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EXPERIMENTAL ARTICLES

Spatial Distribution and Species Composition of Prosthecate Bacteria in Lake Baikal

N. A. Lapteva[a,1], N. L. Bel'kova[b], and V. V. Parfenova[b]

[a] *Institute of Biology of Inland Waters, Borok, Yaroslavl obl., 152742 Russia*

[b] *Limnological Institute, Siberian Division, Russian Academy of Sciences, ul. Ulan-Batorskaya 3, 664033, Russia*

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Abstract—From the water column of Lake Baikal, several strains of prosthecate bacteria belonging to the genera *Caulobacter* and *Brevundimonas* were isolated. In this article, the methods applied for their isolation and cell number determination are described; the occurrence frequency and spatial distribution of these microorganisms in the lake are demonstrated. Characterization of the species composition of cultivable and uncultivable prosthecate bacteria was carried out using the methods of traditional and molecular microbiology, respectively. A comparative phylogenetic analysis of the DNA sequences of uncultivable bacteria, which showed homology to the members of the alpha subclass of proteobacteria, was carried out. It was demonstrated that the lake water column is inhabited by uncultivable alpha-proteobacteria of uncertain phylogenetic affinity, in addition to representatives of the species *Caulobacter vibrioides* and *C. leidyi*, which were detected by traditional microbiological methods.

Key words: prosthecate bacteria, distribution, Lake Baikal, phylogeny.

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The widespread occurrence of prosthecate bacteria, their unique cell morphology, and the complexity of their life cycle have aroused considerable scientific interest in this group of bacteria. Prosthecate bacteria differ from other known groups of microorganisms by the presence of prosthecae, outgrowths of the cell wall containing the cytoplasm. Over many years, the ecology of prosthecate bacteria has been of interest to microbiologists [1–5]. During recent years, the taxonomy of prosthecate bacteria has received intense scientific attention. A taxonomic revision [6] of the genus *Caulobacter* based on the 16S rRNA gene analysis has led to a transfer of some of its species to the genus *Brevundimonas*. This transfer has been officially confirmed, and, according to *Bergey's Manual* (2005) [7], the genus *Caulobacter* now includes the following species: *C. vibrioides; C. fusiformes* Poindexter, 1964; *C. henricii* Poindexter, 1964, *C. segnis* Urakami et al., 1999; and the newly declared species *C. tundrae*, for which the description has yet to be published. Other species, such as *C. bacteroides, C. subvibrioides, C. intermedius*, and *C. variavilis*, were transferred to the genus *Brevundimonas.* The species *C. leidyi* remains to be classified; it can presumably be classified into the family *Sphingomonadaceae.*

Among prosthecate bacteria, members of the genus *Caulobacter* are most often found in soils and aquatic habitats of different trophic levels [1, 4]. These microorganisms have been discovered in polluted rivers, air tanks, and precipitation tanks of woodworking enterprises, where they prevailed over other prosthecate bacteria [3]. Caulobacters are abundant in the water column and neuston; they are often found attached to detritus particles, dead algal cells, other bacterial cells, and stems of higher plants [1, 2, 4]. They always accompany cellulose-fermenting bacteria [8–10]. In the majority of water bodies, members of the genus *Caulobacter* dominate and comprise a considerable fraction of the total bacterial number and of the number of facultative oligotrophs [1, 11]. *Bergey's Manual* [12] and a number of separate publications [1, 2, 4] include data on the trophic heterogeneity of stalked bacteria. No strict confinement of the majority of species to ecological and trophic groups has been revealed. A considerable number of *Caulobacter* species are facultative oligotrophs [1, 2].

Importantly, affiliation with species of the genus *Caulobacter* was based on the morphological properties (cell shape and size), as well as on physiological and biochemical properties. The difference in the physiological and biochemical properties of microorganisms is based on their requirements for growth factors (carbohydrates, vitamins, amino acids, and organic acids), which vary depending on environmental conditions and cultivation techniques and may entail the

[1] Corresponding author. E-mail: lap@ibiw.yaroslavl.ru.

morphological variability of the cells. The specific classification of this genus inhabiting Lake Baikal requires additional data, including molecular genetic data.

The goal of this work was to study the spatial distribution of prosthecate bacteria in Lake Baikal, as well as to determine their species composition using methods of traditional and molecular microbiology.

MATERIALS AND METHODS

Our collection of strains was obtained in August–September 1981, 1982, and 1985 in the course of the study of prosthecate bacteria in Lake Baikal. Water samples were taken throughout the whole area of the lake at the central stations of 11 standard transects. The deep water was sampled with a sterile bathometer and, at a depth of 5 m, with a Frantsev bathometer (1974). The total number of microorganisms (*N*) was determined on erythrosine-stained 0.2–0.3 μm membrane filters. In order to detect these microorganisms by transmission electron microscopy, copper grids were placed in the water samples [1, 11].

Sampling for molecular biological studies of uncultivable bacteria inhabiting Lake Baikal was carried out in the 1995–1998 summer seasons at the central stations of two transects: Listvyanka settlement–Tankhoi settlement (the southern lake basin) and Cape Ukhan–Cape Tonkii (the middle lake basin). Water samples were collected with a sterile bathometer from different depths, from the water surface to the bottom layer.

To isolate the studied bacteria in pure culture and to determine their cell numbers, the obtained samples were inoculated into sterile lake water. The water was then supplemented with yeast extract (5 mg/l) and acid hydrolyzed casein (5 mg/l) (DIFCO, United States). The water was inoculated by titration with replicate tenfold dilutions and incubated at 20–25°C for 14 days. Prosthecate bacteria were detected in media inoculated with the terminal dilutions; the cells were then isolated in pure culture by repeated plating onto a solid nutrient medium. The cell morphology (cell shape and size, prostheca location and size), as well as the cultural and physiological properties of pure cultures, were studied according to V.B. Skerman [13].

The species were identified and classified using *Bergey's Manual* [1, 12] and according to J.S. Poindexter [8]. A total of 23 strains were isolated and determined.

Isolation of the total bacterial DNA from the obtained deep-water samples and its molecular analysis was carried out by the techniques described in [5, 14]. Comparative analysis of 302 fragments of the 16S rRNA gene sequences obtained by cloning of the PCR amplification products from total DNA using the highly conserved bacterial primers 500L (514–533) and 1350R (1389–1407) was carried out (the nucleotide numbering of *Escherichia coli* is given in brackets). The length of the obtained sequences varied from 207 to 878 base pairs (bp). All the obtained sequences were grouped according to the results of single-letter sequencing [15]. The members of 33 groups (63 sequences) have shown maximum homology to the alpha-subclass of proteobacteria. Nucleotide sequences of alpha-proteobacteria (more than 200 bp) are available from the EMBL bank under the accession numbers X99983–X99984, X99988–X99989, AJ001425–AJ001427, AJ289940–AJ289941, AM422761–AM422768, and AM489778–AM489784. The nucleotide sequences of the 16S rRNA gene fragments (422 bp) were aligned using the CLUSTAL W 1.7 software package; the phylogenetic tree was constructed by the neighbor-joining method implemented in the TreeConW software package.

RESULTS AND DISCUSSION

Morphological, physiological, and biochemical analysis

Both the electron microscopic examination of submerged grids and inoculation of water samples into a low-nutrient liquid medium revealed prosthecate bacteria at eight out of eleven stations. The isolated cultures were classified with the genera *Caulobacter* and *Brevundimonas* (Table 1). Most of them were classified as *C. vibrioides* and *B. bacteroides* with occurrence frequency of 50% and 25%, respectively, of the total number of isolated cultures. The average ratio of *C. vibrioides* in the total bacterial population of the water samples collected at all the stations was 0.03%; the average ratio of *B. bacteroides* cells was 0.06%.

It should be noted that 14 of the 23 strains were isolated from water samples collected in the middle lake basin. In the hypolimnion, their cell numbers (*NP*) were low and varied from 5.0 to 1.0×10^3 cells/ml. The highest numbers of the above-mentioned bacteria were observed at the central station of the Ukhan–Turka transect in the upper water layer and in the 50-m layer of the thermocline. In different years, various species of prosthecate bacteria were found to be dominant in the trophogenic layer at this station. The *NP*/*N* ratio was 0.1–0.17%. At the other stations of the middle lake basin, where the *NP*/*N* ratio was 0.003–0.06%, their numbers varied from 5 to 100 cells/ml. In the southern lake basin, at the central station of the Polovinka–Murino transect, prosthecate bacteria ($0.01–0.6 \times 10^3$ cells/ml) were a part of the dominant community of facultative oligotrophs (FO). In the northern lake basin, prosthecate bacteria were dominant at three out of four stations. These bacteria were abundant at the central stations of the Baikal'sk–Cape Turali and Tyya–Nemnyanka transects (the northern extremity of the lake). The vibrioid cells of *C. vibrioides* and *B. subvibrioides* were predominant. These microorganisms (50–500 cells/ml) were isolated from the upper layer of the hypolimnion. The *NP*/*N* ratio was 0.1–0.5. The highest number of their cells was observed in the 50-m

Table 1. Spatial distribution of prosthecate bacteria in Lake Baikal

Central station of the transect	Depth, m	Species	*NC** cells/ml	*N*** $\times 10^6$ cells/ml	*NC*/*N*, %
Polovina–Murino	25	*C. vibrioides* *(C. crescentus)*	100	1.0	0.01
		C. leidyi	500	0.08	0.62
Anga–Sukhaya	100	*B. bacteroides* *(C. bacteroides)*	50	0.4	0.013
	500	*C. vibrioides* *(C. crescentus)*	50	0.08	0.06
	1000	*C. vibrioides* *(C. crescentus)*	5.0	0.04	0.013
Ukhan–Turka	0	*B. bacteroides* *(C. bacterides)*	1000	1.0	0.1
		C. vibrioides	1000	1.0	0.1
		B. subvibrioides *(C. subwibrioides)*	1000	1.0	0.1
	15	*C. fusiformis*	1000	0.8	0.13
	30	*C. vibrioides* *(C. crescentus)*	50	0.2	0.025
	30	*C.* sp.	50	0.2	0.025
	50	*B. bacteroides* *(C. bacteroides)*	100	0.06	0.17
	1000	*C. henricii*	5.0	0.2	0.0025
Solnechnyi–Ushkan'i islands	15	*C. vibrioides*	50	1.4	0.04
Baikal'sk–Turali	25	*B. subvibrioides* *(C. subvibrioides)*	500	0.5	0.1
Tyya–Nemnyanka	50	*C. vibrioides*	50	0.01	0.5
	100	*C. vibrioides* *(C. crescentus)*	100	0.2	0.05

Notes: * NC, amount of *Caulobacter* cells,
** N, total amount of microbial cells. Species names according to the old nomenclature are given in parentheses.

water layer at a *NP*/*N* ratio of 0.13% and in the hypolimnion at a *NP*/*N* ratio of 0.03%. Electron microscopic observations also revealed their constant presence at the surface and in the 50-m layer of the middle lake basin (the Ukhan–Turka transect). Filamentous forms of prosthecate bacteria with short prosthecae were detected at the central station of the Polovinka–Murino transect, as well as in the near-bottom layers of the Selenga shoal.

According to the cell morphology, all strains may be divided into four groups: vibrioid, spindle-shaped, bacteroid, and slightly vibrioid (Table 2). The cell size of the first two groups of the genus *Caulobacter* was 0.8–1.9 × 0.2–0.3 μm; the cell size of the other groups of the genus *Brevundimonas* was 0.8–3.0 × 0.3–0.4 μm. The prostheca sizes depended on the concentration of digestible organic matter (DOM). The typical cell morphology of the isolated cultures of prosthecate bacteria was observed at DOM concentrations of 100–500 mg/l. Ovoid or lemon-shaped cells were most often present in young cultures of dividing bacteria. Comparative analysis of their physiological and biochemical properties has shown that the isolated strains belonging to the genera *Caulobacter* and *Brevundimonas* can actively utilize carbohydrates (mono- and disaccharides) and simple alcohols (Table 3). Growth on organic acids of the Krebs cycle is weaker; some cultures are not able to utilize these acids. All the isolated cultures grew well on

Table 2. Morpological properties of prosthecate bacteria of the genera *Caulobacter* and *Brevundimonas*

Species	Cell shape	Number of strains	Size, μm	
			cell	prosthecae
C. vibrioides	vibrioid	5	0.8–1.9	2.0–5.0
	slightly vibrioid	3	0.8–1.8	1.0–3.0
	oval or lemon-shaped	2	0.8–1.3	1.0–5.0
C. henricii	vibrioid	1	0.8–1.2	2.0–4.0
C. fusiformis	spindle-shaped (tapered)	1	1.0–1.4	2.0
C. leidyi	slightly vibrioid	1	1.0–1.2	0.2–0.5
B. bacteroides	rodlike with rounded ends	3	0.7–3.0	2.0–2.5
	rodlike (cylindrical)	1	0.5–3.0	0.9–2.5
	rodlike (during division, close to lemon-shaped)	1	0.7–3.0	0.5–0.9
B. subvibrioides	slightly vibrioid	2	1.0–1.8	2.0–2.5

media with casein hydrolysate and amino acids (alanine, proline, and glutamine). Some strains of both genera exhibited amylolytic, proteolytic, and catalase activities. The colony pigmentation (dull or bright yellow and orange) was observed in some strains grown on rich media. It was established that they grew better within a temperature range of 26–30°C and in the pH interval from 6.5 to 7.5.

The quantitative analysis of the spatial distribution of the studied bacteria indicated that, within the given period, they were dominant in the cultured heterotrophic microbial community. Judging from the occurrence of the representatives of this genus in the water samples, only members of the following species were predominant: *C. crescentus, C. vibrioides, C. fusiformis*, and *C. henricii.*

Molecular and genetic analysis

The results of microbiological analyses of prosthecate bacteria inhabiting the water column revealed their great diversity in the southern and middle lake basins. Based on these data, we decided to perform a molecular genetic investigation of the water samples collected at these sites.

Members of the alpha subgroup of proteobacteria were detected in all the deep-water samples, except for the samples collected at a depth of 25 m (Table 4). Nucleotide sequences homologous to those of various representatives of prosthecate bacteria were found in the samples collected in different years at depths of 400 and 1200 m from the southern lake basin (maximal depth, 1450 m), as well as from the middle lake basin (maximal depth, 1637 m) at depths of 1400 and 1600 m. Hence, our molecular genetic studies showed that prosthecate bacteria occur both in the surface and deep-water communities of Lake Baikal.

To construct a phylogenetic tree, we used nucleotide sequences of Lake Baikal alpha-proteobacteria, phylogenetically close to various prosthecate bacteria (X99988, AJ289940–AJ289941, and AM422761–AM422768), as well as the 16S rRNA gene sequences of the main species of the family *Caulobacteraceae* and *Sphingomonadaceae.* The results of the phylogenetic analysis (Fig. 1) demonstrate that only two sequences (1200-4 and 1200-25) fell into the cluster with the known species *C. vibrioides* and *C. leidyi* (the latter species is closely related to the family *Sphingomonadaceae*). Other studied sequences of Baikal strains are clustered together with strain sequences of uncertain species affiliation (1204-42, 1405-56, 1405-59, and 1405-60) or form separate clusters (1602-14 and 1602-1007; 1204-37 and 1405-69; 400-6). For instance, we failed to generate a more representative cluster for two sequence groups, 1602-14–1602-1007 (which is close to the members of the genus *Caulobacter*) and 1204-37–1405-69 (close to the members of the genus *Sphingomonas*). Moreover, the sequence 400-6 forms an independent branch, which we also failed to attribute to any phylogenetic group. The sequence 400-6 may belong either to a bacterium of the genus *Caulobacter* or to a member of the genus *Asticcacaulis*.

Hence, the results of molecular biological investigations confirmed that prosthecate bacteria of such well-known species as *C. vibrioides* and *C. leidyi* are present in the water column of Lake Baikal. In addition, some sequences of uncultivable prosthecate bacteria may belong to new species of the genus *Caulobacter.* On the other hand, using the methods of traditional microbiol-

Table 3. Physiological properties of prosthecate bacteria isolated from Lake Baikal water samples in 1981–1985

Property	Amount of cultures exhibiting the property (arranged in groups)					
	1		2		3	4
	C. vibrioides (*C. crescentus*)	*C. henricii*	*C. fusiformis*	*C. leidyi*	*B. bacteroides* (*C. bacteroides*)	*B. subvibrioides* (*C. subvibrioides*)
Number of strains	10	1	1	1	5	2
Carbon sources:						
Carbohydrates	8	1	0	1	3	2
Amino acids	9	1	1	1	5	2
Simple alcohols	8	1	0	1	3	2
Organic acids	8	1	0	1	2	2
Growth on potato tubers	2	0	0	0	2	2
Growth on potato agar	5	1	1	0	2	1
Mineral forms of nitrogen	7	1	1	0	2	2
Urea	5	1	0	0	2	2
Caseinase (growth on milk)	0	0	0	0	0	1
Protease (gelatinase)	0	0	0	1	1	1
Amylase	1	0	0	0	0	0
Catalase	1	0	0	0	1	0
Peroxide production	2	0	0	0	0	0
Reduction of NO_3 to NO_2	3	0	0	1	2	1

Table 4. Distribution of prosthecate bacteria at different depths of the central stations of the southern and middle lake basins of Lake Baikal according to the results of molecular genetic analysis of the clone library of the uncultivable microorganisms isolated from Lake Baikal

Phylogenetic group	Southern Baikal				Middle Baikal			Total
	25 m	400 m	1200 m	1400 m	25 m	1400 m	1600 m	
Eubacteria	22	68	86	26	17	29	54	302
Proteobacteria	3	39	62	6	1	17	37	165
α-proteobacteria	0	16	25	6	0	5	11	63
Prosthecate bacteria	0	1	11	0	0	5	2	19

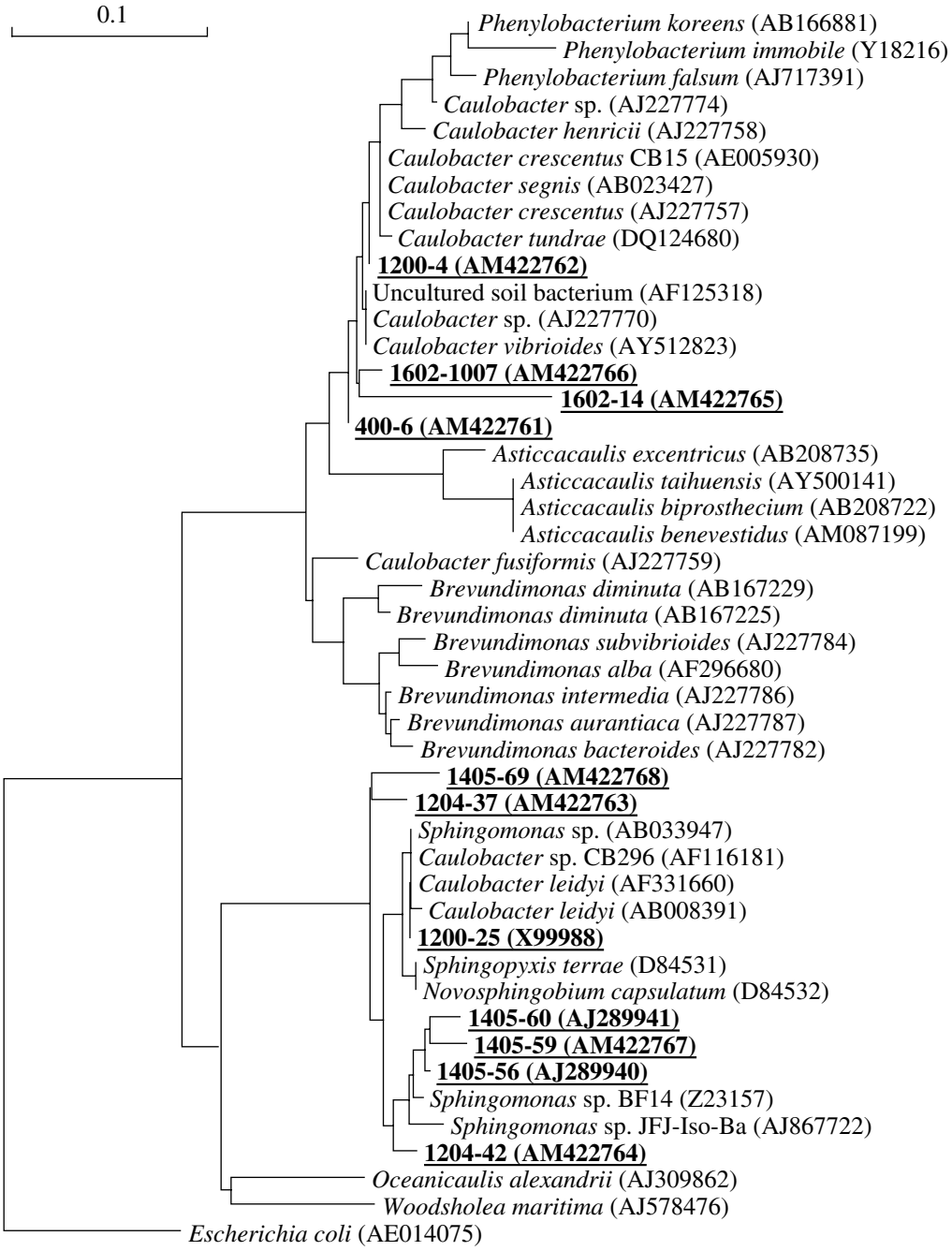

Fig.1. Phylogenetic tree constructed on the basis of the 16S rRNA gene fragments of uncultivable proteobacteria detected in the water of Lake Baikal and of their close relatives from the EMBL database. Scale bar, 10 nucleotide substitution for each 100 nucleotide base pairs.

ogy, we showed that the culturable species *C. crescentus, C. vibrioides, C. fusiformis*, and *C. henricii* are predominant in the community of *Caulobacter* species inhabiting Lake Baikal.

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