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EXPERIMENTAL ARTICLES

The Chitinolytic Activity of *Bacillus* Cohn Bacteria Antagonistic to Phytopathogenic Fungi

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Abstract—Among the 70 tested *Bacillus* spp. strains antagonistic to phytopathogenic fungi, 19 were found to possess chitinolytic activity when grown on solid media with 0.5% colloidal chitin. The chitinolytic activity of almost all of these 19 strains grown in liquid cultures ranged from 0.1 to 0.3 U/ml. One of the 19 strains exhibited exochitinase activity. In addition to chitinase, two strains also produced chitosanase and one strain, β-1,3-glucanase. No correlation was found between the antifungal activity of the bacillar strains studied and their ability to synthesize extracellular chitinase. Among the 19 chitinolytic strains, the correlation between these parameters was also low ($r_{x,y}$ = 0.45), although the enzymatic preparations of most of these strains inhibited the growth of the phytopathogenic fungus *Helminthosporium sativum.*

Key words: antagonism, antifungal activity, mycolytic enzymes, chitinase, bacilli, biocontrol.

The microbial producers of mycolytic complexes are of great importance in the biocontrol of phytopathogenic fungi. Chitinase (EC 3.2.1.14), one of the key enzymes of the mycolytic complexes of soil saprophytes, provides them with nutrients. Some researchers believe that chitinase is involved in the suppression of phytopathogenic fungi by antagonistic bacteria [1–5]; however, relevant data in the literature is ambiguous. On the one hand, there is evidence that some bacterial chitinases can efficiently suppress the fungal diseases of agricultural plants [2, 3]. On the other hand, other bacterial chitinases were reported to exhibit low, if any, antifungal activity [6–8]. This discrepancy can be explained by the different susceptibility of fungal strains (even of one species) to chitinases and the use of crude chitinase preparations in researches, in which case it is difficult to take into account the effect of other hydrolytic enzymes and low-molecular-weight antibiotic substances likely to be present in such preparations [4, 9–10].

Of great interest is the role of chitinases in the competitive interactions of soil bacteria (such as the well-known chitinolytic bacteria *Bacillus* Cohn) with soil micromycetes. Unfortunately, relevant investigations have primarily dealt either with the antagonistic properties of bacilli or with their chitinolytic activity [11, 12], but have disregarded the study of correlation between these parameters. The rare exception is the work of Frändberg and Schnürer [13], who analyzed the antifungal and chitinolytic activities of diverse bacterial isolates and found that there was no correlation between these activities when the bacterial isolates and fungi were grown on the same plates. Only 4% of the chitinolytic isolates exhibited antifungal activity, all susceptible fungi being streptomycetes.

The aim of this work was to study the correlation between the chitinolytic and antifungal activities of a large group of *Bacillus* Cohn bacteria.

MATERIALS AND METHODS

Experiments were carried out using 70 *Bacillus* spp. strains with antifungal activity, which were obtained from the collection at the Institute of Biology, Ufa Research Center. Forty-eight of these strains belonged to the species *Bacillus subtilis*; thirteen, to the species *B. polymyxa*; and nine, to *Bacillus* spp. unidentified at the species level. The description and some characteristics of these strains can be found in the report [14]. Nineteen of the 70 bacillar strains (18 *B. subtilis* strains and *Bacillus* sp. IB-30) were found to possess chitinolytic activity.

The antagonistic activity of the bacillar strains was evaluated using phytopathogenic micromycetes from the aforementioned collection and the collection at the All-Russia Institute of Plant Protection: *Helminthosporium sativum* Pam., King et Bakke (= *Drechlera sorokiniana* (Sacc.) Subram. et gain or *Bipolaris sorokiniana*), *Fusarium solani* Mart. App. et Wr., *F. oxysporum* (Schlecht.) Snyd. et Hans., *F. culmorum* (W.G. Smith) Sacc., *F. moniliforme* Sheild., *F. graminearum* Schwabe, *F. avenaceum* (Fr.) Sacc., *F. sporotrichiella* Bilai var. *sporotrichioides* (Sherd.) Bilai, *F. nivale* (Fr.) Ces., and *F. sambucinum* Fuck. The antifungal activity

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Table 1. The chitinolytic and antifungal activities of various bacillar strains grown on agar plates

Strain	D_z/D_c on the 6th day**	Increase in D_z (mm/day)*	Area (in mm^2) of sterile zone around bacillar colonies on an *H. sativum* lawn**	Number of inhibited fungal species
B. subtilis IB-14	2.7	3	115.6	4
B. subtilis IB-21	2.1	5.3	150.9	13
B. subtilis IB-23	1.1	5.7	–	ND
B. subtilis IB-24-2	1.9	4	–	2
B. subtilis IB-26-a	1.4	5.7	120.9	6
B. subtilis IB-26-b	2.0	3.7	91.8	2
Bacillus sp. IB-30	2.0	2.3	143.5	7
B. subtilis IB-31	2.2	3	157	3
B. subtilis IB-33-1	1.6	3.7	65.3	6
B. subtilis IB-33-2	3.0	4	136.6	12
B. subtilis IB-34-a	2.1	3.7	129.5	7
B. subtilis IB-34-b	1.2	3.7	–	ND
B. subtilis IB-34-c	2.9	1.7	75.4	1
B. subtilis IB-34-d	2.3	3.7	96.8	ND
B. subtilis IB-35	2.2	2.3	144.2	12
B. subtilis IB-36	2.3	3	75.6	9
B. subtilis IB-39-1	1.5	4	133.4	9
B. subtilis IB-39-3	1.6	5	38.3	2
B. subtilis IB-47	1.3	5.3	150.1	5

Note: D_z/D_c is the ratio of the diameter of the zone of colloidal chitin hydrolysis around a colony to the colony diameter (a measure of the relative chitinolytic activity of strains). ND stands for "not determined." * and ** mark data obtained on agar plates with 0.5% colloidal chitin and potato–glucose agar, respectively.

of chitinase preparations was mainly estimated using the fungus *H. sativum* Pam., King et Bakke.

The bacillar strains were maintained on a solid nutrient medium containing (g/l) insoluble potato starch, 10.0; yeast extract, 3.0; peptone, 3.0; corn extract, 3.0; $(NH_4)_2HPO_4$, 2.0; KH_2PO_4, 2.0; and agar, 16.0.

The phytopathogenic fungi were maintained on Czapek agar and potato–glucose agar (PGA).

The antifungal activity of the bacillar strains was assayed by inoculating them onto PGA plates, which were preliminarily inoculated with spores of a test fungus. The plates inoculated with the test fungus and one of the tested bacillar strains were incubated at 28°C for 3–7 days, after which the fungistatic activity of the tested bacillar strain was evaluated as the diameter of the zone of inhibited fungal growth around bacillar colonies.

The chitinolytic activity of the bacillar strains was evaluated both on plates with the agar medium supplemented with colloidal chitin and in liquid cultures containing colloidal chitin as the major carbon source. These methods are described in detail in our previous publication [15]. The composition of the chitinolytic complexes of the bacilli and the activity of their components were studied using the following chromogenic derivatives of *N*-acetyl-D-glucosamine and chitooligosaccharides: *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, *p*-nitrophenyl-*N*,*N*'-diacetyl-β-D-chitobiose, and *p*-nitrophenyl-*N*,*N*',*N*''-triacetyl-β-D-chitotriose (Sigma, United States). For this purpose, 50 μl of an enzymatic preparation in 50-mM phosphate–citrate buffer (pH 6.0) was mixed with 10 μl of a 5-mM substrate solution, and the mixture was incubated at 50°C for 10 min. The reaction was stopped by adding 3 ml of 0.2 M KOH. The extinction of the colored product of the reaction (*p*-nitrophenol) was measured spectrophotometrically at 405 nm. The concentration of the *p*-nitrophenol formed in the reaction was determined using a calibration curve constructed with the aid of *p*-nitrophenol purchased from Sigma (United States). One unit of enzymatic activity (U) was defined as the amount of enzyme which catalyzes the formation of 1 μmol *p*-nitrophenol per minute.

The antifungal activity of chitinases produced by antagonistic bacilli was determined using filter-paper disks impregnated with 20–25 μl of the culture liquids (CLs) of the bacilli grown in the liquid medium with

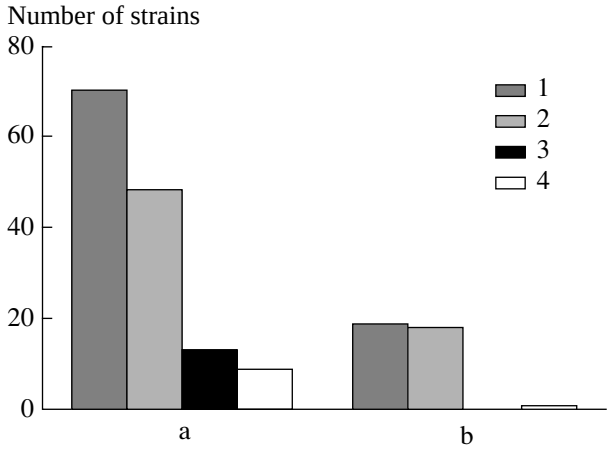
colloidal chitin. Czapek agar plates were inoculated with spores of a test fungus and overlaid with the CL-impregnated filter-paper disks. The plates were incubated at 28°C for 3–7 days. The antifungal activity of the CLs was evaluated on the third day of incubation as the diameter of the growth inhibition zone around the disks.

The data obtained were statistically processed with the aid of Excel-97 software. The empirical coefficient of correlation was calculated by the formula $r_{x,y} = [1/n\sum_{i=1}^{n}(x_i - x_{av})(y_i - y_{av})]/S_xS_y$. Taking into account the fact that the data sample was small ($n < 30$), we used a correction coefficient specified by the formula $[1 + (1 - r^2)/(2(n-3))]$. The correlation coefficient was determined for a 5% level of significance.

RESULTS AND DISCUSSION

The *Bacillus* spp. strains studied differed in the range of inhibited fungi. More than one-third of the strains inhibitory to *H. sativum* were unable to inhibit the growth of *Fusarium* fungi. At the same time, the other bacilli under study inhibited from two to ten fungal species. Among the 70 strains with antifungal activity, 19 strains (i.e., 27%) possessed chitinolytic activity (figure). Almost all of these 19 strains (specifically, 18 strains) belonged to the species *B. subtilis*, while only one strain (*Bacillus* sp. IB-30) was close to *B. circulans* in its physiological and biochemical properties. The correlation analysis of the whole cluster of 70 strains showed that their chitinolytic activity and antifungal activity against *H. sativum* are not correlated ($r_{x,y} = -0.095$), the most active antifungal strains *B. subtilis* IB-4, IB-9, IB-52, and IB-54 exhibiting no chitinolytic activity at all.

In the second set of experiments we performed, the correlation analysis of the cluster of 19 chitinolytic strains with allowance made for their relative chitinolytic activity (evaluated as the ratio of the diameter of the zone of colloidal chitin hydrolysis around a colony to the colony diameter), the mean daily increase in the diameter of this zone, the area (expressed in mm^2) of the growth inhibition zone on a lawn of *H. sativum*, and the number of inhibited phytopathogenic fungi (Table 1) showed no correlation between the different parameters of chitinolytic and antifungal activities either, as is evident from the low values of the correlation coefficients (0.01 and 0.05, respectively). We were also unable to reveal any correlation between the chitinase activity of the bacillar strains and the range of inhibited fungi ($r_{x,y} = 0.1$ in both cases) or between the chitinase and antifungal activities of the bacillar strains evaluated using solid and liquid nutrient media ($r_{x,y} = 0.05$) (Tables 1, 2). The high negative value of correlation between the specific chitinase activity of strains and the mean daily rate of chitin hydrolysis ($r_{x,y} = -0.63$) indicates that the latter quantitative parameter of chitinase activity is inappropriate.



(a) The species composition and (b) the number of chitinase producers in the group of *Bacillus* spp. strains antagonistic to phytopathogenic fungi: (1) the total number of strains; (2) the number of *B. subtilis* strains; (3) the number of *B. polymyxa* strains; (4) the number of other strains.

In the third set of experiments, we attempted to find correlation between the antifungal activity of the strains and the activity of the particular enzymes of their chitinolytic complexes. Almost all of the strains under study had *N*-acetylglucosaminidase activity within 0.1–0.2 U/ml. The proportion between the chitobiosidase and chitotriosidase activities of the strains was typically about 2 : 1. The only exception was *Bacillus* spp. strain IB-30, which exhibited very high relative exochitinase activity, as is evident from the high value of the above proportion equal to 8 : 1 (Table 2). This is in agreement with the fact that only this strain is characterized by a clear-cut zone of colloidal chitin hydrolysis around its colonies, while the other strains had diffuse zones of chitin hydrolysis. Furthermore, IB-30 was the only strain that synthesized constitutively extracellular β-1.3-glucanase at a level of 3.55 U/ml in the medium with colloidal chitin. Chitosanase activity was detected in two strains, *Bacillus* spp. IB-30 and *B. subtilis* IB-26-a. The low constitutive level of this activity (0.01–0.02 U/ml) increased tenfold to twentyfold when the medium contained chitosan or fungal biomass, indicating that the synthesis of chitosanase is inducible.

The crude chitinase preparations of antagonistic bacilli (specifically, their culture liquids) were tested for their ability to inhibit the growth of *H. sativum* (the causal fungus of root rot) on Czapek agar. As a rule, culture liquids with high chitinase activity exerted a profound antifungal effect (Table 2); however, the correlation between these parameters was weak ($r_{x,y} = 0.45$).

Thus, it is unlikely that the antifungal activity of the bacillar strains under study can be explained by their chitinolytic activity; rather, it depends mainly on antibiotic substances possibly synthesized by these strains. For this reason, the activity of chitinase and other mycolytic enzymes cannot serve as a reliable criterion for

Table 2. The chitinolytic and fungistatic activities of the culture liquids of bacillar strains

Strain	Chitinase, U/ml	Hydrolysis rates (in μmol *p*-nitrophenol per ml per min) of			Diameter (in mm) of the growth inhibition zone of *H. sativum**
		GlcNAc	$(GlcNAc)_2$	$(GlcNAc)_3$	
B. subtilis IB-14	0.21	115	210	75	0
B. subtilis IB-21	0.34	159	302	129	25
B. subtilis IB-23	0.19	134	363	296	17
B. subtilis IB-24-2	0.33	157	257	145	27
B. subtilis IB-26-a	0.22	144	167	104	4
B. subtilis IB-26-b	0.33	164	275	124	22
Bacillus sp. IB-30	0.10	165	883	115	5
B. subtilis IB-31	0.28	123	242	111	20
B. subtilis IB-33-1	0.19	162	161	82	33
B. subtilis IB-33-2	0.22	89	151	94	14
B. subtilis IB-34-a	0.24	92	152	88	15
B. subtilis IB-34-b	0.31	95	250	89	16
B. subtilis IB-34-c	0.22	164	158	91	0
B. subtilis IB-34-d	0.22	102	149	84	21
B. subtilis IB-35	0.19	76	112	86	0
B. subtilis IB-36	0.17	116	302	ND	24
B. subtilis IB-39-1	0.20	212	358	ND	14
B. subtilis IB-39-3	0.18	213	255	ND	4
B. subtilis IB-47	0.15	231	369	ND	10

Note: ND stands for "not determined." * The zone diameter (a measure of the fungistatic activity of the culture liquid) was determined on the third day of the incubation of CL-impregnated filter-paper disks on a lawn of *H. sativum.*

the antifungal activity of bacilli. For instance, *Bacillus* spp. IB-30 likely possesses the most active complex of mycolytic enzymes, but it is far from being the most active antagonistic strain (Tables 1, 2). Nor can the high chitinolytic activity of *Bacillus* strains serve as a criterion for the possibility of using them for the biocontrol of phytopathogenic fungi, although the ability of some strains to produce extracellular mycolytic enzymes should increase the competitiveness of these strains in the environment due to a wider range of possible nutrient sources.

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