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EXPERIMENTAL ARTICLES

Character of Interactions of Saprophytic Soil Microflora via Gaseous Metabolites

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Abstract—The character of interaction between saprophytic soil bacteria via gaseous metabolites was studied. It was established that, at the metabolic level, a diverse character of interspecies interrelationships between bacteria exist, directly influencing their reproduction and preservation in soil. Volatile compounds produced by bacteria are able to act as both intra- and interspecies regulators of microbial communities. The soil microbiocenosis composition may be therefore regulated by volatile products of metabolism of saprophytic soil bacteria. Methanol released by bacteria into the environment plays an important role in this process.

Key words: saprophytic soil microflora, gaseous metabolites, interaction, methanol.

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Soil is a multiple-factor system with a variety of diverse species of microorganisms and relationships between them; the study of all the components of a microbial ecosystem is therefore complicated. The study of the mechanism of regulation of activity of soil microorganisms is one of the central problems of soil microbiology. The presence of microorganisms in a given natural zone is determined not only by the environmental conditions but also by the existence of control performed by other members of the biocenosis. Such a control is one of the causes of microbial associations being formed in natural ecosystems.

Soil microbiocenosis is one of the most complicated biological communities. Various interrelationships occur between bacteria in the process of their activity, including those at the metabolic level [1–8]. The interactions between populations via metabolites [9, 10], including gaseous substances [11–13], are of crucial importance for maintaining the stability of microbial communities and the control of their species composition and production capacity. Both the stimulating and inhibiting action of volatile compounds of microbial origin on bacterial growth has been noted [14, 15].

Considering the fact that volatile compounds produced by microorganisms are able to act as intra- and interspecies regulators of microbial communities [11, 12, 16–18], we attempted to study the character of the interaction between saprophytic soil bacteria by means of gaseous metabolites.

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MATERIALS AND METHODS

To study the interactions between soil bacteria from established microbial associations of brown forest and brown podzolic soils (the south of the Far East of Russia), we isolated saprophytic bacteria. The microorganisms were isolated and grown for the experiments on peptone agar (PA) (1% peptone (Mikrogen, FGUP NPO, Ministry of Health of the Russian Federation) and 0.5% NaCl in distilled water with 2% agar; pH 7.4). In order to compare the effect of gaseous metabolites on the growth of microorganisms, nutrient agar (NA) and plant agar (PA) [19] were used.

A total of 20 strains of microorganisms differing in their cultural and biochemical properties were isolated. They were assigned to the genera *Agrobacterium, Acinetobacter, Aeromonas, Micrococcus* (*M. roseus*), *Pseudomonas* (*P. fluorescens, P. aerugonosa, P. putida*), *Flavobacterium,* and *Bacillus* (*B. cereus, B. mesentericus*) according to Bergey's Manual of Determinative Bacteriology (1997) by means of API (Analytical Profile Index) tests (BioMerieux, France).

The method proposed by Tirranen [20], modified by us, was used for the quantitative assessment of the action of volatile biologically active substances produced by saprophytic soil microflora. The culture whose volatile substances were studied is further referred to as the study culture; the culture on which this action was tested is referred to as the test culture. All saprophytic bacteria were alternately tested as the study and test cultures. In order to obtain suspensions of the study cultures, the cells were washed off solid

media. Petri dishes with PA medium were inoculated with this suspension and incubated at 20°C for 72 h. Suspensions of test cultures were prepared and distributed into the wells of an immunological plate. A multichannel pipette was used to prepare replicas (0.1 ml each) on the surface of sterile media (NA or PA, depending on the experimental variant). Since the amount of medium in a petri dish may affect the results, the medium was distributed uniformly (15 ml per dish). One half of a petri dish with a lawn of the study culture was covered with the other half with the freshly applied replicas of the test cultures and incubated in the thermostat at 20°C. The growth of the test organisms depended on the action of the volatile metabolites produced by the study culture grown on the opposite side of the dish.

The reactions of the test cultures to the effect of volatile metabolites of the study cultures were assessed by the difference in the colony size of the test organisms in the experiment and control on the second or third day of incubation, when the growth of the test culture colonies in the control was well-pronounced. The dishes with the test cultures that were not subjected to the action of the volatile products of metabolism of the study organisms served as controls. The statistical data processing was carried out according to F.G. Lakin [21]. The mean of the colony diameter and the mean error were considered. The assessment criterion was the normalized standard deviation value to which the real value of this criterion was compared for the 95% level of significance.

The effect of the study culture on the test culture was assessed as positive (stimulating) or negative (inhibitory) when the test colony size in the experiment was increased or decreased by 20% or more, respectively, compared to the control. The effect of the study culture was assessed as zero if the colony sizes in the experiment did not differ from the control ones by more than ±20%. The results of the comparison of the colony sizes were expressed in millimeters. A total of 400 variants of the experiment with five repeats were performed.

The analysis of the volatile metabolites of saprophytic bacteria with gas–liquid chromatography (GLC) was performed using a Shimadzu-16A gas chromatograph (Japan); helium was the carrier gas, the column temperature was 35°C, the vaporizer temperature 100°C, the flame ionization detector temperature 100°C, and the hydrogen and air pressure on the detector was 0.5 kg/cm^2. The chromatogram calculation and processing were carried out using a specialized Shimadzu-cR-4A computer (Japan). The qualitative composition of volatile organic substances was determined by comparing the chromatographic peaks obtained to the peaks acquired during the chromatographic analysis of pure volatile organic substances (references) without any impurities.

For chromatographic analysis, bacteria were grown in tightly capped vessels on the NA medium. After 24, 48, and 72 h of incubation, 1 ml of air was sampled from the vessels through the rubber caps with a syringe and introduced into the chromatograph dispenser.

RESULTS AND DISCUSSION

The results obtained enabled us to assess the degree of influence of the volatile metabolites of one species of saprophytic bacteria on the growth of other species during their interaction. Among them, 42% were negative (the volatile metabolites of the study cultures inhibited the growth of the test cultures), 30% were positive (the volatile metabolites of the study cultures stimulated the growth of the test cultures), and the remaining results (28%) were neutral. The zero interactions observed in the experiments may be weak positive or negative effects (less than 20% of the control), which were not determined by the method of investigation used.

The experimental results presented in the table demonstrate that among the bacteria studied an interaction exists via gaseous metabolites. Most of the study cultures released inhibitory volatile substances, which had a negative effect on the growth of the test cultures. The stimulating, i.e., positive effect of the cultures occurred less frequently.

All the study cultures exerted a selective, both inhibitory and stimulating, effect on the growth of the test cultures. Bacteria probably produce a range of volatile compounds, their spectrum of action varying from broad to narrower. Of all the strains of the study cultures, the bacteria of the genera *Pseudomonas* and *Acinetobacter* revealed the highest inhibitory activity in relation to the test cultures (Fig. 1). The stimulating action of these bacteria was observed in no more than 28% of cases. The volatile metabolites of *Aeromonas* exhibited the greatest stimulating activity. The inhibitory action of these bacteria was observed in no more than 8% of cases. Hence, it may be suggested that it is pseudomonads and aeromonads that exert the most significant effect on the growth and development of microflora in the soils studied.

It should be noted that the volatile metabolites of the *Flavobacterium* and *Bacillus* (*B. cereus*, *B. mesentericus*) strains investigated in the experiment did not appreciably influence the growth of the saprophytic microflora of soils (table).

The reaction of the test cultures to the volatile metabolites of the study microorganisms was diverse. Thus, most of the cultures (*P. aeruginosa* strain 2; *P. fluorescens* strains 6, 14; *Micrococcus* strain 3; *Aeromonas* strain 10; *Acinetobacter* strains 8, 17; and *Flavobacterium* strain 16) exhibited a mainly negative reaction. An interesting feature was noted in relation to *Micrococcus* strain 9, which revealed a neutral reaction to the action of volatile substances in 50% of cases. No strains showing a surge of growth in most cases were revealed.

Analysis of the experimental data revealed direct correlation (for all the strains studied, except bacilli and

Influence of the volatile metabolites of one species of saprophytic soil bacteria on the growth of others

Study cultures	Test cultures																		
	P. aeruginosa strain 11	*P. aeruginosa* strain 2	*P. aeruginosa* strain2	*P. fluorescens* strain 7	*P. fluorescens* strain 13	*P. fluorescens* strain 14	*P. fluorescens* strain 6	*Micrococcus* strain 3	*Micrococcus* strain 9	*Aeromonas* strain 1	*Aeromonas* strain 10	*Aeromonas* strain 15	*Aeromonas* strain 20	*Acinetobacter* strain 8	*Acinetobacter* strain 17	*Bacillus*strain 15	*Bacillus* strain 19	*Bacillus* strain 4	*Flavobacterium* strain 16	*Flavobacterium* strain 18
P. aeruginosa strain 11	0	–	–	+	–	–	–	–	+	0	–	+	–	–	–	–	–	–	–	–
P. aeruginosa strain 2	+	–	–	+	–	–	–	–	+	0	–	0	–	–	–	–	+	–	–	–
P. aeruginosa strain 12	0	–	–	+	–	–	–	–	+	–	0	+	–	–	–	–	–	–	–	–
P. fluorescens strain 7	–	–	–	–	–	0	–	+	–	–	–	–	+	–	–	–	–	–	–	+
P. fluorescens strain 13	–	–	+	–	–	–	–	+	–	–	–	–	0	–	–	–	–	+	–	–
P. fluorescens strain 14	–	–	–	–	–	–	–	+	–	–	0	–	–	–	–	–	–	0	–	+
P. fluorescens strain 6	–	–	+	–	–	–	–	+	–	–	–	–	0	–	–	–	–	0	–	+
Micrococcus strain 3	+	–	+	–	+	–	0	–	+	–	–	–	+	–	–	+	0	+	–	–
Micrococcus strain 9	+	–	+	–	+	–	0	–	–	–	–	–	+	–	–	+	0	+	–	+
Aeromonas strain 1	+	+	+	–	+	+	+	+	0	+	+	+	+	+	+	+	+	–	+	+
Aeromonas strain 10	+	+	+	+	+	+	0	+	0	+	+	+	–	+	+	+	+	+	+	+
Aeromonas strain 5	+	–	+	0	+	+	+	+	0	+	+	+	–	0	+	+	+	+	+	+
Aeromonas strain 20	+	+	+	0	+	+	+	+	0	+	+	+	–	+	+	+	+	+	+	+
Acinetobacter strain 8	–	+	0	–	0	–	–	+	+	+	–	–	+	+	–	–	–	–	–	–
Acinetobacter strain 17	–	+	0	–	0	–	–	+	0	+	–	–	+	+	–	–	+	–	–	–
Bacillus strain 15	0	0	0	0	0	0	+	0	0	–	0	0	0	0	0	0	–	0	0	0
Bacillus strain 19	0	0	0	0	+	0	0	0	0	0	+	0	0	0	0	0	0	0	0	–
Bacillus strain 4	0	0	0	0	0	0	+	0	0	+	0	0	0	0	0	0	0	0	0	0
Flavobacterium strain 16	0	0	0	–	0	0	0	0	0	0	0	0	0	+	0	0	0	0	0	0
Flavobacterium strain 18	0	0	0	0	0	+	0	0	0	0	–	0	0	+	0	0	0	0	–	0

Note: 0, no action; +, the action is positive; –, the action is negative.

flavobacteria, which showed a neutral effect) between the inhibiting and stimulating effect of the culture; the absence of stimulation correlated with the presence of inhibition.

The action of the strain volatile metabolites on themselves was also established. The bacteria reacted mainly negatively to "proprietary" volatile metabolites: 50% of negative, 30% of positive, and 20% of zero effects were noted. The self-action of pseudomonads and micrococci was inhibitory, while aeromonads and acinetobacters stimulated their growth. Bacilli and flavobacteria exhibited a neutral reaction.

It is known that the growth rate of bacteria in different media may be different. Bacterial metabolites accumulated on different media may differ both in their qualitative and quantitative composition. We therefore studied the influence on bacterial growth of the gaseous metabolites of the same cultures grown on nutrient agar and plant agar. These comparative studies did not reveal a substantial difference between the action of the volatile metabolites of the cultures grown on different media (the difference is statistically insignificant, $p > 0.05$).

Thus, the stimulating and inhibiting effect of the volatile metabolites of saprophytic soil bacteria on each other was noted. Some gaseous substances released by one species of bacteria may serve as nutrients for other bacteria. For example, D.G. Zvyagintsev [22] stated that certain components of volatile metabolites of microbial origin (acetaldehyde, ethanol) could act as nutrients for microorganisms. Larionov [23] demonstrated that long-term cultivation of *Pseudomonas pseudomallei* in soil extracts resulted after six months in the activation of the fermentative properties and the synthesis of an inhibitor of concomitant microflora. In the opinion of Tirranen [20], the interaction of microorganisms by means of their gaseous metabolites is a widespread phenomenon, which may play a certain ecological role in natural habitats. The qualitative composition of volatile metabolites may influence posi-

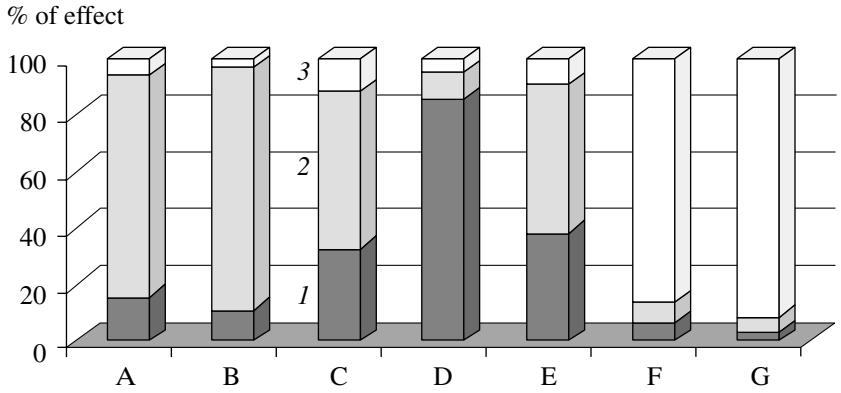

Fig. 1. Character of interrelationships of saprophytic soil microflora via gaseous metabolites. A, *Pseudomonas aureus*; B, *Pseudomonas fluorescens*; C, *Micrococcus*; D, *Aeromonas*; E, *Acinetobacter*; F, *Bacillus*; G, *Flavobacterium.* Positive effect (*1*), negative effect (*2*), zero effect (*3*).

tively or negatively the process of consumption of organic compounds.

In order to determine the relationship between growth of the bacteria studied and the qualitative composition of their volatile metabolites, we analyzed the volatile samples by gas–liquid chromatography.

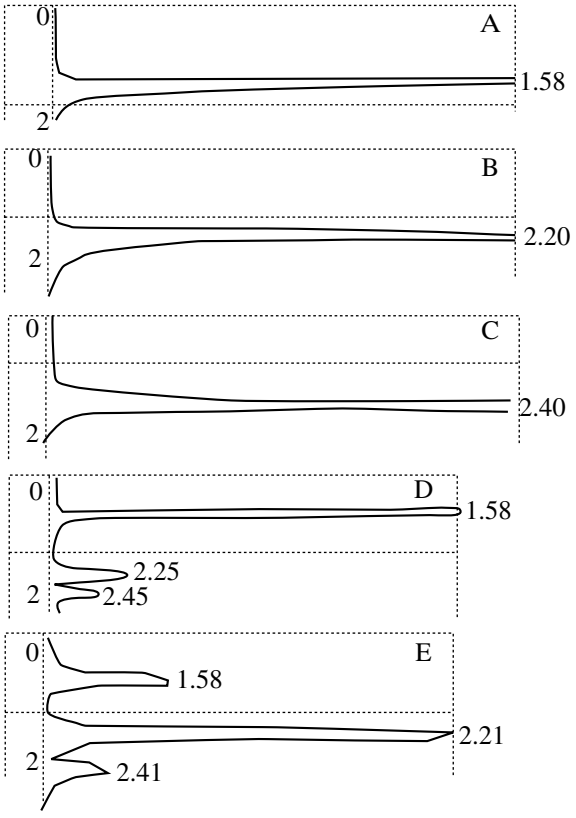

Fig. 2. Data of the chromatographic analysis of volatile metabolites of the bacteria of the genus *Pseudomonas* (D) and the genus *Aeromonas* (E). Control: A, acetaldehyde; B, methanol; C, ethanol.

Chromatographic analysis of the volatile metabolites of *Pseudomonas* and *Aeromonas* revealed relatively high content of acetaldehyde (1 μl/ml in pseudomonads and 0.55 μl/ml in aeromonads), methanol (0.43 and 1.2 μl/ml, respectively), and ethanol (0.16 and 0.2 μl/ml, respectively) (Fig. 2). A greater content of methanol was noted in the composition of the volatile metabolites of aeromonads, compared to pseudomonads. It may be suggested that methanol is utilized by the saprophytic soil microflora as a carbon source, since some microorganisms are known to grow on C_1 compounds, including methanol [24]. A greater content of acetaldehyde, which inhibits bacterial growth, was noted in the composition of the volatile metabolites of pseudomonads.

The data obtained allow us to assert that, at the metabolic level, a diverse character of interspecies interrelationships is observed between the bacteria, directly influencing their growth and preservation in soils. The volatile compounds produced by microorganisms may act as both intra- and interspecies regulators of microbial communities. In this regard, the composition of soil microbiocenosis may be regulated by the products of metabolism of saprophytic soil bacteria. An important role in this process is played by methanol released by saprophytic bacteria into the environment.

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