Microbiology, Vol. 73, No. 6, 2004, pp. 710–715. Translated from Mikrobiologiya, Vol. 73, No. 6, 2004, pp. 825–831.

EXPERIMENTAL ARTICLES

Bacteria Associated with the Roots of Epiphytic Orchids

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Received September 30, 2003; in final form, November 25, 2003

Abstract—This work is the first to report the isolation and identification of bacteria colonizing the roots of the tropical epiphytic orchids *Acampe papillosa* (Lindl.) Lindl. and *Dendrobium moschatum* (Buch.–Ham.) Swartz. and bacteria inhabiting inner layers of the aerial and substrate roots of *A. papillosa.* We showed by the example of this epiphyte that associative bacteria are present in large amounts on the aerial but not the substrate roots. We isolated and identified bacteria from the substrate roots of *D. moschatum* and from its growth substrate (pine bark). The structure of the intercellular matrix of the associative bacteria was studied.

Key words: associative bacteria, tropical orchids, intercellular matrix.

At the present time, microbial interactions with plants are receiving more and more attention. Associative microorganisms have a great and often favorable impact upon plant development due to fixation of atmospheric nitrogen, phytohormone synthesis, improvement of water regime and mineral nutrition, and production of fungicidal and bactericidal substances reducing the number of phytopathogens [1]. Associative microorganisms are considered to attach to the root surface with an intercellular matrix rather than form specialized structures such as nodules on plant roots [2].

It was not until recently that attention was drawn to orchid-associated bacteria. In the late 1980s, Australian scientists isolated endotrophic bacteria from the inner layers of the substrate roots of ground orchids [3, 4]. These bacteria were assigned to the genera *Pseudomonas, Bacillus, Xanthomonas, Arthrobacter*, and *Kurthia*, and some strains belonged to enterobacteria. We failed to find any other information on bacterial microflora of orchids.

In our previous studies [5–7], we found that greenhouse tropical orchids are closely associated with phototrophic and heterotrophic bacteria and micromycetes. The urgency of studies of orchid–microbial interactions is determined by the absence of information on localization of bacteria and their number and taxonomic composition, as well as on the bacterial influence on the growth and development of the host plant. Such information is valuable when cultivating rare and endangered orchid species in botanic gardens.

The aim of the present work was to determine the generic composition of the bacterial association colonizing aerial and substrate roots of greenhouse tropical orchids and to study the composition of the intercellular matrix of the associative bacteria.

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MATERIALS AND METHODS

We studied aerial and substrate roots of *Acampe papillosa* (Lindl.) Lindl. and substrate roots of *Dendrobium moschatum* (Buch.–Ham.) Swartz. The plants were obtained from the Collection Greenhouse of the Tsitsin Central Botanical Garden, Russian Academy of Sciences, Moscow. In their native environments, in Southeast Asia, these orchids inhabit branches of high deciduous trees. Epiphytes form aerial roots, which function as the ordinary roots of ground plants upon penetrating a substrate. In greenhouses, they are grown in pots with the use of pine bark as a substrate. A detailed description of the cultivation conditions was given earlier [5–7].

Associative microorganisms were isolated on the day of sample collection. Experiments were performed with roots aged one year and older. Substrate and root samples were collected using sterile tweezers and spatulas and placed in sterile jars. Substrate roots were mechanically cleaned of pieces of pine bark, washed with sterile tap water, cut into small pieces (of 1–2 mm), and ground in a sterile mortar into a suspension that was then successively diluted. Each dilution was plated in a volume of 0.1 ml onto Czapek agar supplemented with 50 μg/ml nystatin for the prevention of fungal growth. Incubation was performed in the dark at 30°C until single colonies could be detected. Pure bacterial cultures were obtained from individual colonies through inoculation onto twice-diluted nutrient agar and maintained on nutrient agar, twice-diluted nutrient agar, and starch agar.

Isolation of endotrophic bacteria from the inner layers of the roots was performed in accordance with the modified techniques described earlier [3]. *A. papillosa* roots were cleaned, cut into fragments (approximately 10 mm long), sterilized for 20 min in a 1% sodium

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hypochlorite solution, and thrice washed with sterile distilled water for 5–10 min. The prepared suspension was successively diluted, and each dilution was plated onto solid medium as described above.

To isolate the bacterial complex from the substrate of *D. moschatum*, pine bark was cut into small pieces with a scalpel, ground in a mortar, and suspended in sterile tap water (0.1 g of substrate per 100 ml). The resultant suspension was then successively diluted, and each dilution was plated in triplicate onto solid medium.

Bacterial cells inhabiting the surface of orchid roots were enumerated by the Koch plating method [8]. The dilutions of suspensions of substrate and aerial *A. papillosa* roots were plated onto nutrient medium as described above. The bacterial numbers were converted to 1 g of dry root weight. The experiment was conducted in five replicates.

Generic identification of bacteria was carried out by routine methods [9, 10] based on morphological, cultural, physiological, and biochemical characteristics. We took into consideration the data of microscopic examinations, the presence of oxidase and catalase, denitrifying ability, sugar fermentation, growth at pH 4.5, oxidation/fermentation of glucose, phenylalanine deaminase activity, amylolytic and proteolytic activity, acid resistance (for mycobacteria), and spore formation. Actinomycetes were identified using Gauze medium and oat agar (to observe mycelium formation). More comprehensive study of chemotaxonomic criteria was not performed as we did not aim to determine the species affiliation of the isolated bacteria.

The carbohydrate composition of the bacterial capsules and matrix was established in a qualitative reaction with 2,3,5-triphenyltetrazolium [11]. A microorganism culture was grown for 2 or 3 days on nutrient agar, collected with a spatula, and used to prepare a cell suspension. One milliliter of the cell suspension was placed in a tube and mixed with 0.5 ml of 3% 2,3,5-triphenyltetrazolium bromide solution in water and 0.5 ml of 2 N NaOH. The mixture was heated on a water bath for no more than 1 min to obtain a cherry coloration and acidified with 0.5 ml of 2 N acetic acid. Then, the suspension was examined under a microscope. In the case of cell damage caused by thermal treatment, the time of heating was reduced.

The acidic (polyanionic) composition of the capsules and matrix was established in a reaction with cetrimide [11]. One drop of a warm cell suspension of a moderate density was put on a preheated slide, and one drop of a reagent (also preheated to 40–50°C) was added (cetyltrimethylammonium bromide). If acidic groups were present, formation of flocks and strands consisting of a complex with cetrimide was observed. If acidic groups were present in the matrix in low quantities, formation of the complex could be observed only under a microscope at a magnification of 30× or 100×.

For the quantitative determination of proteins and carbohydrates in the bacterial capsules and matrix, cultures were grown on nutrient agar supplemented with 2% glucose, washed off with sterile physiological saline using a glass spatula to obtain a suspension of low viscosity, and centrifuged at 10000 *g* for 15 min. The supernatant was concentrated by dialysis for 12 h [12]. The carbohydrate content was determined by the phenol–sulfuric method [3], and the protein content was determined by the Lowry method [8].

To estimate the degree of polymerization of extracellular polysaccharides, they were precipitated with various volumes of 96% ethanol. One volume of supernatant obtained as described above was mixed with two volumes of 96% ethanol with stirring (both components were precooled to 4–5°C). The mixture was left in the cold until sedimentation of polysaccharides. If sedimentation was not observed, two additional volumes of 96% ethanol were added.

RESULTS AND DISCUSSION

In our previous works [5, 7], we compared photographs of the surface of aerial and substrate roots of epiphytic orchids obtained by means of scanning electron microscopy and found that aerial roots were more densely colonized by bacteria than substrate ones. This was confirmed by a quantitative study of the *A. papillosa* bacterial complex. Thus, 1 g of aerial roots of *A. papillosa* contained 213.5×10^6 bacterial cells, whereas 1 g of substrate roots contained 7.6×10^6 bacterial cells. This can possibly be determined by greater concentrations of root exudates on the surface of aerial roots (on substrate roots, watering results in their dilution), as well as substrate specificity (pine bark) or even absence of substrate (when epiphytes are grown on blocks of artificial inert materials). Evidently, it is the rhizoplane that is the zone of maximal microbial activity in epiphytic orchids and not the rhizosphere as in other plants.

The results obtained after isolation and identification of the bacterial complexes from roots of tropical epiphytic orchids are presented in Table 1. We isolated bacteria of the genera *Acinetobacter, Bacillus, Cellulomonas, Gluconobacter, Mycobacterium, Pseudomonas* (two strains), *Rhodococcus* (four strains), and *Streptomyces* (two strains) from the substrate roots of *A. papillosa* and bacteria of the genera *Bacillus, Flavobacterium, Micrococcus* (three strains), *Pseudomonas* (four strains), *Rhodococcus* (six strains), *Streptomyces*, and *Xanthomonas* from the aerial roots of this orchid.

Bacteria of the genera *Acinetobacter, Aquaspirillum* (two strains), *Bacillus, Pseudomonas* (eight strains), and *Rhodococcus* (four strains) were isolated from the substrate roots of another epiphytic orchid, *D. moschatum. Mycobacterium* sp., *Pseudomonas* sp., *Aquaspiril-*

Table 1. Bacteria associated with the roots of tropical epiphytic orchids

Orchids	Bacteria	Orchids	Bacteria	Endotrophic bacteria
D. moschatum, Aerial roots*	*Bacillus*	*A. papillosa*, Aerial roots	*Bacillus*	*Bacillus*
	Flavobacterim		*Flavobacterium*	*Flavobacterium*
	Nocardia		*Micrococcus*	*Pseudomonas*
	Pseudomonas		*Pseudomonas*	*Rhodococcus*
	Curtobacterium		*Rhodococcus*	*Xanthomonas*
	Rhodococcus		*Streptomyces*	
	Xanthomonas		*Xanthomonas*	
D. moschatum, Substrate roots	*Acinetobacter*	*A. papillosa*, Substrate roots	*Acinetobacter*	*Alcaligenes*
	Aquaspirillum		*Bacillus*	*Bacillus*
	Bacillus		*Cellulomonas*	*Gluconobacter*
	Pseudomonas		*Gluconobacter*	*Pseudomonas*
	Rhodococcus		*Mycobacterium*	
			Pseudomonas	
			Streptomyces	

* The data on the bacterial community of the aerial roots of *D. moschatum* were obtained earlier [5].

lum sp., and *Nocardia* sp. were isolated from the substrate used to grow this orchid.

Earlier, we showed that the microbial population of the ground orchid *C. vestita* Lindl. var. *rubro-oculata* differs from that of the surrounding substrate [5]. This also applies to *D. moschatum.*

It is commonly known that the all orchids produce phytoncidal substances with a high bactericidal activity [15]. At the same time, we observed active bacterial colonization of the orchid rhizoplane. This allows the suggestion to be made that specific phytoncide-resistant bacterial complexes are formed on the root surface of these plants.

Our data show that the qualitative composition (generic diversity) of the microbial community of epiphytic orchids differs from that of the ground orchid *C. vestita* [5]. The roots of epiphytic orchids are inhabited by rhodococci, micrococci, *Flavobacterium* spp., and *Xanthomonas* spp. (Table 1), which were not found on *C. vestita* roots. At the same time, the presence of a large number of *Pseudomonas* strains is characteristic of all types of the roots studied. This taxonomic group is most frequent in the rhizosphere and rhizoplane of agricultural plants [15, 16]. Bacteria of the genera *Bacillus*, *Micrococcus, Streptomyces, Flavobacterium*, and *Xanthomonas* also colonize the near-root zone and root surface of various plants [16–18].

Although the two studied orchids were grown in one greenhouse in the same climate and substrate, the qualitative structure of the bacterial communities on their aerial and substrate roots was not identical.

There is no information available on bacterial complexes of the rhizoplane of wild and greenhouse orchids. However, Wilkinson *et al.* showed the prevalence of *Pseudomonas* and *Bacillus* spp. and frequent occurrence of *Arthrobacter, Xanthomonas*, and *Kurthia* spp. for endotrophic bacterial associations of 12 different Australian ground orchids [3, 4]. In order to confirm the data of scanning electron microscopy on bacterial colonization of the inner layers of *A. papillosa* aerial roots [7], we conducted a number of experiments on the isolation of endotrophic bacteria from the aerial and substrate roots of this orchid.

Alcaligenes sp., *Gluconobacter* sp., *Bacillus* sp., and *Pseudomonas* sp. (two strains) were isolated from the inner layers of the substrate roots, and bacteria of the genera *Bacillus, Flavobacterium, Pseudomonas, Rhodococcus* (three strains), and *Xanthomonas* (two strains) were isolated from the inner layers of the aerial roots (Table 1). The strains isolated from the inner layers and rhizoplane of the aerial roots were identical.

Australian researchers suggested that orchids are characterized by limited specificity in choosing their bacterial partners [3]. The presence of bacteria of the genera *Pseudomonas, Bacillus*, and *Xanthomonas* in the inner layers of *A. papillosa* roots confirms this supposition. The above authors stress that the bacterial community of a ground orchid of the genus *Pterostylis* is dominated by gram-negative bacteria, whereas that of an orchid of the genus *Diuris* is dominated by gram-positive bacteria [4], which may result from the differences in the morphological and physiological characteristics of these two orchids. We also tend to the conclusion that the composition of the associative bacterial communities is partly determined by the environmental characteristics of the ecological niche that an orchid occupies. Thus, rhodococci isolated from the inner layers of the aerial roots of the orchid *A. papillosa* were not

Table 2. Presence of acidic groups and carbohydrates in matrices of associative bacteria

Orchid species	Root type	Bacteria	Matrix composition	
			Presence of acidic groups*	Presence of carbohydrates
C. vestita	substrate	*Mycobacterium* sp. 1	+–	+–
		Arthrobacter sp. 4	+	++
		Bacillus sp. 12	+	+–
		Pseudomonas sp. (two strains)	+	++
		Pseudomonas sp. 22	+	+–
D. moschatum	aerial	*Xanthomonas* sp. (two strains)	–	–
		Bacillus sp. 24	+	+–
		Rhodococcus sp. 25	+	+–
		Pseudomonas sp. 18	+	+–
		Rhodococcus sp. 23	+	–
	substrate	*Pseudomonas* sp. 50	+	+–
		Bacillus sp. 3	+	+–
		Rhodococcus sp. 1	+	+
		Aquaspirillum sp. (two strains)	+	++
		Pseudomonas sp. (two strains)	+	+–
Substrate of *D. moschatum* (pine bark)		*Mycobacterium* sp. K1	+	+–
		Aquaspirillum sp. K2	+	++
		Pseudomonas sp. K3	+	++
		Nocardia sp. 15	+	–
A. papillosa	aerial	*Pseudomonas* sp. (two strains)	+	++
		Pseudomonas sp. 42	+	+–
		Pseudomonas sp. 43	+	–
		Rhodococcus sp. 36	+	+–
	substrate	*Micrococcus* sp. 8	+	–
		Rhodococcus sp. (two strains)	+	+–
		Rhodococcus sp. no. 16	–	++
		Pseudomonas sp. no. 24	+	+–
		Cellulomonas sp. no. 23	+	–

* "+" Stands for "high content," "+–" stands for "low content," and "–" stands for "not present."

found in the inner layers of its substrate roots, and bacteria of the genera *Gluconobacter* and *Alcaligenes*, isolated from the inner layers of its substrate roots, were not found in the inner layers of the aerial roots.

In our previous works, we studied the surface of the aerial and substrate roots of orchids using scanning electron microscopy. We found that bacteria are attached to the root surface by an intercellular matrix and excrete slime, which, as a binding agent, favors the formation of bacterial agglomerates [5, 7]. We studied the structure of the intercellular matrix of many bacterial isolates, including those obtained from the roots of the ground orchid *C. vestita.* Typically, the bacterial matrix is composed of acidic polysaccharides and glycosylphosphate-containing polymers (i.e., teichoic acids) or, less frequently, glycoproteins and peptides [19]. We found that most matrices studied (90%) were polyanionic, i.e., contained a large number of acidic groups. Bacteria of the genus *Xanthomonas* and one species of the genus *Rhodococcus* (*Rhodococcus* 16) produced neutral matrices (Table 2).

The carbohydrate fraction of the matrices of most bacteria was found to consist mainly of sugars (Table 2), which is in accordance with the available data [19]. A high polysaccharide content was revealed for bacteria of the genera *Arthrobacter, Pseudomonas,* and *Aquaspirillum* and for *Rhodococcus* sp. 16. Moderate-intensity (+) or pale (+–) staining with triphenyltetrazolium was characteristic of mycobacteria, bacilli, rhodococci, and *Pseudomonas* spp., which indicates minor

Table 3. Structure of certain bacterial matrices

Bacteria	Degree of polymerization		Carbohydrate/protein ratio in the matrix
	Ethanol : CL (2 : 1)	Ethanol : CL (4 : 1)	
Rhodococcus sp. 1	+	+	1.5
Aquaspirillum sp. K2	4+	4+	11.5
Aquaspirillum sp. 7	3+	4+	3.5
Aquaspirillum sp. 5	3+	3+	2.4
Bacillus sp. 3	+	2+	1.2
Nocardia sp. K5	+–	+	0.6
Pseudomonas sp. 18	+	+	0.5
Pseudomonas sp. 22	–	+	1.0
Cellulomonas sp. 23	+	2+	0.3
Pseudomonas sp. 24	2+	3+	0.6
Pseudomonas sp. 42	–	+	0.9

Note: CL, culture liquid; "–" means that no polysaccharide sediment was formed; "+–" characterizes scarce amounts of polymers precipitated; "4+" means abundant sediment throughout the entire volume of the solution; "+," "2+," and "3+" stand for intermediate patterns of sedimentation.

quantities of polysaccharides in their matrices. No reducing sugars (–) were found in matrices of bacteria of the genera *Xanthomonas, Micrococcus* or in *Pseudomonas* sp. 43.

In some bacteria belonging to different genera, we also studied the characteristics of the polymers of their matrices and the carbohydrate and protein content of the matrices (Table 3). Gram-negative bacteria are known to produce the most massive exopolysaccharide matrices [20]. Precipitation of polysaccharides of *Aquaspirillum* spp. and *Pseudomonas* sp. 24 with ethanol resulted in quick sedimentation, which is a sign of an abundant high-molecular polymeric matrix. *Pseudomonas* sp. 42 and *Pseudomonas* sp. 22 produced low-molecular matrices since larger volumes of ethanol were required for sedimentation of polysaccharides (four instead of two volumes). An intermediate pattern was characteristic of *Bacillus* sp. 3, *Nocardia* sp. K5, *Cellulomonas* sp. 23, and *Pseudomonas* sp. 18.

The matrix of *Aquaspirillum* sp. contained a substantial proportion of polysaccharides. The matrices of *Rhodococcus* sp. 1 and *Bacillus* sp. 3 were also mainly composed a of carbohydrates. *Cellulomonas* sp. 23 produced a protein matrix. The proportion of proteins was somewhat larger than that of sugars in the matrices of *Nocardia* sp. K5, *Pseudomonas* sp. 24, *Pseudomonas* sp. 18 and *Pseudomonas* sp. 42, whereas the matrix of *Pseudomonas* sp. 22 contained equal quantities of proteins and carbohydrates. Thus, the data on sugar and protein proportions in bacterial matrices are in accordance with the results of qualitative carbohydrate analysis (Table 2).

The intercellular matrix of a bacterial colony is a multifunctional formation that plays structural and communicative roles, as various exometabolites and signaling compounds are excreted into it [12, 20]. It protects bacterial cells from antibiotics and predaceous protozoa, as well as from dehydration and numerous physical and chemical impacts [19]. Bacteria also produce various pigments for protection from the adverse influence of UV radiation. The bacterial colonies isolated from the roots of *A. papillosa* (particularly from the aerial roots) were brightly colored yellow, orange, ginger, red, and crimson. Similar data were obtained for another epiphyte, *D. moschatum* (this and previous [5] works). Apparently, pigments protect bacterial cells from solar radiation since epiphytes grow under constant illumination of high intensity.

Thus, the excretion of an intercellular matrix (most often of a carbohydrate nature) by the bacteria isolated confirms its crucial role in the formation of associations of microorganisms with a host plant, particularly in the bacterial attachment to the root surface, which we showed previously with scanning electron microscopy [5, 7]. Bacteria form communities on the roots of tropical epiphytic orchids by means of the matrix. The qualitative structure of a community is determined by the type of ecological niche preferred by microorganisms (aerial or substrate roots), as well as by morphological and physiological characteristics of the host plant. However, the data that we obtained do not reflect quantitative characteristics of bacterial communities: proportions of different species in a consortium and the nature of dominant, moderately abundant, and minor species. To obtain such information, further studies on the structure and dynamics of associative microbial communities of orchids are required.

ACKNOWLEDGMENTS

We are grateful to L.V. Lysak for help in bacterial identification, to I.V. Botvinko for help in determination of the characteristics of bacterial matrices and to G.L. Kolomeitseva for providing samples of orchid roots.

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