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EXPERIMENTAL ARTICLES

Oil-Oxidizing Potential of Associative Rhizobacteria of the Genus *Azospirillum*

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Abstract—The oil-oxidizing potential of associative rhizobacteria of the genus *Azospirillum* was studied under laboratory conditions. After screening, *A. brasilense* strain SR80 was chosen for further investigation. The strain was capable of degrading 56.5% of crude oil (added in a concentration of 1%) over 14 days in a medium containing malate as an additional source of carbon and energy. Studies of associative properties showed that the strain had positive chemotaxis to wheat root exudates, colonized wheat roots, and produced indole-3-acetic acid. The synthesis of indole-3-acetic acid was not inhibited by oil. Under hydroponic conditions, crude oil stimulated growth of *A. brasilense* SR80, which promoted development of the wheat root system in the presence of oil and enhanced the level of oil degradation by the plant–microbial association.

Key words: azospirillum, oil degradation, plant–microbial association.

The use of plants for remediation of environmental objects (phytoremediation) has become an area of intense research in the last years. Studies in the mechanisms of phytoremediation have great potential for the ecobiotechnology market and encourage research in fundamentals of the interaction of plants and microorganisms.

One of the primary phytoremediation mechanisms is microbial degradation of a pollutant in the plant rhizosphere [1, 2]. It has been shown that it is the way that soil is purified from most organic pollutants, oil hydrocarbons in particular [3].

It is known that oil pollution of soil affects different groups of soil microorganisms in different ways. According to [4, 5], oil inhibits the activity of nitrifiers and cellulolytic microorganisms but stimulates ammonifiers, denitrifiers, and nitrogen-fixing microorganisms, the activity of which may be specifically or nonspecifically connected with the plant [1]. Presumably, oil pollution limits access of oxygen into soil, thus protecting microbial nitrogenase from O_2 and activating the process of nitrogen fixation carried out by associative and symbiotic microorganisms. Thus, the presence of a hydrocarbon-oxidizing potential in nitrogen-fixing bacteria may give them a significant advantage over other groups of microorganisms and is very fruitful in terms of soil bioremediation. Furthermore, stabilization of the population of such bacteria in soil by additional factors (e.g., by an associative plant resistant or adapted to a pollutant) should ensure phytoremediation results.

We studied an azospirillum–wheat association as a model. Bacteria of the genus *Azospirillum* are natural inhabitants of the rhizosphere of many cereals. They are well-studied associative microorganisms capable of nonsymbiotic nitrogen fixation. Their activity results in pronounced stimulation of growth and development of the host plant [6].

The goal of our work was to scan the genus *Azospirillum* for oil-oxidizing properties, to study the revealed oil degraders in association with wheat, to investigate the effects of oil pollution on the azospirillum–wheat association, and to study oil biodegradation by this association and its members.

MATERIALS AND METHODS

We studied the ability to degrade crude oil in 33 azospirillum strains from the collection of rhizosphere microorganisms of the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences: 9 *Azospirillum lipoferum* strains, 15 *A. brasilense* strains, and 9 unidentified azospirilla.

Experiments were carried out with a high-sulfur crude oil of the following composition (according to data of our analysis), %: alkanes, 22.3; naphthenes, 47.4; mono- and biaromatic carbohydrates, 10.5; polycyclic aromatic hydrocarbons, 4.5; alcohol–benzene gums, 4.1; asphaltenes, 11.9.

For investigation of biodegradative properties, cultures were grown in minimal salt medium M9 [7], min-

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eral medium with trace elements (MSM) [8] and yeast extract (0.05) for growth stimulation, medium Nfb for azospirilla [9], and medium for plant growth (MCP) containing (g/l distilled water) K_2HPO_4, 0.02; KCl, 0.075; $MgSO_4$, 0.3; $CaCl_2$, 0.1; $Ca(NO_3)_2$, 0.01, EDTA, 0.02.

The ability of microorganisms to use crude oil as the source of carbon was studied by the MacClung method [10]. Cultures were grown on solid media of the composition described above with the addition of crude oil.

For quantitative estimation of degradative abilities, cultures were grown in flasks containing media with the addition of oil (1%) on a shaker (100 rpm) at 29°C for 14 days. Then, residual oil concentrations were determined.

For studies of the effect of the nitrogen source on the degradation of crude oil by *A. brasilens*e strain SR80, we grew the strain on Nfb medium without nitrogen-containing salts or replaced NH_4Cl with KNO_3 in equimolar quantities.

The increase in biomass of *A. brasilense* SR80 in liquid medium was estimated by inoculation of serial dilutions of a microbial suspension onto plates with Nfb medium and calculation of the number of colony forming units (CFU).

We used wheat (*Triticum aestivum* L.) of the sort Saratovskaya 29. Before germination, seeds were sterilized with a mixture of ethanol and hydrogen peroxide (1 : 1) for 5 min. For sterility control, seeds were incubated on nutrient agar plates for 2 days at 28°C. Only sterile seedlings were used.

Chemotaxis of *A. brasilense* strain SR80 was studied according to [11] on Petri plates with Nfb medium containing 1% agar. To obtain the necessary attractants, sterile wheat seeds were grown in a hydroponics chamber for 7 days. Then, roots were aseptically separated and ground. Sterile hollow plastic cylinders were placed in the center and periphery on plates with Nfb medium. The central cylinder was inoculated with two drops of a 48-h *A. brasilense* SR80 culture grown in liquid Nfb medium supplemented with 6 g/l peptone (or two drops of sterile water as a control). Peripheral cylinders were filled with the attractants under study: crude oil, root homogenate, mineral medium after growing of wheat, or mineral medium for plant growth. The results were first estimated after 2 days. Chemotaxis was judged from the diameter and time of appearance of a growth zone.

To study the production of indole-3-acetic acid (IAA), *A. brasilense* strain SR80 was grown in 0.25-l flasks containing 50 ml of Nfb medium supplemented with 0.2 g/l DL-tryptophane as a phytohormone precursor. To study the effect of oil pollution on IAA synthesis, the medium was supplemented with 1% crude oil. The culture was incubated at 29°C under passive aeration for 14 days. All experiments were conducted in triplicate.

For analysis of the concentrations of IAA and tryptophane in the cultivation medium, oil was extracted from the medium with chloroform, microbial cells were sedimented by centrifugation (8000 g, 20 min), and samples were analyzed on an HPC 500 chromatograph (Czech Republic) equipped with a UV-detector.

Bacterization of 48-h wheat seedlings was carried out with an *A. brasilense* SR80 suspension containing 10^6 cells/ml. Untreated seedlings were used as a control. Seedlings were placed in a number of 10 on a steel grid in 0.25-l chambers closed with cotton plugs and containing 50 ml of sterile medium for plant growth supplemented with 1% crude oil or without addition of oil. Chambers were incubated at 24°C for 7 days. All the experiments were performed in four replicates. After incubation, the length of roots was measured.

To study root colonization, a root homogenate prepared from 9-day wheat seedlings and diluted 1 : 10 was plated onto nutrient agar and Nfb medium that contained Congo-rot indicator for an easier cell count [12].

In all experiments, oil content in the medium before and after cultivation was determined by the gravimetric method [13] after extraction with chloroform. Oil degradation was expressed in per cent of the initial concentration.

RESULTS AND DISCUSSION

Data on the ability of azospirilla to degrade xenobiotics are very scarce. We found only some data on the respiratory activity of azospirilla in the presence of phenolic compounds [14]. At the same time, azospirilla are well studied as associative bacteria that stimulate growth and development of the host plant, which was our reason to study pollutant degradation by these microorganisms.

The results (Table 1) showed that most strains were capable of using crude oil if grown on Nfb medium containing malate as an additional source of carbon and energy. Eighteen of 33 cultures studied showed considerable growth on this medium. However, only two strains grew on M9 medium supplemented with oil: *Azospirillum* sp. SR4 and *Azospirillum* sp. SR66.

Quantitative estimations of the oil-degradative ability of azospirilla showed that the best results were achieved on Nfb medium. Maximal degradative activity was shown by *A. lipoferum* SR42 and *A. brasilense* SR80, which degraded 57.5 and 56.5% of crude oil (added in a concentration of 1%) over 14 days.

The highest degradative activity was shown on a medium containing malate. In the rhizosphere, root exudates may serve as a source of malate. This demonstrates the importance of the associative properties of the studied microorganisms, which are capable of consuming root exudates as cosubstrates. Taking into account all the above, for further experiments we chose strain SR80, which showed the most pronounced growth in the presence of oil.

Table 1. Growth and degradative activity of azospirilla on different media supplemented with crude oil (1%)

no.	Strain	M9 medium		MSM medium		MSM medium + yeast extract		Nfb medium	
		Growth	Degradation, %	Growth	Degradation, %	Growth	Degradation, %	Growth	Degradation, %
1	*Azospirillum* sp. SR3	–		+	35	–		–	
2	*Azospirillum* sp. SR4	+	20.5	+	43	+	13	±	
3	*Azospirillum* sp. SR5	–		±		+	7	–	
4	*Azospirillum* sp. SR14	–		–		±		+	55
5	*Azospirillum* sp. SR16	±		+	36	±		±	
6	*Azospirillum* sp. SR35	–		–		–		–	
7	*Azospirillum* sp. SR45	–		+	12	+	13	–	
8	*Azospirillum* sp. SR55	–		+	21	+	0	–	
9	*Azospirillum* sp. SR98	–		±		±		–	
10	*A. lipoferum* SR33	–		–		±		+	36
11	*A. lipoferum* SR42	–		–		–		+	57.5
12	*A. lipoferum* SR43	–		–		±		–	
13	*A. lipoferum* SR54	–		–		–		+	0
14	*A. lipoferum* SR59BT	±		±		±		–	
15	*A. lipoferum* SR66	+	16	+	28	+	41	–	
16	*A. lipoferum* SR85	–		+	27	–		±	
17	*A. lipoferum* SR94	–		+	0	±		+	0
18	*A. lipoferum* SR99	±		+	31	±		±	
19	*A. brasilense* Sp7 (T)	–		±		±		+	9.5
20	*A. brasilense* SR7	–		–		–		+	31
21	*A. brasilense* SR8	±		±		+	22	+	16
22	*A. brasilense* SR15	–		–		±		+	0
23	*A. brasilense* SR56	–		–		±		+	15
24	*A. brasilense* SR75	–		±		±		+	33
25	*A. brasilense* SR80	–		–		+	25	+	56.5
26	*A. brasilense* SR88	–		–		±		+	19
27	*A. brasilense* SR96	±		±		±		+	18
28	*A. brasilense* SR100	–		–		–		+	6
29	*A. brasilense* SR103	–		±		±		+	0
30	*A. brasilense* SR109	–		–		–		+	43
31	*A. brasilense* SR111	±		±		±		±	
32	*A. brasilense* SR115	±		+	9	±		+	11
33	*A. brasilense* Sp245	–		±		±		+	22

Note: "–" stands for absence of growth on agar-containing medium with oil; "±" stands for weak growth; "+" stands for considerable growth. Degradative activity was measured only for strains exhibiting considerable growth on the oil-supplemented media.

Studies of the effects of nitrogen and an additional source of carbon on the oil degradation by *A. brasilense* SR80 showed that the biomass growth was highest on media containing malate as an additional source of carbon and energy, irrespective of the source of nitrogen (Table 2). However, oil degradation was not pronouncedly stimulated by malate, nitrogen source being more crucial. Maximal level of oil degradation was achieved on media containing ammonium nitrogen (56.4 and 40% in the presence and absence of malate, respectively). Higher level of degradation in the presence of malate resulted from a threefold higher biomass increase.

In the presence of nitrate nitrogen in combination with malate, the degradative activity of *A. brasilense*

Table 2. Effect of nitrogen and malate on the growth and degradative activity of *Azospirillum brasilense* strain SR80 towards crude oil (1%) on Nfb medium

Source of nitrogen	Additional source of carbon	Growth on solid medium	Growth in liquid medium	Oil degradation, %
–	Malate	+	3.0×10^8	27.8
–	–	±	1.1×10^8	34.3
KNO_3	Malate	+	2.6×10^9	36.4
KNO_3	–	+	2.1×10^8	27.6
NH_4Cl	Malate	+	6.8×10^8	56.4
NH_4Cl	–	+	2.2×10^8	40.0

Note: Dose of inoculum was 2.16×10^7 cells/ml. Oil content decrease in an abiotic control was 16–21%.

SR80 was much lower (36.4%), but the biomass yield was maximal. Presumably, nitrate is a more preferable source of nitrogen for malate utilization. Without a cosubstrate, the level of oil degradation dropped to 27.6% (which was close to oil dissipation in the abiotic control). This allowed us to conclude that the level of degradation on a medium for plant growth would be also quite low.

Remarkably, strain SR80 grew and degraded oil on a nitrogen-free medium. In the presence of malate, a biomass increase was detected. Without a cosubstrate, cells used crude oil for growth, which resulted in an increase in the level of degradation (34.3% compared to 27.8%) but minimal biomass growth.

Thus, we proved the ability of *A. brasilense* strain SR80 to degrade crude oil (including nitrogen-free conditions) and the crucial role of malate for microbial growth and degradation of pollutants in the presence of ammonium and nitrate nitrogen; i.e., *A. brasilense* SR80 may participate in the degradation of crude oil in an association with an appropriate cereal culture supplying additional organic substrate (e.g., malate).

Thus, we studied an association of *A. brasilense* SR80 and wheat and the effects of oil pollution on this association.

Positive chemotaxis to root exudates is considered to be the first step in the formation of a plant–microbial association. It is known that azospirilla are highly motile and exhibit positive chemotaxis to organic acids, sugars, and root exudates [6].

Our experiments also demonstrated the ability of *A. brasilense* SR80 to move toward root exudates. As Table 3 shows, attractant properties were most pronounced for the homogenate of wheat roots. Growth around the cylinder with this attractant was visible on the 2nd day of the experiment. The strain also showed positive chemotaxis toward root exudates: growth around the cylinder with the broth from plant cultivation was detected on the 7th day of the experiment. Chemotaxis toward oil was detected on the 10th day of the experiment, which is an extra evidence of the oil-degrading activity of *A. brasilense* SR80. The data obtained allow a conclusion that oil pollution does not impede formation of an associative symbiosis of wheat and azospirilla. Moreover, the presence of oil in the plant rhizosphere may fasten root colonization by degradative strains. This is proved by our data presented in Table 4. The number of azospirillum cells in the rhizosphere of oil-polluted plants was significantly higher compared to nonpolluted plants.

Bacteria of the genus *Azospirillum* are well-known for their plant-growth-stimulating properties [6, 15, 16]. Initially, the interest of the researches in azospirilla was connected to their ability of fixing molecular nitrogen, but more profound research showed that the positive influence of azospirilla on plant growth and development is of a multiple-factor nature. Azospirilla produce growth-stimulating substances (auxins, gibberellins, and cytokinins), which plays an important role in the interaction with the host plant [6]. There is evidence that the leading factor in the growth-stimulating activity of azospirilla is their ability to produce indole-3-acetic acid (IAA) and thus to replenish the hormone pool of the plant [6, 17]. Therefore, we studied synthesis of indole-3-acetic acid by *A. brasilense* SR80 as an associative property of this microorganism towards wheat and the effects of crude oil on this process.

We showed that strain SR80 was capable of producing more that 20 μg/ml IAA over 14 days of cultivation (figure). Addition of 1% oil to the medium did not result in a decrease in phytohormone synthesis. Analysis of

Table 3. Chemotaxis in *A. brasilense* SR80

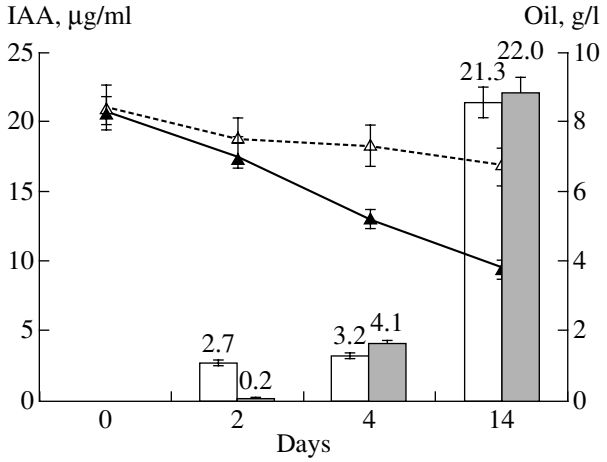
Attractant	Growth zone diameter, mm	Time of growth zone development, days
Root homogenate	2.6	2
Root exudate-containing mineral medium after plant growth	1	7
Mineral medium for plant growth	–	–
Crude oil	2	10

Table 4. Degradation of oil by an azospirillum–wheat association and the effect of *A. brasilense* strain SR80 on the development of the plant root system under hydroponic conditions with and without pollutant

Experimental variant	Development of the plant root system		Root colonization by the microorganism, cells/g of fresh roots	Oil degradation, %
	Number of roots	Length of roots, cm		
Medium without oil				
Inoculated plant	6	9.57 ± 0.39	$1 \times 10^8 \pm 1.2 \times 10^7$	–
Sterile plant	6	7.91 ± 0.30	–	–
Medium with 1% crude oil				
Inoculated plant	3	0.97 ± 0.03	$7 \times 10^8 \pm 9.1 \times 10^7$	28.1 ± 5.5
Sterile plant	2	0.75 ± 0.03	–	20.8 ± 7.96

microbial degradation of oil in these experiments also proved the advantages of Nfb medium. As the figure shows, the level of oil degradation on Nfb medium supplemented with tryptophane was 54% after 14 days. Thus, oil pollution did not result in a decrease in the growth-stimulating activity of the microorganism under study in terms of IAA synthesis.

Evidently, this explains the positive influence of the microorganism on the development of the plant root system that we observed while growing wheat seedlings in a hydroponic chamber in the presence of oil (Table 4). The addition of oil to the nutrient solution resulted in an approximately tenfold inhibition of root development in comparison with the control (a decrease in the number and length of the developing roots). However, preliminary bacterization of seedlings with a suspension of *A. brasilense* SR80 resulted in stimulation of root development. Moreover, the effect was more pronounced in the presence of oil, 23% stimulation in comparison with 17% stimulation without oil. This, apparently, resulted from a sevenfold enhancement of the bacterial population in the presence of oil as compared to the control (a pronounced stimulating effect of the pollutant on the bacterial population was evident).



Effect of crude oil on synthesis of indole-3-acetic acid by *A. brasilense* strain SR80 on Nfb medium without (▭) and with (▬) oil: (△) oil decrease in abiotic control; (▲) oil degradation by strain SR80.

Our studies of oil degradation by an azospirillum–wheat association showed that under hydroponics conditions, sterile wheat seedlings alone eliminated more than 20% of oil from the medium. To answer the question whether this oil was degraded or consumed by the plant, additional studies are required. In the case of bacterization, the oil content of the medium decreased by 28.1% after cultivation. We concluded that it was the bacterial partner in the association that provided the extra decrease in oil concentration. Thus, the presence of azospirilla in the plant rhizosphere resulted in a decrease of the toxic effect of oil on the plant, promoting plant survival and development under oil pollution. Although we conducted our experiments with a model association and the observed increase in the level of oil degradation was not large (only 9%), the revealed mechanisms and methods may be extended to other associations more promising for phytoremediation.

Studies of associative degradation of hydrophobic substrates under hydroponic conditions cannot adequately represent the process of phytoremediation via rhizosphere microbial degradation of a pollutant. Nevertheless, the experiments conducted allowed us to reveal certain ecological plant–microbial interactions under conditions of oil pollution. For the first time it was shown that bacteria of the genus *Azospirillum* are capable of degrading oil hydrocarbons. It was also shown that azospirilla degrade hydrocarbons mostly in the presence of organic acids and that production of indole-3-acetic acid by azospirilla is not inhibited by hydrocarbon pollution. Bacterization of a plant with associative oil-degrading rhizobacteria stimulated the development of the plant root system.

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REFERENCES

1. Siciliano, S.D. and Germida, J.J., Mechanisms of Phytoremediation: Biochemical and Ecological Interactions between Plants and Bacteria, *Environ. Rev.*, 1998, vol. 6, pp. 65–79.
2. Walton, B.T., Hoylman, A.M., Perez, M.M., Anderson, T.A., Johnson, T.R., Guthrie, E.A., and Christman, R.F., in *Bioremediation through Rhizosphere Technology*, Anderson, T.A. and Coats, J.R., Eds. Washington: Am. Chem. Soc., 1994, pp. 82–92.
3. Gunther, T., Kirsche, B., and Fritsche, W., in *Bioremediation of Contaminated Soil*, Wise, D.L., *et al.*, Eds., New York: Marcel Dekker, 2000, pp. 285–293.
4. Ismailov, N.M., Microflora and Enzyme Activities in Oil-contaminated Soils, *Vosstanovlenie neftezagryaznennykh pochvennykh ekosistem* (Bioremediation of Oil-contaminated Soil Ecosystems), Glazovskaya, M.A., Ed., Moscow: Nauka, 1988.
5. Kireeva, N.A., *Mikrobiologicheskie protsessy v neftezagryaznennykh pochvakh* (Microbiological Processes in Oil-contaminated Soils), Ufa: Bashk. Gos. Univ., 1994.
6. Steenhoudt, O. and Vanderleyden, J., *Azospirillum*, a Free-Living Nitrogen-Fixing Bacterium Closely Associated with Grasses: Genetic, Biochemical and Ecological Aspects, *FEMS Microbiol. Rev.*, 2000, vol. 24, pp. 487–506.
7. Miller, J.H., *Experiments in Molecular Genetics*, Cold Spring Harbor: Cold Spring Harbor Lab., 1972.
8. Tarrand, J., Krieg, N., and Dobereiner, J., A Taxonomic Study of the *Spirillum lipoferum* Group, with Descriptions of a New Genus *Azospirillum* gen. nov. and Two Species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov., *Can. J. Microbiol.*, 1978, vol. 24, pp. 967–980.
9. Dobereiner, J. and Baldani, V.L.D., Selective Infection of Maize Roots by Streptomycin-Resistant *Azospirillum lipoferum* and Other Bacteria, *Can. J. Microbiol.*, 1979, vol. 25, pp. 1264–1269.
10. MacClung, S., Recent Developments in Microbiological Techniques, *Annu. Rev. Microbiol.*, 1949.
11. Koronelli, T.V. and Komarova, T.I., The Chemotactic Reactions of a Paraffin-oxidizing *Pseudomonas aeruginosa* Strain, *Mikrobiologiya*, 1982, vol. 51, no. 4, pp. 689–690.
12. *Metody izucheniya pochvennykh mikroorganizmov i ikh metabolitov* (Methods for Studying Soil Microorganisms and Their Metabolites), Krasil'nikova, Ed., Moscow: Mosk. Gos. Univ., 1966.
13. Yakovlev, A.D., *Poroshkovye kraski* (Powdered Paints), Leningrad: Khimiya, 1987.
14. Barkovskii, A.L., Boullant, M.-L., and Balandeau, J., 1979 Phenolic Compounds Respired by Bacteria, in *Bioremediation through Rhizosphere Technology*, Anderson, T.A. and Coats, J.R., Eds., Washington: Am. Chem. Soc, 1994, pp. 28–42.
15. Okon, Y. and Vanderleyden, J., Root-Associated *Azospirillum* Species Can Stimulate Plants, *ASM News*, 1997, vol. 63, pp. 366–370.
16. Umali-Garcia, M., Hubbell, D.N., Gaskins, M.H., and Dazzo, F.B., Association of *Azospirillum* with Grass Roots, *Appl. Environ. Microbiol.*, 1980, vol. 39, pp. 219–226.
17. Barbieri, P. and Galli, E., Effect on Wheat Root Development of Inoculation with an *Azospirillum brasilense* Mutant with Altered Indole-3-Acetic Acid Production, *Res. Microbiol.*, 1993, vol. 144, pp. 69–75.