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EXPERIMENTAL ARTICLES

Bacteria of the Genus *Burkholderia* as a Typical Component of the Microbial Community of *Sphagnum* Peat Bogs

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Abstract—Bacteria of the genus *Burkholderia* are a typical component of the microbial complex of *Sphagnum* peat bogs and constitute a substantial portion of the aerobic chemoorganotrophic isolates which are routinely obtained from these environments on an acidic nutrient media. The ecophysiological characteristics of the 27 strains of such organisms, which were isolated from the peat of acidic *Sphagnum* bogs of the boreal and tundra zones of Russia, Canada, and Estonia, were investigated in the present study. Most of the *Burkholderia* strains isolated from these bogs were phylogenetically close to the species *B. glathei, B. phenazinium, B. fungorum*, and *B. caryophylli*, the typical inhabitants of soil and plant rhizosphere. The bog isolates utilized a broad range of substrates as carbon and energy sources, including organic acids, sugars, polyalcohols, and certain aromatic compounds. All the strains studied were capable of growth on nitrogen-free media. They developed in the pH range of 3.5 to 7.4 and from 3 to 37°C, with the optima at pH 5–7 and 11–23°C, respectively. They were therefore moderately acidophilic, psychroactive, dinitrogen-fixing microorganisms well adapted to the conditions of acidic northern *Sphagnum* bogs.

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Key words: bacteria of the genus *Burkholderia*, *Sphagnum* bogs.

The genus *Burkholderia* comprises gram-negative, aerobic or microaerophilic chemoorganotrophic bacteria which are capable of utilizing a broad range of organic compounds as growth substrates [1–3]. This genus was described in 1992 as the result of reclassification of seven *Pseudomonas* species based on the comparative analysis of 16S rRNA sequences, DNA-DNA homology, and a number of phenotypic and chemotaxonomic characteristics [1]. The genus *Burkholderia* of the class *Betaproteobacteria* comprises presently 34 species, with *B. cepacia* as the type species. A number of the representatives of this genus are known to be phytopathogenic or pathogenic for animals and humans (*B. cepacia, B. mallei, B. pseudomallei*); many other *Burkholderia* species are the typical inhabitants of soil, plant rhizosphere and rhizoplane [3, 4]. The strains of various *Burkholderia* species are used as efficient biodegrading agents and for the biological control of phytopathogenic microorganisms and for the stimulation of plant growth [5–7]. Traditionally pathogenic representatives of *Burkholderia* have attracted the special attention [3]. Only recently, a number of works dealing with the ecology of this group of organisms has been published [4, 7, 8]. Bacteria of the genus *Burkholderia* were shown to inhabit a wide range of ecological niches with diverse physicochemical characteristics. The general picture of the distribution and functions of these bacteria in natural ecosystems is, however, still far from clear.

We have been working on assembling the collection of acidophilic chemoorganotrophic bacteria inhabiting acidic *Sphagnum* bogs for several years. The identification of the isolates, by determination of the partial sequences of 16S rRNA genes, revealed that about 30% belonged to the genus *Burkholderia*. Since nothing was known previously concerning the abundance of these bacteria in *Sphagnum* bogs, this investigation was undertaken. The goal of the present work was to study the ecophysiological characteristics which determine the adaptation of bacteria of the genus *Burkholderia* to the conditions of acidic northern bogs.

MATERIALS AND METHODS

Twenty-seven bacterial strains belonging to the genus *Burkholderia* were isolated from the *Sphagnum* bogs of the boreal and tundra zones of Russia, Canada, and Estonia for the purposes of our investigation (Table 1). Twelve of these strains were isolated by direct plating of dilutions of the native peat on agar MB medium of the following composition (g/l): glucose, 0.5; KNO_3, 0.5; KH_2PO_4, 0.1; $MgSO_4$, 0.1; $CaCl_2$, 0.02;

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Table 1. Strains of the genus *Burkholderia* isolated in the present work from the *Sphagnum* peat bogs of different geographical locations

Source of isolation	Strain	Cultured organism with the highest 16S rRNA gene similarity	Identity, %	Sequence accession no. in GenBank
Peat of the Kurovskoe bog, Moscow oblast	SB1	*B. fungorum*	98	AJ544690
″	K1	*B. glathei*	98	AY154374
″	K(N-)1	*B. fungorum*	98	AY568512
Peat of a tundra bog, Vorkuta	SB5	*B. glathei*	99	AY154374
″	SB6	*B. phenazinium*	96	AY154376
″	SB7	*B. phenazinium*	96	AY154376
Peat of the Bakcharckoe bog, Tomsk oblast	PB1	*B. fungorum*	98	AJ544690
Peat of a bog from the Bryansk Forest preserve	B5	*B. phenazinium*	99	U96936
″	B8	*B. phenazinium*	97	AB021394
″	B(N-)1	*B. phenazinium*	98	AB021394
Peat of Mer Bleue bog, Ontario, Canada	C1	*B. fungorum*	98	BFU544690
Peat of Risti bog, Estonia	Y	*B. phenazinium*	98	AY154375
The cellulolytic community isolated from the Kurovskoe bog	F4	*B. fungorum*	98	BFU544691
″	F4W	*B. phenazinium*	98	AY154375
The cellulolytic community isolated from the Bakcharskoe bog	PC3	*B. terricola*	98	AY040362
The methanotrophic community isolated from the Sosvyatskii Mokh bog, Tver oblast	S1	*B. 'phytofirmans'* *B. caryophylli*	98 97	AY497470 U91570
″	S14	*B. caryophylli* *B. 'phytofirmans'*	97 98	U91570 AY497470
The methanotrophic community isolated from the Krugloe bog, Tomsk oblast	S2	*B. 'phytofirmans'* *B. caryophylli*	98 97	AY497470 U91570
″	S3	*B. 'phytofirmans'* *B. caryophylli*	98 97	AY497470 U91570
″	S6	*B. cepacia*	100	AB051408
″	S8	*B. cepacia*	99	AF097530
The methanotrophic community isolated from the Bakcharskoe bog	S4	*B. xenovorans*	100	U86373
″	S7	*B. tropicalis*	97	AY128104
″	S10	*B. tropicalis*	96	AY128104
″	S5	*Burkholderia* sp.	99	AY571292
″	S9	*B. caryophylli*	97	U91570
″	S11	*B. caryophylli*	97	U91570

Note: The nonvalidated taxa are marked by single quotes.

yeast extract, 0.1. After sterilization, the pH of the medium was adjusted to 3.5–4.2 with 0.1 M H_3PO_4. Peat samples from the following bogs were used for inoculation: (1) the Kurovskoe oligo-mesotrophic ombrotrophic *Sphagnum* bog, Losinoostrovskoe forest, Pushkin district, Moscow oblast (55° N, 38° E), bog water pH 4.7–5.2; (2) oligotrophic ombrotrophic *Sphagnum* bog of the Bryansk Forest national preserve (54° N, 32° E), bog water pH 4.5–4.7; (3) oligo-mesotrophic ombrotrophic *Sphagnum* bog Bakcharskoe, Tomsk oblast, South Vasyugan'e, the Plotnikovo field station of the Institute of Soil Science and Agricultural Chemistry, Russian Academy of Sciences, Siberian department (56°53′ N, 82°50′ E), bog water pH 4.0–4.5; (4) the tundra ombrotrophic oligotrophic sedge-*Sphagnum* bog, Tal'nik station, Vorkuta (67° N, 63° E), bog water pH 4.5–4.8; (5) Mer Bleue ombrotrophic *Sphagnum* bog, Ontario, Canada (45°24′ N, 75°30′ W), bog water pH 3.8–4.3; (6) Risti

Table 2. Utilization of various substrates as carbon and energy sources by the *Burkholderia* isolates

Substrates	Strain				
	F4	SB5	Y	C1	SB1
Arabinose	–	+/–	+	+/–	+/–
Galactose	+	+	+	++	++
Glucose	+	+	++	+	++
Xylose	++	+	++	+	++
Lactose	–	+	+/–	–	–
Maltose	+/–	+	+	+/–	+
Rhamnose	+/–	++	+	+	+
Raffinose	–	+/–	–	–	+/–
Sucrose	+/–	+	+/–	–	+
Trehalose	+	++	+	–	+
Fructose	++	++	++	+	++
Cellobiose	++	+/–	+	–	+
Acetate	+	+	+/–	+	+
Butyrate	++	+/–	+	++	++
Valerate	++	+/–	++	++	++
Gluconate	++	++	+	+	++
Caproate	+	+/–	+	+	+
Malate	+	+	++	++	+
Oxalate	+/–	+/–	+/–	–	+/–
Pyruvate	+/–	++	+	+	++
Propionate	+	+	+	++	+
Succinate	+	+	+	+	++
Fumarate	+	–	+/–	+/–	+/–
Itrate	++	+	++	++	+
Methanol	–	–	–	–	–
Dulcitol	–	++	–	–	–
Inulin	–	+/–	–	–	+/–
Mannitol	+	++	+	+	++
Meso-inositol	+	+	++	+	++
Sorbitol	+	++	++	+	+
Protocatechuic acid	+	+	–	+	+/–
Coumarin	–	–	–	–	–
m-Hydroxybenzoic acid	–	++	–	+/–	+/–
Salicin	–	–	–	+	–
Amino acids hydrolysate	++	++	+	++	++

Note: –, OD_{410} below 0.03; +/–, OD_{410} 0.03–0.06; +, OD_{410} 0.06–0.3; ++, OD_{410} above 0.3.

ombrotrophic *Sphagnum* bog, Estonia (58°59′ N, 24°4′ E), bog water pH 4.0.

The other twelve *Burkholderia* strains were isolated on the MB media from the previously described acidophilic methanotrophic communities obtained from the Sosvyatski Mokh (Tver oblast), Krugloe, and Bakcharskoe (Tomsk oblast) bogs [9]. Three strains, F4, F4W, and PC3 were isolated from acidophilic cellulolytic microbial communities obtained from the peat of Kurovskoe (Moscow oblast) and Bakcharskoe (Tomsk oblast) *Sphagnum* bogs [Pankratov et al., unpublished data]. The isolates were obtained by inoculating the cells of the communities on the agarized, twofold diluted ST5 mineral medium [10], pH 4.5–5.0, with 0.05% glucose.

The effect of pH on the growth of the isolates was determined on a liquid MB medium in the pH range 2.8 to 7.8. To achieve the required pH value, KH_2PO_4 of the original version of MB medium was substituted by a mixture of H_3PO_4, KH_2PO_4, K_2HPO_4, and K_3PO_4, with the total phosphate concentration equal to that in the original MB medium. These media were inoculated with the exponential phase cells grown at pH 5.0; the cells were centrifuged and washed with respective media. The cultures were incubated at 28°C in shaken 500 ml flasks with 100 ml medium for one to two days. In the periodically taken samples, OD_{410} was determined using Specol (Carl Zeiss, Germany), and pH of the culture liquid, using a 211 pH meter (Hanna Instruments, Germany). The initial OD_{410} of 0.07–0.08 was the same for all the experimental flasks. The growth rate μ was calculated from the changes in OD_{410} per unit time during the logarithmic growth phase; it was correlated with the current pH value.

The temperature growth range of the isolates was determined on an agar MB medium. The effect of temperature on the growth rates of the cultures was studied in more detail by monitoring the dynamics of OD_{410} at 2, 7, 11, 15, 23, and 30°C under static conditions in liquid media. The initial OD_{410} in the experimental flasks was 0.06–0.07.

To determine the spectrum of the substrates utilized by the isolates as carbon and energy sources, growth was assessed on the liquid medium containing (g/l): $(NH_4)_2SO_4$, 0.5; KH_2PO_4, 0.25; yeast extract, 0.05. The compounds listed in Table 2 were added in 0.04% concentrations. Growth was assessed as OD_{410} after three days of incubation at 25°C. Growth on the same medium without the added substrate was used as a control. The ability of the strains to grow on nitrogen-free media was determined on a liquid MB medium without nitrate.

For DNA extraction from the isolates, a modification of the previously described method based on the use of sodium dodecyl sulfate (SDS) as a lysing agent was applied [9]. The PCR amplification of 16S rRNA genes used universal *Bacteria*-specific primers [11] on the PE GeneAmp PCR System 9700 thermocycler (Perkin-Elmer Applied Biosystems, United States). The nucleotide sequences of the amplified fragments were determined using ABI 377A sequencer (Perkin-Elmer Applied Biosystems, United States). For the analysis of

the nucleotide sequences and for constructing phylogenetic trees, ARB software package was used (http: //www.arb-home.de). The statistical confidence of the phylogenetic trees was determined using Phylip software package by bootstrap analysis, constructing 1000 alternative trees. The complete 16S rRNA gene sequences of the *Burkholderia* isolates obtained from *Sphagnum* bogs were deposited in GenBank under accession nos. AJ971346–AJ971354.

RESULTS

Morphological characteristics. The cells of the isolates obtained from *Sphagnum* bogs were gram-negative coccobacilli or rods, 0.5–0.8 × 0.8–2.5 μm, single or in chains (Fig. 1); the cells were surrounded with capsules with sizes varying, depending on the growth conditions. The cells of most of the cultures (excluding strains B5, B8, and S11) were nonmotile. The cultures isolated from bogs formed convex, semitransparent, slimy colonies, which were 2–6 mm in diameter on agar media. The color varied from white or cream-colored for most of the cultures to bright yellow or greenish-yellow for a group of strains (Y, F4, F4W, SB1, and PB1). The latter strains also excreted into the medium a pigment of the same color as their colonies. Growth in liquid media was homogeneous; surface films were not formed.

The spectrum of the substrates utilized. The bog isolates were capable to utilize a wide range of substrates, including organic acids, sugars, polyhydric alcohols, and certain aromatic compounds as carbon and energy sources (Table 2). The preferable substrates for all the strains were: salts of organic acids (gluconate, valerate, butyrate, pyruvate, malate, and citrate); certain sugars (fructose, xylose, glucose, and galactose); certain polyhydric alcohols (mannitol, meso-inositol, and sorbitol); and amino acids hydrolysate, a complex organic substrate. Some strains were capable of growth on such aromatic compounds as protocatechuic and hydroxybenzoic acids, salicin. No growth occurred in controls without added substrates.

The growth characteristics of the isolates were investigated for strains SB1 and C1 isolated from *Sphagnum* bogs of the boreal zone, and strain SB5 from a tundra zone bog. Glucose or sodium gluconate, butyrate, and acetate were used as growth substrates. For the isolates SB1 and C1, the maximal growth rates with acetate, sodium gluconate, and glucose were close, 0.24–0.27 h^{-1}, while with sodium butyrate they were 0.13–0.14 h^{-1}. A similar growth pattern was determined for strain SB5 with all the substrates tested; the growth rates, however, did not exceed 0.13 h^{-1}.

All the strains investigated were capable of exponential growth in a liquid MB medium without a source of bound nitrogen. On nitrogen-free medium, the growth rates were 0.08–0.15 h^{-1}.

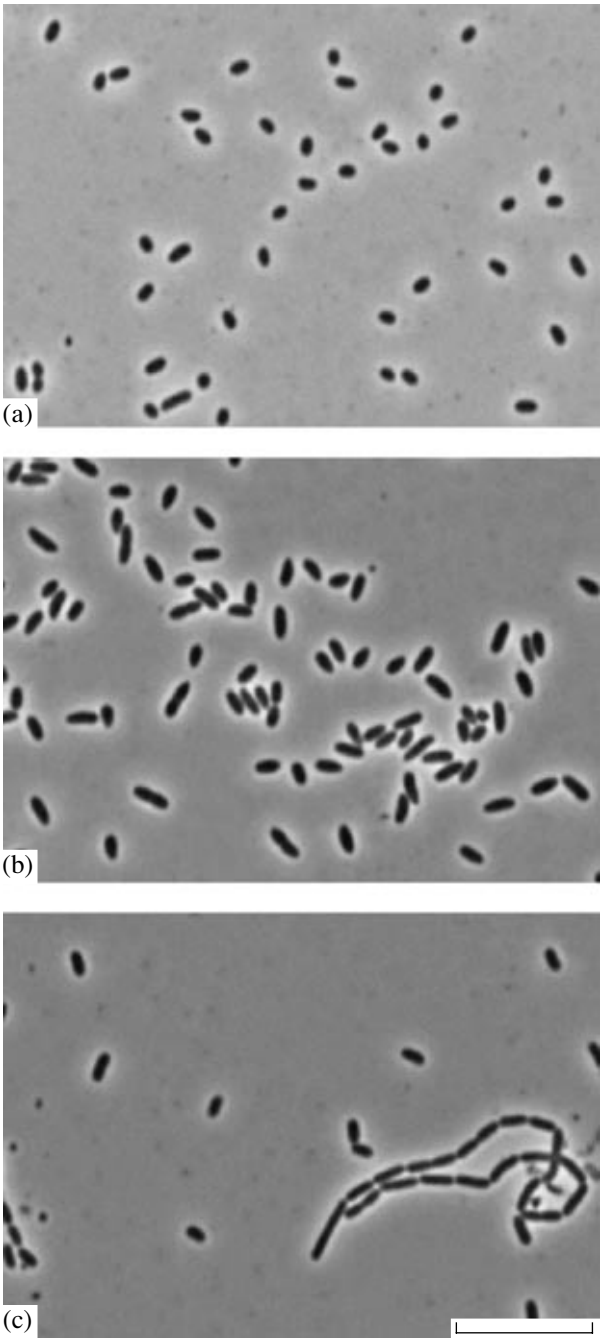

Fig. 1. Morphology of the cells of the *Burkholderia* strains isolated from *Sphagnum* peat bogs, phase microscopy: (a) coccobacilli, strain C1; (b) rods, strain SB5; (c) chains of rod-shaped cells, strain PC3. Scale bar, 10 μm.

Temperature range of growth. The bog isolates were capable of growth in the range 3 to 37°C. The growth rate (μ) under static conditions at 3°C was 0.003–0.01 h^{-1}. The curves of μ dependence from temperature were similar for different strains; they indi-

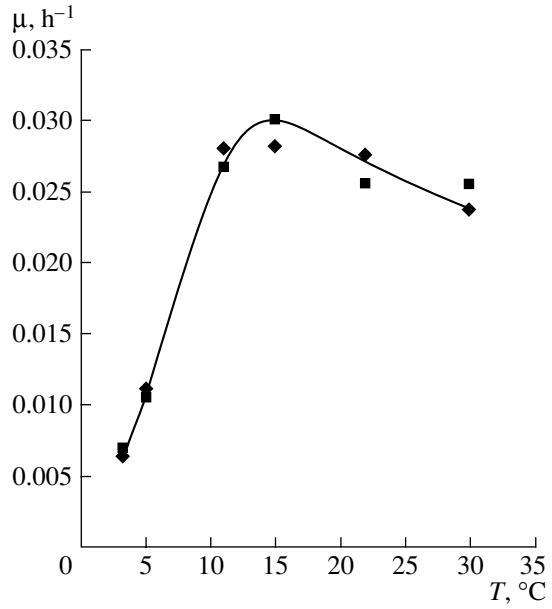

Fig. 2. Growth rates of the *Burkholderia* isolates (μ) as dependent on the temperature for strains Y (squares) and C1 (rhombs).

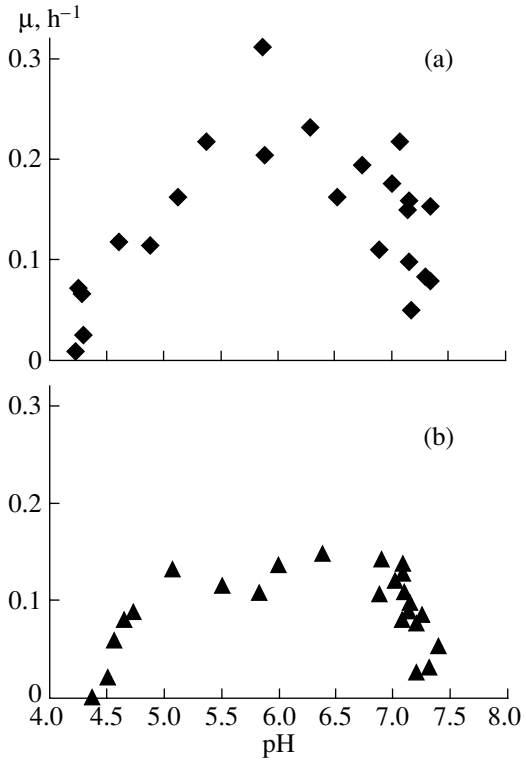

Fig. 3. Growth rate (μ) of the *Burkholderia* isolates depending on the pH of the medium: (a) strain SB1; (b) strain SB5.

cated the physiological optimum for the bog isolates in the range 11 to 23°C (Fig. 2).

Effect of pH on growth rates. The effect of pH on the growth of the cultures, strains SB1 and SB5, isolated from the boreal and tundra zone bogs, was studied. These cultures could grow both on liquid and on agar media with a pH 3.5–3.6. At such pH values, however, growth in liquid culture resulted in the formation of cell aggregates, therefore determination of growth characteristics was difficult. Homogenous growth in a liquid media occurred only at a higher pH, from 4.2 to 7.4, with the optimum in the pH range 5.0 to 7.0 (Fig. 3). For strain SB1, the highest growth rate of 0.31 h^{-1} was detected at pH 5.9–7.0, though active growth with a rate of 0.12 h^{-1} occurred at pH 4.6–4.8 (Fig. 3a). For strain SB5, no pronounced maximum was found; the highest μ values (0.14 h^{-1}) were observed within the pH from 5 to 7 (Fig. 3b).

Phylogenetic analysis. It was mentioned above that the determination of the partial sequences of 16S rRNA genes of the isolates (ca. 500–600 nucleotides) revealed that they belong to the genus *Burkholderia*. The strains isolated from the *Sphagnum* bogs were phylogenetically close (97–100% identity of 16S rRNA gene sequences) to the species *B. glathei, B. phenazinium, B. fungorum, B. caryophylli, B. cepacia, B. terricola*, and *B. tropicalis* (Table 1). Almost complete sequences of 16S rRNA genes were determined for nine isolates; their phylogenetic position among the other representatives of the genus *Burkholderia* is shown in Fig. 4. The group of yellow-pigmented isolates (strains PB1, SB1, F4W, F4, and Y) from the bogs of different geographic localization form a separate cluster and possibly represent a new *Burkholderia* species.

DISCUSSION

The ecophysiological characteristics determined in this study for isolates of the genus *Burkholderia* suggest that these bacteria are moderately acidophilic, psychroactive, dinitrogen-fixing microorganisms well adapted to the conditions of northern acidic *Sphagnum* bogs. Little is known about the physiological optimum of the representatives of this genus. The research traditionally focused rather on the pathogenic properties of burkholderia; the issues of their physiological optimum and growth range seldom attracted the attention of researchers. As a result, neither the temperature, nor the pH growth ranges of the representatives of the genus *Burkholderia* were presented in *The Prokaryotes* [3]. The same shortcoming, with rare exceptions [12], is characteristic for the descriptions of numerous representatives of this genus. Only a narrow temperature range from 30 to 42°C was used for the differentiation between the *Burkholderia* species; therefore we could not find any data concerning the development of these organisms at low temperatures. Our data indicate that the representatives of *Burkholderia* inhabiting *Sphagnum* bogs are capable to grow in a wide temperature range, from 3 to 37°C, with a growth optimum at 11–23°C.

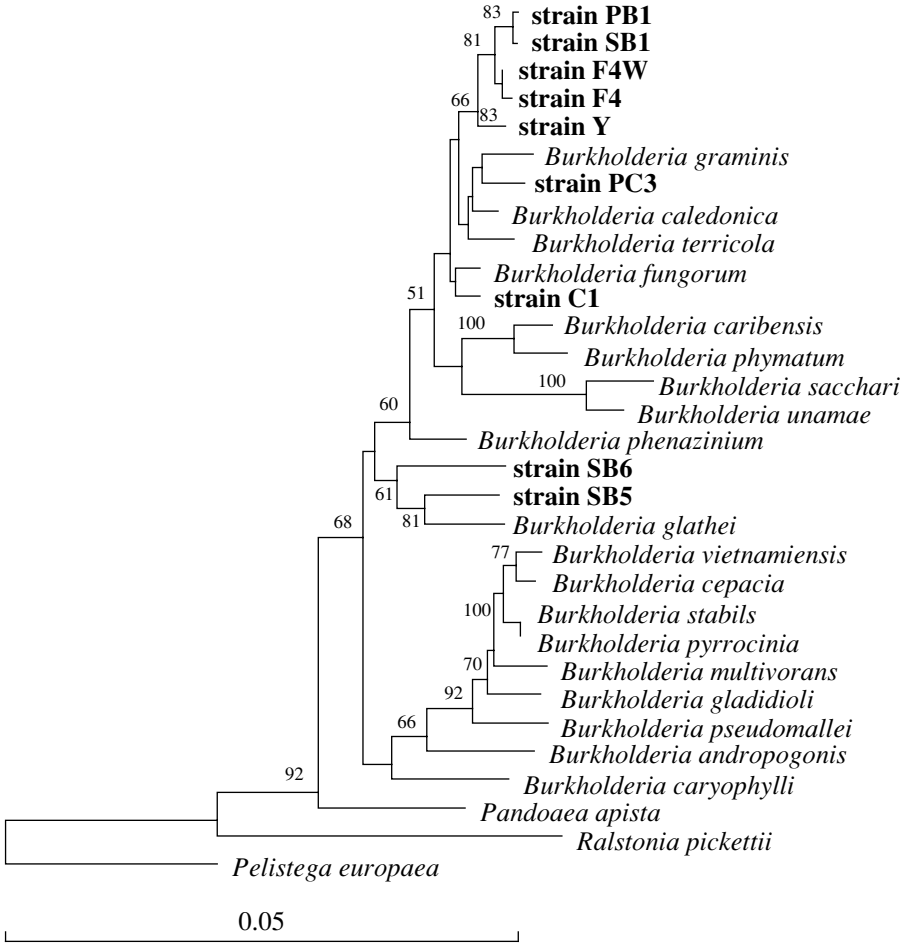

Fig. 4. 16S rRNA-based dendrogram showing the phylogenetic relationships of peat bog isolates to known representatives of the genus *Burkholderia*. Scale bar, 0.05 substitutions per base.

Somewhat more is known about the growth of burkholderia at low pH values and of their distribution in acidic environments. 16S rRNA gene sequences belonging to the representatives of *Burkholderia* were found in the acidic (pH 3–4) thermal soils of New Zealand [13] and in acidic soil (pH 4.8) from agrocenoses of Holland [8]. Several *Burkholderia* strains capable of growth in the pH range 3.5 to 8.0 were isolated from the acidic soils (pH 2.9) of North Carolina coniferous forests [14]. The representatives of the species *B. unamae* were found in the rhizosphere and rhizoplane of maize, sugar cane, and coffee plants grown in soils with a pH of 4.5–7.1, though not in soils with a pH above 7.5 [12]. The burkholderia isolated by us from a *Sphagnum* bog were phylogenetically closely related to a number of the known representatives of this genus. The capability to survive and grow in an acidic media is therefore probably the feature shared by many representatives of *Burkholderia*.

Utilization of a wide range of organic compounds as carbon and energy sources, which was observed for the isolates from *Sphagnum* bogs, is characteristic for all burkholderia [2, 3]. Many of the substrates used in the present paper are intermediate decomposition products of the polymeric organic compounds of peat. The capability of the isolated strains to utilize aromatic compounds is of special importance since various phenolic compounds and derivatives of benzoic acid are a major group of organic compounds produced by mosses and accumulated in *Sphagnum* bogs [15, 16]. The stable presence of burkholderia in methanotrophic and cellulolytic communities isolated from *Sphagnum* bogs and maintained for several years under laboratory conditions also deserves consideration. Since none of the isolates were capable to utilize methanol, burkholderia probably survives in methanotrophic communities by the utilization of polysaccharides from the capsule material of methanotrophs, or of the organic matter from their dead cells. In cellulolytic communities, burkholderia form the trophic stage following the organisms performing the initial stage of cellulose decomposition.

We have previously demonstrated that the representatives of the class *Betaproteobacteria* are a numerically significant component of the microbial community of *Sphagnum* bogs [17]. The number of their cells

varies within the range from 10^5 to 10^7 cells per 1 g of wet peat. Further research is necessary in order to determine the portion of *Burkholderia* in this group. The isolates obtained in the present work, judging from terminal dilutions of the peat, represented the population of 10^3–10^5 cells per 1 g of wet peat; however, the total number of all the representatives of this genus in peat may be substantially higher.

Thus *Sphagnum* bogs are one of the natural ecological niches for bacteria of the genus *Burkholderia*. The high degree of adaptation to the specific conditions of *Sphagnum* bogs, together with the high frequency of isolation of these organisms from acidic peat, with their diversity, indicate that burkholderia are a typical component of the microbial complex of such ecosystems.

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