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Bacillus subtilis and Phenotypically Similar Strains Producing Hexaene Antibiotics

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Abstract—We studied metabolites synthesized by *Bacillus subtilis* strains, including the type strain of *B. atrophaeus* and phenotypically similar cultures. These metabolites were represented by polyene antibiotics with conjugated double bonds. Hexaenes from the strains under study inhibited the growth of phytopathogenic fungi *Fusarium culmorum*, *F. sporotrichiella*, *F. oxysporum*, *Botrytis sorokiniana*, *Alternaria tenui*, and *Phytophthora infestans*. The degree of growth inhibition depended on the test fungus.

Polyene antibiotics constitute the main group of antibiotics with antifungal activity [1]. Specimens of the genus *Streptomyces* are major producers of polyenes [2, 3]. The polyene bacillen is synthesized by *B. subtilis* strain ATCC 55422, exhibits selective bacteriostatic activity, and has no effect on *Saccharomyces cerevisiae* and *Candida albicans* [4].

The species of *B. subtilis* (Ehrenberg 1835) Cohn 1872 produces a variety of polypeptide antibiotics that affects mainly gram-positive and gram-negative bacteria [5]. Some polypeptide or peptide antibiotics synthesized by several strains of *B. subtilis* (antibiotics of the bacillomycin group, iturin, fungistatin, mycobacillin, mycosubtilin, etc.) impair the development and inhibit the growth of phytopathogenic fungi [5, 6].

This work was designed to study antibiotics synthesized by cultures of *B. subtilis* and *B. atrophaeus* and phenotypically similar strains.

MATERIALS AND METHODS

Experiments were performed with cultures obtained from the All-Russia Collection of Microorganisms (VKM), belonging to the same phylogenetic cluster: type strain of *B. subtilis* VKM B-501, VKM B-759, VKM B-721, VKM B-722, and *B. atrophaeus* Nakamura 1989 VKM B-763. Phenotypically similar strains of *Bacillus* sp. (s-1c, s-2t, s-4, and 21/2) were obtained from the working collection.

Strains s-1c, s-2t, s-4, and 21/2 were routinely characterized by cultural, morphological, physiological, and biochemical traits [7–10].

To study antifungal metabolites, bacilli were cultivated in a liquid nutrient medium used in growing *B. subtilis* strain ATCC 55422. It produces the hexaene antibiotic bacillen. The medium included 15 g/l heated soybean flour, 15 g/l soluble starch, 50 g/l glucose, 0.005 g/l $CoCl_2 \cdot 6H_2O$, 10 g/l $CaCO_3$, and 1 l distilled water [4]. Cultivation was performed in 750-ml flasks with 50 ml nutrient medium using a stir plate at 28°C and 180 rpm for 72 h. The titer of microbial cells in the culture liquid (CL) was estimated by inoculation of nutrient agar (BBL, Sigma) with serial dilutions. It corresponded to $4.4 \pm 0.6 \times 10^5$ cells/ml.

To isolate the hexaene-containing fraction, CL was centrifuged at 4500 *g* for 15 min. The supernatant was divided into three parts. The first part was acidified with diluted HCl (ratio 1 : 1) to pH 2.5–3.0. The second part was alkalized with ammonia to pH 8.5–9.0. And the third part was treated with ammonia to pH 7.0. Polyenes were extracted with ethyl acetate. The pellet of the biomass was suspended in water and centrifuged. Ethyl acetate was added to washed cells. Metabolites were extracted for 1 h under shaking.

Extracts were dried above anhydrous sodium sulfate, filtered, and stripped to dryness on a rotor evaporated at a temperature of below 40°C. The content of polyenes was determined by measuring the absorption of methanol-treated samples on a UV-160A spectrophotometer (Shimazu, Japan) at 382 nm.

Antifungal activity of strains was studied by the method of paper discs. Activity of extracts was estimated by the method of diffusion in agar [10].

Antifungal activity of polyenes was studied with extracts obtained from CL at an acid pH value. The absence of protein components in samples was verified by the method of Bradford. An aliquot part of the concentrate containing 7×10^{-3} mg hexaene was placed on the disc. A similar extract from the nutrient medium served as the control.

Saccharomyces cerevisiae VKM Y-375 and phytopathogenic fungi *Fusarium culmorum* strain 27, *F. sporotrichiella* strain 3s, *F. oxysporum* strain 11, *Botrytis sorokiniana* strain 1, *Alternaria tenui* strain 148/1, and *Phytophthora infestans* strain T.21 were obtained from the All-Russia Institute of Phytopathology (Rus-

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sian Academy of Agricultural Sciences) and served as the test objects. They were incubated at 24°C for 4 days and maintained at room temperature over 30 days to study the stability of antifungal agents. The effects of CL and extracts were estimated by the area of the growth inhibition zone for the test fungus.

RESULTS AND DISCUSSION

Strains *s*-1c, *s*-2t, *s*-4, and 21/2 were phenotypically similar to the species of *B. subtilis* by their cultural, morphological, physiological, and biochemical characteristics. The strains under study were presented by gram-positive single and chained rods (0.7–0.8 × 2–3 μm) with the nonswollen sporangium and exocentrically localized oval spores. On meat–peptone agar they formed dense small-wrinkle colonies with incised edges. Growth on a liquid nutrient medium at 28°C for 24 h was accompanied by the formation of a film on the medium surface and flaky suspension (or flaky pellet). Physiological-and-biochemical studies showed that these strains produce proteases (hydrolysis of casein and fluidization of gelatin), amylase (hydrolysis of starch to dextrins), lysin decarboxylase, arginine dehydrolase, ornithine decarboxylase, phenylalanine deaminase, urease, and β-galactosidase [8, 10]. They reduced nitrates to nitrites. Acetone was revealed in the Voges–Proskauer test. The strains under study used citrate as a carbon source (not propionate). They grew in the medium with 7% NaCl but not under anaerobic conditions.

The strains under study possessed antifungal properties. They completely or partially inhibited the growth of phytopathogenic fungi tested (fungal growth as individual colonies in agar). The area of the growth inhibition zones varied from 7 to 65 mm. Growth of *B. sorokiniana* and *A. tenui* was inhibited most significantly.

We studied the metabolites of the strains. They produced polyene antibiotics with six conjugated double bonds. This was confirmed by the location of absorption maxima in the UV spectrum. The UV spectrum of metabolites was similar for the strains under study and included three bands with λ_{max} 343, 362, and 382 nm (Fig. 1). Similar results were obtained in previous studies of bacillen [4].

Hexaenes from the strains were extracted from acid, neutral, and alkaline solutions with ethyl acetate. They fluoresced upon exposure to UV light with a wavelength of 365 nm. These compounds were revealed by means of thin-layer chromatography (system of solvents containing chloroform and methanol in the 4 : 1 ratio) on Silufol plates at $R_f \times 100$ and 60–70 nm. A functional study showed that molecules of study compounds do not contain carbonyl groups (test with 2,4-dinitrophenylhydrazine), amino acid residues (test with ninhydrin), or aldoses (test with benzidine).

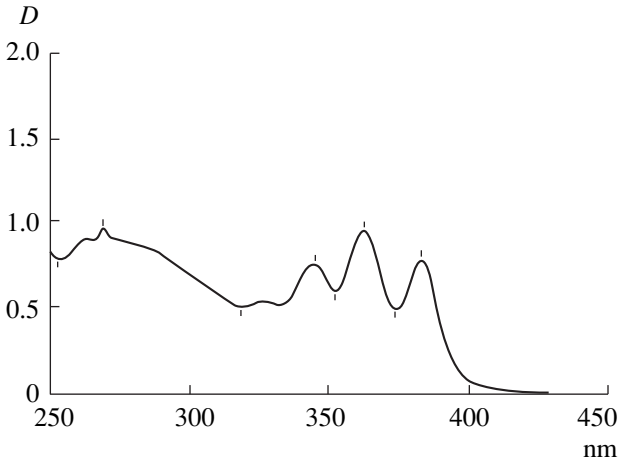

Fig. 1. UV spectrum of hexaenes from the type strain of *B. subtilis* VKM B-501.

Since hexaenes were revealed in the nutrient medium, it may be suggested that the molecules of these hydrophobic compounds incorporate carboxyl and/or hydroxyl groups. Previous studies showed that bacillen has the carboxyl and two ester groups [4]. Some polyene antibiotics synthesized by representatives of the genus *Streptomyces* have a macrocyclic lactone ring, considerable numbers of hydroxyl groups (including those conjugated with the double bond), hydrocarbon radicals and, sometimes, carbohydrates attached to the macrolide via glycoside bonds [2, 3].

Polyene antibiotics are unstable. The structure of most compounds, including bacillen, remains unknown. Hexaenes were preserved in CLs of various strains at 0°C over the period of study (6 months). However, purification of compounds was accompanied by their partial degradation, which was manifested in a decrease in the amount of substances and the appearance of additional bands in the UV spectrum of samples

Table 1. Concentration of hexaenes in cells and supernatant of strains under study on day 4 of cultivation

Strain	Hexaene concentration, mg/l	
	cells	supernatant
VKM B-501	5	6
VKM B-759	6	19
VKM B-763	12	20
s-2c	11	24
s-2t	18	48
s-4	10	44
21/2	10	6

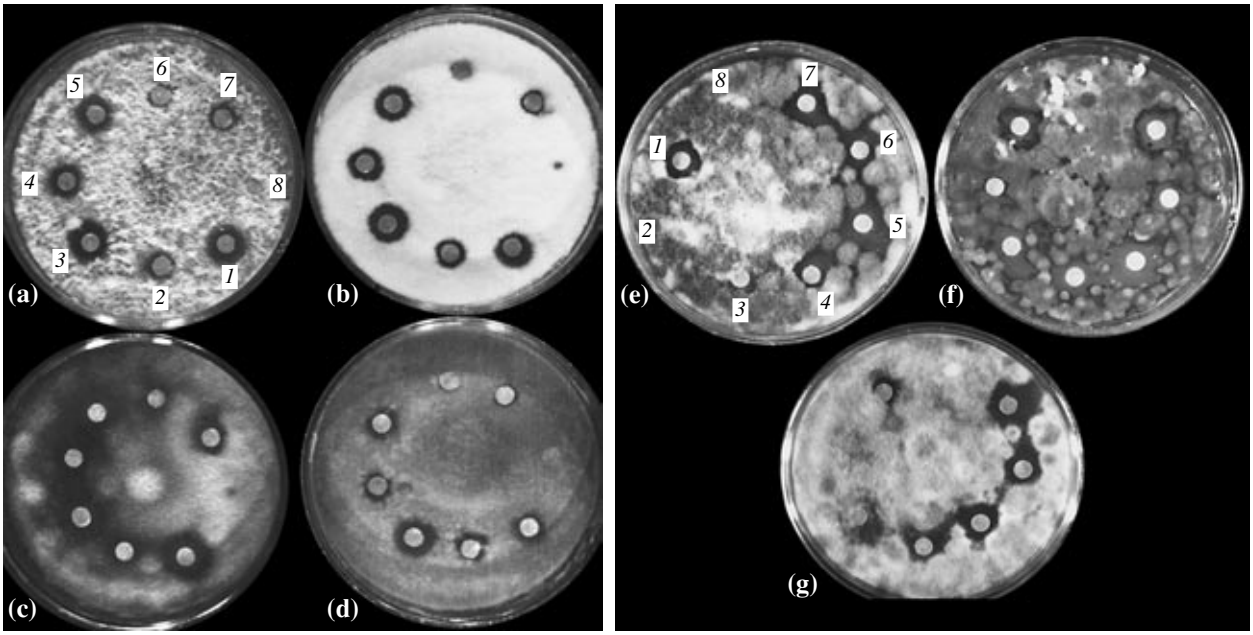

Fig. 2. Antifungal effect of polyene antibiotics from strains (*1*) s-4, (*2*) VKM B-763, (*3*) VKM B-759, (*4*) s-2c, (*5*) s-2t, (*6*) VKM B-501, and (*7*) 21/2 on test fungi (a) *P. infestans*, (b) *A. tenui*, (c) *F. oxysporum*, (d) *F. culmorum*, (e) *A. tenui*, (f) *B. sorokiniana*, and (g) *F. sporotrichiella* on day 4 of cultivation. (*8*) Control.

(not typical of hexaene). Hexaenes from strains *s*-2t and *s*-1c were particularly unstable. Due to the absence of pure hexaenes from each study strain, it was impossible to determine whether we isolated one compound or a group of related metabolites. However, the long-wavelength region in the UV spectrum of compounds included a system of bands typical of hexaenes. Other metabolites do not appear in this area. This allowed us to study these compounds in solutions and perform a qualitative analysis.

The concentration of hexaenes produced by the cultures under study was calculated taking into account the data for pentaene filipin (MM 571, $E^{1\%}_{1cm}$ 1330 at λ^{MeOH}_{max} 355) [12] and bacillen (MM 580) [4].

Study strains grown in the nutrient medium did not release hexaenes completely. Part of these compounds remained in the cells (Table 1). The productivity of strain s-2t was the highest. The content of hexaenes reached 66 mg/l on day 4 of cultivation. The type strain of VKM B-501 synthesized the lowest amount of hexaenes.

The specific antifungal effect of polyene antibiotics is associated with the mechanism for their biological activity. Polyenes react with ergosterol in the fungal cytoplasmic membrane and impair its molecular structure [1].

Assessment of extracts from the strains under study in petri dishes by the method of paper discs showed that they do not inhibit the growth of *S. cerevisiae* ascomycetes (similarly to bacillen, Table 2) [4]. However, these extracts exhibit activity in mycelial fungi (Table 2). Zones of complete or partial inhibition of the growth of the phytopathogenic fungi tested were observed on day 4 of cultivation. Antifungal activity of the samples varied, depending on the test fungus and the producer strain. This was probably related to the specific chemical structure of hexaenes. Growth of *B. sorokiniana*, *A. tenui*, and *F. culmorum* was inhibited most significantly. Study of strain *s*-2t showed that the zone of *B. sorokiniana* growth inhibition reaches 29 mm. The extract of polyene antibiotics from strain VKM B-759 produced a stronger antifungal effect on *F. culmorum*. The growth-inhibition zone was 25 mm. Study strains had the same effect on *A. tenui*.

The effect of some extracts became less significant by the end of observations (30 days). This was manifested in an increase in the growth inhibition zone for fungi. These changes were probably associated with a decrease in the concentration of antibiotics due to partial degradation (Table 2).

Our results indicate that strains of the species *B. subtilis* (including the type strain), *B. atrophaeus*, and phenotypically similar cultures of *Bacillus* sp. synthesize polyene antibiotics (hexaenes) with activity against phytopathogenic fungi *Fusarium culmorum*, *F. sporotrichiella*, *F. oxysporum*, *Botrytis sorokiniana*, *Alternaria tenui*, and *Phytophthora infestans*. The degree of growth inhibition depended on the test fungus.

Table 2. Antifungal activity of polyene antibiotics from strains under study after 4-day incubation and 30-day storage at room temperature

Test fungus	Growth inhibition zones for test fungus, mm															
	VKM B-501				VKM B-759				VKM B-763				s-2c			
	4 days		30 days		4 days		30 days		4 days		30 days		4 days		30 days	
	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II
F. culmorum	11	17	–	6	25	–	23	–	19	–	17	–	–	8	–	8
F. sporothiella	11	–	9	–	10	–	9	–	–	11	–	8	–	13	–	11
F. oxysporum	–	11	–	11	11	–	–	11	–	11	–	11	–	11	–	11
B. sorokiniana	15	–	15	–	11	–	10	–	8	–	8	–	14	–	14	–
A. tenui	–	15	–	15	10	–	10	–	14	–	14	–	11	–	10	–
P. infestans	–	11–15	–	12	15	–	15	–	15	–	15	–	–	12	–	10

Test fungus	Growth inhibition zones for test fungus, mm											
	s-2t				s-4				21/2			
	4 days		30 days		4 days		30 days		4 days		30 days	
	I	II	I	II	I	II	I	II	I	II	I	II
F. culmorum	13	–	11	–	13	–	10	–	19	–	19	–
F. sporothiella	–	11	–	9	–	7	–	5	–	7	–	–
F. oxysporum	–	9	–	9	–	10	–	7	–	9	–	7
B. sorokiniana	29	–	28	–	14	–	14	–	24	–	24	–
A. tenui	14	–	14	–	11	–	9	–	11	7	7	4
P. infestans	–	8–9	–	8–9	15	9	9	–	–	8–10	–	9

Note: I, zone of complete inhibition of fungal growth; II, zone of partial inhibition of fungal growth; –, absence of fungal growth inhibition zone.

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