ISSN 0006-2979, Biochemistry (Moscow), 2012, Vol. 77, No. 8, pp. 910-916. © Pleiades Publishing, Ltd., 2012.
Original Russian Text © A. B. Duzhak, Z. I. Panfilova, T. G. Duzhak, E. A. Vasyunina, M. V. Shternshis, 2012, published in Biokhimiya, 2012, Vol. 77, No. 8, pp. 1105-1112.

Role of Prodigiosin and Chitinases in Antagonistic Activity of the Bacterium *Serratia marcescens* against the Fungus *Didymella applanata*

A. B. Duzhak[1]*, Z. I. Panfilova[1], T. G. Duzhak[2,3], E. A. Vasyunina[1], and M. V. Shternshis[4]

[1]*Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences, pr. Lavrent'eva 10, 630090 Novosibirsk, Russia; E-mail: abduzhak@mail.ru*
[2]*The University of Kansas, 2121 Simons Drive, Lawrence, KS 66047 USA*
[3]*International Tomography Center, Siberian Branch of the Russian Academy of Sciences, ul. Institutskaya 3a, 630090 Novosibirsk, Russia*
[4]*Novosibirsk State Agrarian University, ul. Dobrolubova 160, 630039 Novosibirsk, Russia*

Received March 21, 2012

Abstract—The molecular features of antagonism of the bacterium *Serratia marcescens* against the plant pathogenic fungus *Didymella applanata* have been studied. The chitinases and the red pigment prodigiosin (PG) of *S. marcescens* were isolated and characterized. Specific antifungal activity of the purified PG and chitinases against *D. applanata* was tested *in vitro*. The antagonistic properties of several *S. marcescens* strains exhibiting different levels of PG and chitinase production were analyzed *in vitro* with regard to *D. applanata*. It was found that the ability of *S. marcescens* to suppress the vital functions of *D. applanata* depends mainly on the level of PG production, whereas chitinase production does not provide the bacterium with any competitive advantage over the fungus.

DOI: 10.1134/S0006297912080123

Key words: *Serratia marcescens*, *Didymella applanata*, antagonism, chitinase, prodigiosin

Antagonistic relationships in microbial communities in many respects not only determine the structure and vital functions of these communities, but they also influence the state of biosphere as a whole. The forms of antagonism (competition, parasitism, and predation) may be considerably different depending on environmental conditions and on the types of interacting microorganisms (bacteria, protozoa, fungi, etc.). Therefore, the study of the entire diversity of these interactions, particularly their molecular specificity, may be important for both fundamental and applied research.

The soil bacterium *Serratia marcescens* is an efficient antagonist (competitor) of various microorganisms including fungi [1-6]. It is known that the antifungal activity of *S. marcescens* is generally determined by the ability of chitinases (poly[1,4-β(2-acetamido-2-deoxy-D-glucoside)] glycan hydrolases, EC 3.2.1.14) [7] to cleave chitin molecules in the fungal cell walls and thereby to suppress their vital functions [8, 9].

Serratia marcescens produces a complex of chitinolytic enzymes comprising the secreted chitinases (exochitinases ChiA and ChiB, endochitinase ChiC, and chitin-binding protein CBP21) [10, 11] and intracellular *N*-acetylglucosaminidase [12]. Due to the fact that these enzymes synergistically intensify each other's effects [10], the bacterium *S. marcescens* is capable of highly effective competition with many fungi. However, some fungi such as *Fusarium oxysporum* [6], *Trichoderma reesei*, *Phycomyces blakesleeanus* [13], and *Didymella applanata* [14] are relatively resistant *in vitro* to the effect of *S. marcescens* chitinases.

In addition to chitinases, considerable contribution to the antifungal activity of *S. marcescens* is probably made by some secondary metabolites, especially the red pigment prodigiosin (PG) (2-methyl-3-pentyl-6-methoxyprodigiosen) possessing a broad spectrum of antibiotic activity [2, 15]. However, just as in the case with chitinases, there are fungi resistant to the effect of PG [2]. On the other hand, it is known that the presence of chitinases synergistically increases the antifungal activity of PG against *Botrytis cinerea* [3] and *Fusarium oxysporum* [6]. Therefore, the synergistic effects of these metabolites

Abbreviations: CA, chitinase activity; PG, prodigiosin.
* To whom correspondence should be addressed.

may be also essentially important for inhibition of the growth of some fungi. Thus, the roles of chitinases and PG in inhibition of the vital functions of various fungal species by *S. marcescens* bacteria are significantly different. In this context, broadening the concepts of the molecular specificity of interactions between *S. marcescens* and different microorganisms is very important for better understanding of the mechanisms of the fundamental phenomenon of antagonism.

The objects of our research were the previously unstudied interactions between *S. marcescens* and the fungus *Didymella applanata*. The phytopathogenic fungus *D. applanata* (Niessl.) Sacc. (anamorph *Phoma argillacea*) is the pathogen of spur blight, which is one of the most severe diseases of raspberry (*Rubus idaeus* L.) [16]. Our preliminary studies have shown that the bacterium *S. marcescens* can inhibit the growth of the fungus *D. applanata*; however, the inhibitory properties of various bacterial strains were considerably different. Since inhibition of the vital functions of fungi by the bacterium *S. marcescens* is based mainly on the involvement of chitinolytic enzymes and the PG antibiotic [2, 6, 8, 9, 15], we supposed that the inhibitory properties of strains depend on different efficiency of biosynthesis of these metabolites.

The objective of this work was to study the biochemical peculiarities of *S. marcescens* antagonistic activity against the phytopathogenic fungus *D. applanata* under *in vitro* conditions, in particular, to elucidate the role of PG and chitinases in this process. For solution of this problem, chitinases and PG were isolated and their ability to inhibit *in vitro* the growth of *D. applanata* was investigated. The antagonistic properties of several *S. marcescens* strains with different levels of PG and chitinase production against *D. applanata* were analyzed *in vitro*. Conclusions about the role of chitinases and PG in antagonistic interrelationships between *S. marcescens* and *D. applanata* were drawn from comparison of the results.

MATERIALS AND METHODS

The following microorganisms were used in the work: (1) the native prototrophic strain *S. marcescens* B-10 VKM from the Microbial Culture Collection at Irkutsk State University (Irkutsk, Russia). The typical features are the pink color of colonies and the low level of chitinase production [17, 18]; (2) the mutant strain *S. marcescens* M-1 created by treating the synchronized culture of strain B-10 VKM with nitrosomethylurea. The typical features of M-1 are lysine and serine auxotrophy, endonuclease and chitinase superproduction, and the loss of the pink color of colonies [17, 18]. The strain *S. marcescens* M-1 is deposited in the All-Russian Collection of Industrial Microorganisms at the State Research Institute of Genetics and Selection of Industrial Microorganisms, Moscow (deposition number B-3273); (3) nonlysogenic strain *S. marcescens* HY^- W1113 with red color of colonies kindly provided by Dr. U. Winkler [19]; (4) the native strain *Pseudomonas* sp. isolated from forest soil in the vicinity of Novosibirsk. This strain has antagonistic activity against a broad spectrum of fungi [20]. All bacterial strains were stored at −70°C in tubes with LB medium containing 10% dimethylsulfoxide; and (5) phytopathogenic fungus *D. applanata* Niessl. (Sacc.) in the conidial stage of *Phoma idaei* Oudem Da-99 obtained from pseudothecia collected from the stems of diseased raspberries (Novosibirsk) [21]. The fungus was stored in Capek's medium at 4°C.

Isolation of chitinases. The *S. marcescens* M-1 cells were cultivated for 120-150 h in the medium containing 2% powdered chitin [18]. Chitinases were isolated by adsorption on colloidal chitin [22]. Chitinase activity (CA) was assayed by the rate of hydrolysis of a chitin chromogenic substrate [18]. The quantity of the enzyme that catalyzes the formation of 1 μmol of *N*-acetylglucosamine per min at 37°C was taken as a unit of CA.

Protein concentration in the tested preparations was measured by Coomassie brilliant blue G-250 binding [23] with BSA as a calibrator. The composition of the pool of purified chitinases was analyzed by 10% SDS-PAGE. Chitinases were identified by mass spectrometry using a Voyager-DE STR instrument (Applied Biosystems, USA) [22].

Isolation of PG. One colony of the 2-day *S. marcescens* HY^- culture was inoculated in 2 ml of LB and incubated on an orbital shaker (150 rpm) at 28°C for 24 h. A plate (9.5 cm in diameter) with LB agar was inoculated with 0.5 ml of 1-day culture and incubated for 3 days at 28°C in the dark. The bacterial lawn was harvested from the agar surface with a spatula, suspended in 10 ml of saline solution, and centrifuged at 15,000*g* for 30 min at 4°C. The precipitate was suspended in 15 ml of acidified ethanol (95% ethanol/40 mM HCl). PG was extracted by incubating the precipitate suspension on the shaker (150 rpm) at room temperature for 1 h. The extracted red pigment was separated from the cell precipitate by centrifugation at 10,000*g* for 30 min and evaporated dry under vacuum. The dried material was dissolved in chloroform, and the solution was clarified by centrifugation at 10,000*g* for 30 min followed by evaporation dry under vacuum. The PG was finally purified on a Silica gel column [24]. The PG concentration was measured by spectrophotometry (ε_{535} = 112,000 liter/mol·cm) [25]. The isolated pigment was identified as PG by its characteristic absorption spectra in acidic and alkaline solutions [26], molecular weight (323.2 Da), and the ability to nick DNA in the presence of Cu^{2+} ions [27].

The absorption spectra were recorded with a Cary 100 UV/Vis spectrophotometer (Varian, USA).

The effect of nicking was verified by the ability to induce relaxation of the supercoiled DNA with formation

of ring-shaped DNA in the presence of Cu^{2+}. To this end, 1 μl (0.3 μg) of plasmid pUC19 was added to 10 μl of mixture containing 5 μM PG, 10 μM $CuSO_4$, and 10 mM Tris-HCl (pH 7.0), and the mixture was incubated for 30 min at 37°C. The results were analyzed by 1% agarose gel electrophoresis. In the control, the plasmid was incubated in the absence of PG. The linear form of DNA was obtained by hydrolysis of the plasmid with *BamH*I restrictase (SibEnzyme, Russia).

Monoisotopic mass measurement and PG fragmentation were performed in a MALDI-TOF/TOF 4700 Proteomic Analyzer (Applied Biosystems) with a ND-YAG laser (355 nm) in positive ion mode, reflection mode (reflector), and in the mass range 100-800 Da. For PG analysis at 200 Hz and accelerating voltage of 20 kV, the results of 400 laser pulses were accumulated for each registered point (2000-3000 pulses per target); the delay time for ions was 110 nsec. For PG fragmentation, the collision energy in the chamber was 1 kV and the gas (air) pressure in the collision chamber was in the range of $8 \cdot 10^{-5}$ Pa. Fragmented ions were accelerated in the reflector at 15 kV. The resulting spectra were processed with the Data Explorer Version 4.5 software (Applied Biosystems) for the 4700 Proteomics Analyzer. To eliminate the internal signals of the matrix and to improve the noise-to-signal ratio, the mass-spectrometric analysis of PG was performed in the absence of the matrix. The saturated matrix solution of 2,4,6-trihydroxyphenone in 50% acetonitrile and 0.1% trifluoroacetic acid was used in mass spectrometry as a positive control for ionization of the substance.

Analysis of inhibitory effects of chitinase and PG preparations on growth of *D. applanata in vitro*. The inhibitory effect of chitinases on fungal growth was determined by the method of Shternshis et al. [14]. Chitinase solutions (0.4-6.4 U/ml) filtered through a sterilizing filter (0.22 μm) were added in 1.5 ml to a sterile double medium (1.5 ml) cooled to 42°C and immediately poured into 3.5-cm Petri dishes. After solidification of the agar, the dishes were inoculated with the suspension of *D. applanata* spores (15-20 spores) and incubated at 28°C in the dark. The control experiment was performed in the same manner but without chitinases. After 3 days of incubation, the diameter of *D. applanata* colonies was measured under a microscope (8×). The average diameter of colonies in the control was taken as 0% inhibition. The mean values of colony diameters were divided into the control value and multiplied by 100% to calculate the percentage inhibition of the growth of fungal colonies. All experiments were performed in two parallels and three repeats. The data were analyzed by the pairwise *t*-test with a confidence level of 0.05.

The antifungal activity of PG was assayed analogously except for the fact that the PG solution (0.14-4.6 μmol/ml) in 20 μl dimethylsulfoxide was added to 3 ml of the sterile medium. In the control experiment, 20 μl of dimethylsulfoxide was added to the medium.

Determination of chitinase activities and PG content in culture liquid of strains *S. marcescens* HY$^-$, B-10, and M-1. Colonies of 2-day *S. marcescens* cultures (one colony of each strain) were inoculated in LB medium (2 ml) and incubated on the orbital shaker (150 rpm) at 28°C for 48 h. The cells were harvested by centrifugation at 30,000*g* for 30 min at 4°C. Cell precipitates containing PG were used for determining the level of PG production. Chitinase activity was assayed in the supernatants containing the secreted chitinases [18].

Prodigiosin was extracted from cell precipitates by suspension in 2 ml acidified ethanol (95% ethanol/40 mM HCl) and incubation on the shaker (150 rpm) at room temperature for 1 h. Prodigiosin extracts were separated from cell precipitates by centrifugation at 10,000*g* for 30 min. The amount of prodigiosin was determined by spectrophotometry [25]. Prodigiosin content was recalculated in nmol per ml of the initial culture liquid.

Analysis of antagonistic effect of the bacterium *S. marcescens* against *D. applanata* under *in vitro* conditions. The pseudothecia of *D. applanata* were put onto a Petri dish with Capek's medium and incubated for 2 weeks at 25°C to obtain mycelium. A small mycelium fragment was placed into the center of a 9.5-cm Petri dish with the LB agar. The overnight cell suspensions of *S. marcescens* strains HY$^-$, B-10, M-1, and *Pseudomonas* sp. were inoculated in 5 μl 2.5 cm from the center of the dish. The dishes were incubated in the dark at 28°C for 3-4 days. The presence of antagonistic effect was estimated by the dimensions of fungal growth inhibition zone. Inhibition was considered strong if a zone free from the fungus was formed between the bacterial colony and *D. applanata*. Under weak inhibition, the *D. applanata* colony reached the edge of the bacterial colony but did not grow on top of it. Inhibition was absent if the fungal colony grew on top of the bacterial colony.

RESULTS AND DISCUSSION

Characteristics of chitinase and PG preparations. Four proteins corresponding to the extracellular chitinases ChiA, ChiB, ChiC, and CBP21 were found in the isolated chitinase preparation using electrophoresis and mass spectrometry [22]. Such a set of chitinases is typical for different *S. marcescens* strains [10, 11]. The preparation possessed a specific activity of 40 U/mg of protein. No other enzyme activities (protease, lipase, β-1,3-glucanase) were registered in the preparation [14].

The isolated red pigment was identified as typical PG of the strain *S. marcescens* HY [28] based on the following characters. Its absorption spectrum had the maximums typical of PG: at 535 nm under acidic conditions and at 470 nm under alkaline conditions [24]. The pigment was capable of introducing breaks into supercoiled DNA in the presence of Cu^{2+} ions (Fig. 1).

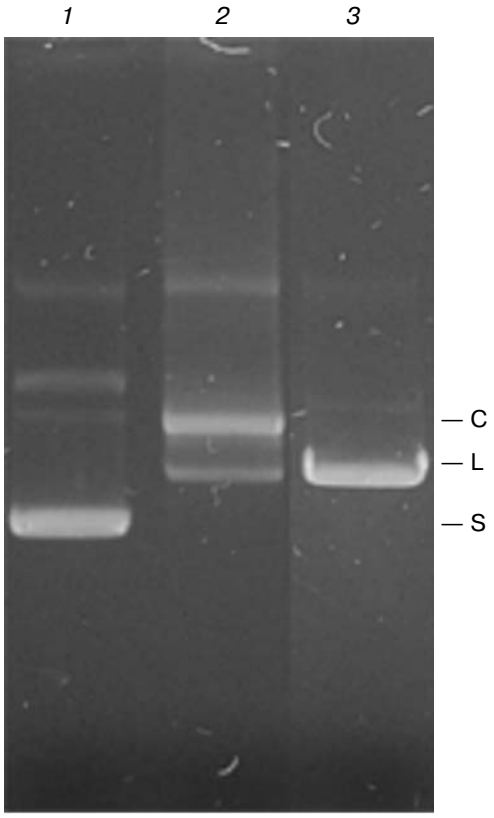

Fig. 1. Ability of PG to relax supercoiled DNA. Electrophoresis in 1% agarose gel: *1*) supercoiled form of plasmid pUC19; *2*) plasmid pUC19 treated with PG in the presence of Cu^{2+}; *3*) linear form of plasmid pUC19. The arrows on the right show the mobility of the circular (C), linear (L), and supercoiled (S) forms of pUC19.

Study using MALDI TOF/TOF mass spectrometry showed that the pigment preparation had one monoisotopic peak with molecular weight of 324.2 ($M + H^+$) Da (Fig. 2a). Induced dissociation of the "parent" ion of PG (324.2 Da) revealed the following monoisotopic fragments of the molecular ion: 149.08, 175.08, 252.11, 266.05, 292.23, and 309.23 Da (Fig. 2b). The molecular weight of the "parent" ion of the pigment and the weights of the fragments exactly corresponded to the molecular structure of typical PG (2-methyl-3-pentyl-6-methoxyprodigiosen) with elemental composition of $C_{20}H_{25}N_3O$ (Fig. 2c). Exactly the same type of PG is produced by most of the *S. marcescens* strains [15], including the strain B-10 used in this work (our data). PG was identified and fragmented by the MALDI TOF/TOF method in the absence of matrix. This phenomenon, which we revealed and used for the first time, clearly requires additional study beyond the scope of the present work. Thus the observed characteristics of the "parent" PG ion and its fragments are analogous to the results obtained by electrospray ionization mass spectrometry (ESI-MS) (our unpublished data and [24, 29]).

Inhibitory effect of chitinase and PG preparations on growth of *D. applanata in vitro*. Figure 3a shows the data on the inhibition of *D. applanata* growth by the preparation of *S. marcescens* chitinases. The inhibitory effect of the preparation at a concentration below 1.5 U/ml was negligible, which is consistent with previously published results [14]. Statistically significant inhibition of *D. applanata* growth by chitinases was observed at concentrations of 1.6 U/ml and more. Chitinase concentration reducing the diameter of the fungal colonies to 50% of the control (IC_{50}) was 2.5 U/ml.

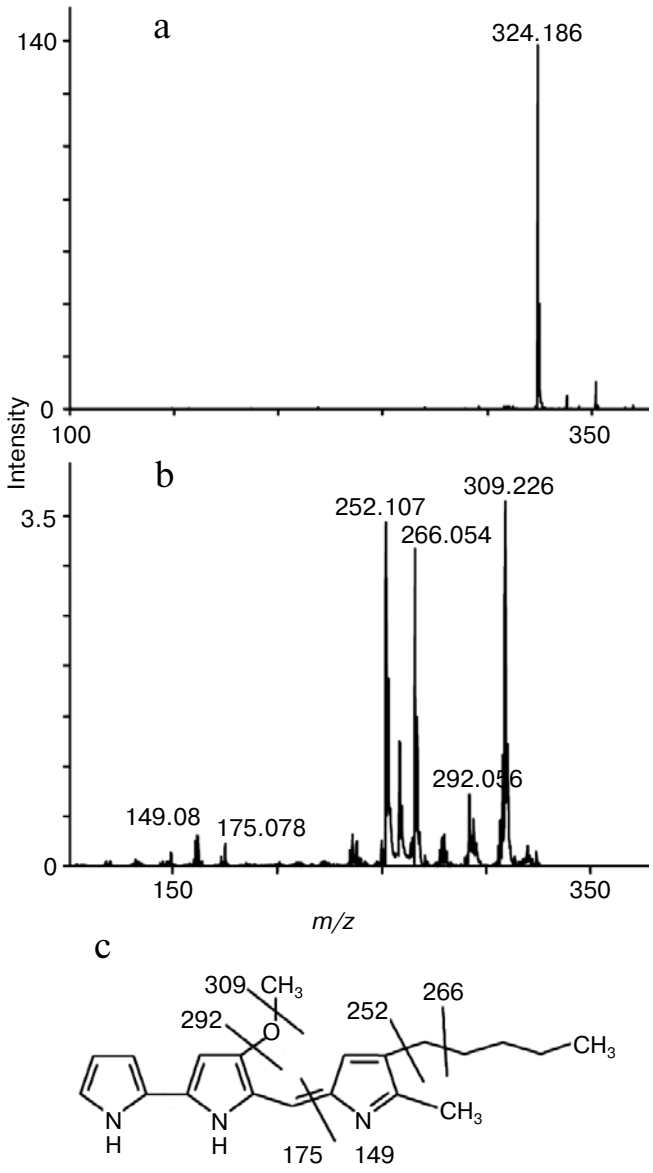

Fig. 2. Analysis and identification of the red PG pigment by MALDI-TOF/TOF mass spectrometry. a) Mass spectrum of PG; b) fragmentation spectrum of PG; c) PG molecule and position of bonds whose breakage results in formation of the respective fragments. Y-axis, relative signal intensity. X-axis, mass-to-charge ratio.

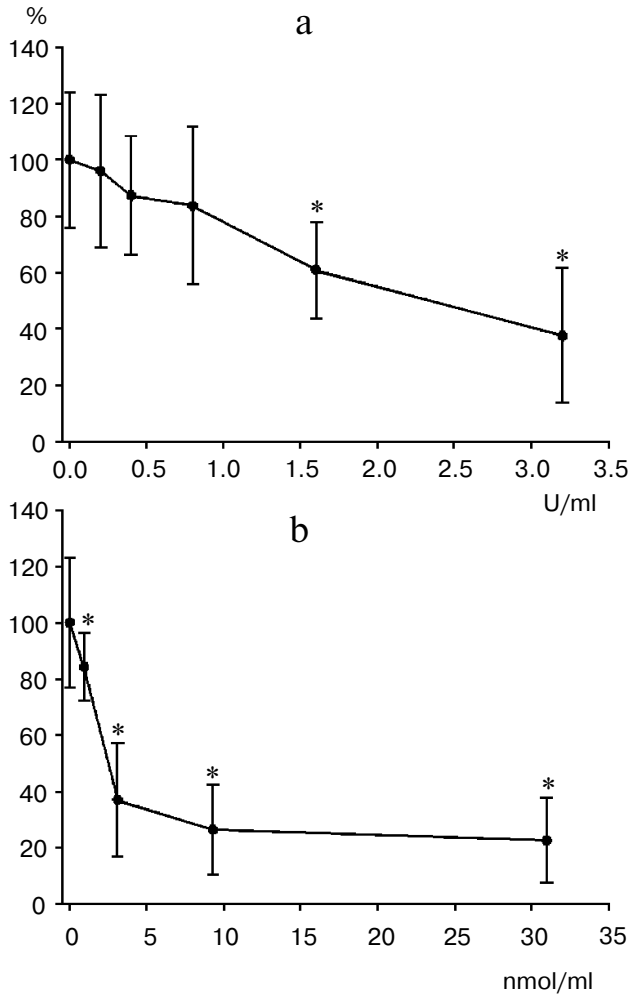

Fig. 3. Effects of chitinases (a) and PG (b) on growth of *D. applanata*. Fungal spores (15-20 spores) were grown on LB agar containing chitinase or PG preparations. Y-axis, mean size of fungal colonies, % of control. X-axis, concentration of the preparation. Vertical bars show the mean root square deviation from the mean. The asterisks mark the values of colony sizes significantly differing from control ($P < 0.05$).

Prodigiosin effectively inhibited the growth of *D. applanata* (Fig. 3b) beginning from the minimum tested PG concentration (0.9 nmol/ml). The IC_{50} value was 2.5 nmol/ml.

Antagonistic effect of *S. marcescens* against *D. applanata* under *in vitro* conditions. As shown above (Fig. 3), PG and chitinases inhibit the growth of the fungus *D. applanata*. However, it is still unclear to what extent the inhibitory abilities (IC_{50}) of each of these metabolites can give *S. marcescens* the advantage over *D. applanata* in their competitive interrelationships.

We have analyzed the productivity of different native strains of *S. marcescens*. We established that the maximum CA (0.2 U/ml) in the culture liquids of all studied strains was much lower and the maximum content of PG (10 nmol/ml) was much higher compared to the respective IC_{50} values. Hence, we suggested that the ability to produce PG, in contrast to chitinases, may provide *S. marcescens* with considerable competitive advantage during it competition with *D. applanata*. Hypothetically, chitinases could also make a certain contribution to the antagonistic properties of *S. marcescens* against *D. applanata* due to possible synergism in their action with PG [3, 6]. Therefore, we studied the possibility of intensifying the inhibition of *D. applanata* growth with joint application of PG and chitinases of *S. marcescens*. However, we did not find any synergism in the action of these metabolites. This gave us additional grounds to believe that the ability of *S. marcescens* to suppress the vital activity of *D. applanata* is determined mainly by the level of PG production, while the presence of chitinases gives no substantial advantage in the competitive antagonistic relationship with *D. applanata*.

For experimental verification of this assumption, we studied the dependence of antagonistic activity of the bacterium *in vitro* on its PG and chitinase productivity. The following *S. marcescens* strains with different levels of PG and chitinase production (table) were selected for the analysis: (1) native strain HY^- with high PG productivity and low CA; (2) native strain B-10 with low PG productivity and low CA; and (3) mutant strain M-1 with the high level of CA and the absence of PG.

Additionally, the *Pseudomonas* sp. strain [20] with low level of chitinase production (<0.01 U/ml) and absence of PG (our data) was used as a model antagonist bacterium with pronounced antifungal activity. Figure 4

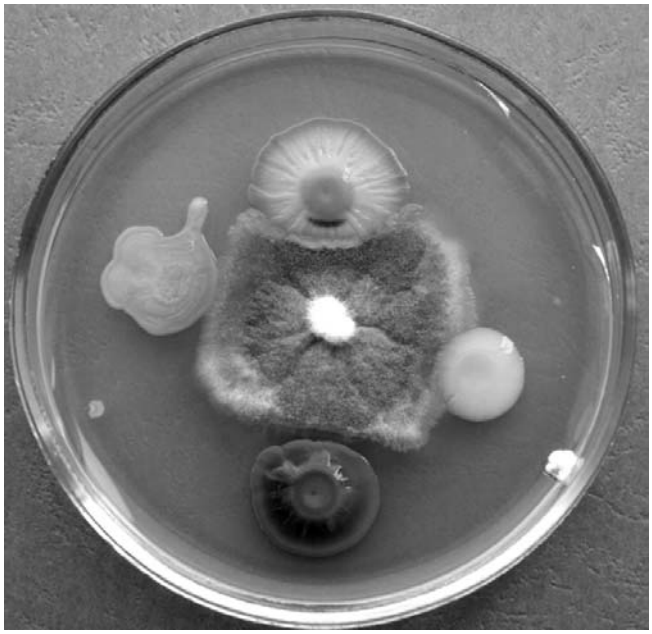

Fig. 4. Antagonistic effects of strains *S. marcescens* HY^- (bottom), M-1 (right), B-10 (top), and *Pseudomonas* sp. (left) on *D. applanata* (center).

Inhibitory activity of PG and chitinases against *D. applanata* and content of these metabolites in culture liquids of strains *S. marcescens* M-1, HY⁻, and B-10

Metabolite	IC_{50}	*S. marcescens* strains		
		M-1	HY⁻	B-10
Chitinases, U/ml	2.5	0.72 ± 0.10	0.11 ± 0.05	0.06 ± 0.03
Prodigiosin, nmol/ml	2.5	0.0	6.7 ± 1.0	0.4 ± 0.1

shows the results of analysis of the antagonistic effects of the bacterial strains on *D. applanata* growth. The coloring of *S. marcescens* colonies resulting from the biosynthesis of the red pigment correlated with PG content in culture liquids of the respective strains. The results confirm the above suggestion about the primary role of PG in the antagonistic activity of *S. marcescens* against *D. applanata*. Strain HY⁻ with PG concentration in the culture liquid 2.7-fold higher than IC_{50} (table) had the maximum inhibitory effect on *D. applanata*. The clear zone between the bacterial and fungal colonies indicates the complete inhibition of *D. applanata* growth. The bacterium *Pseudomonas* sp. inhibited fungal growth to a lesser extent than strain HY⁻. Strain B-10 with the low ($<IC_{50}$) PG content also had relatively low inhibitory activity. The growth of *D. applanata* was inhibited, but a zone of complete growth suppression was absent. It is possible that the amount of PG produced by strain B-10 (0.4 nmol/ml) was enough for partial inhibition of *D. applanata* growth. A similar situation with low inhibitory activity was observed also for mutant strain M-1. The ability to produce PG was lost by the cells of this strain, and the acquired enhanced chitinolytic activity was insufficient for significant inhibition of *D. applanata* growth. Even the relatively high level of CA (0.72 U/ml) in the mutant strain was not only 3.5 times lower than IC_{50}, but also noticeably less than the minimum inhibitory CA (1.6 U/ml) (Fig. 3a). It is unlikely that chitinase production gives the bacteria any substantial competitive advantage over *D. applanata*. In our opinion, the resistance of *D. applanata* cells to the action of *S. marcescens* chitinases *in vitro* may be associated with specific properties of the cell walls of this fungus, which are still little studied. It is probable that chitin molecules are screened by other polysaccharides preventing the direct action of chitinases. This assumption is indirectly confirmed by the previously published data on the efficient inhibition of *D. applanata* growth *in vitro* by the joint action of chitinase and β-1,3-glucanase of *Streptomyces* sp. [14].

Thus, the results of testing of the inhibitory activity of *S. marcescens* strains (table and Fig. 4), as well as the purified PG and chitinase preparations (Fig. 3), convincingly show that antifungal activity of the bacteria depends on their ability to produce PG, which is a peculiar feature of the antagonism of *S. marcescens* against *D. applanata*.

The ascertained antagonistic activity of *S. marcescens* against *D. applanata* and the association between its efficiency and the level of PG production may be a basis for development of new methods for biological control of spur blight. The data presented in this work and the results of our previous studies [14] give grounds for further work in this field.

The authors are grateful to A. A. Belyaev and T. V. Shpatova (Novosibirsk State Agrarian University) for kindly providing *D. applanata* isolates and for their invaluable assistance and collaboration.

REFERENCES

1. Kalbe, C., Marten, P., and Berg, G. (1996) *Microbiol. Res.*, **151**, 433-439.
2. Okamoto, H., Sato, Z., Sato, M., Koizo, Y., Iwasaki, S., and Isaka, M. (1998) *Ann. Phytopathol. Soc. Jpn.*, **64**, 294-298.
3. Someya, N., Nakajima, M., Hirayae, K., Hibi, T., and Akutsu, K. (2001) *J. Gen. Plant Pathol.*, **67**, 312-317.
4. Lavania, M., Chauhan, P. S., Chauhan, S. V. S., Singh, H. B., and Nautiyal, C. S. (2006) *Curr. Microbiol.*, **52**, 363-368.
5. Roberts, D. P., McKenna, L. F., Lakshman, D. K., Meyer, S. L. F., Kong, H., de Souza, J. T., Lydon, J., Baker, C. J., Buyer, J. S., and Chung, S. (2007) *Soil Biol. Biochem.*, **39**, 2275-2288.
6. Someya, N., Tsuchiya, K., Yoshida, T., Noguchi, M. T., Akutsu, K., and Sawada, H. (2007) *Biocontrol Sci.*, **12**, 1-6.
7. Monreal, J., and Reese, E. T. (1969) *Can. J. Microbiol.*, **15**, 689-696.
8. Chet, I., Ordentlich, A., Shapira, R., and Oppenheim, A. (1990) *Plant Soil*, **129**, 85-92.
9. Someya, N., and Akutsu, K. (2005) in *PGPR: Biocontrol and Biofertilization* (Siddiqui, Z. A., ed.) Springer, Netherlands, pp. 297-312.
10. Suzuki, K., Taiyoji, M., Sugawara, N., Nikaidou, N., Henrissat, B., and Watanabe, T. (1999) *Biochem. J.*, **343**, 587-596.

11. Horn, S. J., Sorbotten, A., Synstad, B., Sikorski, P., Sorlie, M., Varum, K. M., and Eijsink, V. G. H. (2006) *FEBS J.*, **273**, 491-503.
12. Toratani, T., Shoji, T., Ikehara, T., Suzuki, K., and Watanabe, T. (2008) *Microbiology*, **154**, 1326-1332.
13. Roberts, W. K., and Selitrennikoff, C. P. (1988) *J. Gen. Microbiol.*, **134**, 169-176.
14. Shternshis, M. V., Beljaev, A. A., Shpatova, T. V., Duzhak, A. B., and Panfilova, Z. I. (2006) *BioControl*, **51**, 311-322.
15. Bennett, J. W., and Bentley, R. (2000) *Adv. Appl. Microbiol.*, **47**, 1-32.
16. Jennings, D. L. (1988) *Raspberries and Blackberries: Their Breeding*, *Diseases and Growth*, Academic Press, London-San Diego.
17. Panfilova, Z. I., and Salganik, R. I. (1983) *Mikrobiologiya*, **52**, 974-978.
18. Duzhak, A. B., Panfilova, Z. I., and Vasyunina, E. A. (2002) *Prikl. Biokhim. Mikrobiol.*, **38**, 248-256.
19. Winkler, U., and Timmis, K. (1973) *Mol. Gen. Genet.*, **124**, 197-206.
20. RF Patent. 1999. No. 2130261.
21. Shpatova, T. V., Shternshis, M. V., and Belyaev, A. A. (2003) *Prikl. Biokhim. Mikrobiol.*, **39**, 43-46.
22. Duzhak, A. B., Panfilova, Z. I., Duzhak, T. G., and Vasyunina, E. A. (2009) *Biokhimiya*, **74**, 257-263.
23. Spector, T. (1978) *Anal. Biochem.*, **86**, 142-146.
24. Alihosseini, F., Ju, K.-S., Lango, J., Hammock, B. D., and Sun, G. (2008) *Biotechnol. Prog.*, **24**, 742-747.
25. Llagostera, E., Soto-Cerrato, V., Joshi, R., Montaner, B., Gimenes-Bonafe, P., and Perez-Tomas, R. (2005) *Anti-Cancer Drugs*, **16**, 393-399.
26. Melvin, M. S., Tomlinson, J. T., Park, G., Day, C. S., Saluta, G. R., Kucera, G. L., and Manderville, R. A. (2002) *Chem. Res. Toxicol.*, **15**, 734-741.
27. Melvin, M. S., Tomlinson, J. T., Saluta, G. R., Kucera, G. L., Lindquist, N., and Manderville, R. A. (2000) *J. Am. Chem. Soc.*, **122**, 6333-6334.
28. Wasserman, H. H., McKeon, J. E., Smith, L. A., and Forgione, P. (1966) *Tetrahedron*, **22**, Suppl. 8, 647-662.
29. Lee, J. S., Kim, Y.-S., Park, S., Kim, J., Kang, S.-J., Lee, M.-H., Ryu, S., Choi, J. M., Oh, T.-K., and Yoon, J.-H. (2011) *Appl. Environ. Microbiol.*, **77**, 4967-4973.