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EXPERIMENTAL ARTICLES

The Production of Antifungal Metabolites by *Pseudomonas chlororaphis* Grown on Different Nutrient Sources

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Abstract—It was found that the antifungal activity of *Pseudomonas chlororaphis* SPB1217 is due to phenazine-1-carboxylic acid, phenazine-1-carboxamide, and two unidentified exometabolites. The carbon source used for the growth of this bacterial strain and iron ions present in the medium considerably influenced the proportion between the antifungal metabolites. The maximum production of phenazines was observed in the media enriched in amino acids and iron ions. The absence of correlation between the production of phenazines and antifungal activity indicates that phenazines are not the only antifungal metabolites of the strain. Organic acids as nutrient sources provide for more intense production of exometabolites and for a higher level of antifungal activity than sugars.

Key words: *Pseudomonas chlororaphis*, nutrient source, antifungal metabolites, antifungal activity.

Rhizosphere bacteria are a good alternative to chemically synthesized pesticides used for control of phytopathogenic fungi. To be efficient, such bacteria must produce antagonistic factors, such as antibiotics, siderophores, and other secondary metabolites [1]. In this regard, of great interest are pseudomonads, which adapt well to rhizosphere conditions and produce a wide range of secondary metabolites. The production of antibiotics and siderophores by pseudomonads largely depends on their carbon and mineral nutrition, the effect of some nutrient sources being strain-specific [2, 3]. It is also known that the synthesis of antibiotics by rhizosphere pseudomonads depends on the host plant species [3]. The root exudates of host plants are the main source of carbon and energy for rhizosphere bacteria. The qualitative and quantitative composition of the exudates varies, depending on the plant species, its physiological state, and growth stage [4].

This work aimed to study the effect of cultivation media (either nutrient-rich media or mineral media supplemented with the individual compounds present in the root exudates of various plants) on the production of antifungal metabolites by *Pseudomonas chlororaphis* SPB1217.

MATERIALS AND METHODS

The *Pseudomonas chlororaphis* strain SPB1217 used in this work was isolated from agricultural soil in the northwestern part of Russia. The strain is antagonistic in vitro to a wide range of phytopathogenic fungi [5].

The phytopathogenic fungus *Fusarium culmorum* strain 258, isolated from the wheat *Triticum aestivum* L. rhizosphere, was obtained from the collection at the All-Russia Research Institute of Agricultural Microbiology.

The bacterium was grown in four different media. The potato medium contained (g/l) potato, 200; succinic acid 2.5; and sucrose 2.5 (pH 6.8–7.0). The LC medium was a modification of Luria–Bertani broth [6], containing (g/l) tryptone, 10; yeast extract, 5; NaCl, 8; $MgSO_4 \cdot 7H_2O$, 2.46; and 1 mM Tris (pH 6.8–7.0). The KB medium (King B medium) contained (g/l) peptone, 15; glycerol, 10; $MgSO_4 \cdot 7H_2O$ – 1.02, KH_2PO_4, 1.0; and, in some cases, 0.1 mM Fe,Na-EDTA (pH 6.8). The MM medium (mineral medium) was a modification of the standard SSM medium [7] containing (g/l) KH_2PO_4, 0.4; K_2HPO_4, 0.1; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; $(NH_4)_2SO_4$, 1.5; 0.04 mM Fe,Na-EDTA; 0.5 mg/l thiamine; 0.01 mg/l biotin; and 1 mg/l nicotinic acid (pH 6.8). The MM medium was either supplemented with succinic acid or not. Organic acids and sugars were added to the MM medium at a final concentration of 10 g/l. D,L-tryptophan was added to the media in an amount of 20 mg/l. When required, the media were solidified with 15 g/l Difco agar.

To prepare bacterial inoculum, cells grown in the potato medium on a shaker were harvested by centrifugation and resuspended in 0.9% NaCl. The inoculum (0.5 ml) was added to a nutrient medium (50 ml) in 250-ml flasks and incubated at 28°C for 4 days without shaking. After determining the culture density, cells were removed by centrifugation, and the supernatant

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(also called culture liquid) was extracted with an equal volume of ethylacetate or chloroform.

Bacterial exometabolites were analyzed using a JASCO LC-900 HPLC system (Jasco Int., Japan) equipped with a (250 × 4.6 mm) C_{18} LiChrosorb RP-18 reversed-phase column. The elution scheme was as follows: 0 min, acetonitrile–water–acetic acid (20 : 80 : 0.1, v/v); 10 min, acetonitrile–water–acetic acid (20 : 80 : 0.1); 55 min, acetonitrile–water–acetic acid (70 : 30 : 0.1). The flow rate was 0.9 ml/min. The column was kept at 33°C.

Eluted peaks were collected, concentrated, and tested for antifungal activity toward the fungus *F. culmorum*. Phenazines were identified based on their retention times (T_R) and UV spectra, which were compared with those of the authentic samples of phenazine-1-carboxamide and phenazine-1-carboxylic acid. The amount of these compounds was determined by comparing their peak areas with those of the standard solutions.

The antifungal activity of bacterial exometabolites was determined using Czapek agar plates contaminated with fungal conidia. Wells (4 mm in diameter) made in the plates were filled in with 40 μl of the exometabolite samples dissolved in methanol. The control wells were filled in with 40 μl methanol (this amount of methanol was not toxic to the test fungus).

To study the effect of carbon sources on the antifungal activity of live bacterial cultures, an aliquot (20 μl) of a culture was spread over the surface of an agar plate, and the plate was incubated at 28°C for 4 days. Then agar blocks about 6 mm in diameter were cut from the plate and placed onto the surface of a twofold diluted Czapek agar contaminated with fungal conidia at a density of 10^5 per ml. After incubation at 28°C for 4 days, antifungal activity was assessed from the diameter of the growth inhibition zone of the test fungus around the agar blocks.

Bacterial growth was evaluated by plating aliquots of 4-day-old liquid cultures onto agar medium and estimating the number of grown colonies.

All the experiments were carried out at least in triplicate. Variance analysis was performed with the calculation of the least significant difference at a 0.95 confidence level [8].

RESULTS AND DISCUSSION

To study the effect of nutrient source on the production of antifungal metabolites by *P. chlororaphis* SPB1217, this strain was grown on the nutrient-rich media (potato, KB, and LC) that are typically used for the cultivation of pseudomonads and on mineral MM medium supplemented with individual compounds typically present in the root exudates of various plants. The KB medium, which favors pigment synthesis in pseudomonads, was chosen for the investigations because of the existence of a correlation between the syntheses of antibiotics and pigments in pseudomonads.

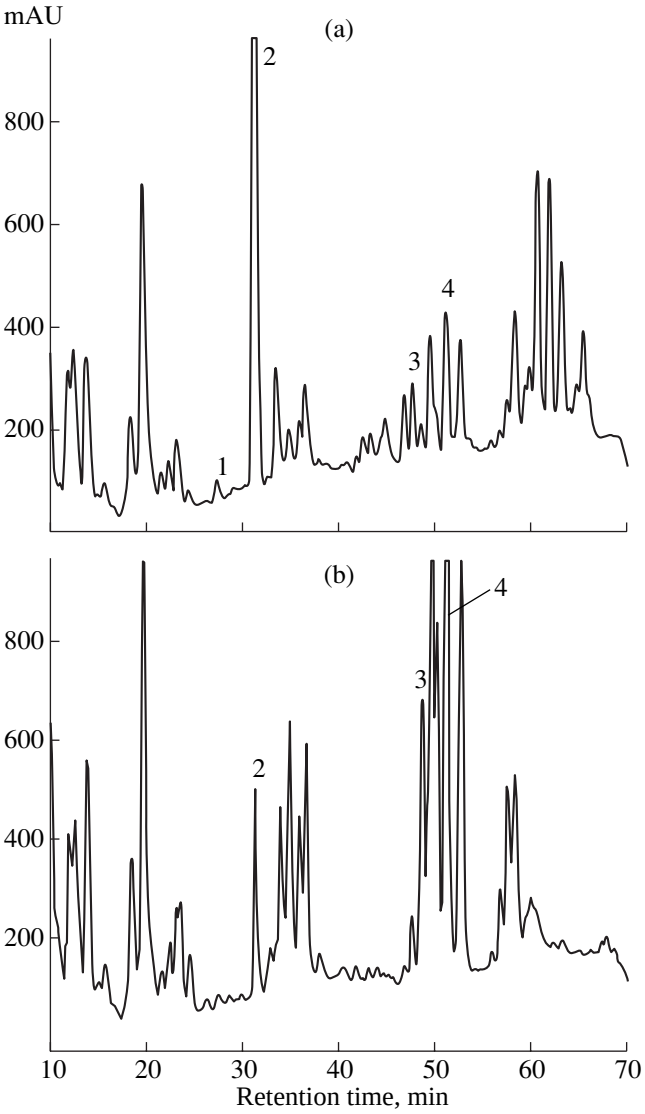

Fig. 1. The HPLC analysis of the culture liquid of *P. chlororaphis* SPB1217 grown in a King B medium with Fe(III). The culture liquid was extracted with (a) chloroform and (b) ethylacetate. Figures 1 through 4 mark the chromatographic peaks of compounds with antifungal activity.

The growth of *P. chlororaphis* SPB1217 on a KB medium with Fe(III) was accompanied by the intense synthesis of a red–orange pigment, which could belong to phenazines [2]. There is evidence that phenazines may be responsible for the ecological competence [9] and the biocontrol activity [10] of pseudomonads.

HPLC analysis (see Fig. 1) showed that *P. chlororaphis* SPB1217 grown on a KB medium with Fe(III) synthesized phenazine-1-carboxamide (peak 1 in the chromatogram) and phenazine-1-carboxylic acid (peak 2). The substances that produced these peaks were identified from their retention times and UV spectra, which

Table 1. The amounts of phenazine-1-carboxylic acid and phenazine-1-carboxamide in the culture liquid of *P. chlororaphis* SPB1217 grown in different nutrient media

Nutrient medium	Phenazine-1-carboxylic acid		Phenazine-1-carboxamide	
	μg/ml	μg/10^9 cells	μg/ml	μg/10^9 cells
Potato medium	traces	–	0	0
Potato medium with tryptophan	0	0	0	0
LC	18.1 ± 4.0	22.9 ± 5.0	0	0
KB	0	0	0	0
KB + Fe(III)	12.7 ± 2.7	6.7 ± 1.4	0.1 ± 0.05	–
KB + Fe(III) + tryptophan	3.1 ± 1.0	1.5 ± 0.5	0	0
MM + succinic acid	0.4 ± 0.2	2.0 ± 0.8	0.1 ± 0.05	0.4 ± 0.1
MM + malic acid	1.0 ± 0.2	11.3 ± 1.3	0.8 ± 0.2	4.9 ± 1.0
MM + fructose	0	0	0	0
MM + xylose	0	0	0	0

Note: Data in Tables 1 and 2 are the means of triplicate measurements ± the standard deviation.

proved to be identical to the T_R and UV spectra of the authentic samples of phenazine-1-carboxamide and phenazine-1-carboxylic acid. The zones of the inhibited growth of *F. culmorum* elicited by the phenazine-1-carboxamide and phenazine-1-carboxylic acid fractions had almost the same size, although the concentrations of these compounds in the culture liquid of *P. chlororaphis* SPB1217 were different (Table 1). This observation is in agreement with the data of other researchers [11].

Two unidentified compounds that produced peaks 3 and 4 in the chromatogram with retention times close to 50 min also possessed antifungal activity. Unlike the phenazines, which were better extracted by the nonpolar solvent chloroform (Fig. 1a), compounds 3 and 4 were better extracted by the polar solvent ethylacetate (Fig. 1b). Consequently, these unidentified compounds are unlikely to be phenazine derivatives. When the wavelength of the UV detector was adjusted to 280 nm, the heights of peaks 3 and 4 increased, indicating that compounds 3 and 4 may have a heterocyclic or aromatic structure.

Among the cultivation media under study, only LC, KB + Fe(II), and MM supplemented with organic acids maintained the synthesis of phenazines (Table 1). *P. chlororaphis* SPB1217 cells grown on an LC medium synthesized only phenazine-1-carboxylic acid (Table 1), peaks with T_R close to 50 min being very small. On the other hand, the potato medium predominantly maintained the synthesis of compounds with T_R close to 50 min.

The KB medium without Fe(III) failed to maintain the synthesis of phenazines and only slightly maintained the synthesis of compounds 3 and 4. At the same time, this medium promoted the synthesis of a pigment with yellow-green fluorescence, which was absent when *P. chlororaphis* SPB1217 was grown in a KB medium with Fe(III). The absorption spectrum of the culture liquid had a maximum at 400 nm, which shifted to 375 nm in response to the addition of Fe(III). Such spectral properties are typical of the fluorescent siderophore pyoverdine [10]. Bacterial siderophores with a high constant of iron binding can inhibit the growth of phytopathogenic fungi by depleting iron ions from the medium [12]. The KB medium with Fe(III) maintained the synthesis of phenazine-1-carboxylic acid and compounds with T_R close to 50 min.

When grown on organic acids, strain SPB1217 produced phenazine-1-carboxylic acid, phenazine-1-carboxamide, and three new compounds other than compounds 3 and 4. When strain SPB1217 was grown in an MM medium with malate, the height of all the chromatographic peaks was much higher than in the case of an MM medium with succinic acid. On the other hand, in the latter case, the strain produced a yellow–green fluorescent pigment, which was absent in the culture grown in an MM medium with malate. The MM medium with sugars failed to maintain the synthesis of pigments, so that no chromatographic peaks were recorded.

The amount of phenazines synthesized by the strain strongly depended on the composition of the medium. Media enriched in amino acids and Fe(III) were able to maintain a more intense synthesis of phenazines than the MM medium with a single carbon source. There was a correlation between the intensity of phenazine synthesis and the bacterial biomass accumulated in the particular medium, except that the strain grew well in a KB medium without Fe(III) but did not produce phenazines in this medium. These data can be accounted for by the fact that some bacterial metabolites can suppress the synthesis of phenazines or that the enzymes involved in phenazine synthesis are very sensitive to reactive oxygen species, whose elimination requires the Fe-dependent superoxide dismutase [13]. The strain

grown on succinic acid produced less phenazine-1-carboxylic acid and especially phenazine-1-carboxamide than when it was grown on malate, although the biomasses accumulated on these two substrates were almost the same. Taking into account the fact that bacterial growth on succinate was accompanied by enhanced fluorescence, the difference between the malate- and succinate-containing media may lie in that the latter medium is more deficient in iron than the former medium [7].

It is known that tryptophan, a component of the root exudates of various plants [14], is a precursor of the antifungal agent pyrrolnitrin [15] and may influence the biosynthesis of phenazines [2]. In our experiments, the addition of tryptophan to a potato medium and to a KB medium with Fe(III) augmented the intensity of some peaks with T_R close to 50 min. At the same time, in the case of the latter medium, tryptophan acted to diminish the yield of phenazines and to enhance the fluorescence of the culture liquid. These data suggest that one of the peaks with T_R close to 50 min may be pyrrolnitrin. The effect of tryptophan on the production of antifungal metabolites depended on the composition of the nutrient medium.

The experimental data concerning the effect of the nutrient source on the antifungal activity of the culture liquid of *P. chlororaphis* SPB1217 are presented in Table 2. As can be seen from this table, the composition of the nutrient medium influenced the intensity of bacterial growth; however, there was no distinct correlation between the growth rate of *P. chlororaphis* on a particular medium and its antifungal activity, except that the poor bacterial growth on xylose correlated with the absence of antifungal activity.

The maximum antifungal activity of strain SPB1217 was observed when it was grown in a potato medium. The high antifungal activity of this strain was also observed on LC and KB media. The enrichment of the KB medium with Fe(III) lowered antifungal activity. The addition of tryptophan to the nutrient media virtually did not influence the antifungal activity of the strain. The absence of correlation between antifungal activity and the level of phenazine synthesis indicated that the antifungal activity of strain SPB1217 could be due not only to phenazines but also to some other bacterial exometabolites, including compounds with T_R close to 50 min. It should be noted that the in vitro testing of antifungal activity revealed a correlation between this activity and the occurrence of the yellow–green fluorescence of the culture liquid, which could be due to pyoverdine.

In general, the antifungal activity of strain SPB1217 grown in an MM medium with organic acids was higher than when it was grown in an MM medium with sugars. Although sugars suppressed the biosynthesis of phenazines, the antifungal activity of strain SPB1217 grown on glucose was quite high.

Table 2. The antifungal activity of *P. chlororaphis* SPB1217 grown in different nutrient media

Nutrient medium	Bacterial growth, log[CFU/ml]	Diameter of the growth inhibition zone of *F. culmorum*, mm
Potato medium	9.2	41
Potato medium with tryptophan	9.3	40
LC	8.9	27
KB	9.4	26
KB + Fe(III)	9.3	18
KB + Fe(III) + tryptophan	9.5	20
MM + succinic acid	8.2	23
MM + malic acid	8.4	19
MM + citric acid	8.1	22
MM + fructose	7.1	14
MM + xylose	5.4	0
MM + glucose	7.3	13
$LSD_{0.05}$	0.5	3

Note: CFU is colony-forming unit; LSD is the least significant difference.

Thus, the carbon source and iron ions present in the medium considerably influence the qualitative and quantitative composition of antifungal metabolites produced by *P. chlororaphis* SPB1217. The absence of a correlation between the antifungal activity of this strain and the level of phenazine synthesis indicates that the antifungal activity may be due not only to phenazines but also to some other bacterial exometabolites. The data obtained suggest that the biosynthesis of antifungal metabolites may be induced in the plant rhizosphere, where a large quantity of organic acids are exuded by the plant roots. This fact should be taken into account when selecting plants resistant to diseases caused by phytopathogenic fungi and introducing biocontrol bacteria into the plant rhizosphere.

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