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EXPERIMENTAL ARTICLES

Role of the Surface and Extracellular Substances of the Phytopathogenic Bacterium *Xanthomonas campestris* in Its Interactions with Cabbage Plants

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Abstract—Changes in some physiological and biochemical characteristics of cabbage (cv. Slava) seedling roots in response to inoculation with the phytopathogen *Xanthomonas campestris* and its surface and extracellular substances were evaluated. Seven days after the inoculation, the growth of the roots was slightly suppressed and they contained increased amounts of peroxidase. The effect of the lipopolysaccharides stripped from the cell surface or isolated from the culture liquid of *X. campestris* was similar to that of the whole cells of the phytopathogen. The bacterial lectin isolated from the cell surface material did not induce any defense response in cabbage plants but, presumably, could play a role in the contact interactions between bacteria and plants.

Key words: *Xanthomonas campestris*, infection, lectins, lipopolysaccharides.

Specific interactions between phytopathogens and plants are due to various reactions between their metabolites, in which the surface and extracellular substances of the phytopathogen play a key role.

Among the surface substances involved in the interactions between bacteria and plants, of great importance are lectins, whose specific binding to the carbohydrate surface receptors of cells is one of the primary events of cell recognition, intercellular communication, and interspecies relationship. It is generally accepted that the lectins of host plants are essential in the interactions between microorganisms and plants (as in the legume–rhizobium symbiosis) [1], although bacterial lectins also play a role in these interactions [2]. The best studied bacterial lectins are those of enteric bacteria, whose enterotoxins contain lectins as indispensable components [3]. There is evidence that the surface substances of the phytopathogenic bacterium *Xanthomonas campestris* possess hemagglutinating activity and may be involved in the process of plant colonization [4].

Xanthomonads lack cellular capsules but, under certain conditions, they produce extracellular polysaccharides [5]. Ramirez *et al.* [6] selected the morphological variants of *X. campestris* which differed in the phytopathogenicity and the ability to produce the exopolysaccharide xanthan and established a correlation between the virulence of particular variants and the viscosity of their xanthans. This exopolysaccharide was found to induce specific reactions in host cabbage plants leading to the rolling and drying of cabbage leaves [7]. As for the lipopolysaccharides (LPSs) of xanthomonads, little is known about their composition, structure, and role in bacterial interactions with plants [8, 9].

The aim of the present work was to isolate and characterize the lectins and LPSs of *X. campestris* pv. *campestris* and to study their effect on the growth and defense response of host plants.

MATERIALS AND METHODS

The *Xanthomonas campestris* strains B 610 and B 611 used in the present study were obtained from the All-Russia Collection of Microorganisms (VKM).

The strains were grown at 25°C for 24 h on a solid medium containing (%) yeast extract, 0.3; bactopeptone, 0.5; glucose, 0.5; and agar, 2. Cells were washed off of the surface of the medium with a sterile physiological saline solution (0.85% NaCl), harvested by centrifugation, and washed severalfold with the same solution. The concentration of cells in suspensions was determined turbidimetrically [10].

The cabbage cv. Slava seeds were obtained from the Sortsemovoshch seed growing company (Saratov). The seeds were washed in a detergent solution, sterilized in 2% $Ca(OCl)_2$ for 20 min, washed with sterile water, kept at 4°C for 18 h, and then washed with water again. The sterilized seeds were germinated in petri dishes on wet filter paper at 22–24°C for 5–7 days, and the seedlings were aseptically transferred to test tubes with a semiliquid mineral medium containing (g/l) K_2HPO_4,

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0.7; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $Na_2MoO_4 \cdot 2H_2O$, 0.02; Na_2SO_4, 0.1; and NH_4Cl, 0.134 (pH 7.0).

The seedlings were infected with *X. campestris* cells in two ways: either by injecting the cells in the lower part of the 7-day-old seedlings or by adding them, at a concentration of 10^8 cells/ml, to the growth medium of the seedlings. In separate experiments, this medium was supplemented with the lectins (5–10 μg/ml) or lipopolysaccharides (0.2 mg/ml) isolated from the culture liquid of the xanthomonads. The cabbage seedlings not inoculated with xanthomonads and grown in the medium without lectins and lipopolysaccharides served as the control.

To determine the content of peroxidase in the seedlings, they were ground in cold citrate–phosphate buffer (pH 4.5), and the homogenate was mixed with the same buffer in a proportion of 1 mg wet plant material per 10 ml of the buffer. After extraction at 4°C for 1 h, the mixture was centrifuged at 10000*g* for 15 min, and 0.5 ml of the supernatant was mixed with 5 ml of a 0.05% solution of *o*-phenylenediamine in the citrate–phosphate buffer and with 5 μl of 33% hydrogen peroxide. After 20 s, the reaction was stopped by adding 0.5 ml of concentrated sulfuric acid. The optical density of the reaction products was measured at 492 nm using an SF-46 spectrophotometer (LOMO, Russia). The reference cuvette contained the same reaction mixture except that hydrogen peroxide was omitted. The content of peroxidase was determined from the calibration curve constructed using the known amounts of horseradish peroxidase and expressed in μg of peroxidase per ml of cabbage extract.

Lectins were stripped from the surface of xanthomonads with 1 M NaCl (pH 6.0) [4]. The lectin preparation was fractionated by adding ammonium sulfate to 50% saturation. To obtain purer lectin preparations, the ammonium sulfate precipitate was dissolved in phosphate buffer, dialyzed against this buffer, and the ballast proteins were removed by adjusting the pH of the solution alternately to 4.0 and to 9.0. After the final centrifugation, the pH of the supernatant was adjusted to 6.0, and the proteins present in the supernatant were precipitated by adding ammonium sulfate to 80% saturation. The precipitate was dissolved in the phosphate buffer.

The purification process was controlled by subjecting intermediate fractions to SDS–PAAG electrophoresis [11] with the molecular weight protein markers (Sigma, the United States). Total protein concentration was determined by the Bradford method [12].

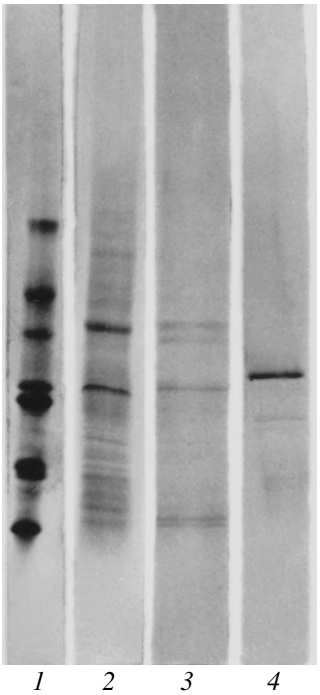
Hemagglutinating activity (HAA) was assayed using multiwell plates [13]. The activity was evaluated visually after incubating the plates at room temperature for 1 h. The carbohydrate specificity of lectins was determined by incubating them with 0.6 M carbohydrates and assaying the changes in their hemagglutinating activity.

Table 1. Effect of the inoculation of 7-day-old cabbage cv. Slava seedlings with *X. campestris* pv. *campestris* cells on the growth of the seedlings roots and their peroxidase content (data are the means for ten replicated seedlings)

Strain	Parameter			
	infected plants, %	root length, cm	root weight, mg	peroxidase content, μg/ml
Control	0	3.7 ± 0.7	15.1 ± 2.6	15.0 ± 4.0
B 610	64	2.6 ± 0.4	11.4 ± 1.4	24.8 ± 3.5
B 611	74	3.6 ± 0.4	14.7 ± 2.9	34.8 ± 3.1

Bacterial lipopolysaccharides were extracted by the Westphal and Jann method [14]. After fractionating the extract with a phenol–water mixture, the lipopolysaccharides, which were localized in the phenol phase, were dialyzed against tap water for 5 days and purified further by chromatography on a column with Sepharose CL-4B (Pharmacia, Sweden). The lipopolysaccharides were eluted in a void volume of the column.

The content of carbohydrates, protein, nucleic acids, and 2-keto-3-deoxyoctonate in the freeze-dried lipopolysaccharide preparation was determined as described earlier [15]. Data were statistically processed as described in the handbook [16].



SDS–PAAG electrophoresis of the lectin stripped from the surface of *X. campestris* pv. *campestris* cells. Lanes: *1*, molecular weight markers; *2* and *3*, lectin preparations from intermediate purification steps; and *4*, purified lectin.

Table 2. Effect of the lectin and lipopolysaccharide stripped from the surface of *X. campestris* pv. *campestris* cells on the growth of the cabbage cv. Slava seedling roots and their peroxidase content (data are the means for ten replicated seedlings)

Preparation added	Parameter		
	root length, cm	root weight, mg	peroxidase content, μg/ml
Control	2.2 ± 0.3	5.7 ± 0.6	5.9 ± 0.8
Bacterial lectin	2.3 ± 0.3	6.4 ± 0.4	6.2 ± 0.9
Control	5.6 ± 0.5	6.7 ± 1.6	10.0 ± 0.8
Second control (dextran)	5.6 ± 0.6	18.1 ± 1.2	12.0 ± 1.0
Bacterial lipopolysaccharide	4.9 ± 0.4	13.9 ± 1.2	22.0 ± 4.0

RESULTS AND DISCUSSION

The infection of cabbage plants by injecting *X. campestris* cells with a syringe is a time-consuming and unefficient procedure: the first result of such an injection (a dark necrotic spot) becomes visible only 7–10 days afterwards, and as low as 64–74% of the inoculated plants become really infected. The infection of cabbage plants by coincubating them with xanthomonads is a more efficient procedure; however, changes in the infected plants are not well pronounced even 2 weeks afterwards.

For this reason, the role of peroxidase in the metabolism and defense reactions of infected plants attracts the interest of researchers, since the increase in the peroxidase activity of plants correlates with their resistance to infection and is believed to be one of the primary plant responses to the action of phytopathogens [17]. With the biochemical test developed in our laboratory, the infection of cabbage seedlings by xanthomonads was tested by assaying peroxidase activity in the seedlings 7–8 days after their inoculation with the phytopathogen (actually, the increase in peroxidase activity became noticeable 3–4 days after the inoculation of strain B 611, being at a maximum between 7 and 10 days after the inoculation). As can be seen from the data presented in Table 1 (7 days after the inoculation of cabbage plants with *X. campestris*) the content of peroxidase in plant tissues increased by 1.5–2 times, indicating the cabbage plant infection.

The subsequent plant withering and rotting can be related not only to the active reproduction of the phytopathogen in the plant xylem and adjacent tissues but also to the toxic action of some bacterial compounds, such as lectins or lipopolysaccharides.

In view of this, we evaluated the hemagglutinating activity of the *X. campestris* strains B 610 and B 611 cells using native rabbit erythrocytes and found that strain B 611 possessed a higher hemagglutinating activity than strain B 610. For this reason, further studies were carried out using the lectin and lipopolysaccharide preparations derived from strain B 611.

In the course of purification of the lectin stripped from the surface of *X. campestris* cells, its specific hemagglutinating activity, expressed as HAA titer/(mg protein ml), increased from 1 (the specific hemagglutinating activity of the crude preparation of lectin was arbitrarily taken as unity) to 3 (the step of ammonium sulfate precipitation at 50% saturation), 16 (the step of the removal of ballast proteins by shifting pH), and, finally, to 170 (the step of ammonium sulfate precipitation at 80% saturation).

The hemagglutinating activity of the lectin preparation was obviously associated with proteins, since it was partially inactivated by proteases such as trypsin and chymotrypsin, and completely suppressed by heating at 100°C for 15 min.

The SDS–PAAG electrophoresis of the purified lectin revealed a protein band with a molecular weight of 29–30 kDa (see figure). Mucin and *N*-acetyl-D-glucosamine, one of the ten carbohydrates tested (D-glucose, D-galactose, D-mannose, D-xylose, L-fucose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, lactose, raffinose, and maltose), inhibited the hemagglutinating activity of lectin.

The lectin stripped from the strain B 611 cells had no appreciable influence on the growth of cabbage seedlings, estimated as the length and weight of the seedling roots, nor did it markedly increase the content of peroxidase in the roots (Table 2). These data suggest that the lectins of xanthomonads do not play a key part in the formation of the plant's response to their invasion. On the other hand, the thin-layer chromatographic analysis [15] of the root exudates and washings from 14-day-old seedling roots showed the presence of galactose and glucosamine. These data, together with the above finding that bacterial lectin is specific for glucosamine, suggest that the lectin may play a role in the contact interactions between xanthomonads and the cabbage seedling roots.

During the extraction of *X. campestris* B 611 cells with a hot phenol solution [18], lipopolysaccharides, which are lipophilic in nature, almost completely passed to the phenol phase with a yield of 0.5 wt % of the dry biomass. Analysis showed that the LPSs of xanthomonads contained 30.5% carbohydrates, 5% protein, less than 1.0% of nucleic acids, and 1.1% of 2-keto-3-deoxyoctonate, the indispensable component of the gram-negative bacterial LPSs.

When added to the growth medium of cabbage seedlings and incubated with them for 7 days, the LPSs of xanthomonads inhibited the growth and development of the cabbage seedlings and increased their peroxidase content by more than two times (Table 2); i.e., the LPSs produced changes comparable with those induced by the whole cells of the phytopathogen. These data demonstrate that the phytopathogenic properties of xanth-

omonads are due to their lipopolysaccharides rather than to their lectins.

The importance of lipopolysaccharides in the infection of plants with xanthomonads is consistent with the data available in the literature that virulent phytopathogenic strains synthesize elevated amounts of extracellular LPSs. The role of extracellular LPS is similar, but not identical, to that of membrane LPSs [19]. Together with exopolysaccharides, extracellular LPSs bind to the cell-wall lectins of plants, thus preventing the LPSs of the bacterial surface from interactions with the plant lectins. This must promote the reproduction of free bacterial cells in the neighborhood of plants [8]. The lipid moiety of LPSs may be responsible for the induction of the defense response reactions of the affected plants [20]. The relevant studies are in progress in our laboratory.

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