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EXPERIMENTAL ARTICLES

Sorption of Humic Acids by Bacteria

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Abstract—Capacity for sorption of humic acid (HA) from water solutions was shown for 38 bacterial strains. Isotherms of HA sorption were determined for the cells of 10 strains. The bonding strength between the cells and HA (k) and the terminal adsorption (Q_{max}) determined from the Langmuir equation for gram-positive and gram-negative bacteria were reliably different. Gram-positive bacteria sorbed greater amounts of HA than gram-negative ones ($Q_{max} = 23 \pm 10$ and 5.6 ± 1.2 mg/m^2, respectively). The bonding strength between HA and the cells was higher in gram-negative bacteria than in gram-positive: $k = 9 \pm 5$ and 3.3 ± 1.1 mL/mg, respectively.

Keywords: gram-positive and gram-negative bacteria, cell wall, humic acids, sorption

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Although the contact of humic acids (HA) with living cells including bacterial ones is accompanied by a wide spectrum of response reactions of the organisms [1, 2], specific mechanisms of this process are poorly understood. The interaction of HA with bacteria obviously occurs only on the surface of the cell walls [3].

In water solutions surrounding microbial cells in soil, HA are able to bind metal ions and xenobiotics into neutral complexes, thus decreasing their impact on the organisms [4]. Similar reactions may proceed at the cell surface in the layer formed by the HA molecules sorbed onto the cell wall [5, 6]. In our opinion, many biological processes which are associated with the HA contact with the microbiota can be explained by the HA localization on the cell walls, although the sorption of HA by living and dead organisms is scarcely studied [7].

At present, the only information available on the HA sorption is concerns the bacteria *Bacillus subtilis* and *Escherichia coli* [8, 9]. The HA sorption onto bacterial cells was studied as a function of pH and duration of the cell interaction with the adsorbate. The authors did not attempt extrapolation of the obtained results to other prokaryotes; moreover, it seems to be hardly possible.

There is more data on bacterial sorption of metal ions and organic compounds of molecular mass up to 3 kDA. The observed differences in the sorption capacity of gram-positive and gram-negative bacteria were associated with different structure of their call walls [10–12].

The goal of the present work was to determine the quantitative parameters of the HA sorption by strains of gram-positive and gram-negative bacteria.

MATERIALS AND METHODS

Bacteria. The study was carried out with bacteria isolated from the upper layers of pale-podzolic soil under the wood sorrel spruce forest (the Central Forest State Nature Biosphere Reserve, Tver oblast), sod-podzolic soil (experimental ground of the All-Russia Scientific Research Institute of Phytopathology, Russian Academy of Agricultural Sciences, Golitsyno), ordinary chernozem (Kursk), and dark chestnut soil (Saratov), as well as with bacterial strains obtained from the Culture Collections of the Faculty of Soil Science, Moscow State University and from the Institute of Gene Biology, Russian Academy of Sciences.

The isolation of gram-negative bacteria was performed on the MacConky medium. Gram-positive bacteria were isolated on the glucose-peptone-yeast extract agar (GPY); soil samples were pretreated with 0.2 N NaOH for 15 min for the lysis of gram-negative bacteria [13]. Colonies grown on petri dishes were treated with 0.1 M NaOH to determine the affiliation of bacteria to one or another group. As a result, 19 gram-positive and 19 gram-negative strains were isolated (Table 1). Identification of bacteria was performed either by molecular genetic methods or on the basis of their morphological and physiological charac-

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Table 1. List of tested bacteria

Strain number	Name	Source
	Gram-negative bacteria	
1	1 ns	Golitsyno
2	2 ns	"
3	3 ns	"
4	4 ns	Saratov
5	5 ns	"
6	6 ns	"
7	7 ns	Kursk
8	8 ns	"
9	9 ns	Golitsyno
10	10 ns	Kursk
11	11 ns	"
12	12 ns	"
13	*Cytophaga* sp.	Collection
14, 15	*Escherichia coli, E. coli* (BW2952)*	"
16	*Pseudomonas putida*	"
17	*Pseudomonas* sp. (HF913575)*	"
18	*P. fluorescens* (HF913573)*	"
19	*Serratia marcescens**	"
20	*Chromobacterium* sp.*	"
	Gram-positive bacteria	
21	1 ps	Collection
22	2 ps	Golitsyno
23	3 ps	Kursk
24	4 ps	"
25	5 ps	"
26	6 ps	Tver
27	7 ps	Collection
28	8 ps	Kursk
29	9 ps	Tver
30	10 ps	"
31	*Arthrobacter atrocyaneus**	Collection
32	*A. globiformis**	"
33, 34	*Bacillus megaterium, B. megaterium**	"
35	*B. pumilus*	"
36	*B. subtillis*	"
37	*Bacillus* sp. (HF913574)*	"
38	*Rhodococcus luteus**	"

* Isotherms of the HA sorption were constructed; "ns" stands for "gram-negative unidentified strains"; "ps" means "gram-positive unidentified strains".

teristics. PCR amplification of the 16S rRNA gene sequences was carried out using primers 27F (AGA GTT TGA TCCTGG CTC AG) and 1492R (ACG GCT ACC TTG TTA CGA CTT).

The nucleotide sequences were deposited in GenBank under accession numbers HF913573, HF913574, and HF913575. Twenty-two bacterial strains were not identified.

Humic acid. The HA used (Merck, Germany) had an average molecular mass of 20 kDa and the following composition (%): C, 40; H, 37.8; N, 4.2; O, 18 [14]. HA was dissolved in 0.1 M sodium phosphate buffer (pH 7.0) and sterilized through a 0.2-μm filter (Millipore, United States).

Microscopy. The number and size of bacterial cells in suspensions were determined with the use of bright field microscopy without preliminary specimen staining in a Biomed 6 microscope (Russia) under Köhler illumination at magnification of 1000×; planachromatic oil immersion lens with oil immersion (100×) was used; photo fixation was performed on a DCM 510 camera using the ScopePhoto 3.0 program (China).

After incubation of bacteria with the HA solutions, an aliquot of preliminary diluted cell suspension (20 μL) was spread onto 4 cm^2 of a microscope slide and fixed over flame.

The length (h), diameter (d), and the number of cells were determined from micrographs (measurements were performed in six replicates from ten microscopic fields). The number of cells and their parameters were calculated, taking into account the area of the microscopic field. The area of the cell surface (S_{bact}) was taken as a surface of a cylinder or ball in the case of rods (equation 1) or cocci (equation 2), respectively,

$$S_{bact} = 2\pi r(h + d/2), \quad (1)$$

$$S_{bact} = 4\pi(d/2)^2. \quad (2)$$

Qualitative test for the cell capacity for HA bonding. Bacteria were incubated in 200-mL conical flasks with 100 mL of GPY medium on a Unimax 2010 shaker (Heidolph, Germany) (120 rpm) at 25°C for 8–14 h. The cells were precipitated on a Minispin centrifuge (Eppendorf, Germany) at 12000 rpm for 10 min, resuspended in 0.1 M Na phosphate buffer (pH 7.0), and centrifuged on a Multi-Vortex V-32 device (Biosan, Latvia) at the maximal number of revolutions for 5 min. The washing procedure was repeated twice. The supernatant was removed; 750 μL of HA solution in 0.1 M Na phosphate buffer (0.1 mg HA/mL) was added to the cell precipitate. In the control, 750 μL of 0.1 M Na phosphate buffer was added to the cells. The cell suspensions were vigorously stirred on a Multi-Vortex V-32 shaker for 30 min at the maximal number of revolutions and separated on a Minispin centrifuge (Eppendorf, Germany) (12000 rpm, 10 min). The supernatant was removed, the cells were resuspended

Table 2. Linear dimensions and cell surface areas of the studied bacteria (average data from measuring of 60 microscopic fields)

Bacteria	Length, μm	Radius, μm	Area, μm^2
Rhodococcus luteus	3.4 ± 0.2	0.5 ± 0.1	12.9 ± 0.4
Bacillus sp. HF913574	4.5 ± 0.2	0.4 ± 0.1	12.4 ± 0.4
B. megaterium	5.2 ± 0.2	0.9 ± 0.2	34.6 ± 0.5
Arthrobacter globiformis	3.0 ± 0.1	0.5 ± 0.1	10.6 ± 0.1
A. atrocyaneus (rods)	3.4 ± 0.1	0.5 ± 0.1	13.2 ± 0.1
A. atrocyaneus (cocci)		0.6 ± 0.1	3.8 ± 0.1
Escherichia coli BW2952	4.7 ± 0.2	0.5 ± 0.1	17.1 ± 0.4
Pseudomonas sp. HF913575	3.4 ± 0.2	0.4 ± 0.1	9.5 ± 0.4
P. fluorescens HF913573	3.2 ± 0.1	0.7 ± 0.2	17.2 ± 0.2
Serratia marcescens	3.1 ± 0.1	0.4 ± 0.1	9.8 ± 0.1
Chromobacterium sp.	3.1 ± 0.2	0.5 ± 0.1	11.9 ± 0.4

in 50 μL of Na phosphate buffer, and the optical density was determined on a Sunrise spectrophotometer (Tecan, Switzerland) at 450 nm. The relative intensity of the cell staining with HA (ΔA_{cells}) was calculated from equation (3):

$$\Delta A_{cells} = (A_{HA} - A_{without\ HA})/A_{without\ HA}, \quad (3)$$

where A_{HA} is optical density of the cell suspension incubated with HA; $A_{without\ HA}$ is optical density of the cell suspension incubated in phosphate buffer without HA.

Isotherms of HA sorption by bacterial cells. Isotherms of HA sorption were constructed for five strains of gram-positive and five strains of gram-negative bacteria. Bacterial cultures were incubated in liquid GPY medium at 25°C for 8–14 h on a shaker (100 rpm). The cells were washed off from the medium as described above and resuspended in 50 mL of 0.1 M Na phosphate buffer (pH 7.0). An average concentration of cells in the suspensions was 10^8–10^9 cells/mL. An aliquot of the cell suspension (5 mL) was added to 5 mL of sterile HA solutions containing 0.125, 0.25, 0.5, and 1.2 mg HA/mL. In the control, the cell suspension (5 mL) was added to 5 mL of 0.1 M Na phosphate buffer.

The obtained cell suspensions were stirred for 30 min at 1000 rpm, and aliquots (1–2 mL) were used for determination of the number and size of bacterial cells. The remaining cell suspension was separated on a Centrifuge 5804 (Eppendorf, Germany) at 6000 rpm for 15 min; optical density of the supernatant was measured in triplicate for each HA concentration on a Specord 50 (Analytic Jena, Germany). The equilibrium concentration of HA ($C_{HA\ equil}$) was determined from the optical density of solutions using a calibration curve of A_{350} vs. C_{HA}, where A_{350} is optical density of HA solution at 350 nm and C_{HA} is concentration of HA in the solution. The amount of HA sorbed onto the unit of the cell surface (Q_p, mg/m^2) was calculated from equation (4):

$$Q_p = (C_{HA\ initial} - C_{eq})/(N_{bact} \times S_{bact}), \quad (4)$$

where $C_{HA\ initial}$ is the initial concentration of HA, mg/mL; C_{eq} is the equilibrium concentration of HA, mg/mL; N_{bact} is the number of bacterial cells incubated with HA, cells/mL; S_{bact} is the surface area of a single bacterial cell, m^2.

The isotherms of HA sorption showing Q_{eq} as a function of C_{eq} were constructed with the use of the Langmuir equation (5) [15]:

$$Q_{eq} = Q_{max} \times k \times C_{eq}/(1 + k \times C_{eq}), \quad (5)$$

where Q_{eq} is the value of the equilibrium sorption of HA, mg/m^2; Q_{max} is the value of terminal adsorption, the maximum amount of HA sorbed onto the cells, mg/m^2; k is the equilibrium constant of the adsorption process, which characterizes the strength of HA bonding onto the cell surface, mL/mg; C_{eq} is the equilibrium concentration of HA, mg/mL.

The values of k and Q_{max} were calculated from the linearized Lengmuir equation [16]:

$$1/Q_{eq} = 1/Q_{max} + (1/(Q_{max} \times k)) \times 1/C_{eq}. \quad (6)$$

The dependence $1/Q_{eq} = f(1/C_{eq})$ represents an equation of the straight line, in which the tangent of its angle of inclination is equal to $1/Q_{max}$, and the intercept on an axis corresponds to $1/(Q_{max} \times k)$. Coefficients of the equation were obtained by linear regression from the available data.

RESULTS AND DISCUSSION

Staining of bacterial cells with HA. In the course of cell incubation with HA, gram-positive bacteria became more intensely colored than gram-negative

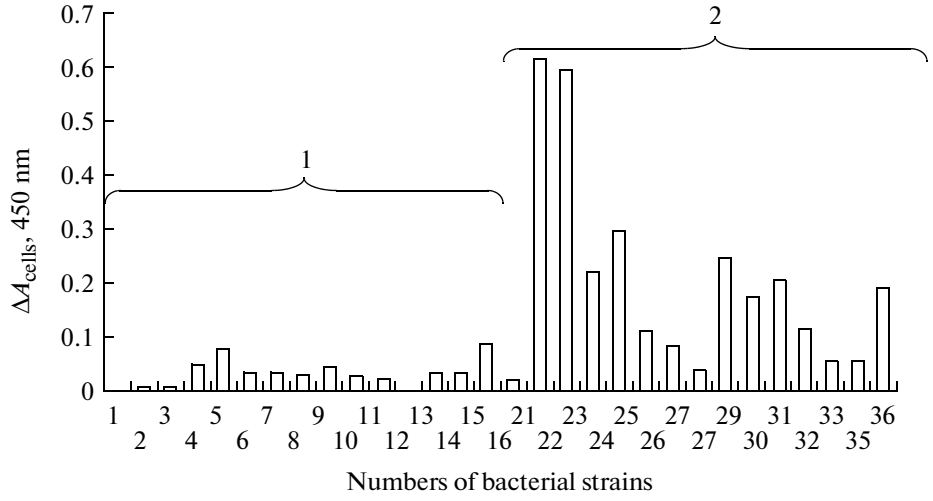

Fig. 1. Relative changes in the cell staining (ΔA_{cells}) of gram-negative (*1*) and gram-positive (*2*) bacteria incubated with HA.

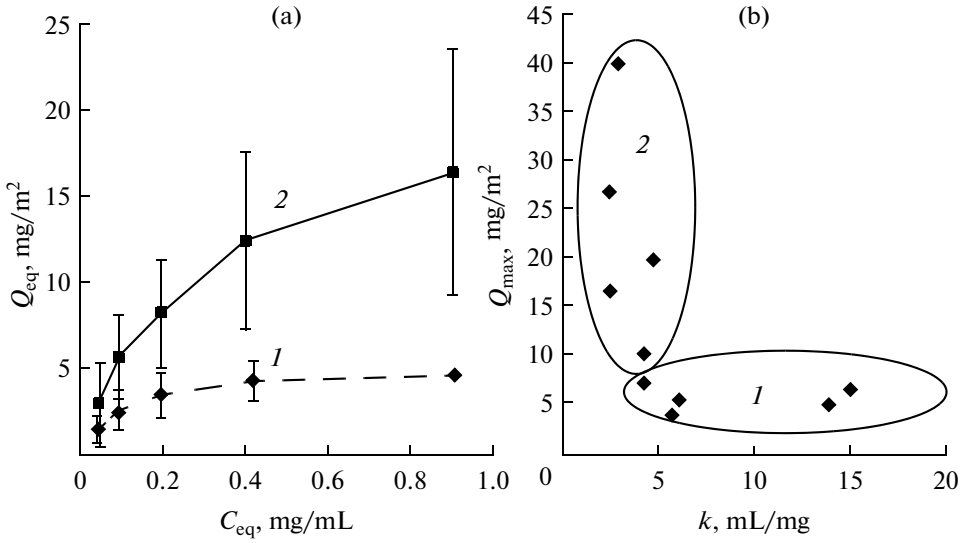

Fig. 2. Averaged isotherms of HA sorption (a) and the coefficients (Q_{max}, k) determined from the Langmuir equation (b) for five strains of gram-negative (*1*) and five strains of gram-positive (*2*) bacteria.

ones (Fig. 1). The average values of ΔA_{cells} for gram-positive and gram-negative bacteria were reliably different (at $p = 0.95$) comprising 0.26 ± 0.1 and 0.05 ± 0.02, respectively.

The capacity of the strains for HA binding varied considerably within the groups of gram-positive and gram-negative bacteria (Fig. 1). No considerable difference in the values of ΔA_{cells} between these groups of bacteria was revealed; nevertheless, the average values of variation in the relative cell staining with HA for gram-positive and gram-negative bacteria were reliably different. We were the first to show the HA binding by a wide range of bacteria characterizing by different structure of the cell walls; the importance of these findings for ecology should be appraised.

Isotherms of the HA sorption by bacterial cells. The curves of the HA sorption by the cells (Fig. 2a) exhibited common patterns: a sharp slope at low values of the equilibrium HA concentration of and gradual transition to the plateau with increasing HA concentrations that is indicative of gradual saturation of the adsorbent (bacterial cells) surface. Such curve shape is typical of the sorption process described by the Langmuir equation [17]. The values of sorbed HA at the plateau level agreed well with those calculated from

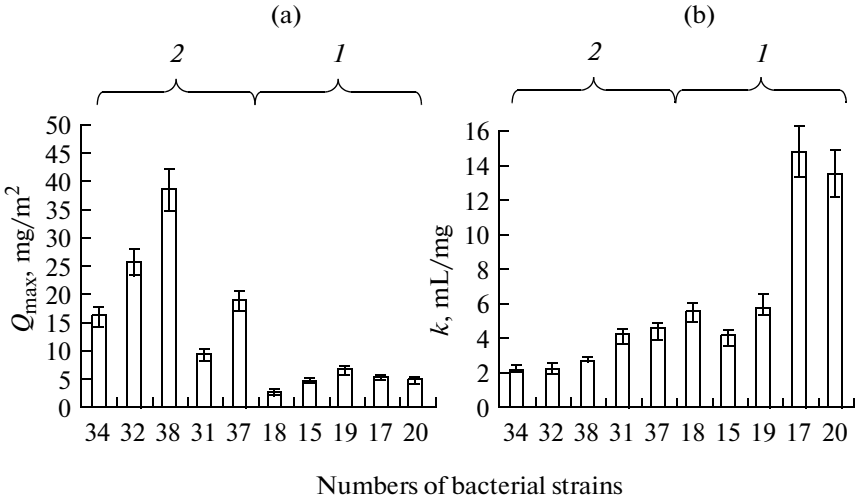

Fig. 3. Terminal values of HA adsorption onto the cells (a) and the binding strength between HA and the cells (b) for gram-negative (*1*) and gram-positive (*2*) bacteria.

the Langmuir equation (Q_{max}), which confirmed the correctness of using this equation for description of the sorption process.

The average values of the parameters k and Q_{max} obtained for the studied groups of microorganism were reliably different (Fig. 2b). Gram-positive bacteria showed higher values of the terminal adsorption (Q_{max}) than gram-negative bacteria (23 ± 10 and 5.6 ± 1.2 mg/m^2, respectively). However, the binding strength between the cells and HA was higher in gram-negative bacteria (9 ± 5 mL/mg) than in gram-positive bacteria (3.3 ± 1.1 mL/mg). The largest values of Q_{max} were revealed for strains *Rhodococcus luteus* and *Arthrobacter globiformis*; the maximum values of k were found for *Pseudomonas* sp. (HF913575) and *Chromobacterium* sp. (Fig. 3).

We think that low values of HA sorption onto the cells of gram-negative bacteria as compared with those observed for gram-positive bacteria can be explained by the fact that the outer lipopolysaccharide layer of the cell wall in gram-negative bacteria hampers the penetration of HA with an average molecular mass over 20 kDa into deeper layers of the cell wall. In particular, it was shown that the outer membrane of gram-negative bacteria prevented penetration into the cells of pesticides [11], heavy metals [18], and the bactericidal compound, nisin, with molecular mass of about 3 kDa [12]. It is known that HA are amphiphilic compounds [19], whereas the outer cell membrane is hydrophilic [20]; therefore, the transfer of HA molecules through the thick outer membrane is mainly restricted by their sizes. In gram-positive bacteria, the outer cell membrane is absent, therefore HA can diffuse easier into the peptidoglycan layer surrounding the cell. It is possible that in this case, HA molecules are absorbed in the whole volume of the cell wall.

At neutral pH value in 0.1 M Na phosphate buffer (pH 7.0), both the peptidoglycan layer and the cell wall had a negative charge due to the dissociation of the carboxylic groups of their polymers [21]. Under these conditions, HA molecules also had a negative charge because of dissociation of their COOH groups [22]. However, no complete extrusion of the similarly charged HA molecules from the pores of bacterial cell walls occurred, possibly because of the low density of negatively charged carboxylic groups located both in the cell wall volume and at the surface of HA molecules, which can be bound with peptides and polysaccharides of the cell wall by numerous hydrogen bonds between O- and N-containing groups, as well as by means of hydrophobic interactions.

In the studies on the sorption of metals and pesticides onto the cell walls of bacteria [23], the role of polysaccharide capsules in these reactions was shown. In the studies on HA sorption onto bacterial cells, microscopic examinations of bacteria in the presence of the Indian ink particles did not reveal light layers at the cell/medium interface that indicated the absence of polysaccharide capsules. The dominant role of the structure of the cell wall surface in the processes occurring at the interface boundary was indirectly confirmed by the finding of D.G. Zvyagintsev that adhesion of the living and dead bacterial cells to the soil solid phases was not different [24].

The phenomenon of HA sorption onto bacterial cells may be used for several purposes: (a) in the non-waste technologies for purification of environmental colored water; and (b) for the introduction of microorganisms into unfavorable environments, e.g., it was found that growth of the oil-degrading strain of gram-positive bacterium *Acinetobacter* sp. was accelerated in the presence of HA [25].

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