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EXPERIMENTAL ARTICLES

Peculiarities of Adhesion of Epiphytic Bacteria on Leaves of the Seagrass *Zostera marina* and on Abiotic Surfaces

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Abstract—A comparative study of the adhesion of epiphytic bacteria and marine free-living, saprophytic, and pathogenic bacteria on seagrass leaves and abiotic surfaces was performed to prove the occurrence of true epiphytes of *Zostera marina* and to elucidate the bacterium–plant symbiotrophic relationships. It was shown that in the course of adhesion to the seagrass leaves of two taxonomically different bacteria, *Cytophaga* sp. KMM 3552 and *Pseudoalteromonas citrea* KMM 461, isolated from the seagrass surface, the number of viable cells increased 3–7-fold after 60 h of incubation, reaching $1.0–2.0 \times 10^5$ cells/cm^2; however, in the case of adhesion of these bacteria to abiotic surfaces, such as glass or metal, virtually no viable cells were observed after 60 h of incubation. Such selectivity of cell adhesion was not observed in the case of three other bacterial species studied, viz., *Vibrio alginolyticus* KMM 3551, *Bacillus subtilis* KMM 430, and *Pseudomonas aeruginosa* KMM 433. The amount of viable cells of *V. alginolyticus* KMM 3551 adsorbed on glass and metal surfaces increased twofold after 40 h of incubation. The cells of saprophytic *B. subtilis* KMM 430 and pathogenic *P. aeruginosa* KMM 433 adsorbed on three studied substrata remained viable for 36 h and died by the 60th hour of incubation.

Key words: epiphytic bacteria, seagrass *Zostera marina,* adhesion.

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The family *Zosteraceae* (its representatives are frequently termed "seagrasses") belongs to a very interesting group of flowering plants adapted to growth in the saline water of seas and oceans. From 1940 to the present day, seagrasses of the family *Zosteraceae* have attracted the interest of researchers as unique renewable sources of a number of pharmacologically important compounds, such as flavonoids with antioxidant activity, higher fatty acids exhibiting hypocholesterolemic properties, and pectins, which are used as antiulcer agents and detoxicants [1]. Seagrasses may be used for the production of iodine preparations applied for the prophylaxis and therapy of sclerosis and thyroid gland diseases and as fodder in animal and poultry husbandry [1].

Zostera marina L. is the most abundant seagrass in seawater of the middle and high latitudes of the Northern Hemisphere. The zostera fields are among the most productive plant bodies, with the primary production of 0.2 to 1.0 mg C/(g dry weight h) [2]. The bulk of the *Z. marina* mass in the Peter the Great Bay of the Sea of Japan was estimated as 16000 tons of wet weight [1].

The productivity of seagrass fields depends on various biotic and abiotic factors. Usually, seagrasses grow in nutritionally deficient water bodies and are inhabited by epiphytic communities composed of algae, bacteria, fungi, and protozoa. The structure of such epiphytic communities is determined by diverse trophic interactions between inhabitants. Epiphytes reduce carbon turnover and illumination at the leaf surface and can have a considerable effect on the productivity of the macrophytes. Bacterial growth is limited mainly by nutrient supply [3]. Bacterial epiphytes of *Z. marina* have been shown to obtain organic compounds containing carbon, phosphorus, and nitrogen from the seagrass leaves [4, 5]. This requires the existence of functional symbiotrophic interrelations between certain heterotrophic epiphytes and *Z. marina*. The elucidation of the microorganism–plant interactions in marine environments and of the ecological role of epiphytic microbial populations is of great importance for the development of biotechnological approaches to the regulation of the studied ecosystem and for evaluation of the adaptive potential of microbial communities under anthropogenic impact.

The aim of this work was to study the characteristics of adhesion of epiphytic bacteria and free-living saprophytic and pathogenic bacteria to seagrass leaves and abiotic surfaces.

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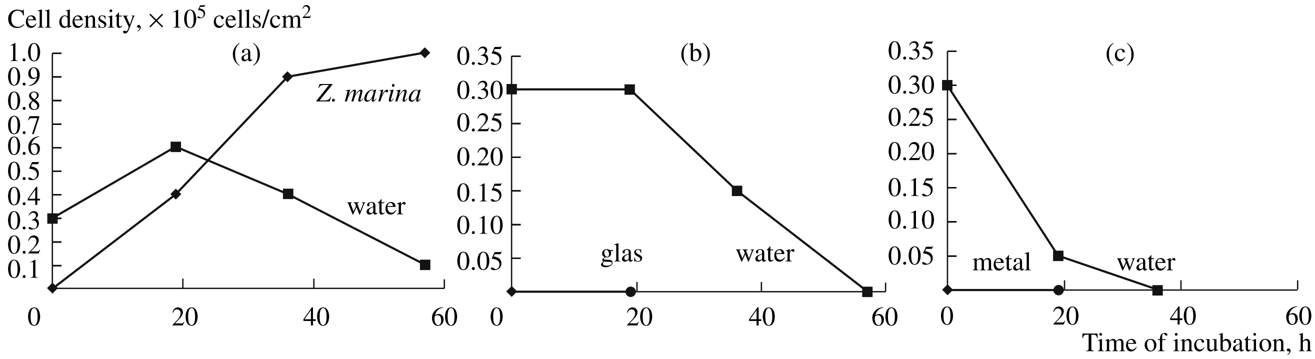

Fig. 1. Time course of adhesion of an epiphytic bacterium *Cytophaga* sp. KMM 3552 on (a) leaves of seagrass *Zostera marina* and abiotic surfaces (b) glass and (c) metal.

MATERIALS AND METHODS

The study was carried out with bacteria *Cytophaga* sp. KMM 3552 and *Pseudoalteromonas citrea* KMM 461 isolated in July 1998 by the replica-plating method from the middle-layer leaves of *Z. marina* grown in the Troitsa Bay of the Peter the Great Bay, and *Vibrio alginolyticus* KMM 3551 isolated from a seawater sample from the seagrass ecotope. Bacteria were isolated from individual colonies grown for seven days at 28°C on agarized medium 1 containing (g/l): peptone, 5.0; yeast extract, 2.0; glucose, 1.0; K_2HPO_4, 0.2; $MgSO_4$, 0.05; agar, 20.0; distilled water, 500 ml; seawater, 500 ml; pH 7.8. Bacteria were identified by the standard methods [6, 7]. Bacteria *Bacillus subtilis* KMM 430 and *Pseudomonas aeruginosa* KMM 433 were obtained from the Collection of Marine Microorganisms (KMM) of the Pacific Institute of Bioorganic Chemistry, Far East Division, Russian Academy of Sciences. Pure cultures were maintained both on semisolid medium 1 (5 g agar/l) under mineral oil at 4°C and in liquid medium 1 supplemented with 20% glycerol at –80°C.

In model experiments performed in three parallel series, 6- to 7-cm-long pieces of the middle-layer leaves of seagrass were thoroughly washed in sterile seawater and placed into sealed beakers containing 200 ml of sterile seawater together with sterile glass and stainless steel plates with the surface area of 1 cm^2. The beakers were inoculated with 200 ml cell suspension (10^6 cells/ml) containing a mixture of five bacterial species, viz., *B. subtilis* KMM 430, *V. alginolyticus* KMM 3551, *P. aeruginosa* KMM 433, and two strains of the seagrass epiphytes, *Pseudoalteromonas citrea* KMM 461 and *Cytophaga* sp. KMM 3552. The beakers were incubated at 11.5°C. The amount of microbial cells after 19, 36, and 57 h of incubation was determined in water samples (200 μl), in scrapes from a 1-cm^2 area of the leaves (preliminarily washed with sterile seawater), and in replica from one side of glass and metal plates. The adhesion of bacteria to *Z. marina* leaves, glass, and metal was evaluated by estimating the number of colony-forming units (CFU) after direct plating of samples on petri dishes with agarized medium 1.

RESULTS AND DISCUSSION

In model experiments, we studied the time courses of adhesion of epiphytic bacteria *Cytophaga* sp. 3552 (Fig. 1) and *Pseudoalteromonas citrea* KMM 461 (Fig. 2), as well as *Vibrio alginolyticus* KMM 3551 isolated from seawater (Fig. 3), saprophytic *Bacillus subtilis* KMM 430 (Fig. 4), and pathogenic bacterium *P. aeruginosa* KMM 433 (Fig. 5) on both the *Zostera marina* leaves and biologically inert surfaces, such as glass and stainless steel plates. It was revealed that by the 60th hour of incubation, the number of viable cells of two taxonomically different strains isolated from the seagrass surface increased by 3–7-fold and reached 1.0–2.0 × 10^5 cells/cm^2 of the leaf, whereas viable cells were virtually absent on glass and metal surfaces (Figs. 1 and 2). It should be noted that when strains of different taxa were incubated in sterile seawater, the number of viable cells ranged from 0 to 0.1–0.4 × 10^5 cells/ml after 40 h; after 60 h of incubation, viable cells were usually completely absent.

Bacteria *V. alginolyticus* KMM 3551, *B. subtilis* KMM 430, and *P. aeruginosa* KMM 433 showed no selectivity of adhesion to either biotic or abiotic surfaces (Figs. 3–5). At the same time, in the case of adhesion to glass and metal surfaces of *V. alginolyticus* KMM 3551, an inhabitant of marine ecosystems, the number of viable cells increased twofold after 40 h of incubation, whereas cells of saprophytic *B. subtilis* KMM 430 and pathogenic *P. aeruginosa* KMM 433 adsorbed on the three studied substrata remained viable for 36 h and died by the 60th hour of incubation. Adhesion of these strains appeared to be nonspecific and based on physicochemical binding of cells with substrata of diverse chemical structure [8].

The results obtained demonstrate that bacteria possessing certain adhesive and metabolic properties exhibited selectivity in adhering to hard substrata. The studied epiphytic bacteria belonged to two different

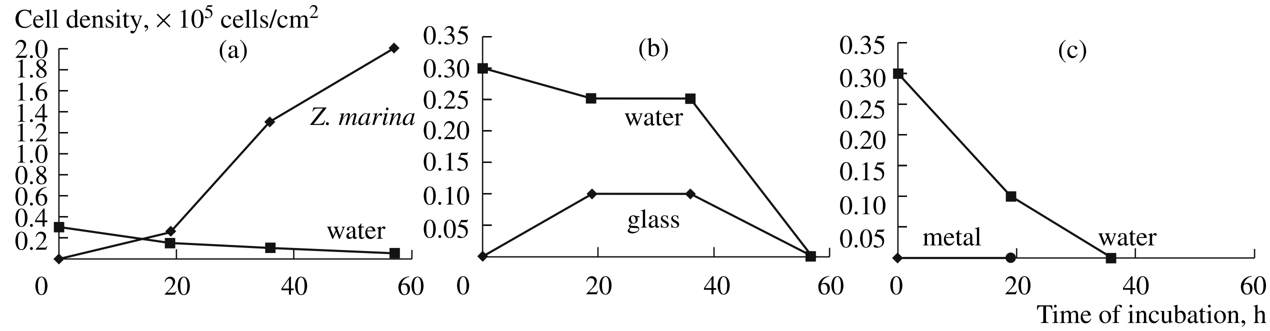

Fig. 2. Time course of adhesion of an epiphytic bacterium *Pseudoalteromonas citrea* KMM 461 on (a) leaves of seagrass *Zostera marina* and abiotic surfaces (b) glass and (c) metal.

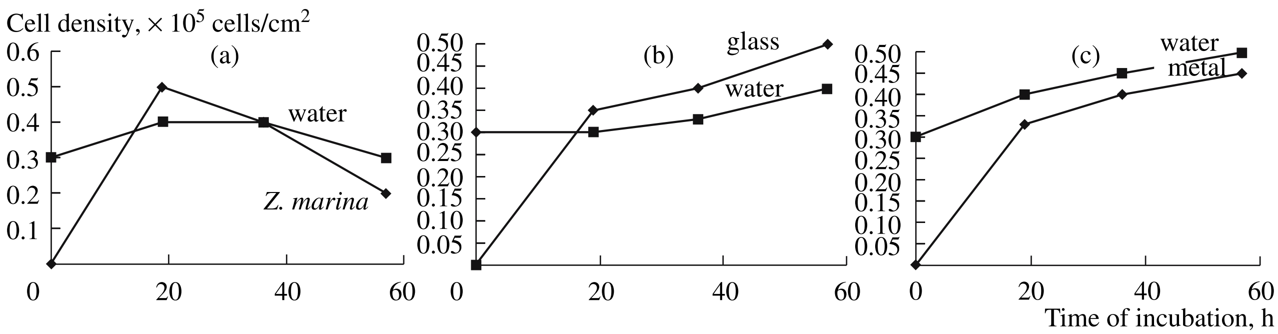

Fig. 3. Time course of adhesion of a free-living bacterium *Vibrio alginolyticus* KMM 3551 on (a) leaves of seagrass *Zostera marina* and abiotic surfaces (b) glass and (c) metal.

taxa, viz., the gamma-subclass of *Proteobacteria* and the *Flavobacterium–Cytophaga–Bacteroides* phylogenetic cluster. Nevertheless, these taxonomically different bacteria isolated from leaf surfaces, unlike the three other bacterial species tested, were able not only to attach to the leaf surface, but also to survive in an oligotrophic medium. Most likely, epiphytic bacteria were capable of effective utilization of organic substances produced by *Z. marina.* In our opinion, this finding is evidence of symbiotrophic relations between epiphytic bacteria and seagrass and agrees well with the data on direct transfer of carbon and nitrogen from zostera leaves to epiphytes [9]. It was found that ^{14}C-labeled HCO_3^- and ^{15}N-labeled NO_3^-, NH_4^+, and $(NH_4)_2CO_3$ were consumed by plant roots from the solution and transferred to all plant parts and, subsequently, to the leaf-inhabiting epiphytes. These data confirm the transport of nitrogen and carbon from the bottom sediments to the epiphytic community of rooted macrophytes due to the consumption of plant-secreted organic and inorganic compounds by epiphytes.

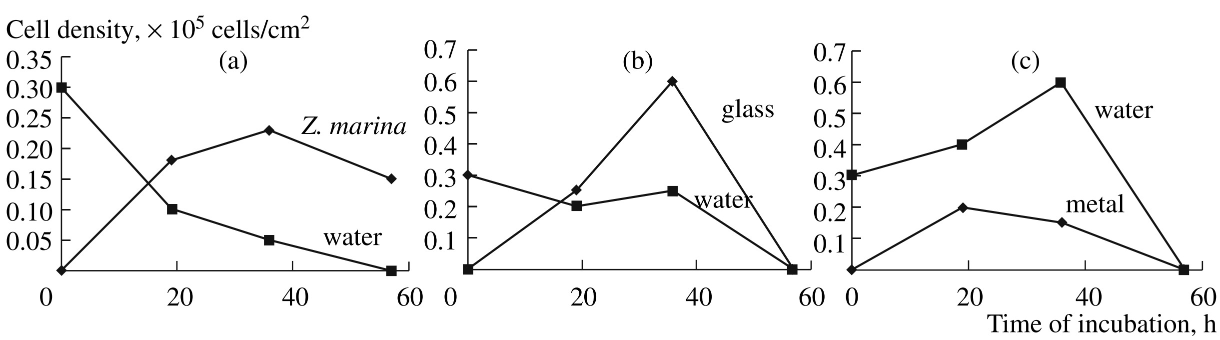

Fig. 4. Time course of adhesion of a saprophytic bacterium *Bacillus subtilis* KMM 430 on (a) leaves of seagrass *Zostera marina* and abiotic surfaces (b) glass and (c) metal.

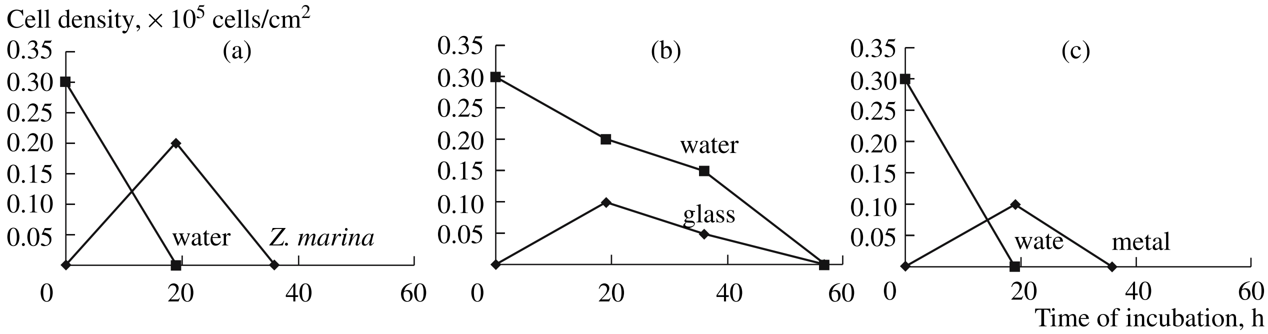

Fig. 5. Time course of adhesion of a pathogenic bacterium *Pseudomonas aeruginosa* KMM 433 on (a) leaves of seagrass *Zostera marina* and abiotic surfaces (b) glass and (c) metal.

The results of this study agree well with the aforementioned literature data and provide indirect support for the existence of a symbiotrophic relationship between epiphytic bacteria and *Z. marina*. Selective adhesion of epiphytic bacteria to *Z. marina* leaves seems to be determined by their metabolic characteristics, which allow them to utilize organic substances containing carbon, phosphorus, and nitrogen from the seagrass leaves [4, 5].

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