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EXPERIMENTAL ARTICLES

Isolation of Bacteria of the Family *Enterobacteriaceae* from Plant Tissues

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Abstract—Bacteria of the family *Enterobacteriaceae* were isolated from the tissues of a number of wild and cultivated plants. All the cultures isolated had a broad spectrum of resistance to antibiotics and were highly adhesive to human erythrocytes. The studies conducted indicate the possibility of a concentration of microorganisms pathogenic for humans in plant tissues.

Key words: plant tissue, enterobacteria, antibiotics, adhesion.

The historically established division of microorganisms into phytopathogenic, pathogenic for animals (humans), and nonpathogenic seems, from a contemporary standpoint, quite conventional, as has been demonstrated by recent studies of the genetics of pathogenicity [1] and by the results of practical observations. For example, certain plant pathogens (*Erwinia* and *Agrobacterium*) have been revealed in human clinical material [2]. The fact that many free-living microorganisms that were earlier thought to be nonpathogenic are becoming increasingly important in human infectious pathology is undoubted [3, 4].

At the same time, the problem of interrelationships between plant organisms and human pathogens remains almost completely uninvestigated. However, it is known that some bacteria that are not phytopathogenic are capable of penetrating to the depth of plant tissues through the root system or wounds [4–8]. Several studies have been published linking bacterial contamination of fruit and vegetables to the occurrence of enteric infections [9]. The problem of disinfection of vegetable products is being actively developed abroad [10]. Importantly, some microorganisms that can penetrate into plant tissue (*Escherichia coli* in particular) are resistant to the action of many common disinfectants [11, 12].

The present study was concerned with the detection in plant tissues of microorganisms capable of causing infectious diseases in humans and animals, with a focus on the family *Enterobacteriaceae*, whose representatives are widespread in nature and include the causative agents of many human diseases, such as dysentery and pseudotuberculosis.

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MATERIALS AND METHODS

The microbial population was studied during the summer in the following plants: wild pear (*Pyrus pasha*), ash-leaved maple (*Acer negundo* L.), squawbush (*Viburnum opulus* L.), common plantain (*Plantago major*), celandine (*Chelidonium majus*), dandelion (*Taraxacum officinale*), lettuce (the variety Kucheryavets Odesskii), potatoes (the variety Adretta), tomatoes (the variety Sibirskii early ripening), and cucumber (the variety Izyashchnyi). Since it was of interest to identify only the microorganisms present inside the plants, the studied material was preliminarily sterilized with a 0.2% diacid reagent [13], which was prepared by separately dissolving 330 mg of ethanol mercuric chloride and 660 mg of cetylpyridinium chloride in hot water (330 ml), mixing these solutions, adjusting the volume to 1 l, and adding several drops of Tween-80 detergent. After treatment with this sterilizing reagent, the material was washed three times in sterile distilled water. Sterility control was carried out by inoculation of the nutrient media with the last wash solution.

The leaves were then homogenized, mixed with peptone water, and incubated at 37°C. After 24 h, three Petri dishes with nutrient agar and three Petri dishes with Endo medium, which is a differentiating medium for enterobacteria, were inoculated with the samples [14]. The samples were also incubated at 37°C. After 24 h, the number of bacterial colonies that had formed was counted, and the cells were Gram-stained. In the gram-negative cultures, the presence of the enzymes oxidase and catalase was determined. The cultures that were gram-negative, oxidase-negative, and catalase-positive were assigned to the family *Enterobacteriaceae* [15]. Further identification was carried out using tests for glucose, mannitol, sorbitol, inositol, and lac-

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Table 1. Conditionally pathogenic microorganisms isolated from plant tissues (% of the total number of isolates)

Species	%
Providentia stuartii	23
Providentia rettgeri	4
Serratia marcescens	6
Enterobacter aerogenes	44
Morganella morganii	4
Citrobacter koseri	6
Escherichia coli	2
Bordetella parapertusis	2
Salmonella sp.	6
Enterobacter amnigenes	2
Citrobacter farmeri	2

tose fermentation; urea hydrolysis (urease); phenylalanine deamination (phenylalanine deaminase); production of lysine decarboxylase and ornithine decarboxylase; citrate and malonate utilization; formation of indole and hydrogen sulfide; and formation of acetylmethylcarbinol (the Voges–Proskauer test). In addition, the bacteria were assessed for motility. The species were identified using the Bakanalizator computer program.

The cultures were also tested for resistance to the antibiotics cephalothin, ampicillin, polymyxin, tetracycline, streptomycin, levomycetin, kanamycin, rifampicin, gentiomycin, oxacycline, and cephazolin using the standard disk method based on antibiotic diffusion into the nutrient medium. For this purpose, a microbial suspension was layered onto the Petri dishes filled with nutrient agar. After the suspension had been uniformly spread, the dishes were dried in a thermostat at 37°C. Filter paper disks impregnated with antimicrobial preparations (NITsF, St. Petersburg) were then applied to the agar. The antibiotic concentration in the disks was adjusted so that the growth inhibition zone diameters of the standard test organisms were 28–32 mm. After incubation at 37°C for 24 h, the growth inhibition zone diameter was measured. The zone sizes were compared to the values indicated in the instructions, after which the microorganisms were ascribed either to those sensitive or resistant to a given preparation [16].

Table 2. Resistance of the isolates to antibiotics (% of the total number of isolates)

Plant	Oxacycline	Cephazolin	Polymyxin	Ampicillin	Tetracycline	Levomycetin	Cephalothin	Streptomycin	Kanamycin	Rifampicin	Gentiomycin
Pear	50	81	9	64	100	100	50	–	–	–	–
Maple	–	87	43	87	66	–	–	–	–	–	–
Plantain	71	90	45	82	82	100	71	100	100	0	0
Dandelion	100	–	100	100	25	50	100	50	25	50	25
Celandine	–	12	62	12	12	–	–	–	–	–	–
Lettuce	100	100	100	100	0	0	0	–	–	–	–
Tomatoes	100	100	100	40	40	80	100	67	67	67	33
Potatoes	100	100	67	67	0	33	100	–	–	–	–

Note: A dash means that no analyses were performed.

Table 3. Adhesive capacity of the isolates from plant tissues

Adhesion	Number of cultures	Species	Source of isolation
Nonadhesive	0		
Low-adhesive	0		
Moderately adhesive	2	*Providentia stuartii*	Dandelion
	2	*Serratia marcescens*	Dandelion
	2	*Providentia stuartii*	Plantain
Highly adhesive	1	*Serratia marcescens*	Tomatoes
	1	*Morganella morganii*	Tomatoes
	1	*Morganella morganii*	Potatoes
	1	*Escherichia coli*	Potatoes
	1	*Citrobacter koseri*	Potatoes
	1	*Providentia stuartii*	Dandelion

The adhesiveness index of a number of the isolated cultures, i.e., their ability to adhere to animal cells, which is the initial stage of an infectious process, was determined as one of the pathogenicity factors.

The adhesiveness was determined in the following way: cells from a 24-h culture were washed off with phosphate buffer (pH 7.2–7.3), centrifuged at 1500*g* for 10 min, resuspended in phosphate buffer, and adjusted to a concentration of 10^9 cells/ml [17].

Formaldehyde-treated human erythrocytes (group 0 (1), Rh+) served as the adhesion substrate. They were washed twice with phosphate buffer (300*g* for 5–10 min) and diluted so as to adjust their concentration to 10^6 cells/ml.

Bacterial suspension (0.5 ml) was added to 0.5 ml of the erythrocyte suspension. The mixture was incubated at 37°C for 30 min with shaking. The mixture cells were then washed with phosphate buffer (600*g*), and smears were prepared on degreased slides. The smears were dried at room temperature, heat-fixed, and stained with methylene blue. Microscopy was carried out at 90× under the conditions of immersion.

The adhesiveness index of the microorganisms (AIM) was calculated using the formula [18]

$$\text{AIM} = \text{MNM} \times 100/K,$$

where MNM is the mean number of microorganisms adhering to one erythrocyte and *K* is the coefficient of the involvement of the erythrocytes in the adhesive process (% of the erythrocytes with adhered microbes on their surface; the calculation was carried out for 50 erythrocytes).

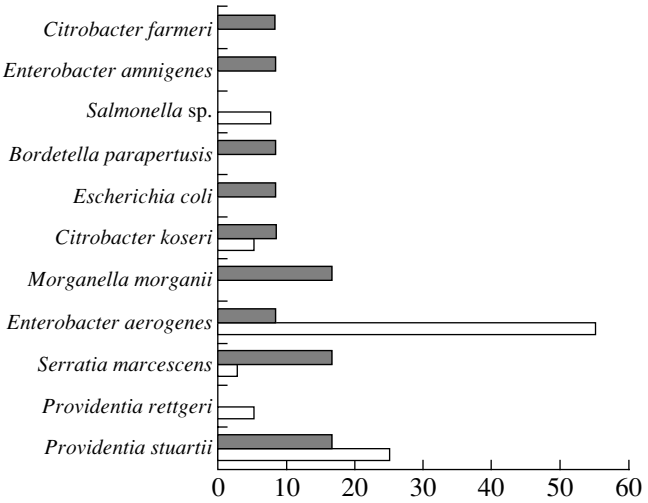
A microorganism was considered to be nonadhesive at AIM ≤ 1.75, low-adhesive at AIM = 1.76–2.5, moderately adhesive at AIM = 2.51–4.0, and highly adhesive at AIM ≥ 4.01.

RESULTS AND DISCUSSION

The study of the microflora of plant tissues revealed that they contained a rather a wide content of conditionally pathogenic microorganisms. Virtually half (47%) of all the samples contained bacteria from this group. Their species composition is presented in Table 1, from which it follows that the prevalent species were *Enterobacter aerogenes* (44%) and *Providentia stuartii* (23%).

Owing to the fact that we studied plants growing within the city precincts as well as outside, it was of interest to compare the species composition of the microorganisms isolated in these zones (figure). *Enterobacter aerogenes* and *Providentia stuartii* dominated among the species obtained from the plants growing within the city precincts. No marked dominance of any of the species was observed among the microorganisms isolated from the plants growing outside the city.

The microorganisms that are pathogenic for humans and animals are known to develop resistance to antibiotics used in clinical and veterinary practice. Therefore,



Comparison of the species composition of bacteria isolated from the tissue of plants taken for analysis in Irkutsk and outside it: □ the territory of the city; ■ outside the city.

it was of interest to test all the cultures isolated for their sensitivity to widely used antibiotics such as oxacycline, ampicillin, cephalothin, cephazolin, polymyxin, levomycetin, tetracycline, streptomycin, kanamycin, rifampicin, and gentiomycin (Table 2). It appeared that the most widespread was resistance to oxacycline (86.8%) and cephazolin (81.4%). The isolated species of enterobacteria were least resistant to gentiomycin (19.3%).

Thus, the high level of resistance to antibiotics in the isolates allows us to make the suggestion that these bacteria are not endogenous to plants but entered them as a result of anthropogenic contamination.

Adhesion to human erythrocytes was determined for 12 isolates. Among them, there were no nonadhesive or low-adhesive strains; however, there were six moderately adhesive strains and six highly adhesive strains (Table 3).

The adhesion factor is present in many saprophytic bacteria [4]. Nevertheless, its presence in virtually all of the bacteria studied in the present investigation provides evidence of their potential capacity to initiate an infectious process when entering the human organism.

The studies conducted by us show the possibility of the presence of conditionally pathogenic microorganisms in the tissues of plants growing on the territory of Irkutsk. Many of the bacteria isolated were resistant to a number of antibiotics commonly used in clinical practice and also revealed high adhesiveness to human erythrocytes. These tests allow us to consider that not only the surface of the plants that are growing under an increased anthropogenic and technogenic load but also their tissues may be carriers of pathogenic and conditionally pathogenic microorganisms.

Further studies in this direction are necessary to reveal the most dangerous foci determining the circula-

tion of pathogenic microorganisms in the environment, including phytocenoses, and to formulate recommendations for reducing the risk of infection for humans and animals from plants used as food.

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