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EXPERIMENTAL ARTICLES

Acidobacteria in Microbial Communities of the Bog and Tundra Lichens

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Abstract—Bacterial communities of the lichens from a *Sphagnum* bog (Karelia) and tundra (Vorkuta oblast) were investigated. Members of the phylum *Acidobacteria* were numerous in the thallus of living and decaying lichens (3.8×10^8 cells/g), constituting 6 to 32% of the total bacterial number. Pure cultures of acidobacteria were isolated from the samples of living and decaying lichen thallus. Ten of them were identified and classified as members of subgroup 1 of the *Acidobacteria*. The hydrolytic activity of two strains isolated from the living and decomposing zones of the thallus was investigated. They were capable of growth on xylan, starch, pectin, laminarin, and lichen extract. Acidobacteria were shown to be a stable and numerous component of microbial communities of the bog and tundra lichens.

Keywords: lichens, *Acidobacteria*, polysaccharide decomposition, *Sphagnum* bogs, tundra.

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Bog and tundra ecosystems constitute a significant part of the Russian boreal landscapes. The estimated area of bogs in Russia is 1.6×10^6 km^2, while the tundra territories occupy 2.4×10^6 km^2, or 13% of the territory [1]. A significant part of this area is covered by fruticose and foliose epigeal lichens. For a long time this group of symbiotic microorganisms has been of interest to microbiologists. The Russian works of the 1930s showed the presence of nitrogen-fixing bacteria, including *Azotobacter,* in the lichen thallus [2]. The introduction of molecular techniques resulted in renewed interest in lichens. In these works, the lichen thallus was then considered as an environment for novel bacterial species [3] and a concentrator of nitrogen-fixing bacteria [4]. In the thallus of *Cladonia arbuscula*, about 13% of the total number of bacterial cells revealed by acridine orange staining were unidentified [5]. Culturable bacteria with chitinolytic, glucanolytic, and proteolytic properties, as well as producers of indole-3-acetic acid, were found in the microbial communities of the same lichen [6].

Hodkinson and Lutzoni [7] detected the 16S rRNA gene sequences of the phylogenetic group *Acidobacteria* in the bacterial clone library of the community associated with *C. arbuscula*.

Acidobacteria are a group of difficult-to cultivate bacteria, which was acknowledged as a separate phylum in 1992, soon after the description of its first member, *Acidobacterium capsulatum* [8]. During the next 18 years, only 6 new genera with 10 species were isolated. Abundance of acidobacterial clones in the ecosystems with a broad range of physicochemical parameters suggested their wide distribution in all types of terrestrial ecosystems [9]. However, the isolation and maintenance of pure cultures of acidobacteria still remains a difficult challenge.

In 2006, we observed extensive red-colored bacterial overgrowth on the decaying thallus of the lichen *Cladonia* sp. collected on a Bakchar *Sphagnum* bog, in the Tomsk oblast. Isolation of pure bacterial cultures from this material resulted in the description of a new genus and species of acidobacteria, *Granulicella paludicola*, which possessed hydrolytic properties. This was the starting point for investigation of the processes of microbial decomposition of the lichen thallus and for the search of hydrolytic acidobacteria in this material.

The goal of the present work was to analyze the bacterial communities of epigeal bog lichens occurring in northern Russia (Karelia and Arctic tundra), isolate pure cultures of acidobacteria, and determine their hydrolytic activity.

MATERIALS AND METHODS

Sampling. Lichen and peat samples were collected in 2009 from the Lake Verkhnee ombrotrophic bog in the vicinity of the White Sea Biological Station (66°33′N, 33°06′E) and in 2010 in the vicinity of the Tal'nik station, Vorkuta oblast (67°19′N, 63°44′E). The whole thalli of the lichens were collected and transported undisturbed in sterile plastic vials. In the laboratory, three parts of the samples (the upper living part of the thallus, the intermediate decaying part, and

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Table 1. Characterization of the samples

No.	Sample	Description	pH of the water extract from the homogenate
1	*Cladonia* sp., uppert	Verkhnee ombrotrophic bog, Karelia	3.8
2	*Cladonia* sp., decomposition zone	"–"	4.0
3	Peat	*Sphagnum* sp., Verkhnee ombrotrophic bog, Karelia	4.0
B7	*Cladonia rangiferina*, uppert	Vorkuta, Tal'nik, site no. 1	4.8
B8	*C. rangiferina,* decomposition zone	"–"	6.7
B9	Peat	*Polytrichum uniperinum,* Vorkuta, Tal'nik, site no. 1	7.2
B10	*C. rangiferina*, uppert	Vorkuta, Tal'nik, site no. 2	5.0
B11	*C. rangiferina*, decomposition zone	"–"	5.1
B12	Peat	*Sphagnum papillosum,* Vorkuta, Tal'nik, site no. 2	5.1
B13	*Sphaerophorus globosa*, uppert	Vorkuta, Tal'nik	4.5
B14	*S. globosa*, decomposition zone	"–"	5.3
B15	Peat	*Sphagnum* sp., Vorkuta, Tal'nik	5.3

the underlying peat) were segregated. After visual examination, each lichen sample was cut with sterile scissors according to the degree of decomposition of the thallus, into the living thallus and the decaying one. The underlying peat was cleared from the thallus fragments and treated separately. Water extracts of the samples had pH from 3.8 to 7.2 (Table 1). The upper, living parts of the lichens had the lowest pH values.

Fixation. The sample material (1 g) with distilled water (20 mL) was placed into a bag for homogenization. Microbial cells were extracted in a MiniMix homogenizer (model 100, Interscience, France) for 10 min using the BagFilter® sterile packs with an internal filtering insert. An aliquot of the suspension (5 mL) was used for pH measurement. Another aliquot (0.5 mL) was centrifuged in a sterile 1.5-mL test tube at 5000 *g* for 5 min. The supernatant was discarded and the pellet was covered with 1 mL of formaldehyde (4% solution in PBS buffer (g/L): NaCl, 8.0; KCl, 0.2; Na_2HPO_4, 1.44; NaH_2PO_4, 0.2; pH 7.0). Fixation was carried out for 1.5 h with periodic shaking. Formaldehyde was then removed by centrifugation (5000 *g* for 5 min) and the pellet was washed twice with the PBS buffer, resuspended in 0.5 mL of 50% ethanol and stored at –20°C.

Total number and FISH. Total bacterial numbers in the samples and hybridization with oligonucleotide probes were carried out as described previously [11]. The cells were enumerated under an Axioplan Imagening (Zeiss, Germany) epifluorescence microscope in 40 fields of view for each sample.

Isolation of pure cultures. Bacterial cultures were isolated by direct plating on LE1 and M3 media. LE1 medium contained the following: extract of the *Cladonia* and *Cetraria* lichens obtained by heating at 115°C under pressure (100 g of dry thalli per 1 l distilled water), gellan-gum, 10 g; $MgSO_4 \cdot 7H_2O$, 0.8 g. M3 medium contained the following (g/L distilled water): starch, 0.5; glucose, 0.5; $MgSO_4 \cdot 7H_2O$, 0.04; KH_2PO_4, 0.1; casamino acids, 0.1; yeast extract, 0.1; $NaNO_3$, 0.02; $Ca(NO_3)_2$, 0.02; gellan-gum, 10. For the polymerization of gellan-gum, $MgSO_4 \cdot 7H_2O$ (80 mg) was added. pH of the media was 4–5.

Identification of the isolates was carried out by 16S rRNA gene sequencing. For DNA extraction, a modification of the previously described method based on sodium dodecyl sulfate (SDS) as a lysing agent was used [12]. PCR amplification of the 16S rRNA genes was carried out using the 9f and 1492r universal eubacterial primers [13] on a PE GeneAmp PCR System 9700 themocycler (Perkin-Elmer Applied Biosystems, United States). Comparison of the 16S rRNA gene sequences of the bog isolates with those from the GenBank database was carried out using the Blast2 software (http://www.ebi.ac.uk/blast2/). The sequences determined in the present work were deposited to GenBank.

Growth on polymers, oligosaccharides, and monosaccharides. The cell suspension (1 mL) with OD_{600} of 0.05–0.1 (determined on a Cary 50-Scan spectrophotometer, Varian) was added to 20 mL of M3 medium. The vials were then supplemented with 1 mL of solutions or water suspensions of colloidal chitin, colloidal cellulose, lichenan, pullulan, laminarin, starch, pectin, or xylan to the final concentration of 0.5–1 g/L. The main components of the M3 medium were added to the lichen extract prepared as described above. Cellobiose, raffinose, maltose, trehalose, acetylglucosamine, and glucose (0.5 g/L) were used to assess capacity for oligosaccharide hydrolysis and growth on monosaccharides. M3 medium without polymers and sugars was used as a negative control. The vials (120 mL) were incubated for two weeks. The capacity for utilization of polymers and sugars as car-

Table 2. Total number of bacterial cells revealed by DAPI staining and the numbers of the major bacterial groups identified by hybridization with the group-specific probes: ALF46 (*Alphaproteobacteria*, AlphaPB), BET42a (*Betaproteobacteria*, BetaPB), HGC69 (*Actinobacteria*), and HoAc1402 (*Acidobacteria*)

No.	Total number (DAPI), $N \times 10^8$	Abundance of the major bacterial groups, ($N \times 10^8$) Share of the total microbial number, %			
		AlphaPB	BetaPB	*Actinobacteria*	*Acidobacteria*
1	44.1 ± 7.3	6.6 ± 1.8 15	0.2 ± 0.1 <1	20.7 ± 9.8 47	3.8 ± 1.1 9
2	79.1 ± 15.3	4.3 ± 1.3 5	0.7 ± 0.3 1	10.9 ± 3.9 14	6.4 ± 2.1 8
3	10.1 ± 1.9	1.1 ± 0.7 9	0.4 ± 0.1 3	12.2 ± 4.2 78	1.3 ± 0.5 10
B7	7.8 ± 1.4	1.8 ± 0.7 22	0.3 ± 0.1 4	0.1 ± 0.1 1.5	1.9 ± 1.2 24
B8	19.8 ± 2.8	2.9 ± 1.0 15	2.0 ± 1.0 11	0.4 ± 0.2 2	1.2 ± 0.8 6
B9	28.45 ± 9.2	3.0 ± 0.9 11	2.3 ± 0.8 8	0.1 ± 0.1 <1	1.6 ± 0.8 6
B10	5.7 ± 1.2	3.4 ± 1.3 59	0.2 ± 0.1 5	0.03 ± 0.03 <1	1.2 ± 0.7 20
B11	11.6 ± 5.4	0.9 ± 0.3 8	0.3 ± 0.2 2	0.2 ± 0.2 2	3.7 ± 1.7 32
B12	17.4 ± 5.9	1.9 ± 1.3 11	0.4 ± 0.3 2	0.2 ± 0.2 1	1.8 ± 1.1 10
B13	12.8 ± 1.9	6.3 ± 2.1 49	0.2 ± 0.1 2	0.04 ± 0.04 <1	1.1 ± 0.4 9
B14	15.5 ± 5.6	4.6 ± 2.4 30	0.4 ± 0.2 2	0.2 ± 0.1 1	3.3 ± 1.3 21
B15	18.2 ± 6.6	2.7 ± 1.3	0.5 ± 0.2	0.2 ± 0.1	1.3 ± 0.5

bon sources was determined by measuring the respiration rates of the growing cultures [14].

RESULTS

Analysis of total abundance. Bacterial numbers in the samples varied depending on the degree of decomposition of the thallus and on the substrates on which the lichen grew. The highest number of bacteria (79×10^8 cells/g) was observed in the decomposing thallus of *Cladonia* sp. from the Lake Verkhnee *Sphagnum* bog and the lowest (5.7×10^8 cells/g), for the living part of the tundra lichen *Cladonia rangiferina* (Table 2). In the bog lichens, a tendency to increased bacterial numbers in the zone of decaying thallus and to their decrease in the *Sphagnum* peat was observed. This difference could reach 12%. In the tundra samples, the total bacterial numbers increased from the upper part of the thallus towards the peat. The highest difference ($\Delta N = 20.65 \times 10^8$ cells/g) was found in the grumous tundra. In this sample, pH varied most drastically, from 4.8 in the living part of the thallus to 7.2 in the substrate (Table 1). The number of bacteria in the undisturbed zone of the bog lichens was 3–8 times higher than in the same zone of the tundra lichens.

FISH detection of the predominant bacterial groups. Group-specific oligonucleotide probes made it possible to reveal the dominant groups of bacteria in the thalli of the lichens and in the underlying substrates. The *Alphaproteobacteria, Actinobacteria*, and *Acidobacteria* were the most numerous groups. The highest numbers of alphaproteobacteria were found in the living part of the lichens from all sites. Their share in the bacterial community varied from 15 to 59% in the bog and tundra lichens, respectively (Table 2). The number of these bacteria in the decaying thallus varied from 0.9 to 4.6×10^8 cells/g, i.e., from 5 to 30% of the total bacterial number. Similar values were found in the pit.

In the samples from the Lake Verkhnee ombrotrophic bog, actinobacteria were the dominant group in the peat (78%) and living thallus (47%). They were less numerous (14% of the total number) in the zone of decomposition. In the tundra lichens, actino-

Table 3. Phylogenetic position of the strains isolated from the lichen samples of the Lake Verkhnee *Sphagnum* bog and the tundra lichen by plating on solid media

Strain	GenBank accession no.	Source of isolation/medium	Microorganisms with the highest similarity of the 16S rRNA gene	
			Species	Similarity, %
CE1	JF490071	**Verkhnee bog** Decomposing part of the lichen *Cladonia*/M3 starch	*Acidobacterium capsulatum*	95
CS23	Not available		*Granulicella aggregans*	100
CS07	Not available		*G. aggregans*	98
C27	JF490072		*G. aggregans*	95
C02	Not available	Decomposing part of the lichen *Cladonia*/M3 lichen extract	*G. aggregans*	99
CE14	Not available		*A. capsulatum*	95
K5	JF490073		*A. capsulatum*	93
CU2	JF290434	Living part of the lichen *Cladonia*/M3 lichen extract	*Acidipila rosea*	94.7
C1	Not available		*G. aggregans*	99
B1415	Not available	**Vorkuta, Tal'nik** Decomposing part of the lichen *Cladonia*/M3 lichen extract	*A. capsulatum*	96

bacteria were a minor component of bacterial communities (1–2%).

The share of the *Betaproteobacteria* was low in all samples. The highest number of the cells revealed by the probe BET42a (2.0×10^8/g or 11% of the microbial population) was found in the decomposition zone of the tundra lichen. The lowest number of betaproteobacteria ($0.2–0.7 \times 10^8$/g) was observed in the *Sphagnum* bog samples (Table 2).

Abundance of the *Bacteroidetes* group varied from 0.2 to 1.0×10^8/g (less than 2% of the total number). Bacteria of the phyla *Gammaproteobacteria* and *Firmicutes* were minor of the association, which in some cases were not reliably identified at all.

The number of metabolically active cells of the *Acidobacteria* was at least 1.8×10^8 cells/g, i.e., 6% of the total cell number. The highest number of acidobacteria (3.8×10^8/g) was detected in the living zone of the *Sphagnum* bog lichen, while the highest share of this group (32%) occurred in the decomposition zone of the tundra sample (Table 2).

Localization and morphology of acidobacteria. The cells of acidobacteria were short rods, either single or organized in aggregates or microcolonies (Figs. 1a–1d). Aggregates adsorbed on the surface of intact or decaying hyphae of the mycobiont occurred most often, while single cells occurred more seldom. The cells were often immersed in a capsular matrix, which formed a "sac" containing the cells of different age (Fig. 1d).

Isolation and characterization of pure cultures. Acidobacteria were isolated from the living and decaying thalli of *Cladonia* by direct plating on media with lichen extract or starch. Numerous small (d = 0.5–2.0 mm) round, mucous colonies were obtained after two weeks of incubation at 15°C. Out of over 100 colonies, 50 were selected, which contained morphologically uniform cells. Hybridization with the probe HoAc1402 was carried out to confirm the purity of the cultures and their relation to the phylum *Acidobacteria*. The homogeneity of 10 most typical and common colonies was further confirmed by plating on solid media. Their phylogenetic position was determined by sequencing the 16S rRNA genes (Table 3). They all belonged to the first physiological group of *Acidobacteria*, with *Acidobacterium capsulatum* and *Granulicella aggregans* being the closest taxonomically describes species. Four of these ten strains were morphologically similar and were related to the recently described species *Granulicella aggregans* [10] (98–100% similarity). Three strains (K5, C27, and B1415) were morphologically and phylogenetically close to the recently isolated strain CCO287 ('*Acidobacterium polymorpha*') from the cellulolytic community in Obukhovskoe, Russia [15]. Strain CU2 (Fig. 1e) exhibited 95–96% similarity to the recently described *Acidipila rosea* [16]. Strains CE1 and CE14 had 99% similarity in 16S rRNA gene sequences but differed phenotypically. The biomass of strain CE1 (Fig. 1f) was colorless, with inhomogeneous growth in liquid medium. The colonies of strain CE14 were pink; the culture grew homogeneously in liquid medium. Both strains were most closely related to *A. capsulatum* (95% similarity).

The hydrolytic capacity of the isolates was studied for two strains, CU2 and CE1. The former was isolated from the upper (living) part of *Cladonia* sp. (*Sphagnum* bog) and the latter, from the decaying *Cladonia* sp. thallus.

Both cultures exhibited the highest hydrolytic activity in the case of starch and xylan. For strain CE1 grown on starch and xylan, the highest respiration

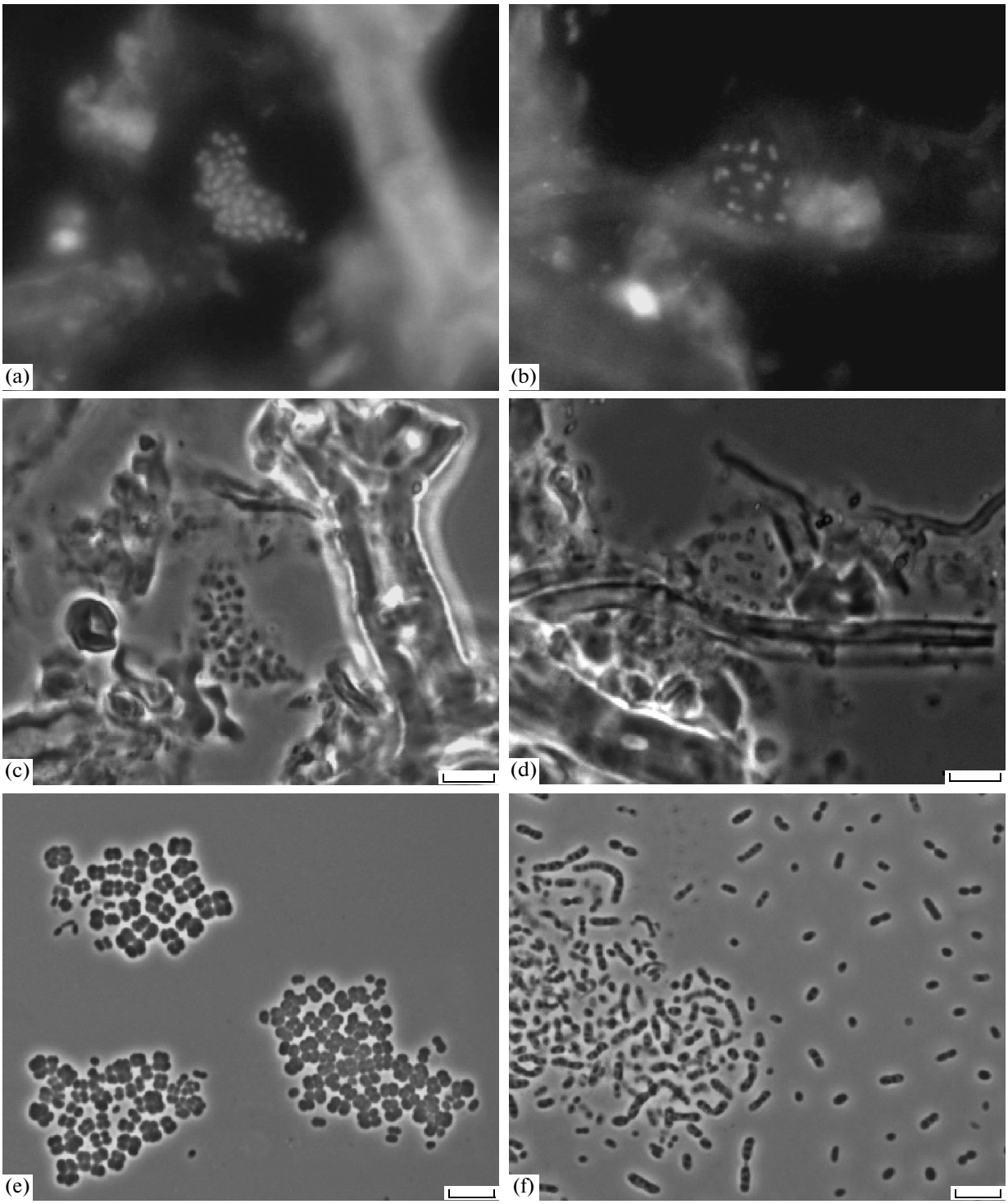

Fig. 1. Microcolonies of acidobacteria in the homogenate of the living (a), (c) and decaying (b), (d) parts of the lichen *Cladonia* sp. Detection with the fluorescently labeled probe HoAc1402 (a), (b); phase contrast (c), (d). Phase contrast photographs of strains CU2 (e) and CE1 (f) grown on xylan. Scale bar, 5 μm.

rates were 2.3 and 3.1 μg $C–CO_2$ mL^{-1} of liquid medium, respectively. The respiration rate of strain CU2 was lower (0.8 μg $C–CO_2$ mL^{-1}). Growth on lichenan, pectin, and laminarin was moderate, while growth on chitin, cellulose, and pullulan was practically absent. Unlike strain CE1, strain CU2 could grow on lichen extract at the same rate as on xylan (0.9 μg $C–CO_2$ mL^{-1}). Capacity for hydrolysis of oli-

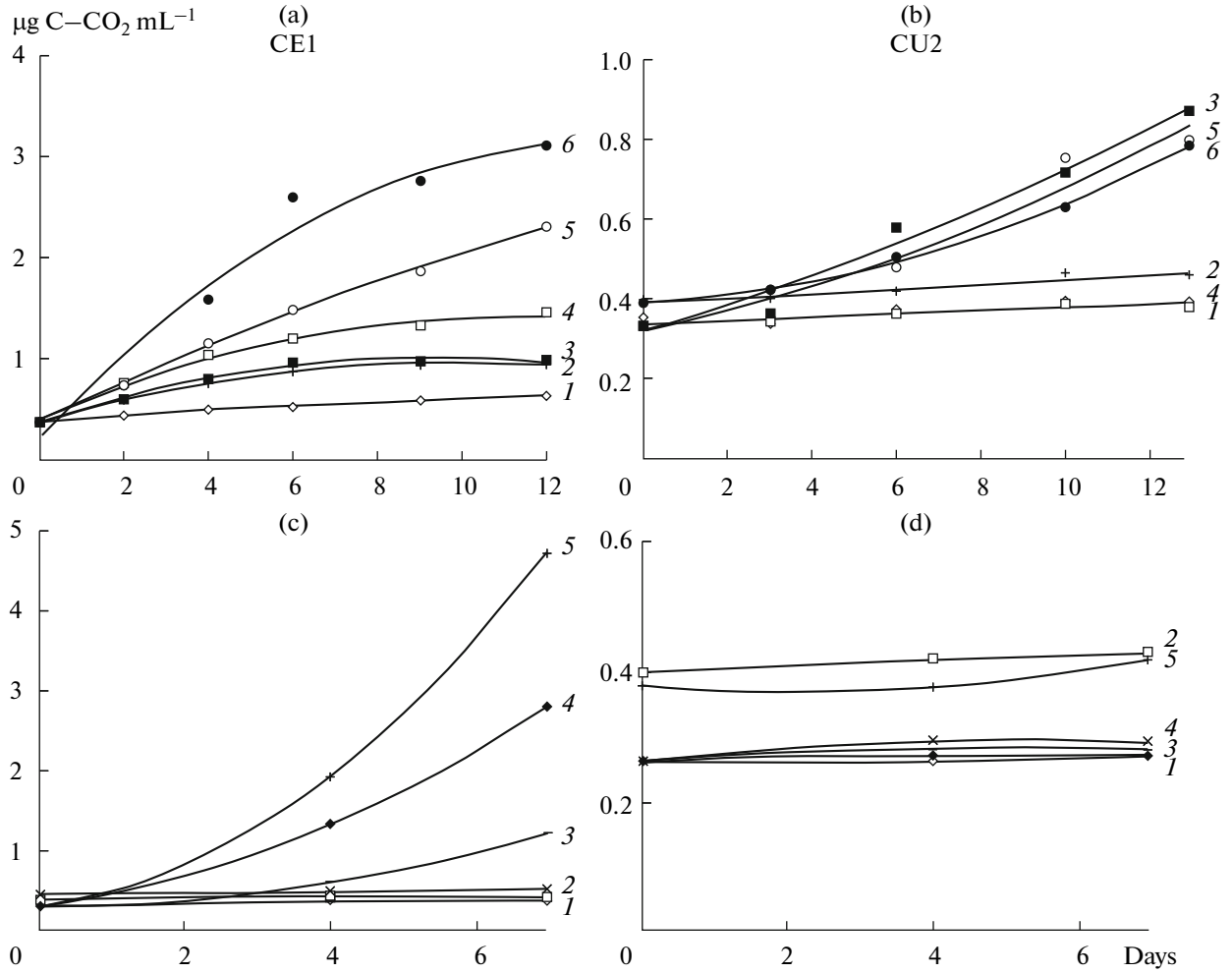

Fig. 2. Cumulative respiration curves for strains CE1 and CU2 grown on polymers (a), (b) and sugars (c), (d). Designations for plates a and b: control (*1*), pectin (*2*), lichen extract (*3*), laminarin (*4*), starch (*5*), and xylan (*6*). Designations for plates (c) and (d): control (*1*), raffinose (*2*), N-acetylglucosamine (*3*), glucose (*4*), and cellobiose (*5*). In all cases, the standard error did not exceed 5% of the average value.

gosaccharides (raffinose, cellobiose, trehalose, and maltose) and for utilization of monosaccharides (glucose and N-acetylglucosamine) was determined. Strain CE1 grew well on cellobiose, N-acetylglucosamine, and glucose (Fig. 2c), while strain CU2 grew only on raffinose with a very low respiration rate (Fig. 2d).

DISCUSSION

The total number of bacteria from bog and tundra lichens was 1 to 2 orders of magnitude higher than the number reported by Cardinale et al. for the *Cladonia arbuscula* samples collected in the Austrian mountains [5]. This result is not surprising, since the water regime of the habitats of these lichen associations is different. The lichens of bogs and grumous tundra are supplied not only by rainwater, but also by capillary water from the bog. This type of humidification results in more active growth of the lichen thallus and more intense decomposition of the dead myco- and phycobionts. An increase in bacterial numbers in the soil or peat of the tundra samples compared to those of the thallus was determined by variations in pH and mineralization of the soil. For example, the number of bacteria in the peat of the swamp samples decreased due to decreased concentrations of salts and available substrates.

The elevated number of bacteria inhabiting the thallus probably implies an increased number of nitrogen-fixing prokaryotes. High abundance of the *Alphaproteobacteria* in all the lichen samples studied confirms the numerous evidence of their predomination [4–7]. We found that they were especially numerous in the growing parts of the thalli, where photosynthesis of the phycobiont was most intense. This is the realm of dissipotrophs, which are known to belong mostly by members of the order *Rhizobiales: Hyphomi-*

crobiaceae, Bradyrhizobiaceae, and *Xanthobacteraceae*.

Abundance of actinobacteria in the sample was also not unexpected [17]. We have previously demonstrated that in *Sphagnum* bogs, actinobacteria are among the most numerous groups and are responsible for decomposition of cellulose and other polymers of the plant mass [18]. The absence or low numbers of the *Gamma-* and *Betaproteobacteria*, as well as the *Firmicutes* were reported earlier [5, 6]. The lichens were shown to contain a variety of biologically active polysaccharides, acids, quinones, and glycopeptides [19, 20]. A combination of these compounds acts as a factor in specific selection of bacteria and in the evolution of microbial communities in such "slow bioreactors" as lichens.

The presence of numerous metabolically active cells of acidobacteria in the lichen microbial communities makes it possible to suggest that low temperatures, low pH values, and the presence of available polysaccharides are the major selective factors for development of this specific group. Acidobacteria were a subdominant group both in the living and decaying thalli of the lichens.

The isolation of pure cultures of acidobacteria from the lichens of the boreal zone demonstrates that their "unculturability" is a consequence of their low growth rate and their substrate specificity in respect of oligo- and polysaccharides. Importantly, the culture isolated from the apical zone of the lichen had significantly less pronounced hydrolytic activity and low growth rates on both sugars and polymers, while the strain from the zone of decomposition had high hydrolytic activity and grew rapidly both on polymers and sugars. The culture isolated from the living zone of the lichen was, however, a more active hydrolyser of the lichen extract than the strain from the zone of decomposition. The generation time of the slowly growing acidobacteria (several days) was comparable with the growth rate of the lichen thallus. Such growth rates are characteristic of many bacterial inhabitants of the lichens, e.g., *Rhizobiales* and actinobacteria. Since lichens are slowly growing symbiotic organisms, the growth rate of the thallus should correlate with the decomposition rate of their dying parts.

This is the first demonstration of high numbers of the members of the phylum *Acidobacteria* in the lichens of the bog and tundra associations. Acidobacteria are a stable and numerous component of bacterial communities which develop both in the living part of the thallus and at the zone of its decomposition. Their high number possibly results from a combination of a number of factors: the growth rate of the lichens, water regime in their growth zone, low pH, variety of the oligo- and polysaccharides in the thallus, and the presence of antibiotic compounds. Detection of numerous metabolically active cells of acidobacteria in the growing and decaying parts of the thalli of the northern Russian lichens is of theoretical and applied interest. Further ecological and taxonomin investigation of *Acidobacteria* in the lichens of the boreal and tundra zones of Russia will be therefore carried out.

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