Microbiology, Vol. 69, No. 1, 2000, pp. 103–108. Translated from Mikrobiologiya, Vol. 69, No. 1, 2000, pp. 120–126.

EXPERIMENTAL ARTICLES

Investigation of the Initial Stages of Interaction of the Bacterium *Azospirillum brasilense* with Wheat Seedling Roots: Adsorption and Root Hair Deformation

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Received January 12, 1999

Abstract—The initial stages of colonization of wheat roots by cells of *Azospirillum brasilense* strains 75 and 80 isolated from soils of the Saratov oblast were studied. The adsorption of azospirilla on root hairs of soft spring wheats rapidly increased in the first hours of incubation, going then to a plateau phase. Within the first 15 h of incubation, exponential-phase cells were adsorbed more intensively than stationary-phase cells. Conversely, stationary-phase cells were adsorbed more intensively than exponential-phase cells, if the period of azospirilla incubation with the wheat roots was extended. As the time of incubation increased, the attachment of azospirilla to the wheat roots became stronger. The effect of cell attachment to root hairs was strain-dependent; the number of adsorbed cells of a given strain of azospirilla was greater in the case of host wheat cultivars. The deformation of wheat root hairs was affected by the polysaccharide-containing complexes isolated from the capsular material of azospirilla. The suggestion is made that common receptor systems are involved in the adsorption of azospirilla on roots and in root hair deformation.

Key words: Azospirillum brasilense, adsorption dynamics, wheat, root hair deformation, selectivity.

Azospirilla, which live in rhizospheres, rhizoplanes, and root tissues [1, 2], are known to have a beneficial effect on host plants. Azospirilla are able to bind to the roots of a variety of plants [3], although they show preferential binding to roots of their host plants [4, 5]. A strong adhesiveness of azospirilla to plant roots serves the better survival of these bacteria in soils. On the other hand, attached azospirillas supply the intercellular space of the root cortex with excretory substances and protect binding sites on the root surface from colonization with deleterious microorganisms [1].

The adsorption of azospirilla on plant roots has been found to proceed in two stages. At the first stage of a weak, active, and reversible adsorption, the key role is played by protein substances, while at the second stage of a strong and irreversible adsorption, the key role is presumably played by extracellular polysaccharides [6]. The failure of heat-inactivated azospirilla to bind to roots indicates that only metabolically active bacterial cells are capable of such binding [7]. Azospirilla can best colonize the elongation zone of roots, their tips, and root hairs [8]. The metabolites of azospirilla increase the number of wheat root hairs and cause their deformation; this exerts a beneficial effect on plants and is considered to be one of the early responses of plants to inoculation with these bacteria [9, 10].

Although the initial stages of interaction of bacteria with cereal roots have recently been extensively studied, the mechanism of azospirilla attachment to the roots remains unclear. In particular, little is known as to how the concentration of bacterial suspensions and the time of their incubation with the roots affect the dynamics of adsorption and the adhesiveness of the bacteria to the roots. Furthermore, data on the selectivity of bacterial adsorption with respect to host plants are often contradictory.

The present work was undertaken to study some aspects of azospirilla adsorption on roots in detail, which is important not only theoretically, but also practically, from the standpoint of an adequate choice of optimal conditions for bacterial inoculation. We also attempted to estimate the selectivity of the relationship of phyto- and microsymbionts at the stage of adsorption and the selectivity of the effect of root hair deformation under the action of the polysaccharide-containing complexes isolated from the cell surface of azospirilla.

MATERIALS AND METHODS

Strains and cultivation conditions. *Azospirillum brasilense* strains 75 and 80, isolated from the roots of the wheat cultivars Saratovskaya 29 and Saratovskaya 49,

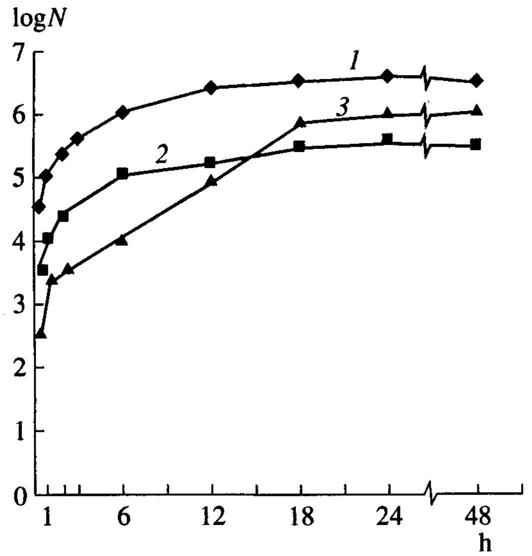

Fig. 1. Dependence of the attachment of *A. brasilense* 75 cells to seedling roots of cv Saratovskaya 29 on cell concentration, growth phase, and incubation time. N is the number of adsorbed cells/cm root. Curve *1* represents the adsorption of exponential-phase 20-h-old cells from a suspension containing 4.0×10^8 cells/ml; curve 2 represents the adsorption of such cells from a suspension containing 2.5×10^7 cells/ml; and curve *3* represents the adsorption of stationary-phase 48-h-old cells from a suspension containing 3.0×10^7 cells/ml.

respectively [11], were obtained from the collection of the Institute of Biochemistry and Physiology of Plants and Microorganisms. The type strain *A. brasilense* Sp7 (ATCC 29145) was a generous gift from D. Janssens, Laboratory of Microbiology, University of Ghent, Belgium.

In bacterial adsorption experiments, cultures were grown in a liquid medium with malate [12] on a temperature-controlled shaker (30°C; 120 rpm) to the end of the exponential phase. Cells were then harvested by centrifugation and washed twice with a phosphate buffered saline (PBS) containing (g/l) KH_2PO_4, 0.43; Na_2HPO_4, 1.68; and NaCl, 7.2 (pH 7.2).

To obtain polysaccharide-containing complexes from the cell surface, azospirilla were grown in an Ankum-2M fermentor (Russia) as described earlier [12].

Plants. Seeds of the soft spring wheat *Triticum aestivum* L. cultivars Saratovskaya 29, Saratovskaya 49, and Saratovskaya 58 were obtained from the Research Agricultural Institute of Southeastern Russia, Saratov.

Inoculation of azospirilla and enumeration of adsorbed cells. Wheat seeds were sterilized as described elsewhere [10]. Sterile seeds were placed on nutrient agar plates and allowed to germinate for 2 days in the dark at 25°C. Seedlings were aseptically transferred to petri dishes containing small amounts of water and incubated over the next 24 h in the dark at 25°C. The adsorption of *A. brasilense* cells was studied using the isolated roots of 3-day-old wheat seedlings. For this purpose, 2-cm segments of roots cut from their apical ends were aseptically transferred to tubes (one segment per tube) containing 4.5 ml of PBS and inoculated with 0.5 ml of a bacterial suspension of a certain optical density. The number of viable cells in the suspension used for inoculation was determined by plating it onto nutrient agar. Roots were incubated with azospirilla on a shaker at 30°C over different time periods (from 0.25 to 48 h), after which root segments were washed thrice with PBS under gentle shaking and assayed for the presence of adsorbed cells as described below.

In bacterial adhesiveness studies, roots with adsorbed cells were subjected either to a gentle stirring or to a vigorous vortexing in an MPW-302 homogenizer (1000 rpm, 50 s), and then the number of adsorbed cells was determined as described below. Vortexing conditions were chosen such as not to damage the root surface and root hairs.

The number of cells attached to roots was determined as follows. Roots with adsorbed cells were homogenized, and the homogenate was suspended in PBS. The suspension was serially diluted, and the dilutions were plated onto agar medium containing malate. The plates were incubated for 3 days at 30°C. The number of viable adsorbed cells was determined by calculating the number of grown colonies. The results of the experiments were statistically processed as described in the handbook [13]. Data are presented for a confidence level of 95%.

Light microscopy. After incubation with azospirilla, wheat roots were washed with PBS, placed in a drop of PBS, covered with a glass slip, and examined under a Biolar PI polarization–interference microscope using either a 20× or 40× objective and a 12× eyepiece.

Technique for investigating the effect of polysaccharide-containing complexes on the morphology of wheat root hairs. The isolation of a lipopolysaccharide–protein complex (LPSPC) and polysaccharide–lipid complex (PSLC) and the slide technique for inves-

Table 1. Time course of the adsorption of *A. brasilense* 80 cells on 3-day-old seedling roots of wheat cv Saratovskaya 58

Incubation time, h	Number of adsorbed bacteria, cells/cm root*
0.25	$(1.10 \pm 0.33) \times 10^4$
1	$(4.27 \pm 1.08) \times 10^4$
2	$(1.10 \pm 0.29) \times 10^5$
3	$(2.27 \pm 0.49) \times 10^5$
4	$(7.72 \pm 2.35) \times 10^5$
6	$(2.07 \pm 0.51) \times 10^6$
9	$(8.21 \pm 1.85) \times 10^5$
12	$(5.61 \pm 1.52) \times 10^5$
24	$(2.98 \pm 0.96) \times 10^5$
48	$(1.99 \pm 0.77) \times 10^5$

Note: Concentration of bacteria in the inoculum was 7.5×10^8 cells/ml.
* Confidence limits are given for a 95% confidence level.

tigating the effect of these complexes on the morphology of wheat root hairs were described in detail in our previous publication [10].

RESULTS AND DISCUSSION

The dynamics of bacterial adsorption on wheat roots was investigated using two pairs of symbionts: *A. brasilense* 75 cells–wheat cv Saratovskaya 29 seedlings and *A. brasilense* 80 cells–wheat cv Saratovskaya 58 seedlings. The results obtained are presented in Fig. 1 (curve *1*) and Table 1. It is evident that the adsorption of exponential-phase cells of *A. brasilense* 75 on wheat roots began as soon as they came into contact and then rapidly increased, so that the number of adsorbed cells reached 3.3×10^4 cells/cm root after 15 min of incubation and 3.9×10^6 cells/cm root after 18 h of incubation (the cell suspension used for the inoculation contained 4.0×10^8 cells/ml). The number of attached cells showed an insignificant increase over the next 6 h and no increase in the course of a longer incubation. In the case of a thinner cell suspension (2.5×10^7 cells/ml), the number of cells attached to roots fell almost by an order of magnitude, but the time course of cell adsorption was the same as in the previous case (Fig. 1, curve *2*).

In both cases, the number of adsorbed cells did not increase after 24 h of incubation, indicating that the cell–root system attained the state of dynamic equilibrium. A similar effect has been observed by other authors in experiments with *A. brasilense* Sp245 [4] and *A. brasilense* Cd [14]. The dilution of the suspension of *A. brasilense* Sp245 cells from 10^7 to 10^4 cells/ml led to a decrease in the number of adsorbed cells. On the other hand, the use of thick cell suspensions containing more than 10^9 cells/ml not only failed to promote cell adsorption [4], but even inhibited the growth of roots [3]. The limited adsorption of bacterial cells on roots has been interpreted by the saturation of bacterial adsorption by virtue of the limited number of binding sites available at the root surface [3, 4, 14]. In our opinion, however, the dependence of bacterial adsorption on the cell concentration in inocula allows an alternative explanation, namely, that the cell suspension used for inoculation was heterogeneous with respect to the adhesiveness of cells to roots. As a result, the most adhesive cells were attached to roots in the early terms of incubation, while the remaining cells were poorly attached to roots.

To elucidate whether the adhesiveness of azospirilla to wheat roots is strain-dependent, we also investigated another pair of symbionts: *A. brasilense* 80 and wheat cv Saratovskaya 58 (Table 1). It can be seen that, after 6 h of incubation of a cell suspension containing 7.5×10^8 cell/ml with roots, the number of attached *A. brasilense* 80 cells reached its maximum value (2.07×10^6 cells/cm root). The number of adsorbed *A. brasilense* 75 cells exceeded the number of adsorbed *A. brasilense* 80 cells throughout the period of their incubation with wheat roots, the difference being more pronounced after 12 h of incubation. Thus, after a 1-h contact with the roots, the number of attached cells was 1.2×10^5 and 4.27×10^4 cells/cm root in the case of *A. brasilense* 75 and *A. brasilense* 80, respectively. After 24 h of incubation, the number of attached cells increased to 4.1×10^6 and 2.98×10^5 cells/cm root, respectively. To properly interpret these data, we must recall that *A. brasilense* strain 75 was isolated from the roots of wheat cv Saratovskaya 29 (and it is this wheat cultivar that was used in our studies on the adsorption of strain 75) and that *A. brasilense* strain 80 was isolated from the roots of wheat cv Saratovskaya 47, but the adsorption of this

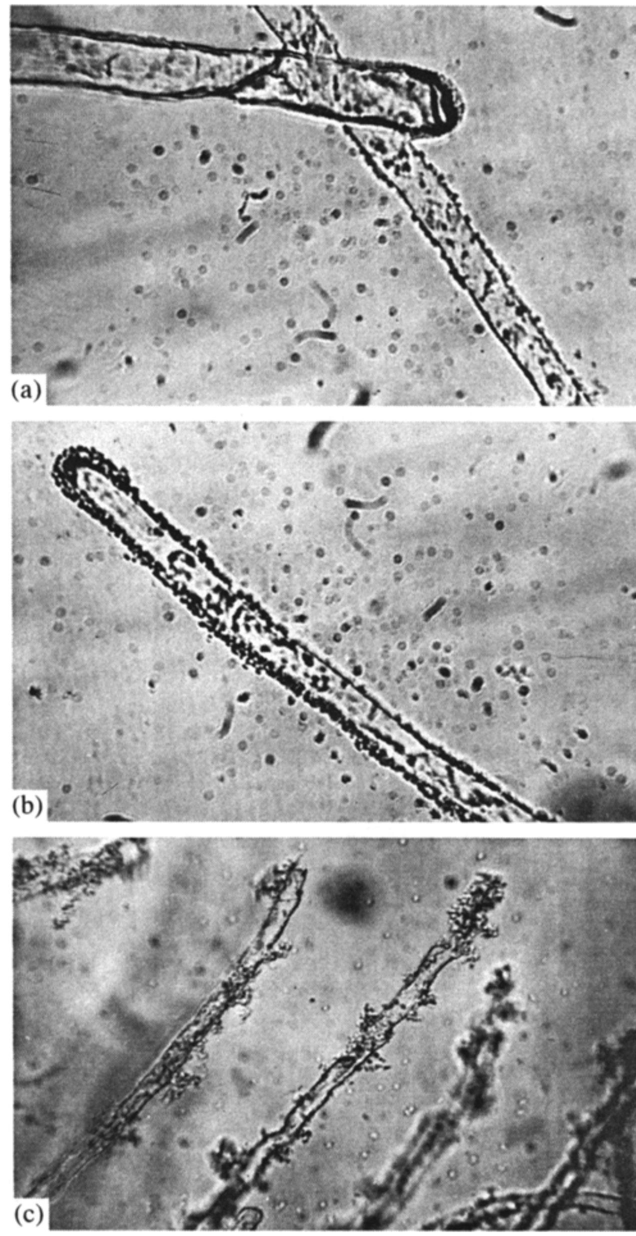

Fig. 2. Micrographs of root hairs colonized with bacterial cells: (a and b) *A. brasilense* 80, incubation time 24 h, magnification 1800×; (c) *A. brasilense* 75, incubation time 48 h, magnification 900×.

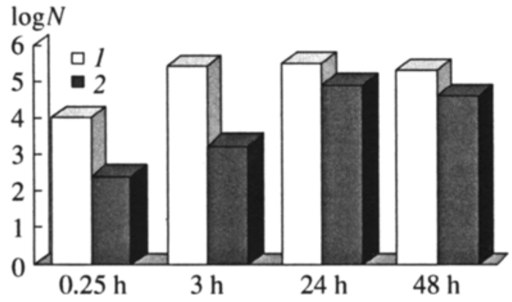

Fig. 3. Attachment of *A. brasilense* 80 cells to seedling roots of wheat cv Saratovskaya 58 as a function of the intensity of stirring and the time of root contact with bacterial cells. *N* is the number of adsorbed cells/cm root. Bars *1* correspond to gentle stirring of roots in PBS (three times for 1 min), and bars *2* correspond to vigorous stirring of roots in the homogenizer for 50 s.

strain was studied with wheat cv Saratovskaya 58. Bearing this in mind, the aforementioned data can be interpreted as indicative of the preferential adsorption of azospirilla on the roots of their host wheat cultivars. The same inference has been made by other authors [5]. The mechanism of such selective adsorption is unknown; we may only state that selective adsorption manifests itself already in the early terms of the interaction of phyto- and microsymbionts.

Light microscopic studies showed that bacterial cells were nonuniformly distributed over the root surface: they preferentially attached to the root tip, elongation zone, and to the base of the root hairs. At the same time, the root hairs themselves typically contained a low number, if any, of adsorbed bacterial cells. The distribution of cells over root hairs was irregular (Fig. 2a). Some root hairs turned out to be extensively colonized with azospirilla, especially in the late terms of incubation (24 and 48 h) (Fig. 2b, 2c). It should be noted that these observations agree with the relevant data available in the literature on the adsorption of other *A. brasilense* strains [3, 8].

Some authors relate the strong bacterium–root interaction to the presence of complementary structures on the surface of contacting partners. Since the surface properties of azospirilla cells change with age [15, 16], it would be reasonable to suggest that the adhesiveness of bacterial cells depends on their growth phase. To validate this supposition, we compared the adsorption of exponential-phase (20-h-old) and stationary-phase (48-h-old) *A. brasilense* 75 cells on wheat seedling roots. In these experiments, inocula were equalized with respect to the cell concentration. The results are presented in Fig. 1, where curves *2* and *3* represent the adsorption of exponential- and stationary-phase cells, respectively. It can be seen that, in early incubation terms, the attachment of stationary-phase cells was weaker than that of exponential-phase cells by approximately an order of magnitude. Conversely, in late incubation terms (15–48 h), the attachment of stationary-phase cells to roots was stronger than that of exponential-phase cells.

Analogous experiments with *A. brasilense* Cd have shown that bacterial cells were best adsorbed if taken from the exponential growth phase, while stationary-phase cells showed a weaker adsorption, and 150-h-old cells were poorly adsorbed [8]. Umali-Garcia et al. [17] have found that the 2-day cells of *A. brasilense* Sp7 showed a better adsorption on millet roots than 0.5-day, 3-day, or 5-day cells. It should be noted that these experiments involved a short-term incubation of bacterial cells with roots, which lasted either 90 min [8] or 60 min [17]. In our experiments, incubation times were widely varied, and we may state that our data agree well with the aforementioned data, unless the time of the bacterium–wheat root contact exceeded 15 h. For longer incubation periods, the situation was the opposite: stationary-phase cells were adsorbed better than exponential-phase cells. Probably, the interaction of the lytic enzymes of partners during their long-term contact (more than 15 h) led to the formation of additional binding sites and the involvement of more efficient mechanisms of sorption.

In connection with this, we studied the attachment of exponential-phase azospirilla to wheat roots as a function of incubation time. It is evident from Fig. 3 that the difference in the numbers of adsorbed cells remaining on roots after their gentle or vigorous stirring in a homogenizer reached two orders of magnitude if the time of the cell–root contact was within 0.25–3 h. As the time of contact was extended to 24 and 48 h, the adhesion of bacterial cells to roots became better. Probably, within the first hours of incubation, azospirilla cells were loosely bound to roots and could be easily detached from them by vigorous stirring in the homogenizer. Increasing the contact time made the binding of bacteria to roots fairly strong, so that cell desorption was insignificant. The nature of strong binding is not perfectly understood. In the case of rhizobia, polysaccharide-containing complexes occurring at the surface of bacterial cells are believed to be involved in such binding [18]. As for azospirilla, their strong attachment (anchoring) to roots is presumably due to the formation of polysaccharide-containing microfibrilla that bind microbial cells to the root surface [6].

The strain dependence of adsorption of azospirilla and their ability to induce root hair deformation have been the subjects of investigation of many researchers [4, 19]. The number of root hair deformations is often considered to be a measure of the responsiveness of plants to bacterial inoculation. Earlier, we showed that many *A. brasilense* strains grown in liquid synthetic media with malate to the late exponential phase produce high-molecular-weight polysaccharide-containing substances, arbitrarily called lipopolysaccharide–protein and polysaccharide–lipid complexes. These complexes are loosely bound to the cell surface and can be detached from it in the course of incubation. Konnova *et al.*

Table 2. Deformation of the root hairs of 2-day-old seedling of wheat and cabbage induced by the polysaccharide-containing complexes of *Azospirillum brasilense*

Cultivar	*A. brasilense* strain	Complex	Number of deformations per 1 cm of root length*		*P***
			Experiment	Control	
Wheat cv Saratovskaya 29	Sp7	LPSPC	10.9 ± 2.1 (43)	1.4 ± 0.8 (23)	<0.01
		PSLC	10.9 ± 3.1 (45)	1.3 ± 1.0 (11)	<0.01
	75	LPSPC	16.0 ± 3.0 (22)	2.4 ± 1.2 (7)	<0.01
		PSLC	16.9 ± 2.9 (20)	5.2 ± 2.9 (11)	<0.01
	80	LPSPC	7.5 ± 1.1 (34)	3.4 ± 0.5 (18)	<0.01
		PSLC	6.4 ± 0.8 (33)	2.7 ± 0.9 (14)	<0.01
Wheat cv Saratovskaya 49	Sp7	LPSPC	10.8 ± 1.0 (35)	4.0 ± 1.0 (18)	<0.01
		PSLC	10.2 ± 1.2 (31)	3.7 ± 0.9 (14)	<0.01
	75	LPSPC	8.7 ± 1.0 (28)	1.8 ± 0.8 (16)	<0.01
		PSLC	10.0 ± 1.6 (24)	2.0 ± 0.9 (15)	<0.01
	80	LPSPC	16.9 ± 2.6 (28)	5.3 ± 1.1 (13)	<0.01
		PSLC	17.3 ± 0.8 (30)	3.6 ± 1.2 (12)	<0.01
Cabbage cv Amager	75	LPSPC	1.8 ± 0.3 (35)	1.4 ± 0.7 (17)	>0.05
		PSLC	1.9 ± 0.7 (20)	2.0 ± 0.3 (17)	>0.05

* Confidence limits are given for a 95% confidence level; parenthesized are the number of replicate experiments.

** *P* is the significance level.

[10] have demonstrated that LPSPC and PSLC are able to induce root hair deformation. The selectivity of the adsorption of *A. brasilense* 75 on roots of its host wheat cv Saratovskaya 29 stimulated our investigation of the effect of polysaccharide-containing complexes isolated from various strains of azospirilla. In these experiments, we used seedlings of two wheat cultivars, Saratovskaya 29 (host cultivar of *A. brasilense* 75) and Saratovskaya 49 (host cultivar of *A. brasilense* 80), and cabbage cultivar Amager seedlings. Polysaccharide-containing complexes were isolated from cells of three bacterial strains, *A. brasilense* 75, 80, and Sp7. The results presented in Table 2 show that the numbers of root hair deformations induced by cells of different strains are of the same order. At the same time, it is evident that both LPSPC and PSLC exhibited a greater activity with respect to the root hairs of host wheat cultivars (16–17 deformations/cm root as compared with 10–11 deformations/cm root in the case of nonhost cultivars). It should be noted that LPSPC and PSLC failed to affect the number of root deformations in cabbage. This result, however, must be regarded as tentative. To obtain more conclusive evidence, experiments should involve variations in the age of test cabbage plants and in the concentration of the polysaccharide-containing complexes to be studied. The selectivity of the adsorption of bacteria on the roots of their host wheat cultivars and the higher activity of the polysaccharide-containing complexes of these bacteria with respect to the morphology of the root hairs of host plants indicate that common receptor systems or some of their components may be involved in these processes.

To conclude, we investigated the effect of three factors on the dynamics and adhesion of azospirilla to wheat roots: the duration of the root–cell contact, the concentration of bacterial cells in inoculating suspensions, and the growth phase of the bacterial cultures used for inoculation. The experiments performed allowed us to reveal strain-dependent differences and to understand some general features of adsorption. Each bacterial strain is characterized by a particular set of conditions promoting its maximum adsorption on wheat roots. This should be taken into account when designing experiments on the inoculation of wheat roots with azospirilla.

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