by the presence of a zone of inhibition of fungal growth surrounding bacterial colonies.

The activity of isolated metabolites towards various phytopathogenic fungi was assessed by the dilution method.

Morphological characteristics of developing phytopathogens were investigated with an Amplival 30-G048a (Carl Zeiss, Germany) light microscope at ×480 magnification.

RESULTS AND DISCUSSION

The *Azotobacter* strain under investigation revealed a wide spectrum of antagonistic activity against phytopathogenic fungi of the genus *Fusarium* (Table 1). Significant inhibition of mycelium development compared to the control and a delay of 24–78 h in spore germination was observed in the case of direct interaction between the azotobacter strain and the test fungi, and the fungal hyphae were characterized by distinct changes in morphology (Figs. 1a and 1b). It is to be emphasized that the intensity of the effect on the pathogen was determined by the radial gradient of the metabolite concentration of antagonist bacteria; normal mycelium developed beyond the effective area of metabolites, and the closer the bacterial colony was, the more anomalies were observed. In the presence of metabolites of the antagonistic strain, a deceleration of the development of growth tubes was observed in fusaria. In turn, impaired hyphae development led to over-branched, often segmented mycelium resembling a string of beads (Fig. 1b). A similar effect was observed by other researchers studying the interaction between *Fusarium* and antagonistic bacilli [7].

Table 1. Antifungal activity of *A. vinelandii*

Phytopathogenic fungi	Diameter of the growth inhibition zone, mm
F. culmorum	19.0 ± 2.5
F. gibbosum	18.0 ± 2.2
F. graminearum	14.4 ± 1.4
F. nivale	9.5 ± 0.5
F. semitectum	13.2 ± 1.1
F. solani	8.0 ± 0.5
F. avenaceum	19.0 ± 2.3
Bipolaris sorokiniana	12.0 ± 2.1
Alternaria alternata	20.0 ± 2.0

As the nature of antifungal metabolites of *Azotobacter* is poorly studied, in the present work we attempted to isolate the active antibiotics of the strain and to study their structure.

In the medium chosen for cultivation of azotobacter, a single low-molecular weight metabolite was synthe-

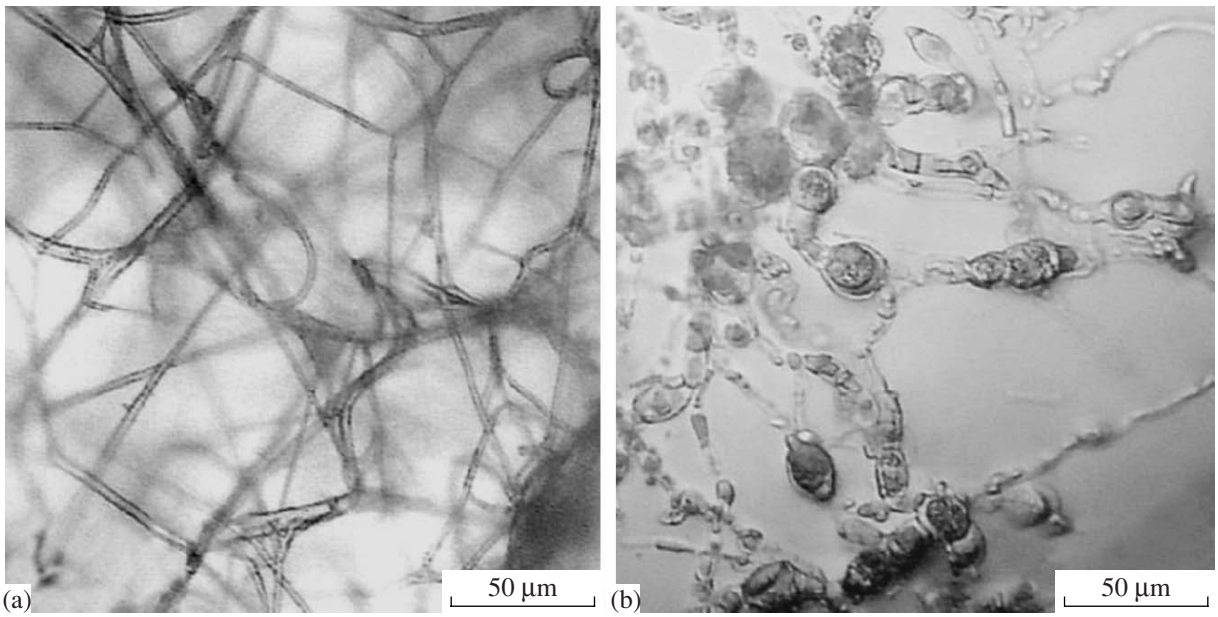

Fig. 1. Effect of *A. vinelandii* metabolites on the development of *F. culmorum*: control (a) and in the presence of metabolite (b).

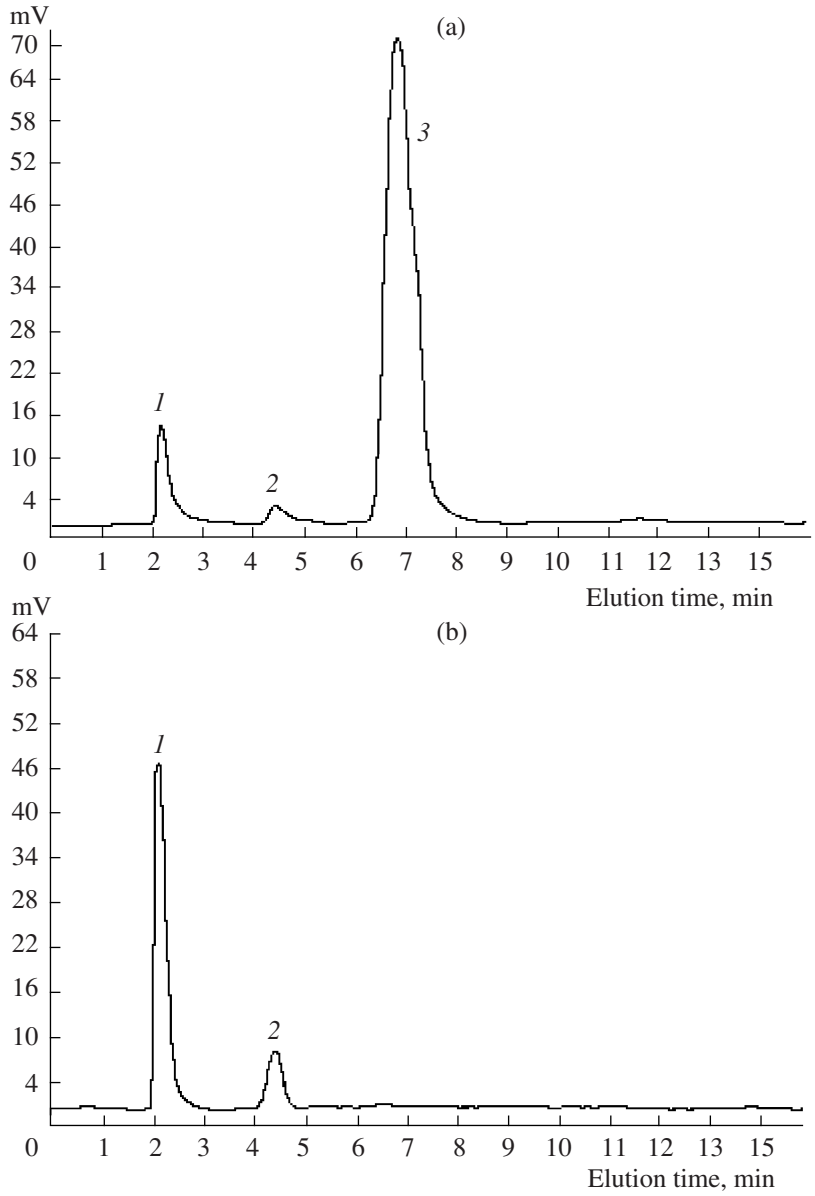

Fig. 2. Characteristic chromatographic profiles of the fractions obtained upon evaporation (a) and of isolated *A. vinelandia* metabolites (b) exhibiting antifungal activity: water (*1*), metabolite (*2*), and residual sucrose (*3*).

sized, which simplified the isolation procedure and chromatographic evaluation of purity of the substance (Fig. 2b) with residual sucrose being the only contaminant. Traces of sucrose were identified based on the characteristic chromatographic profile of the fraction obtained after evaporation (Fig. 2a). The metabolite yield was 1.2 ± 0.2 g/l of the cultivation medium; this value coincided with the maximum solubility of this metabolite in water.

Table 2. Element composition of *A. vinelandii* metabolites exhibiting antifungal activity

Element content, %				
Carbon	Hydrogen	Nitrogen	Sulfur	Phosphorus
3.5	1.7	1.5	5.1	34.2

By method of dilutions, the maximum inhibiting concentration of metabolites against a number of *Fusarium* species was determined as 0.5–0.8 mg/ml, which is comparable to typical inhibiting concentrations for pseudomonad antibiotics [8, 9] and bacillar peptide factors [10]. The effect of metabolites was of the same nature as of the antagonism exhibited by bacteria upon joint cultivation with fungi (Fig. 1b).

The isolated metabolites were investigated by spectroscopy methods. The presence of chemical shifts at 62.2, 63.0, 66.8, 71.7, 71.8, 78.9, and 106.1 ppm in the

Table 3. Spectral analysis data of *A. vinelandia* metabolites exhibiting antifungal activity

^{13}C-NMR data		IR spectroscopy data	
Chem. shift, experimental, ppm	Chem. shift, calculated, ppm	Wave number of the IR spectrum, cm^{-1}	Functional group
62.2	62.2	2800–3000	NH_2–
63.0	62.3	724	C–H
66.8	67.7	1296	CH_3–X (X = P, S)
71.7 and 71.8	71.8 and 71.9	3200–3300	O–H
74.8 and 76.5	74.7		
78.9			
83.4 and 83.8			
94.6	92.3		
106.1	103.2		

^{13}C-NMR spectrum supplemented by the data of elemental analysis is evidence that the major component of the metabolites is a sucrose tetraamine molecule (the presence of amino groups was confirmed by characteristic absorption bands in the IR spectrum at 2800–3000 cm^{-1}). Since no phosphate or sulfate ions were detected in the samples, considering the results of the elemental analysis (Table 2) and bands in the IR spectrum characteristic of P- and S-bonds (1296 cm^{-1}) (Table 3), a polythiophosphate tail was proposed to be a part of the metabolite structure. Thus, the supposed structure of the metabolites is α-D-2,3-diaminoglucopyranosyl-β-D-3,4-diaminofructofuranose polythiophosphates (Fig. 3).

The presence of a polythiophosphate tail is in agreement with the data that *Azotobacter* grown on molecular nitrogen synthesizes significant amounts of high-molecular weight acid-insoluble polyphosphates serving as a phosphate and energy reservoir. Upon activation of biosynthesis or under conditions of phosphate limitation, accumulated polyphosphates may be used in the synthesis of various organic compounds [11], and probably, for antibiotic substances.

Thus, new metabolites consisting of sucrose (α-D-2,3-diaminoglucopyranosyl-β-D-3,4-diaminofructofuranose) tetraamine polythiophosphates exhibiting antifungal activity were isolated from the culture liquid of *A. vinelandii*. The mode of action of the metabolites on the development of fungal phytopathogens is similar to the effect of antifungal antibiotics produced by pseudomonads and bacilli which target the process of the cell wall synthesis in fungi.

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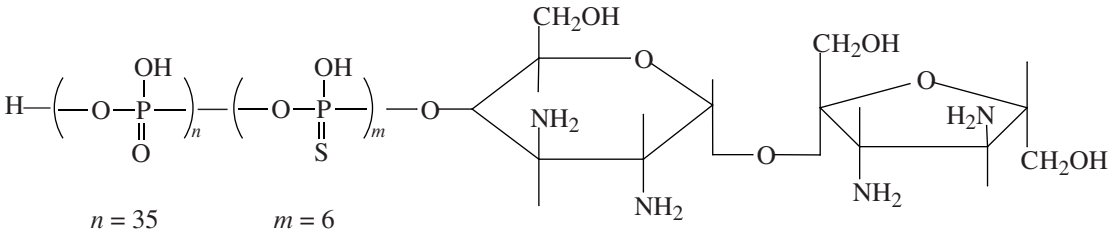

Fig. 3. Possible structure of the metabolites produced by *A. vinelandia* and exhibiting antifungal activity.

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