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EXPERIMENTAL ARTICLES

Evaluation of the Taxonomic Diversity of Prosthecate Bacteria Belonging to the Genera *Brevundimonas* and *Caulobacter* Isolated from Various Eurasian Ecosystems by Analysis of the 16S rRNA Genes

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Abstract—A taxonomic study of 35 cultures of prosthecate bacteria of the genera *Brevundimonas* and *Caulobacter* isolated from various soil and aquatic ecosystems of Eurasia was performed by amplified ribosomal DNA restriction analysis (ARDRA) and 16S rRNA gene sequencing. The most widespread groups of prosthecate bacteria belonging to these genera were revealed; at least two new species belonging to the genus *Brevundimonas* were found. The genus *Brevundimonas* includes both prosthecate and non-prosthecate species; however, it is quite possible that some *Brevundimonas* species may exhibit heterogeneity in such an important taxonomic characteristics as the ability to form prosthecae.

Keywords: *Brevundimonas, Caulobacter,* 16S rRNA, ARDRA.

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Before 1999, bacteria reproducing by nonuniform division and exhibiting a dimorphic life cycle at one stage of which bacterial cells produce polar prosthecae with anchoring disks at their distal ends were assigned to the genus *Caulobacter*. The phylogenetic studies based on 16S rRNA gene sequencing resulted in a taxonomic revision of the genus *Caulobacter* [1]. As a result, the majority of *Caulobacter* species were transferred to the genus *Brevundimonas*. Both prosthecate and non-prosthecate species were described as representatives of the genus *Brevundimonas* [2]; however, no evidence exists concerning the intraspecific heterogeneity in this respect. Three *Caulobacter* strains isolated from marine habitats were redescribed as the representatives of the novel genera, *Maricaulis* [2] and *Woodsholea* [3], of the family *Hyphomonadaceae* [4], whereas *Caulobacter leidyi* should be considered a member of the family *Sphingomonadaceae* [2], according to its phylogenetic properties. Hence, prosthecate bacteria of different genera, families, and orders, which possess a dimorphic life cycle, form a phylogenetically heterogeneous group of prokaryotes; the majority of species belong to two phylogenetically related genera, *Brevundimonas* and *Caulobacter*. Determination of the phylogenetic position of bacterial cultures is an essential element of identification of new isolates of prosthecate bacteria [5].

The available data on the ecophysiological properties of *Brevundimonas* and *Caulobacter* are insufficient for singling out the specific properties of the prosthecate species belonging to these genera, as well as the specifics of their dimorphic life cycle. Prosthecate bacteria have been discovered in diverse habitats, both natural and anthropogenic, as well as in freshwater, brackish water, marine, and soil ecosystems [6–11]. A psychrotolerant strain belonging to the genus *Caulobacter* was isolated from a soil sample collected in the Transpolar tundra [12]. The frequency of their occurrence in clone libraries of 16S rRNA genes may suggest that *Brevundimonas* and *Caulobacter* are widespread in different aquatic and soil ecosystems (the GenBank database contains over 300 *Brevundimonas* clones and about 200 *Caulobacter* clones). Thus far, 6 species of *Caulobacter* and 20 species of *Brevundimonas* have been described (http://www.bacterio.cict.fr/).

The goal of the present work was to continue the ecological and taxonomic studies of prosthecate bacteria exhibiting a dimorphic life cycle, which were carried out in the 1960s to 1990s by Belyaev and Krasil'nikov [6, 7] and Lapteva [8, 9]. Our study made it possible to assess the taxonomic diversity of prosthe-

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Table 1. List of bacterial strains used in this work

Strain number	Original strain designation*	Isolation source
VKM B-1179	BC-101	Chestnut soil, Saratov oblast, Russia
VKM B-1560	BC-107	Chestnut soil, Saratov oblast, Russia
VKM B-1561	BC-112	Chestnut soil, Saratov oblast, Russia
VKM B-1184	BC-131	Chestnut soil, Saratov oblast, Russia
VKM B-1488	BC-140	Chestnut soil, Saratov oblast, Russia
VKM B-1177	BC-19	Brown forest soil, mountain Crimea, Ukraine
VKM B-1180	BC-10	Brown forest soil, mountain Crimea, Ukraine
VKM B-1486	BC-15	Brown forest soil, mountain Crimea, Ukraine
VKM B-1489	BC-20	Brown forest soil, mountain Crimea, Ukraine
VKM B-1191	BC-31	Peat–gley soil, Chashnikovo, Moscow oblast
VKM B-1563	BC-35	Peat–gley soil, Chashnikovo, Moscow oblast
VKM B-1178	BC-26	*Sphagnum* bog, White Sea Biological Station, Murmansk oblast, Russi
VKM B-1182	BC-22	Lake water, White Sea Biological Station, Moscow State University, Murmansk oblast, Russia
VKM B-1183	BC-25	Lake water, White Sea Biological Station, Moscow State University, Murmansk oblast, Russia
VKM B-1186	BC-48	Lake water, White Sea Biological Station, Moscow State University, Murmansk oblast, Russia
VKM B-1188	BC-23	Lake water, White Sea Biological Station, Moscow State University, Murmansk oblast, Russia
VKM B-1986	33	Water, Lake Baikal, north end of lake, Russia
VKM B-1994	9b	Water, Lake Sevan, Armenia
VKM B-1982	9	Water, Lake Sevan, Armenia
VKM B-2583	21a	Water, Lake Sevan, Armenia
VKM B-1981	5	Water, Lake Sevan, Armenia
VKM B-1991	58	Water, Lake Sevan, Armenia
VKM B-2001	57	Water, Lake Sevan, Armenia
VKM B-1992	121	Water, Lake Issyk Kul, Kyrgyzstan
VKM B-1993	213	Water, Lake Issyk Kul, Kyrgyzstan
VKM B-1176	BC-4	Lake water, Borok, Yaroslavl oblast, Russia
VKM B-1988	40	Water, Lake Chashnitskoe, Yaroslavl oblast, Russia
VKM B-2000	51	Water, Lake Kishemskoe, Vologda oblast, Russia
VKM B-2015	23	Water, Lake Beloe, Vologda oblast, Russia
VKM B-2017	227	Water, Lake Teletskoe, mountain Altai, Russia
VKM B-2584	25	Water, Lake Lindlamba, South Karelia, Russia
VKM B-1999	50	Water, Lake Blagoveshchenskoe, Novgorod oblast, Russia
VKM B-1487	BC-3	Water, Volga River, Samara, Russia
VKM B-1181	BC-2	Water, Volgograd Reservoir, Russia
VKM B-1990	56	Water, Rybinsk Reservoir, Yaroslavl oblast, Russia

Note: In the column "Original strain designation," original strain names are given, which have been added to the VKM database. The strains with the BC acronym were obtained from S.S. Belyaev; the strains without the BC acronym were obtained from N.A. Lapteva.

cate bacteria isolated from aquatic and soil ecosystems of several geographical areas of Eurasia, to discover at least two new species of the genus *Brevundimonas*, and to reveal several pairs of closely related prosthecate and non-prosthecate strains in the genus *Brevundimonas*.

MATERIALS AND METHODS

The subjects of study were 35 strains of prosthecate bacteria isolated by Belyaev and Lapteva from different soil and aquatic ecosystems of European Russia, Siberia, Central and Western Asia, and the Crimea (Table 1). All the studied strains are deposited with the

All-Russian Collection of Microorganisms (VKM) and are listed in the VKM Catalogue (www.vkm.ru), which includes the accepted nomenclature of these organisms. The results of the reidentification of prosthecate bacteria now being carried out at the VKM indicate the necessity of renaming some strains and describing new species within the genus *Brevundimonas*. Table 1 therefore includes only the collection numbers of the studied strains without their specific epithets.

DNA extraction. DNA extraction from the *Brevundimonas* and *Caulobacter* cultures grown on the VKM Medium 55 (http:// www.vkm.ru/) at 28°C was carried out as described in [13].

PCR amplification of the 16S rRNA gene was carried out with the universal eubacterial primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3') [14, 15]. The polymerase chain reaction (PCR) was carried out on a GeneAmp PCR System 2700 thermocycler (Applied Biosystems, United States).

The amplified ribosomal DNA restriction analysis (ARDRA) of 16S rRNA genes was performed using the restriction endonucleases *Hpa*II, *Mbo*I, and *Rsa*I (Fermentas, Lithuania). The reaction products were separated by electrophoresis in a 1.3% agarose gel. The restriction profiles were simulated in silico using the VectorNTI 10 software package.

Determination of the 16S rRNA gene sequences was carried out on a CEQ 2000 XL automatic sequencer (Beckman Coulter, United States) according to the manufacturer's instructions.

Phylogenetic analysis. Preliminary analysis of the obtained nucleotide sequences of the 16S rRNA genes was performed using the NCBI BLAST software, National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov). The obtained 16S rRNA gene sequences were aligned with the relevant sequences of the most closely related bacteria using the CLUSTALX software package [16]. The phylogenetic trees were constructed by the methods implemented in the TREECONW v. 1.3b software package. [17].

RESULTS AND DISCUSSION

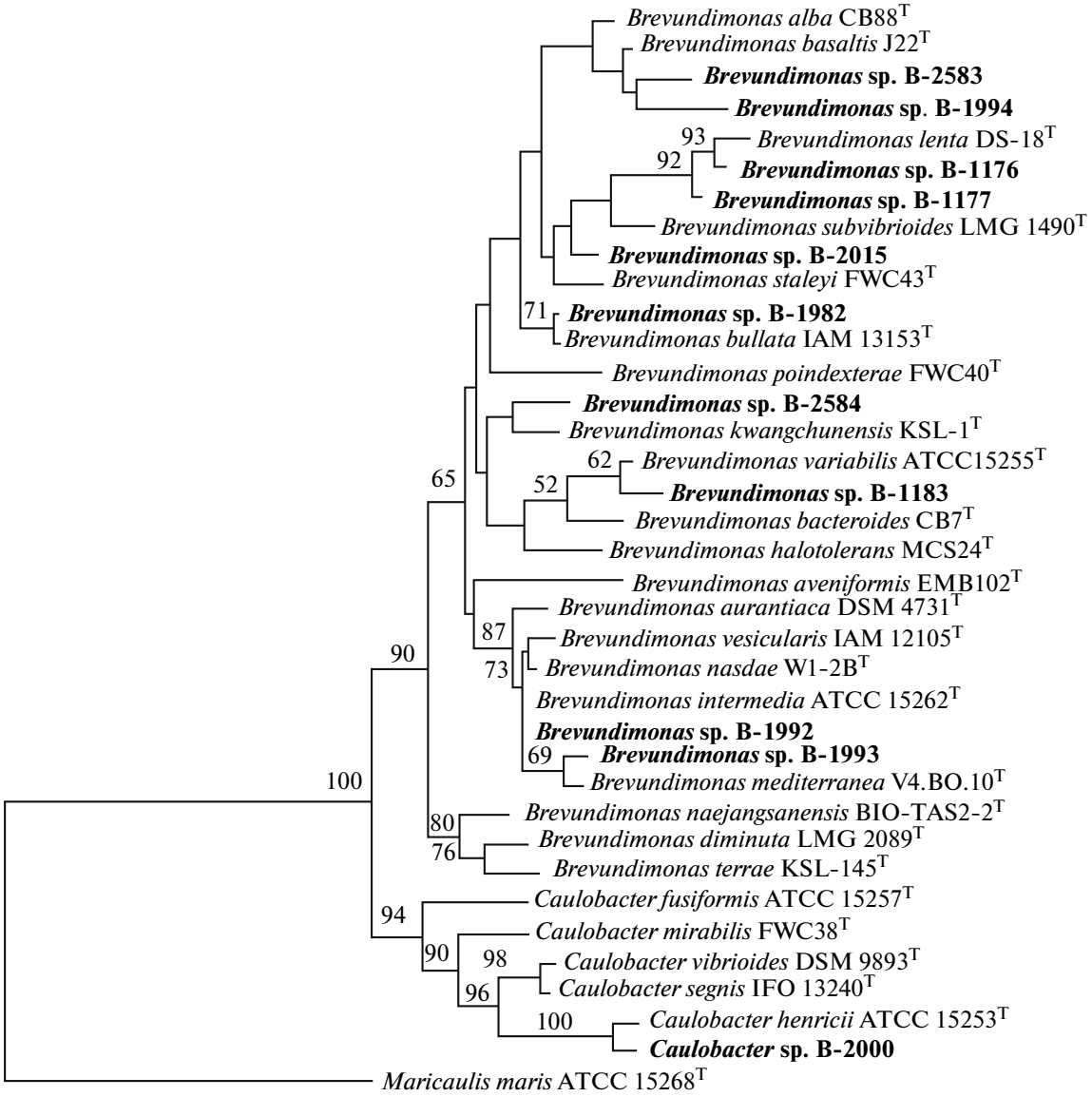
The studied prosthecate strains were classified in the *Brevundimonas–Caulobacter* complex on the basis of their morphology and the typical dimorphic life cycle.

In order to assess the level of 16S rRNA gene polymorphism among 35 prosthecate strains, the method of amplified ribosomal DNA restriction analysis (ARDRA) was applied. Initially, to elucidate the most pronounced differences in restriction profiles, in silico ARDRA analysis of the 16S rRNA gene sequences of all the type strains of the genera *Brevundimonas* and *Caulobacter* (20 and 6 species, respectively) was performed. The levels of 16S rRNA similarity between the species were high and ranged from 96.3 to 99.7% (*Caulobacter*) and from 95.1 to 99.7% (*Brevundimonas*), which significantly complicates the application of 16S rRNA gene analysis for specific identification of the members of these genera.

The *Hpa*II, *Mbo*I, and *Rsa*I endonucleases were found to be acceptable for the intergeneric and interspecific differentiation of the microorganisms belonging to these genera (Table 2). The *Hpa*II endonuclease appeared to be efficient for strain differentiation at the genus level, allowing for distinct differentiation of the type strains of 20 *Brevundimonas* species from 6 type strains of *Caulobacter* species (Table 2). After successive applications of the *Mbo*I and *Rsa*I endonucleases, the following pairs of species with identical restriction profiles and high levels of 16S rRNA similarity remained (%): *B. poindexterae* and *B. aurantiaca* (97.0%), *B. alba* and *B. basaltis* (98.9%), *B. intermedia* and *B. nasdae* (98.6%), and *B. naejangsanensis* and *B. diminuta* (98.7%). However, even in these cases, additional restriction endonucleases (for example, *Alu*I or *Hpy*1881) may be used for differentiation of some pairs of species. Thus, the ARDRA groups obtained from the genera *Brevundimonas* and *Caulobacter* using the *Hpa*II, *Mbo*I, and *Rsa*I endonucleases correspond to the groups of closely related species or even to the interspecific level of relatedness (Table 1). At the same time, the comprehensive ARDRA analysis is counterproductive in most cases, since only preliminary species identification can be achieved even with several restriction endonucleases.

According to the levels of similarity between the restriction profiles of the amplified 16S rRNA genes obtained using the *Hpa*II, *Mbo*I, and *Rsa*I restriction endonucleases, the 35 strains under study were divided into 11 ARDRA groups. The obtained restriction profiles of 20 strains corresponded to those obtained in silico for the type strains of the species *C. henricii, B. basaltis, B. bullata, B. lenta, B. mediterranea,* and *B. staleyi*, and the pair *B. intermedia—B. nasdae* (Table 2). The restriction profiles of the remaining 15 strains differed from those of the type strains of the genera *Brevundimonas* and *Caulobacter* and formed four ARDRA groups, ARDRA group 1 (strains B-2583, B-1179, B-1181, B-1981, B-1986, B-1991, and B-2001), ARDRA group 2 (B-1177, B-1178, B-1180, B-1186, B-1188, and B-1486), ARDRA group 3 (B-2584), and ARDRA group 4 (B-1183) (Table 3).

The 16S rRNA genes of the representatives of all 11 ARDRA groups were sequenced. Figure 1 shows the phylogenetic position of these strains among the type strains of the genera *Brevundimonas* and *Caulobacter*, whereas the levels of similarity between the 16S rRNA gene sequences of these strains and their closest relatives are listed in Table 3. The results of 16S rRNA gene sequencing confirmed the efficiency of ARDRA analysis for the differentiation of prosthecate bacteria at the genus level, as well as for grouping of these



Phylogenetic position of *Brevundimonas* and *Caulobacter* isolates according to the 16S rRNA sequence analysis. The bar shows evolutionary distance, corresponding to two substitutions per 100 nucleotides. The numerals show the significance of the branching order as determined by bootstrap analysis (only bootstrap values above 50% are shown). The 16S rRNA gene sequence of *Maricaulis maris* ATCC 15268^T was used as the outgroup.

strains within the genera *Brevundimonas* and *Caulobacter*.

The only experimentally deduced ARDRA group within the genus *Caulobacter* belongs to *C. henricii* with very high probability (Table 3). Strain VKM B-2000 shows a 99.5% 16S rRNA similarity with the type strain *C. henricii* and a 96.5–98.1% similarity with the type strains of other *Caulobacter* species (*Caulobacter leidyi* is not discussed in this article since, since on the basis of its phylogenetic position, this species is to be considered a member of the family *Sphingomonadaceae*).

The representatives of other ten ARDRA groups form several clusters consisting of *Brevundimonas* species with a high similarity level between their 16S rRNA gene sequences (Fig. 1). The first cluster, consisting of both the prosthecate bacteria *B. alba, B. subvibrioides*, and *B. staleyi* and the non-prosthecate species *B. basaltis, B. bullata*, and *B. lenta*, also contains the majority of the studied prosthecate isolates, including the experimentally deduced ARDRA groups "*B. basaltis–B. alba*", "*B. staleyi*", "*B. bulata*", and "*B. lenta"*; ARDRA group 1; and ARDRA group 2. Table 3 shows that, within these groups, the levels of similarity between the 16S rRNA gene sequences of the studied prosthecate isolates and those of the type strains of the closest non-prosthecate species are high: 99.5% similarity between B-1176 and *B. lenta* DS-18^T,

Table 2. The result of computer simulation of the restriction analysis (*in silico*) of 16S rRNA genes of the type strains of *Caulobacter* and *Brevundimonas*

Strain	Restriction enzyme; length of the restriction fragment (nucleotides)		
	*Hpa*II	*Mbo*I	*Rsa*I
Caulobacter segnis ATCC 21756^T (D13947)	496, 253, 210, 160, 150, 130	1025, 180, 158	422, 266, 236, 233, 171, 120
Caulobacter henricii ATCC 15253^T (AJ227758)			502, 422, 233, 171, 120
Caulobacter mirabilis FWC38^T (AJ227774)	656, 403/401, 210, 132	1023, 241, 160	500, 380, 233, 171, 122
Caulobacter fusiformis ATCC 15257^T (AJ227759)			500, 404, 422, 122
Caulobacter vibrioides SB2^T (AJ227756)	656, 253, 210, 150, 130	–	–
Caulobacter leidyi ATCC 15260^T (AJ227812)	342, 331, 254, 166, 160, 150	–	–
Brevundimonas poindexterae FWC40^T (AJ227797)	403, 387/374, 189, 160, 130/132, 109	1026, 241, 158/160	479, 422, 404, 120
Brevundimonas aurantiaca CB-R^T (AJ227787)			
Brevundimonas kwangchunensis KSL-102^T (AY971368)			826, 479, 120
Brevundimonas alba CB88^T (AJ227785)		708, 318, 241.158/160	826, 479, 120
Brevundimonas basaltis J22^T (EU143355)			
Brevundimonas mediterranea V4.BO.10^T (AJ227801)	547, 439, 189, 130, 109	–	–
Brevundimonas intermedia ATCC 15262^T (AJ227786)	547, 403, 189, 130, 109	684, 318, 234, 158	479, 422, 404, 120
Brevundimonas nasdae W1-2B^T (AB071954)			
Brevundimonas vesicularis IAM 12105^T (AB021414)			826, 479, 120
Brevundimonas terrae KSL-145^T (DQ335215)		684, 282, 241, 158	–
Brevundimonas naejangsanensis BIO-TAS2-2^T (FJ544245)	656, 403, 160, 130/132	684, 282, 241, 158/160	479, 422, 404, 120
Brevundimonas diminuta LMG 2089^T (AJ227778)			
Brevundimonas staleyi FWC43^T (AJ227798)		436, 318, 272, 241, 158	479, 422, 404, 120
Brevundimonas variabilis ATCC 15255^T (AJ227783)			826, 479, 120
Brevundimonas subvibrioides CB81^T (AJ227784)		708, 318, 241, 158	826, 479, 120
Brevundimonas bullata IAM 13153^T (D12785)			479, 422, 404, 120
Brevundimonas aveniformis EMB102^T (DQ372984)	547, 403, 210, 109, 130	–	–
Brevundimonas lenta DS-18^T (EF363713)	496, 403, 189, 160, 130	–	–
Brevundimonas bacterioides CB7^T (AJ227782)	547, 253, 189, 130	–	–
Brevundimonas halotolerans MCS24^T (M83810)	656, 320, 273, 130	–	–

Note: The restriction fragments of less than 100 nucleotides are not presented. "–" designates that this endonuclease was not used for in silico restriction analysis of the relevant strain.

Table 3. The results of the preliminary identification of prosthecate bacteria using restriction analysis and 16S rRNA gene sequencing

Composition of the experimentally deduced ARDRA groups	ARDRA groups	Species most closely related to representatives of ARDRA groups	
		Prosthecate species	Non-prosthecate species
B-1994, B-1184, B-1487, B-1488, B-1560, B-1561, B-1990	"*B. alba*/*B. basaltis*"	*B. alba* (98.7%)	*B. basaltis* (99.0%)
B-2583, B-1179, B-1181, B-1981, B-1986, B-1991, B-2001	ARDRA group 1	*B. alba* (98.3%)	*B. basaltis* (99.1%)
B-1176	*"B. lenta"*	*B. subvibrioides* (98.3%)	*B. lenta* (99.5%)
B-1177, B-1178, B-1180, B-1186, B-1188, B-1486	ARDRA group 2	*B. subvibrioides* (97.9%)	*B. lenta* (99.2%)
B-2015	*"B. staleyi"*	*B. staleyi* (99.3%)	*B. bullat*a (98.9%)
B-1982, B-1182, B-1191, B-1489, B-1563	*"B. bullata"*	*B. staleyi* (99.2%)	*B. bullata* (99.8%)
B-1993	*"B. mediterranea"*	*B. intermedia* (98.5%)	*B. mediterranea* (99.7%)
B-1992, B-1988	*"B. intermedia*/*B. nasdae"*	*B. intermedia* (99.7%)	*B. nasdae* (99.5%)
B-2584	ARDRA group 3	*B. variabilis* (98.0%)	*B. kwangchunensis* (98.6%)
B-1183	ARDRA group 4	*B. variabilis* (98.6%)	*B. kwangchunensis* (96.1%)
B-2000, B-1999, B-2017	*"C. henricii"*	*C. henricii* (99.5%)	–

Note: Representatives of the ARDRA groups are shown in bold; the levels of similarity (%) between their 16S rRNA sequences and those of the type strains of the closely related species are given. "–" designates that all species of the genus *Caulobacter* are prosthecate microorganisms.

99.8% similarity between B-1982 and *B. bullata* IAM^T, 99.1% similarity between B-2583 and *B. basaltis* J22^T, and 99.0% similarity between B-1994 and *B. basaltis* J22^T. The second large cluster, consisting of both the prosthecate bacteria *B. aurantiaca* and *B. intermedia* and the non-prosthecate bacteria *B. mediterranea, B. nasdae*, and *B. vesicularis*, contains two more prosthecate isolates, B-1992 and B-1993. The level of 16S rRNA similarity between the type strain of the non-prosthecate bacterium *B. mediterranea* V4.BO.10^T and the prosthecate isolate B-1993 was very high (99.7%). The level of 16S rRNA similarity between the type strain of the non-prosthecate bacterium *B. nasdae* and the prosthecate isolate B-1992 also was as high as 99.5%.

First of all, the levels of DNA–DNA reassociation should be determined for these six pairs with a similarity level of more than 99% in order to elucidate the species specificity of such important taxonomic properties of members of the genus *Caulobacter* and of many *Brevundimonas*, species as the ability to form prosthecae and the dimorphic life cycle. It is quite possible that some species belonging to these genera are heterogeneous with respect to such an important taxonomic property as the ability to form prosthecae.

Taking into account the high similarity between the 16S rRNA gene sequences of bacteria of the genus *Brevundimonas*, it is possible that strains B-2584 (ARDRA group 3) and B-1183 (ARDRA group 4) represent novel species. The levels of similarity between the 16S rRNA gene sequences of strains B-2584 and B-1183Ñ and those of the closest prosthecate and non-prosthecate species do not exceed 98.6% (Table 3).

The following traits of the studied strains and the data obtained enabled us to draw some taxonomic and ecological conclusions. The strains were isolated from 14 regions of Eurasia; in three regions, soil samples

were collected; and in other regions, water samples were collected (Table 1). The strains have been stored in the working collections of two researchers. Belyaev obtained isolates from both soil and aquatic ecosystems, whereas Lapteva's isolates were obtained only from aquatic ecosystems. The cultures obtained from different samples were isolated using traditional cultural methods and on similar media. According to the results of the 16S rRNA gene analysis, the strains fall into the majority of clusters and subclusters of *Brevundimonas*; however, the phylogenetic diversity of bacteria of the genus *Caulobacter* is less represented.

Of particular interest are the ecological and taxonomic properties of a subcluster consisting of two non-prosthecate bacteria, *B. basaltis* and *B. lenta*, and 21 prosthecate strains of the following ARDRA groups: "*B. basaltis–B. alba*", "*B. lenta*", ARDRA group 1, and ARDRA group 2 (Fig. 1, Table 3). This phylogenetically small group of prosthecate microorganisms exhibited an interspecific level of 16S rRNA similarity (97.6–99.7%) and was detected in Lakes Baikal and Sevan, as well as in lakes of Murmansk oblast, the Rybinsk and Volgograd Reservoirs, and soil samples collected in the Saratov and Moscow oblasts and Crimea (Table 1). Six prosthecate microorganisms from the "*B. bulata*" ARDRA group were also isolated from samples collected in different areas and ecosystems, including soil samples from Moscow oblast and Crimea, Lake Sevan, and several lakes of Murmansk oblast. At the same time, representatives of different species of prosthecate bacteria can be detected in the same geographical area or landscape zone. In this regard, the occurrence frequency of prosthecate bacteria in lakes is indicative. Strains B-1981, B-1991, B-2001, and B-2583 (ARDRA group 1), as well as strains B-1982 and B-1994 (ARDRA groups "*B. bullata"* and "*B. alba/B. basaltis*", respectively) were isolated from Lake Sevan (Table 3). Earlier, Lapteva, when studying the distribution of prosthecate bacteria in Lake Baikal [9], showed the presence of four out of five known species of the genus *Caulobacter*, *C. leidyi, C. henricii, C. fusiformis*, and *C. vibrioides*.

The results obtained and the literature data indicate a considerable taxonomic diversity of prosthecate bacteria of the genera *Brevundimonas* and *Caulobacter* in the aquatic and soil ecosystems of Eurasia. The group of prosthecate bacteria, phylogenetically close to the prosthecate species *B. alba, B. staleyi*, and *B. subvibrioides*, as well as to the non-prosthecate *B. basaltis, B. bulata*, and *B. lenta*, may be among the most widespread groups of prosthecate bacteria inhabiting the aquatic and soil ecosystems of several geographical areas of Eurasia; this conclusion is fully justified, considering the size and formation history of the studied strain collection. However, estimation of the ratios of various species (or groups of species) of prosthecate bacteria in some local ecosystem requires new approaches and, most importantly, identification of the target groups of microorganisms in environmental samples (in situ) using phylogenetic markers.

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