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EXPERIMENTAL ARTICLES

The Chemotactic Response of *Bradyrhizobium japonicum* to Various Organic Compounds

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Abstract—The investigation of the chemotactic response of *Bradyrhizobium japonicum* to amino acids, carbohydrates, multiatomic alcohols, organic acids, and soybean extracts showed that the extracts of some soybean varieties (Chernoburaya and Beskluben'kovaya) contain repellents. This indicates that the soybeans of host plants contain effectors that may play a role at the early stages of their interaction with nodule bacteria.

Key words: chemotaxis, *Bradyrhizobium japonicum*, organic compounds, soybean extracts.

Considerable progress made in the study of bacterial chemotaxis in the second half of the 20th century was related to the Adler capillary method [1]. After introducing this method to research practice, many bacteria have been studied for chemotaxis. Of particular interest is the chemotaxis of nodule bacteria [2–8], which form legume–rhizobial symbiotic complexes. It is believed that the formation of root nodules is largely determined by the positive chemotaxis of bacteria toward chemical substances exuded by the legume roots [3, 4, 6, 7].

The aim of the present work was to study the chemotactic response of *Bradyrhizobium japonicum* to various classes of organic compounds and to soybean extract, which contains a variety of plant metabolites [9].

MATERIALS AND METHODS

Experiments were carried out with slow-growing strains of the nodule bacterium *Bradyrhizobium japonicum*: 634b (industrial strain), 10K (obtained from the Ukrainian Collection of Microorganisms), and 604K (a highly virulent strain, which was kindly donated by N.Z. Tolkachev from the Crimean Branch of the Institute of Soil Microbiology, Ukraine). The strains were grown in the mannitol–yeast extract medium described earlier [8].

Chemotactic responses were studied using some amino acids, carbohydrates, multiatomic alcohols, organic acids, ethanol, and soybean extracts. The chemical substances used were of analytical grade.

Extracts were prepared from soybeans of the varieties Kievskaya-27, Chernoburaya, Soer, Mar'yana, and Beskluben'kovaya. The soybeans were made aseptic by irradiating them with UV light for 48 h and fractionated into cotyledons and coats. The cotyledons and coats were powdered separately. The powders were extracted with a mixture (1 : 1) of 96% ethanol and 10 mM K-phosphate buffer (pH 7.0) for 36 h. The weight percent of the powders in the extracting solution was 10%. After extraction, the solid residue was removed by centrifugation at 2000 *g* for 20 min, and the supernatant was analyzed for the content of carbohydrates by the phenol–sulfuric acid method [10], for the content of proteins by the Bradford method [11], and for the con-

Table 1. The chemotaxis of *B. japonicum* strains toward amino acids

Amino acid	Bacterial propagation zone width, mm		
	Strain		
	634b	10K	604K
L-Arginine-HCl	1.0	0.5	2.0
D,L-β-Phenylalanine	10.0	3.0	6.0
D,L-Lysine-HCl	7.0	2.0	5.6
D,L-Methionine	2.0	3.0	5.0
D,L-Serine	1.0	3.3	6.6
D,L-α-Alanine	1.0	4.0	10.0
L-Glutamic acid	12.0	4.0	7.6
L-Cysteine	2.0	2.6	6.0
D,L-Aspartic acid	2.0	5.0	9.0
L-Isoleucine	2.0	1.2	2.0
L-Histidine-HCl	3.0	3.7	4.3
D,L-Threonine	13.6	1.0	3.0
Control	1.0	1.0	1.0

Note: The concentration of amino acids was 10^{-3} M. Confidence limits did not exceed 10% of arithmetic means.

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Table 2. The chemotaxis of *B. japonicum* toward some carbohydrates, multiatomic alcohols, and organic acids

Substance	Bacterial propagation zone width, mm		
	strain		
	634n	10K	604K
L[+]-Arabinose	10.0	9.3	14.0
L[+]-Rhamnose	3.0	2.0	3.0
D-Glucose	6.0	5.0	6.0
Sucrose	5.0	3.0	6.0
D[+]-Maltose	5.0	6.0	6.0
D[+]-Lactose	0.0	0.0	0.0
Sorbitol	6.0	5.0	7.0
D[-]-Mannitol	8.0	10.0	15.0
Dulcitol	7.0	5.0	9.0
L-Inositol	5.0	4.0	10.0
Succinic acid	5.0	3.0	4.0
Citric acid	10.0	9.0	10.0
Acetic acid	5.0	3.0	6.0
Control	1.0	1.0	1.0

Note: The concentration of chemotactic effectors was 10^{-3} M. Confidence limits did not exceed 10% of arithmetic means.

tent of dry matter by determining the weight difference before and after desiccation at 105°C.

Chemotaxis was studied by the plate and capillary methods. With the plate method, petri dishes were filled with a semiliquid medium containing 0.35% agar, 10 mM K-phosphate buffer (pH 7.0), and a chemotactic effector at a concentration of 1 mM. The control plates contained the same medium without effector. After the agar had hardened, 20-μl aliquots of a microbial suspension containing about 10^8 cells/ml were placed in the agar plate centers. The plates were incubated at 28°C for 72 h and then analyzed for the presence of a chemotactic ring. The width of the zone of microbial propagation was measured from the border of the microbial suspension drop placed at the plate center.

With the capillary method, which was modified as described earlier [8], the soybean extract was diluted 50-, 100-, 500-, 1000-, and 2000-fold. The control media contained the same dilutions of the buffer or the ethanol–buffer mixture. The effect of ethanol on chemotactic responses was estimated from the difference between the number of bacterial cells in the capillary tubes that contained the ethanol–buffer mixture dilutions and the capillary tubes that contained the buffer dilutions. The chemotactic effect due to ethanol was subtracted from the chemotactic effect due to the ethanol–buffer extract of soybeans, so that the results presented in Tables 3–5 reflect the pure chemotactic effect of the soybean substances extractable with 48% ethanol.

To evaluate the effect of the extractable soybean substances on the growth of nodule bacteria, aseptic intact soybeans or soybeans deprived of their outer coat were placed onto the surface of the mannitol–yeast extract agar with a lawn of rhizobia. Alternatively, the wells made in agar plates were filled with the undiluted and diluted (ten- and twentyfold) soybean extract or the ethanol–buffer mixture (in the control). The chemotactic effect of the extractable soybean substances on *B. japonicum* cells was evaluated from the width of the growth inhibition (or stimulation) zone.

The results presented in the paper are arithmetic means at a 5% level of significance.

RESULTS AND DISCUSSION

Of the 12 amino acids studied for their action on the chemotaxis of *B. japonicum* in the semiliquid medium (Table 1), phenylalanine, lysine-HCl, methionine, glutamic acid, and histidine-HCl turned out to be attractants for strains 634b and 10K but not for strain 604K. Unlike strain 634b, strains 10K and 604K exhibited positive chemotaxis toward serine and alanine. Threonine was a strong attractant for strain 634b, a

Table 3. The chemotaxis of the *B. japonicum* strains 634b and 10K toward the soybean extracts of the Chernoburaya variety

Extract dilution	Cell concentration (in 10^8 cells/ml) in the capillar tubes containing			
	soybean coat extract		soybean cotyledon extract	
	634b	10K	634b	10K
1 : 50	0.45 ± 0.09	0.39 ± 0.09	0.37 ± 0.06	0.36 ± 0.07
1 : 100	0.31 ± 0.06	0.34 ± 0.07	0.42 ± 0.08	0.31 ± 0.06
1 : 500	0.26 ± 0.03	0.36 ± 0.06	0.37 ± 0.06	0.34 ± 0.06
1 : 1000	0.13 ± 0.03	0.35 ± 0.06	0.42 ± 0.08	0.35 ± 0.05
1 : 2000	0.38 ± 0.06	0.31 ± 0.04	0.41 ± 0.06	0.29 ± 0.06
Control	0.42 ± 0.06	0.40 ± 0.05	0.39 ± 0.06	0.38 ± 0.06

Note: The control capillar tubes contained K-phosphate buffer.

Table 4. The chemotaxis of the *B. japonicum* strains 634b and 604K toward the soybean extracts of the Beskluben'kovaya variety

Extract dilution	Cell concentration (in 10^8 cells/ml) in the capillar tubes containing			
	soybean coat extract		soybean cotyledon extract	
	634b	10K	634b	10K
1 : 50	0.65 ± 0.09	0.54 ± 0.10	0.63 ± 0.13	0.51 ± 0.09
1 : 100	0.63 ± 0.08	0.52 ± 0.09	0.53 ± 0.11	0.48 ± 0.05
1 : 500	0.44 ± 0.06	0.46 ± 0.09	0.42 ± 0.09	0.43 ± 0.06
1 : 1000	0.45 ± 0.06	0.32 ± 0.06	0.45 ± 0.07	0.34 ± 0.06
1 : 2000	0.60 ± 0.08	0.53 ± 0.07	0.58 ± 0.09	0.52 ± 0.09

Table 5. The chemotaxis of the *B. japonicum* strains 634b and 10K toward the soybean extracts of the Mar'yana variety

Extract dilution	Cell concentration (in 10^8 cells/ml) in the capillar tubes containing			
	soybean coat extract		soybean cotyledon extract	
	634b	10K	634b	10K
1 : 50	0.45 ± 0.09	0.46 ± 0.09	0.63 ± 0.12	0.49 ± 0.08
1 : 100	0.50 ± 0.04	0.43 ± 0.07	0.67 ± 0.10	0.40 ± 0.06
1 : 500	0.51 ± 0.05	0.51 ± 0.08	0.90 ± 0.09	0.37 ± 0.06
1 : 1000	0.64 ± 0.06	0.41 ± 0.06	0.62 ± 0.07	0.36 ± 0.05
1 : 2000	0.44 ± 0.06	0.31 ± 0.06	0.46 ± 0.05	0.34 ± 0.04

weak attractant for strain 604K, and was chemotactically inactive with respect to strain 10K.

Among the carbohydrates tested, arabinose, which is the preferable substrate of slow-growing nodule bacteria [12], was the strongest attractant (Table 2). Glucose, sucrose, maltose, and, to a lesser degree, rhamnose were also positive attractants for the *B. japonicum* strains. At the same time, their chemotaxis toward lactose, which is poorly metabolized in *B. japonicum* [13], was negative.

All the multiatomic alcohols tested (sorbitol, mannitol, dulcitol, and inositol) and organic acids (succinic, citric, and acetic acids) served as attractants for the slow-growing nodule bacteria, the most efficient attractants among the alcohols being mannitol (a universal carbon source for all rhizobia [13]), while citric acid was among the organic acids (Table 2). In the presence of mannitol and citric acid, the propagation zone widths were 8–15 and 9–10 mm, respectively.

Our previous studies showed that the chemotactic response of *B. japonicum* toward glucose increases with its concentration [8]. In the present study, a similar phenomenon was observed for sucrose (Fig. 1), which is present in soybean extracts [9]. The threshold concentration of sucrose for the chemotaxis of strain 10K is 10^{-6} M [1].

It should be noted that sucrose is not metabolized by some slow-growing strains of nodule bacteria [14]. The slow-growing strains used in the present study are likely capable of metabolizing this sugar, since chemotaxis toward a substrate is possible only if this substrate is metabolized, due to which a concentration gradient, the impetus of chemotaxis [15], is produced. When moving toward high sucrose concentrations, *B. japonicum* cells form a chemotactic ring. The formation of this ring again indicates that sucrose is a metabolizable attractant for *B. japonicum.*

The threshold concentration of mannitol for the chemotaxis of strains 634b and 10K was found to be the same as that of sucrose, i.e., 10^{-6} M (Fig. 2).

There are many factors that may influence the behavior of soil bacteria, e.g., pH, oxygen concentration, and the gradients of various substances produced by the soil microflora, microfauna, and plant roots [16, 17]. The plant rhizosphere is characterized by a specific set of conditions, which play an important part in establishing symbiosis between nodule bacteria and leguminous plants. The formation of such a symbiosis suggests the existence of specific molecular interactions between micro- and macrosymbionts [18]. The chemotaxis of bacteria toward the gradients of plant metabolites in the rhizosphere is a prerequisite for such interactions [6]. In some cases, however, plant metabolites may serve as negative attractants (repellents) for rhizobacteria. For instance, Currier and Strobel showed that the nonhost lectins of legumes and peanut are repellents for *Rhizobium meliloti* [19]. Moreover, some sub-

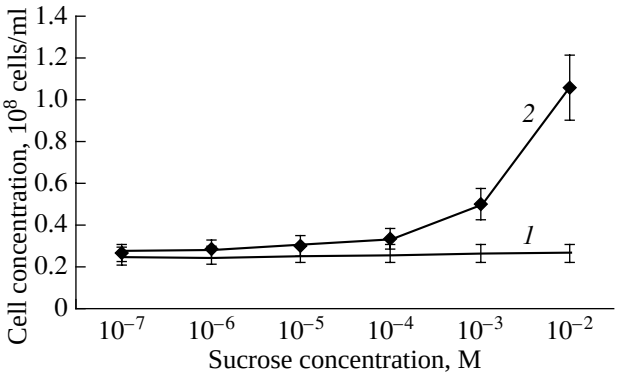

Fig. 1. The effect of sucrose on the chemotaxis of the *B. japonicum* strain 10K. Shown are the concentrations of bacterial cells in the capillary tubes (*2*) with and (*1*) without sucrose.

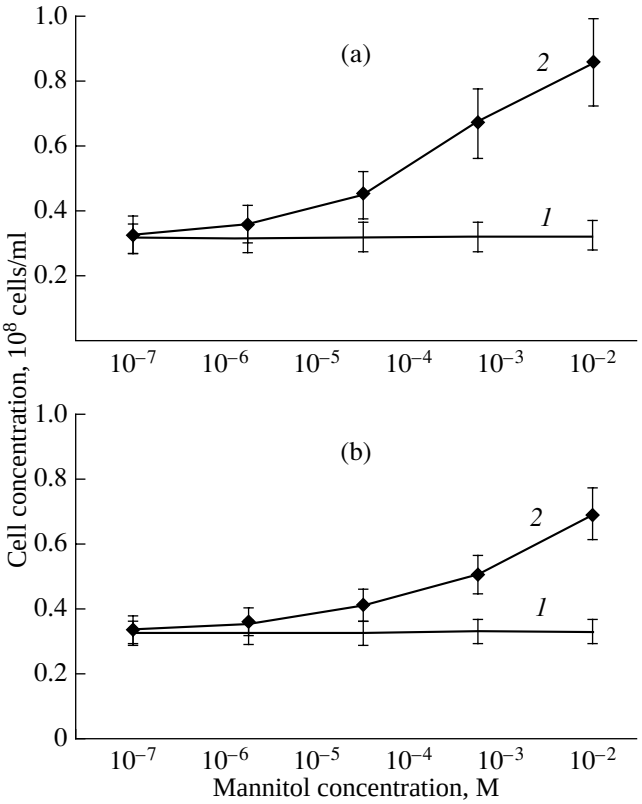

Fig. 2. The effect of mannitol on the chemotaxis of (a) strain 634b and (b) strain 10K. Shown are the concentrations of bacterial cells in the capillary tubes (*2*) with and (*1*) without mannitol.

stances present in peas and soybeans may inhibit the growth of nodule bacteria [20, 21].

Bearing this in mind, we investigated the effect of the extractable soybean substances on the growth and chemotaxis of *B. japonicum*. The experiments showed that the presence of the soybeans of the varieties Kievskaya-27, Soer, Chernoburaya, Mar'yana, and Beskluben'kovaya ("lacking nodules") on a lawn of *B. japonicum* did not exert either a favorable or adverse effect on bacterial growth. Presumably, the concentration of substances exuded by the intact soybeans was insufficient to produce any noticeable effect on bacterial cells.

The extracts of the soybean coats and cotyledons of the varieties Kievskaya-27, Soer, and Mar'yana did not influence the growth of strains 634b and 10K. The ten- and twenty-fold diluted extracts of the soybean coats (but not the cotyledons) of the varieties Chernoburaya and Beskluben'kovaya slightly inhibited the growth of strain 634b (the growth inhibition zone around the wells in agar plates was 2–4 mm wide) and did not influence the growth of strains 10K and 604K. Thus, the soybeans of the varieties Chernoburaya and Beskluben'kovaya likely contain some substances that inhibit the growth of nodule bacteria. The presence of such substances in the soybeans of the Beskluben'kovaya variety, which is unable to produce root nodules, can be easily understood. However, the presence of rhizobial growth–inhibiting substances in the soybeans of the Chernoburaya variety, which is able to produce fully functional root nodules with rhizobia, is a puzzle.

Ethanol, which was used for the extraction of soybeans, served as an attractant at concentrations lower than 0.11 M (strain 10K) or 0.21 M (strain 634b), while it served as a repellent at higher concentrations (Fig. 3).

The chemotactic response of nodule bacteria to the soybean extracts of the rhizobial growth–inhibiting varieties was negative, indicating the presence of repellents. For instance, the extracts of the soybean coats of the Chernoburaya variety induced a negative chemotactic response in strains 634b and 10K (Table 3), and the extracts of the soybean coats and cotyledons of the Chernoburaya variety induced the same response in strains 634b and 10K (Table 4). On the other hand, both types of extracts prepared from the Mar'yana variety soybeans presumably contain attractants for strains 634b and 10K (Table 5).

The investigation of how the dilution degree of the soybean extracts could influence their ability to act as chemotactic effectors showed that the highest negative chemotactic response of strain 634b was induced by the extracts of the soybean coats of the Chernoburaya and Beskluben'kovaya varieties diluted 1000-fold and that the highest negative chemotactic response of strain 604K was induced by the extracts of the soybean coats of the Beskluben'kovaya variety diluted 2000-fold. In the case of cotyledon extracts, the highest positive chemotactic response of strain 634b was induced by the cotyledon extract of the Mar'yana variety diluted 1000-fold and the highest positive chemotactic response of strain 10K was induced by the same extract diluted 50-fold.

Chemical analysis showed that the cotyledon extracts of all the varieties studied contain almost two times greater amounts of proteins, carbohydrates, and dry matter than the extracts of bean coats. The bean coat extracts of the Beskluben'kovaya variety contained the lowest amounts of proteins and carbohydrates (Table 6).

Scher *et al.* [9] showed that soybean extracts contain 18 amino acids and 5 carbohydrates. The investigation of the effect of most of these individual substances (Tables 1 and 2 and Figs. 1 and 2) showed that there is no simple correlation between the chemotactic responses induced in rhizobacteria by soybean extracts and their components. This can be explained by the fact that soybean extracts contain a great amount of substances, which may interfere with each other when interacting with the bacterial receptors responsible for chemotaxis. With the increasing degree of dilution, the interference of particular components in an extract may considerably vary, so that different substances will interact with the chemotactic receptors of bacteria. Moreover, our data and those available in the literature [17] suggest that the chemotactic response of bacteria can be induced by soybean extract components other than proteins or carbohydrates. This suggestion is confirmed by the fact that, of all the extracts studied, the soybean extract of the Beskluben'kovaya variety contains the lowest amounts of proteins and carbohydrates, but can induce a considerable chemotactic response in *B. japonicum*. It should be also noted that the active strains 634b and 10K and the inactive strain 604K exhibited the same chemotactic responses.

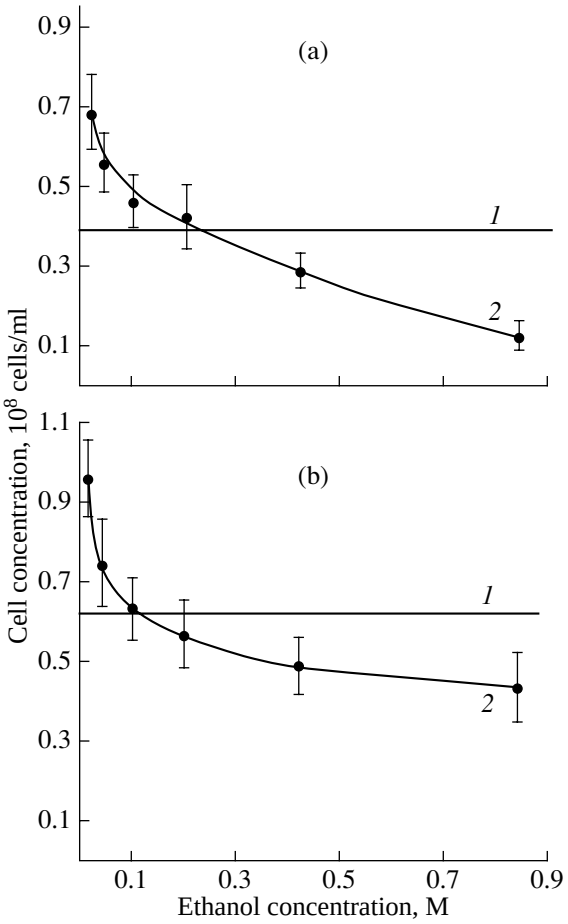

Fig. 3. The effect of ethanol on the chemotaxis of (a) strain 634b and (b) strain 10K. Shown are the concentrations of bacterial cells in the capillary tubes (*2*) with and (*1*) without ethanol.

Thus, the bacterium *B. japonicum* showed positive chemotaxis toward many amino acids, carbohydrates, organic acids, and multiatomic alcohols and negative chemotaxis toward lactose. Ethanol is a repellent at concentrations exceeding 0.11 M, while it is an attractant at lower concentrations. The soybeans of the Chernoburaya and Beskluben'kovaya varieties contain repellents for *B. japonicum*. At the same time, the soybeans of the Mar'yana variety contain attractants for this rhizobacterium. The chemotaxis of nodule bacteria may play a role at the early stages of their interaction with leguminous plants, but this role is not crucial to the establishment of a symbiotic relationship between them. This inference is confirmed by the fact that the soybean extracts of the Chernoburaya variety contain repellents, but this variety is able to form fully functional root nodules with rhizobia.

Table 6. The content of major substances in soybean extracts

Soybean variety	Preparation	Content in mg/ml		
		proteins	carbohydrates	dry matter
Chernoburaya	Soybean coat extract	0.71 ± 0.01	9.25 ± 0.11	14.90 ± 0.12
	Soybean cotyledon extract	1.42 ± 0.03	17.32 ± 0.05	23.85 ± 0.31
Beskluben'kovaya	Soybean coat extract	0.31 ± 0.01	3.50 ± 0.06	7.62 ± 0.23
	Soybean cotyledon extract	1.24 ± 0.02	15.51 ± 0.09	23.60 ± 0.34
Mar'yana	Soybean coat extract	0.93 ± 0.01	7.53 ± 0.10	10.13 ± 0.29
	Soybean cotyledon extract	1.70 ± 0.04	18.15 ± 0.09	23.24 ± 0.32

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