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EXPERIMENTAL ARTICLES

Negative Effect of Alkylresorcinols on Motility of Rhizobacteria *Azospirillum brasilense*

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Abstract—In liquid media, rhizobacteria *Azospirillum brasilense* are motile by means of a polar flagellum and in semisolid media, also by means of additionally synthesized lateral flagella. In the presence of 4-*n*-hexylresorcinol (0.04–0.10 mM), the swimming rate of *A. brasilense* Sp245 decreased significantly. At the concentrations over 0.10 mM, motility was completely suppressed. The presence of 4-*n*-hexylresorcinol in liquid medium also had a negative effect on motility of the cells of Sp245 and other *A. brasilense* strains (Sp7, Cd, and SR75). The concentrations of 5-methylresorcinol which negatively affected the rate of bacterial spreading in semisolid media were an order of magnitude higher than those of 4-*n*-hexylresorcinol. Alkylresorcinols, which belong to alkylhydroxybenzene autoregulators, probably affect the cellular mechanisms (systems) responsible for flagella rotation in azospirilla.

Keywords: Azospirillum brasilense, motility, alkylresorcinols, 4-*n*-hexylresorcinol, 5-methylresorcinol, extracellular autoregulators

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In nature, bacteria of the genus *Azospirillum* (family *Rhodospirillaceae*) occur usually on the roots of higher plants growing under a variety of climatic conditions [1, 2]. These microorganisms are of interest due to their ability to stimulate growth of many plants, including the agriculturally important ones [1].

Colonization of plants by azospirilla depends on their motility in soil and the sensitive system of chemotaxis "tuned" to specific components of the exudates of the preferred partner [1]. Motility of azospirilla in liquid media is due to a single polar flagellum. Additionally synthesized numerous lateral flagella are also responsible for their swarming in semisolid media [1–3].

Azospirilla inhabiting the plant rhizosphere are subject to various environmental factors, including alkylhydroxybenzene derivatives, e.g., alkylresorcinols. These compounds are produced by plants and many microorganisms and act as nonspecific autoinducers with a broad spectrum of adaptogenic and regulatory functions [4–6]. Alkylhydroxybenzenes may affect bacterial metabolism and induce bacterial transition into a dormant state. The mechanisms responsible for these effects include the interaction between alkylhydroxybenzenes and the membrane lipids, resulting in crystallization of the lipid stroma of the membranes, inhibition of their functional activity, including the energy-producing processes, an increase of the membrane permeability for monovalent ions, and dehydration of the protoplast [7, 8]. Moreover, alkylhydroxybenzenes act as low-molecular modifiers affecting the structure of protein macromolecules [9]. Although the effect of these compounds on rhizobacteria is insufficiently studied, alkylresorcinols were found in various parts of the cereal caryopsis and in root exudates [5, 6].

The goal of the present work was to investigate the effect of alkylresorcinols, one of the classes of alkylhydroxybenzenes, on motility of *A. brasilense* in media of different density, a behavioral aspect important for formation of its associations with plants.

MATERIALS AND METHODS

Strains and cultivation conditions. The strains of *A. brasilense* used in the work are listed in the table.

Bacteria were grown in malate salt medium (MSM) [13] at 30°C; when required, relevant concentrations of Bacto agar were added.

Cultivation in liquid media was carried out in Erlenmeyer flasks on a temperature-controlled shaker (180 rpm) or in a thermostat under stationary conditions. Liquid cultures (18 h) grown under the same conditions as the experimental ones were used as inocula. The density of bacterial cultures was assessed using the turbidity standard and adjusted to initial cell

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density of 10^7 cells/mL. Alkylresorcinols 4-*n*-hexylresorcinol and 5-methylresorcinol (orcinol) (Sigma, United States), carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), or amiloride (N-amidino-3,5-diamino-6-chloropyrasine carboxamide) (Sigma, United States) as 1 M stock solutions were added to the medium together with the inoculum (the range of their final concentrations is specified in the Results and Discussion section). The solvents were 96% ethanol for CCCP and 4-*n*-hexylresorcinol, 50% ethanol for 5-methylresorcinol, and dimethyl sulfoxide for amiloride. The medium was supplemented with 50 μg/mL of 2,3,5-triphenyl tetrazolium chloride (Fluka, Switzerland), an indicator on microbial respiratory activity.

Bacterial motility in liquid medium. To investigate motility of the cells, the cultures were grown in a liquid MSM medium supplemented with alkylresorcinols, CCCP, or amiloride. The rate of cell motility was determined using a software package for image analysis as described earlier [14].

Bacterial motility in semisolid medium. Bacterial motility was determined in semisolid media with 0.4% agar. Microorganisms from solid MSM were stab-inoculated into the medium. Morphology of the zones of bacterial spreading was assessed visually.

Number of viable bacterial cells. Growth of the cultures was monitored by optical density (A_{590}) on a Spekol 221 spectrophotometer (Carl Zeiss, Germany). The number of viable cells was determined by enumeration of colony-forming units (CFU). Two parallel dilution series were prepared for each sample and plated on MSM agar medium. Bacterial numbers were calculated per 1 mL of the culture.

Statistical treatment of the results. At least five independent experiments were carried out in each case. All quantitative results were treated using the Microsoft Office Excel SP1 software package (11.6355.5360). The confidence intervals were determined for the 95% confidence level.

RESULTS AND DISCUSSION

Growth and motility in the presence of alkylresorcinols. In liquid medium, the cells of *A. brasilense* Sp245 swim by means of a polar flagellum (Fla); the movement is straight, with random changes of direction. Addition of 0.04 to 0.12 mM 4-*n*-hexylresorcinol did not change the character of the movement, but resulted in a significant decrease in its rate (Fig. 1). The swimming rate was already decreased 1 min after addition of hexylresorcinol (Fig. 1). Motility was completely suppressed in the presence of 0.12 mM hexylresorcinol (Fig. 1). At its lower concentrations (0.04–0.8 mM), bacteria were able to overcome the stress after 18 h, so that their swimming rate increased, although it still remained below the control level. At concentrations of 0.10 mM and higher, the cells remained completely immobilized (Fig. 1). Ethanol used to dissolve hexylresorcinol had no effect on motility (aliquots of ethanol equal to those used to dissolve the inhibitor were added to the culture).

Azospirillum brasilense strains used in the work

Strain	Characterization	Source
Sp7	Wild type isolated from the rhizosphere of crabgrass, Brazil	[2]
Cd	Wild type isolated from the roots of Bermuda grass inoculated with *A. brasilense* Sp7, United States	[10]
Sp245	Wild type isolated from wheat roots, Brazil	[11]
SR75	Wild type isolated from wheat germs, Russia	[12]

For movement on agar media, apart from the polar flagellum, additional lateral flagella are required. Their synthesis commences at agar concentrations of 0.4% or higher [3]. On semisolid MSM medium, hexylresorcinol concentrations from 0.04 to 0.12 mM resulted in a decrease in the diameter of the colonies formed by motile cells of *A. brasilense* Sp245 (Fig. 2). The presence of hexylresorcinol in semisolid medium had a negative effect on the size of colonies formed by all *A. brasilense* strains studied (Sp245, Sp7, Cd, and SR75) (Fig. 3). This effect may indicate the action of hexylresorcinol on both motility and growth of the *Azospirillum* population.

To assess the effect of hexylresorcinol of the viability of azospirilla, bacteria were grown in liquid

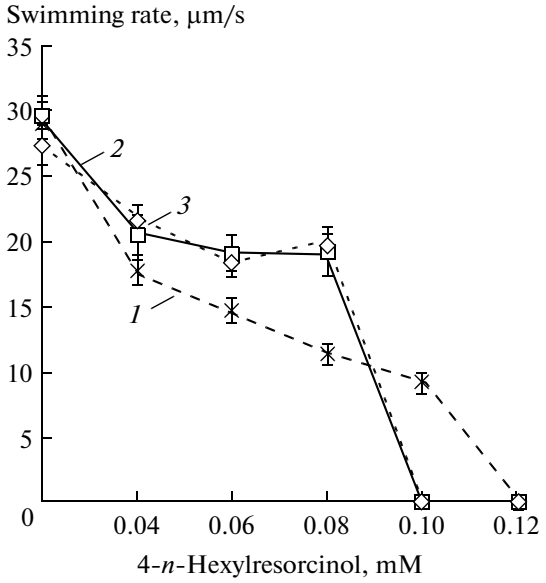

Fig. 1. Effect of 4-*n*-hexylresorcinol on motility of *A. brasilense* Sp245 cells grown in liquid MSM medium under stationary conditions. Incubation duration: 1 min (*1*), 18 h (*2*), and 36 h (*3*).

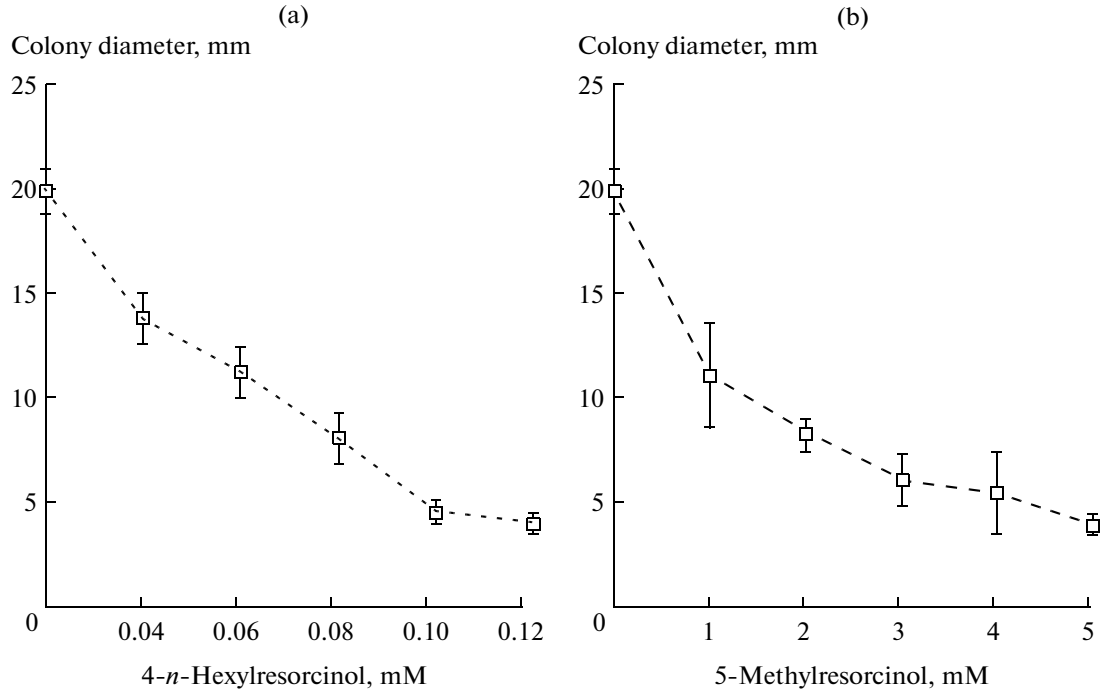

Fig. 2. Diameter of the colonies formed by *Azospirillum brasilense* Sp245 after 36 h in the presence of different concentrations of 4-*n*-hexylresorcinol (a) and 5-methylresorcinol (b) in semisolid (0.4% agar) MSM medium. The inoculation zone was 3.3 ± 0.2 mm in diameter.

medium under stationary conditions. After 36 h of incubation, CFU number in the culture of *A. brasilense* Sp245 with 0.10 mM hexylresorcinol was $(8.6 \pm 1.0) \times 10^5$ cells/mL, i.e., it was significantly lower than $(1.5 \pm 0.2) \times 10^8$ cells/mL found in the control. At lower hexylresorcinol concentrations (0.04, 0.06, and 0.08 mM), CFU numbers were $(1.3 \pm 0.2) \times 10^8$, $(1.6 \pm 0.2) \times 10^8$, and $(1.2 \pm 0.3) \times 10^8$ cells/mL, respectively, which was close to the value in the control. Thus, while 36 h of incubation with 0.04–0.08 mM hexylresorcinol did not affect growth of *A. brasilense* Sp245, it had a negative effect on their motility by means of the polar flagellum (Fig. 1). Diameter of the colonies on semisolid media decreased with increasing concentration of the inhibitor. These findings indicate that the differences in the colony size were probably due to hexylresorcinol affecting the functioning of the lateral flagella.

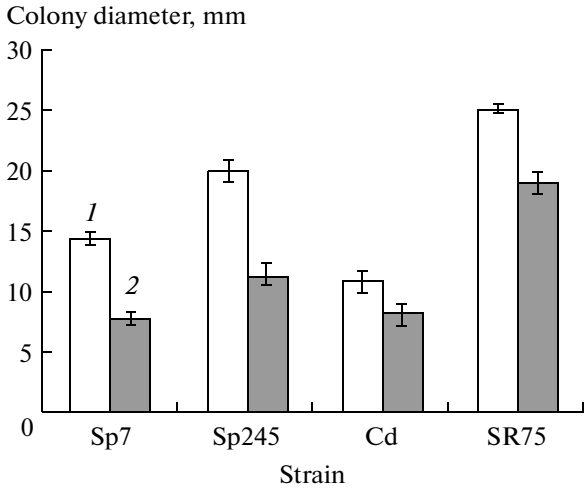

Fig. 3. Diameter of the colonies formed by different *Azospirillum* strains after 36 h in semisolid (0.4% agar) MSM medium (*1*) and in the presence of 0.06 mM 4-*n*-hexylresorcinol (*2*).

The concentrations of methylresorcinol resulting in decreased spreading rates on semisolid media were an order of magnitude higher than the concentrations of hexylresorcinol causing the same effect (Fig. 2). According to the literature data, the biological activity of alkylresorcinols is affected by the length and position of the alkyl radical [4, 15]. The differences in the effect of two alkylresorcinols on bacterial motility are in agreement with these data.

Importantly, azospirilla grown in semisolid medium with or without hexyl- and methylresorcinol (Fig. 2) reduced 2,3,5-triphenyltetrazolium chloride with formation of red formazan. In the presence of this indicator, pink coloration at the point of inoculation became visible after 2–3 h of incubation, while the colonies formed after 18–36 h were bright pink in color. Thus, alkylresorcinols in the concentration range used in our experiments had no significant effect on bacterial respiration in semisolid media.

Our results propose that alkylresorcinols, which belong to alkylhydroxybenzene autoregulators, affect the cellular mechanisms (systems) responsible for the flagella rotation. This effect may be due to the known ability of alkylresorcinols to interact with the membrane lipids [7, 8].

Effect of amiloride, an inhibitor of sodium channels, and of a protonophore carbonyl cyanide *m*-chlorophenyl hydrazone on bacterial motility. The energy required for rotation of bacterial flagella is stored as the H^+ or Na^+ transmembrane potential [16]. Comparison of the effect of amiloride, an inhibitor of sodium channels, a protonophore carbonyl cyanide *m*-chlorophenyl hydrazone, and alkylresorcinols on bacterial motility could reveal the effect of alkylhydroxybenzenes on the cellular mechanisms (systems) responsible for the rotation of flagella. In such bacteria with mixed flagellation as *Vibrio parahaemolyticus*, the polar flagellum is powered by the sodium motor, while the lateral flagella, by the proton motor [17]. The motion of *A. brasilense* Sp7 was found to be arrested by 3 mM of amiloride, an inhibitor of the Na^+ channels [18]. In our experiments, the cells of *A. brasilense* Sp245 lost motility after 30-min incubation with 4 mM amiloride or after 10–18 h of incubation with 0.04 mM amiloride. In the presence of hexylresorcinol, motility was lost after 1 min or 18 h of incubation with similar concentrations of this alkylresorcinol (Fig. 1). Unlike amiloride, which blocks the Na^+ channels of the flagellar motor, the effect of alkylresorcinols on bacterial motility probably stems from their nonspecific action on the functional activity of the membranes, including energy production.

The pumps required to maintain the transmembrane potential of sodium ions function in bacteria at the ambient pH above 8.5 [16]. Growth of *A. brasilense* Sp245 in liquid MSM medium with sodium malate as the carbon source resulted in pH shift from 7.0 ± 0.1 (at the moment of inoculation) to 9.0 ± 0.2 after 18 h of incubation (at the late exponential phase of growth). The swimming rate of *A. brasilense* cells within this pH range did not change (Fig. 1), indicating existence of some labile (rapidly rearranging) systems providing energy for the flagellar motor.

At the ambient pH 7.0–8.5, azospirilla probably use the Na^+/H^+ antiport to maintain the Na^+ transmembrane potential. For example, the cells of *A. brasilense* Sp245 lost motility 1 min after addition of CCCP (2.5 μM). This compound is a weak acid impairing the proton transmembrane gradient [19]. After 18 h of incubation with CCCP, bacteria restored their motility, probably due to a decrease in concentration of the protonated form of the uncoupler due to the pH increase in the course of microbial growth. The swimming rate of the cells grown in the presence of CCCP was lower than that of the bacteria grown without protonophores and did not depend on the concentration of the uncoupler [20]. The low swimming rate could partially result from CCCP inhibiting bacterial respiration [19].

Unlike prolonged incubation with CCCP, motility of *A. brasilensis* cells was not restored when the cells were grown in the presence of hexylresorcinol (Fig. 1). At hexylresorcinol concentrations of 0.10 mM and higher, the cells remained nonmotile. Only within the concentration range from 0.04 to 0.08 mM, the swimming rate increased slightly after 18 h of incubation. Unlike CCCP, which affects the proton transmembrane gradient, the effect of resorcinols on bacterial motility probably resulted from their nonspecific action on the functional activity of the membranes due to their incorporation into the membrane, leading to irreversible impairment of the ion flows [7, 8].

Motility is an indicator of the energetic activity of microorganisms [16]. Autoregulators, including alkylresorcinols, are able to modify bacterial metabolism, inducing transition of a bacterial culture into the stationary phase and then into a dormant state [4]. Our data indicate that decreased motility of the *Azospirillum* cells in the presence of 4-*n*-hexylresorcinol or 5-methylresorcinol may result from modifications in bacterial metabolism affecting the cellular systems responsible for rotation of the flagella [7, 8, 16].

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