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EXPERIMENTAL ARTICLES

Phylogenetic Identification of Three Strains of Rhizosphere Bacteria Based on the Results of 16S rRNA Gene Analysis and Genetic Typing

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Abstract—The rhizosphere nitrogen-fixing bacteria *Herbaspirillum frisingense* B416, *Burkholderia* sp. 418, and *Herbaspirillum huttiense* B601 (degrader of chlorinated s-triazines) were identified by phylogenetic analysis of the 16S rRNA gene sequences, characterization of the 16S–23S intergenic spacer region, Rep-PCR genotyping, and assessment of differentiating phenotypic characteristics. The results obtained indicate that, for correct taxonomic affiliation by comparative analysis of 16S rRNA gene sequences, the ratio between intra- and interspecies variability of these sequences within the group of bacteria closely related to the identified strain should be taken into consideration. If the interspecies differences between 16S rRNA genes are insufficient for differentiation of closely related species, ribotyping and Rep-PCR analysis of genomic DNA can be used for determination of the species affiliation.

Key words: rhizosphere bacteria, phylogenetic identification, 16S rRNA gene, genotyping, *Herbaspirillum, Burkholderia*.

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The rhizosphere is a habitat for a wide variety of bacteria that possess unusual properties or combinations thereof. Identification of these rhizosphere bacteria using the standard phenotypic techniques is often difficult. In bacteriology, reevaluation of the definition of a species ("a species is a category that circumscribes a (preferably) genomically coherent group of individual isolates/strains sharing a high degree of similarity in (many) independent features comparatively tested under highly standardized conditions" [1]) resulted in the leading role of molecular genetic techniques in determination of the taxonomic position of bacteria. As a result, distinct criteria have been established for correct identification of bacterial isolates that hitherto were considered difficult to identify.

The nitrogen-fixing bacterial strains B416 and 418 were previously isolated from the root surface of cocksfoot and barley, respectively [2]; strain B601 carrying the plasmid responsible for the degradation of stable chlorinated s-triazines (herbicides simazine, atrazine, and propazine) was isolated from the corn rhizosphere [3, 4]. The strains B416 and 418 are capable of competitive root colonization, low-temperature nitrogen fixation and plant growth promotion. Strain 418 is used for production of a polyfunctional preparation for vegetable crops in the People's Republic of China [5]. The ease and reliability of reisolation of this strain from the rhizosphere make it a convenient object for ecological studies and for investigation of the mechanisms of root colonization [6–8].

Based on analysis of their phenotypic characteristics, all these strains were identified as *Pseudomonas*-like bacteria [2, 3]. It is well known that, due to the development of the phylogenetic principles of bacterial taxonomy, the genus *Pseudomonas* has been subjected to extensive taxonomic revision [9, 10]. The description of many new bacterial genera belonging to different classes of the *Proteobacteria* was one of the results of this revision. Since the revision was based on comparative analysis of the nucleotide sequences of the 16S rRNA gene, this method became necessary for correct identification of pseudomonads. This technique is a universal, suitable, and very reliable molecular method that allows determination of the generic affiliation of bacteria from various taxonomic groups [11]. Nevertheless, the results of this analysis should be interpreted with caution when determining the species affiliation, taking into account that the similarity between 16S rRNA genes does not necessarily indicate a sufficiently high level of relatedness between bacterial genomes. The resolving power of the typing methods based on the characterization of the 16S–23S intergenic spacer region and on comparative analysis of the obtained Rep-PCR fingerprints makes it possible to perform both inter- and intraspecific differentiation of the strains [12]. The goal of the present work was therefore to carry out phylogenetic identification of strains B416, 418, and B601 of the rhizosphere *Pseudomonas*-like bacteria using both comparative analysis of the nucleotide sequences of the 16S rRNA gene fragments and genetic typing.

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MATERIALS AND METHODS

Bacterial strains and cultivation conditions. The subjects of this study were the previously obtained rhizosphere nitrogen-fixing bacteria "*Pseudomonas*" sp. B416, "*Pseudomonas*" sp. 418 [2], and "*Pseudomonas*" sp. B601 (degrader of chlorinated s-triazines) [3]. The type strains *Herbaspirillum huttiense* DSM 10281^T and *Burkholderia xenovorans* DSM 17367^T were obtained from the German Collection of Microorganisms and Cell Cultures (www.dsmz.de).

The cultures were grown on TY medium (pH 7.0) containing the following (g/l): tryptone (Difco, United States), 10.0; yeast extract (Difco, United States), 1.0; and $CaCl_2$, 0.02. To test their ability to utilize various compounds as the sole carbon source, the cultures were grown on a minimal medium (pH 7.2–7.4) containing the following (g/l): K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; NH_4Cl, 0.5; $CaCl_2$, 0.02; tested compound, 1.0; and bacteriological agar (Difco, United States), 15.0. The growth rate of the cultures was assessed after 2-day incubation at 28°C.

Determination and analysis of the 16S rRNA gene sequences. DNA was extracted using the Genomic DNA Purification Kit (Fermentas, Lithuania) according to the manufacturer's recommendations. The templates for sequencing were obtained by PCR with the primers fD1 and rD1 [13], which allowed amplification of the almost complete 16S rRNA gene sequences (except for the short end sites). The reaction mixture (30 μl) contained the following: 3 μl of 10× reaction buffer (Dialat Ltd., Russia), 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 30 pM of each primer, 1 U of *Taq* polymerase (Dialat Ltd., Russia), and 15 ng of genomic DNA as a template. PCR amplification was carried out on an MJ Mini^{TM} thermocycler (Bio-Rad, United States). The reaction was initiated by incubation of the reaction mixture at 95°C for 4 min followed by 30 cycles (95°C for 30 s, 65°C for 30 s, and 72°C for 2 min). The final elongation was performed at 72°C for 10 min. Analysis of the PCR products was carried out by electrophoresis in 1.2% agarose gel; fragments of about 1500 bp were cut out from the gel and purified using a GFX PCR DNA Kit and Gel Band Purification Kit (Amersham Pharmacia Biotech, United Kingdom) according to the manufacturer's instructions. Sequencing of the 16S rRNA gene fragments was performed on an ABI Prism 310 Genetic Analyzer automatic sequencer (Applied Biosystems, United States) using a BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, United States) according to the manufacturer's recommendations. When carrying out the "sequencing PCR," the internal primers 536f, 1114f, 519r, and 960r were used in addition to fD1 and rD1 [14]. The obtained results were analyzed using the Sequencing Analysis Software v.5.2 (Applied Biosystems, United States). The search for homologous 16S rRNA gene sequences in the GenBank database of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) was performed using the BLAST (Basic Local Alignment Search Tool) software package. The phylogenetic analysis was carried out by the neighbor-joining method using the MEGA4 software package [15].

Genetic typing. In order to assess the genetic affinity between the studied bacteria, comparative analysis of the Rep-PCR genomic fingerprints, as well as analysis of the 16S–23S intergenic spacer region, were carried out [12]. The volume of the reaction mixture and buffer composition were the same as those used for amplification of the 16S rRNA genes.

For Rep-PCR, we used the primers ERIC1R, ERIC2 [16] (60 pmol of each or a mixture), BOXA1R [17] (60 pmol), and 15 ng of genomic DNA as a template. The reaction was initiated by denaturation at 95°C for 4 min followed by 30 cycles which, when carrying out ERIC-PCR, involved incubation: 95°C for 30 s, 51°C for 30 s, and 65°C for 8 min. The reaction was completed with a final elongation performed at 65°C for 16 min. In the case of BOX-PCR, the cycle involved incubation (95°C for 30 s, 60°C for 30 s, and 72°C for 2 min). The final elongation was performed at 72°C for 4 min.

Amplification of the 16S–23S spacer region was carried out using a pair of primers, sp1 and sp2 (30 pmol of each) homologous to the conservative sequences of the 16S and 23S rRNA genes, respectively [18]. The reaction was initiated by denaturation at 95°C for 4 min, followed by 30 cycles that involved incubation (94°C for 1 min, 55°C for 3 min, and 72°C for 2 min) and the final elongation at 72°C for 5 min. The amplification products were separated by gel electrophoresis both directly and after the treatment with a mixture consisting of the *Ava*I, *Bam*HI, *Eco*RI, *Hin*dIII, and *Pst*I restriction enzymes (Fermentas, Lithuania). Electrophoresis was carried out in 2% agarose gel using the standard Tris–borate buffer at half concentration. The gel slabs were stained with ethidium bromide and photographed under UV illumination.

RESULTS AND DISCUSSION

Phylogenetic analysis of the 16S rRNA gene sequences. The obtained 1400-bp nucleotide sequences of the 16S rRNA genes of the strains B416, 418, and B601 were deposited in the GenBank database under accession numbers FJ657665, AY520839, and AY953141, respectively. Comparison of the obtained sequences with the sequences in the GenBank database using the NCBI BLAST software package revealed the greatest similarity of the 16S rRNA genes of strains B416 and B601 to those of members of the genus *Herbaspirillum,* family *Oxalobacteraceae*, while the 16S rRNA gene sequence of strain 418 displayed the greatest similarity to those of members of the genus *Burkholderia*, family *Burkholderiaceae*.

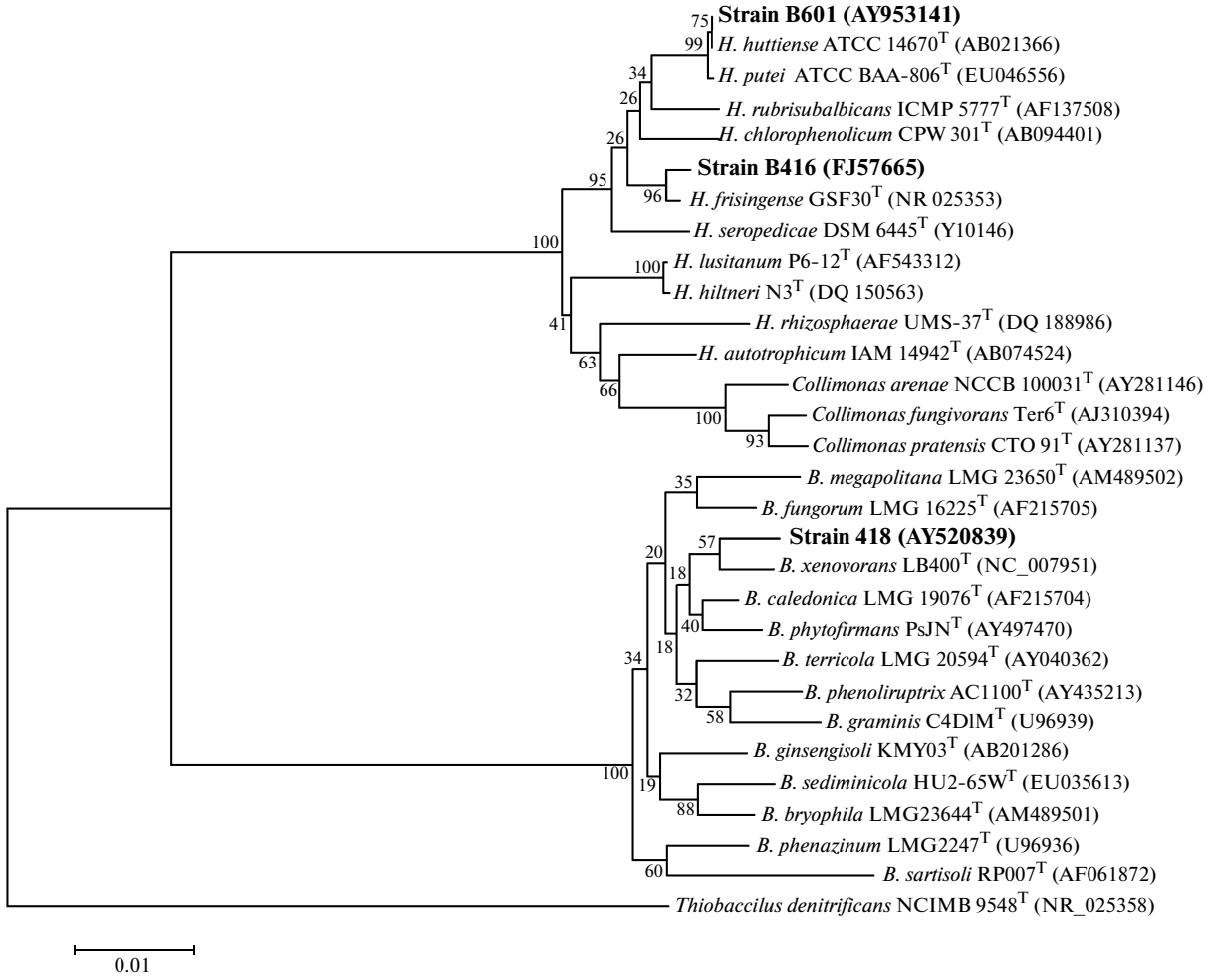

Fig. 1. Phylogenetic tree showing the relatedness between the strains B416, B601, and the type strains of the genus *Herbaspirillum* and between strain 418 and the type strains of the *B. graminis* group. The GenBank accession numbers of the sequences are given in parentheses (www.ncbi.nlm.nih.gov). There are 1257 nucleotides in the comparison dataset. Bootstrap values were calculated based on 1000 resamplings. Scale bar, 0.01 substitution per nucleotide position.

Both families belong to the order *Burkholderiales*, class *Betaproteobacteria*.

The results of pairwise comparison indicated that the 16S rRNA gene fragments of strain B416 displayed the greatest similarity with those of *Herbaspirillum frisingense* GSF 30^T and *H. chlorophenolicum* CPW 301^T (99.7 and 99.1%, respectively). The 16S rRNA gene sequence of strain B601 differed from those of the type strains *H. huttiense* ATCC 14670^T (= DSM 10281^T) and *H. putei* ATCC BAA-806^T (= 7-2^T) by only two and three base pairs, respectively. Moreover, analysis of the 16S rRNA gene sequences revealed a high level of similarity (99.2%) between strain B601 and *H. frisingense* Mb11; the species affiliation of the latter was determined in the course of description of the species *H. frisingense* [19].

In the case of strain 418, the greatest similarity was revealed between the 16S rRNA sequence of this strain and those of the type strains *Burkholderia xenovorans* LB400^T (= DSM 17367^T), *B. phytofirmans* PsJN^T, and *B. fungorum* LMG 16225^T (99.3, 98.8, and 98.8%, respectively). These species belong to the *B. graminis* group, which is phylogenetically distant from the complex adjacent to *B. cepacia,* the type species of the genus *Burkholderia* [20].

Comparative phylogenetic analysis of the 16S rRNA gene sequences of the studied strains, as well as of the type strains of the genus *Herbaspirillum* and the *B. graminis* group, revealed that, as expected, the sequences displaying the greatest similarity, according to the results of pairwise comparison, were most closely related to each other (Fig. 1). High bootstrap support was observed for all pairs (strain B416–*H. frisingense* GSF 30^T, strain 418–*B. xenovorans* LB400^T, and strain B601–*H. huttiense* ATCC 14670^T), which confirms statistical significance of the clustering results. Nevertheless, the obtained pairs differed significantly with respect to their phylogenetic relatedness. The 16S rRNA gene sequences of strains B601

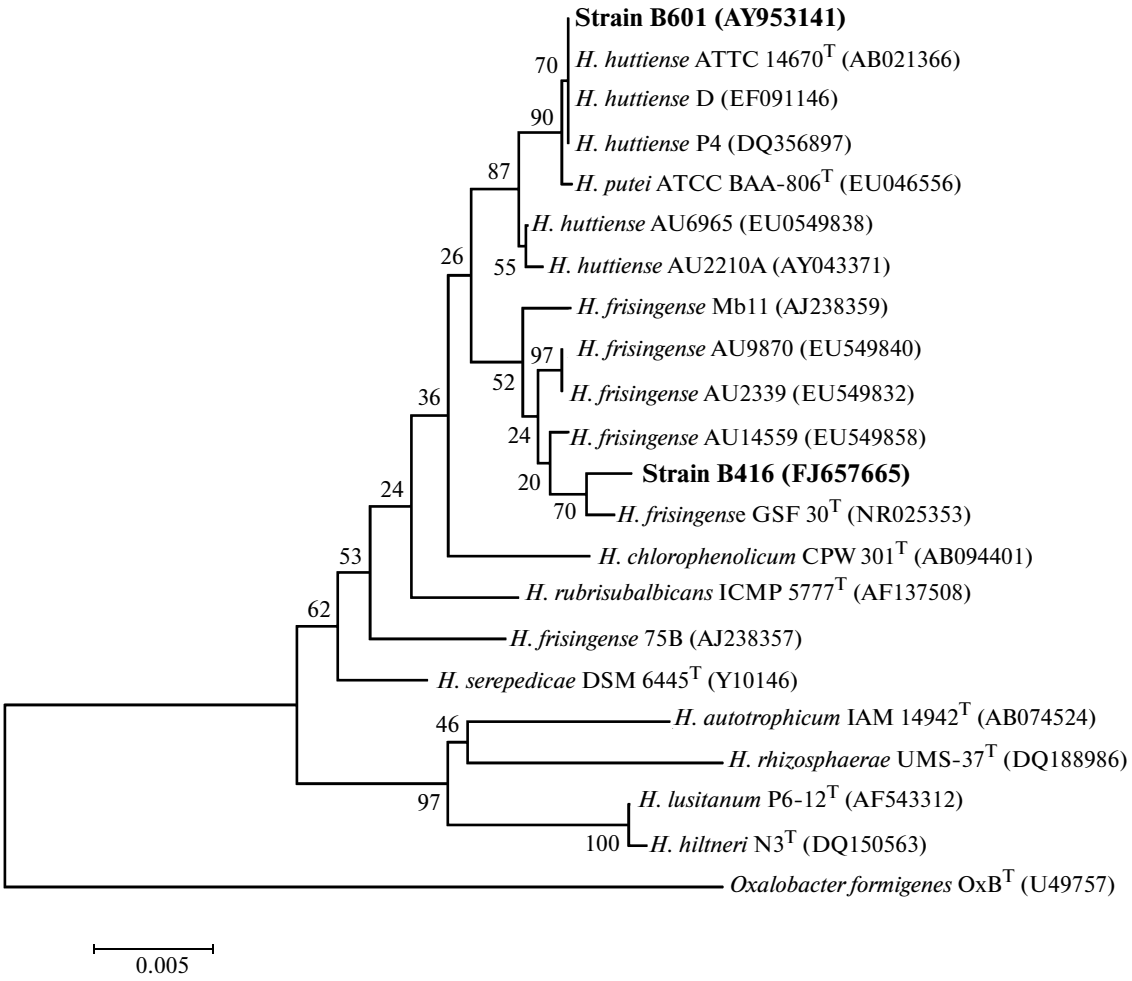

Fig. 2. Phylogenetic tree showing the position of the strains B416 and B601 among bacteria of the species *H. frisingense* and the *H. huttiense–H. putei* group, respectively. The type strains are designated with the letter ^{T} after the strain number. The GenBank accession numbers of the t sequences are given in parentheses(www.ncbi.nlm.nih.gov). There are 1338 nucleotides in the comparison dataset. Bootstrap values were calculated based on 1000 resamplings. Scale bar, 0.005 substitution per nucleotide position.

and *H. huttiense* ATCC 14670^T did not differ within the comparison unit; the difference between these strains and *H. putei* ATCC BAA-806^T was insignificant. On the other hand, the extent of phylogenetic divergence between strain B416 and *H. frisingense* GSF 30^T, as well as between strain 418 and *B. xenovorans* LB400^T, corresponded to that found for many closely related species and significantly exceeded the extent of divergence between the type strains of *H. huttiense* and *H. putei* or *H. lusitanum* and *H. hiltneri* (Fig. 1). All these facts cast doubt on the validity of affiliation of the studied strains to particular species based on the BLAST search results and phylogenetic analysis of the 16S rRNA sequences of the studied strains and closely related type strains.

To enhance the resolution of phylogenetic analysis, the nucleotide sequences of the reference strains available from GenBank belonging to the species most closely related to the studied strains were added to the alignments. Figure 2 shows that bacteria belonging to the species *H. frisingense* and *H. huttiense* (along with *H. putei* ATCC BAA-806^T) formed separate clusters, suggesting the possibility of differentiation of these species based on the results of comparative analysis of their 16S rRNA gene sequences. Strain *H. frisingense* 75B, which did not cluster with any species and formed a separate branch between *H. rubrisubalbicans* and *H. seropedica*, was the only exception. The integrity of the *H. frisingense* cluster was restored after the 16S rRNA gene sequences of the species *H. chlorophenolicum, H. huttiense*, and *H. putei* (which were described after *H. frisingense*) were excluded from the analysis. The phylogenetic separation of strain 75B was quite surprising. Its affiliation to *H. frisingense* was confirmed by the results of comparison of this strain with the type strain *H. frisingense* GSF 30^T, as well as by the high level of DNA–DNA homology (76%). However, despite these facts, there was no similarity between the obtained Rep-PCR fingerprints of these strains [19].

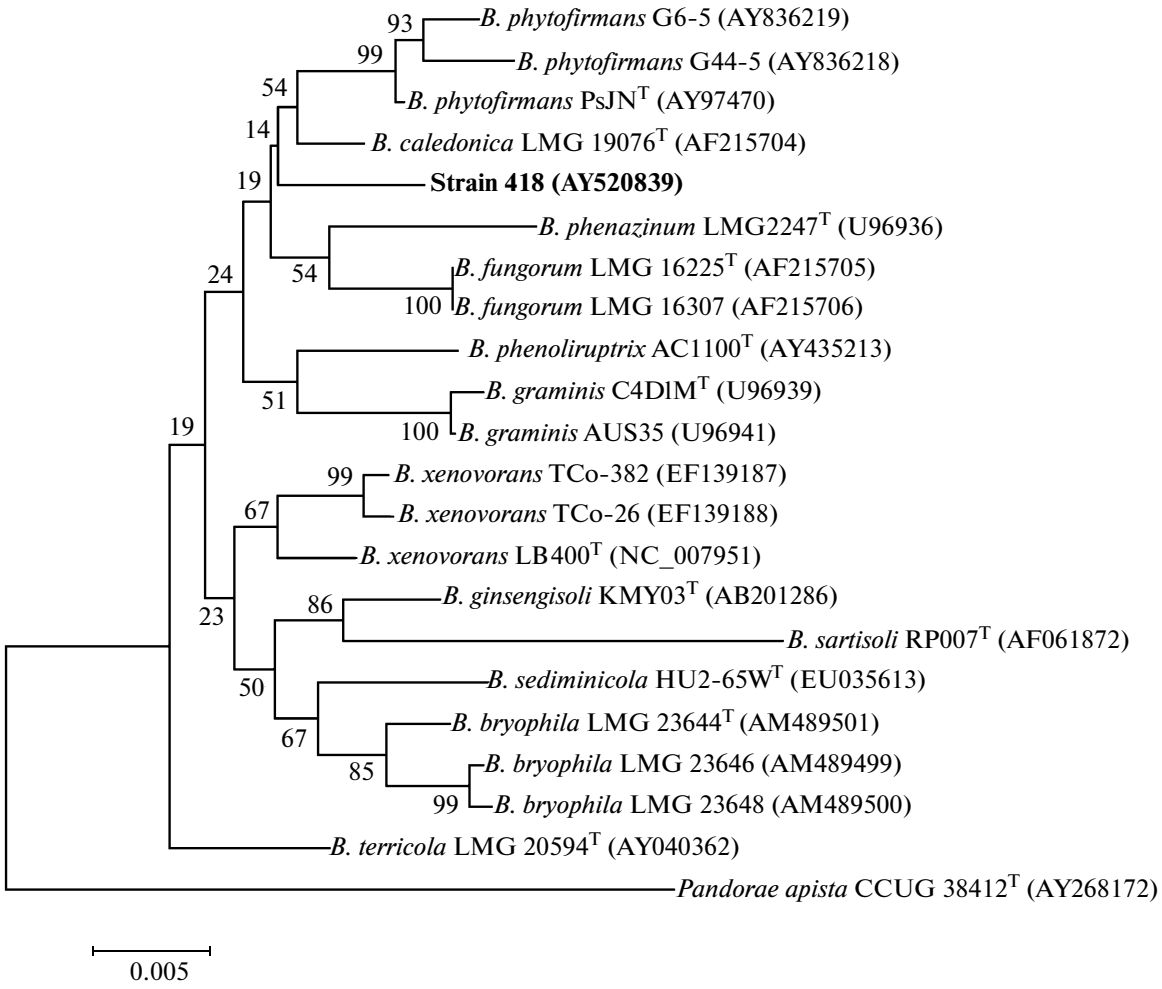

Fig. 3. Phylogenetic tree showing the phylogenetic position of strain 418 among representatives of the *B. graminis* group. The type strains are designated with the letter ^{T} after the strain number. The GenBank accession numbers of the gene fragment sequences are given in parentheses (www.ncbi.nlm.nih.gov). There are 1297 nucleotides in comparison dataset. Bootstrap values were calculated based on 1000 resamplings. Scale bar, 0.005 substitution per nucleotide position.

Within the main cluster formed by *H. frisingense* strains, the 16S rRNA gene sequences of which exhibited higher variability than those of the cluster formed by *H. huttiense*, strain B416 was closely related to the type strain *H. frisingense* GSF30^T. According to these data, strain B416 is phylogenetically closest to the type strain GSF30^T (among all the studied bacteria of the species *H. frisingense* with identified 16S rRNA gene sequences). These results also confirm the validity of affiliation of this strain to *H. frisingense*.

Strain B601 fell into the cluster formed by *H. huttiense* strains and the type strain of *H. putei*, which indicated its affiliation with one of these species and excluded the possibility of its affiliation to *H. frisingense*. Differentiation of the species *H. huttiense* and *H. putei* on the basis of the results of phylogenetic analysis was found to be impossible. Low variability of the 16S rRNA gene sequences within the *H. huttiense–H. putei* subgroup may be a possible cause of this phenomenon; in the case of *H. huttiense*, the level of intraspecific variability was higher than the level of differences between the type strains of these species.

Addition of other reference 16S rRNA sequences to the data unit of the *B. graminis* group resulted in the exclusion of strain 418 from the cluster containing representatives of the species *B. xenovorans* (Fig. 3). In the obtained phylogenetic tree, this strain formed a branch adjacent to the *B. caledonica–B. phytofirmans* cluster. Low bootstrap value (14%) indicated that the position of strain 418 was unstable and could vary depending on the composition of the alignment.

Differentiation by phenotypic tests. To determine the species affiliation of the studied strains, comparative phenotypic analysis of the strains B601 and *H. huttiense* DSM 10281^T was carried out using tests that, as was demonstrated earlier [21, 22], enabled differentiation of the species *H. huttiense, H. putei*, and *H. frisingense*. No differences were revealed between

Table 1. Differentiation of *H. huttiens*e, *H. putei*, and *H. frisingense* based on their phenotypic characteristics

Carbon source	Bacterial strains								
	B416	B601	*H. huttiense* DSM 10281^T			*H. putei* 7-2^T		*H. frisingense* GSF 30^T	
	Data source								
	Experiment			[21]	[22]	[21]	[22]	[21]	[22]
Not added	–	–	–						
α-D-glucose	+	+	+	– !?	+ ?	– ?	+ ?	+	+
D-galactose	+	+	+	– !?	+ ?	– ?	+ ?	+	+
L-arabinose	+	+	+	+	+	– ?	+ ?	– ?	+ ?
D-xylose	+	+	+	nd	nd	nd	nd	nd	nd
D-lactose	–	–	–	nd	–	nd	–	nd	–
D-mannose	+	+	+	– !	– !	± ?	– ?	± ?	+ ?
D-fructose	+	+	+	nd	– !	nd	–	nd	+
D-rhamnose	+	–	–	–	–	–	–	+ ?	– ?
Citrate	+	+	+	nd	+	nd	+	nd	+
Malonate	+	–	–	nd	–	nd	–	nd	+
Tween-80	–	–	–	nd	+ !	nd	–	nd	+
L-leucine	+	–	–	nd	–	nd	–	nd	+
L-serine	–	–	–	nd	–	nd	–	nd	+
L-threonine	+	–	–	nd	+ !	nd	–	nd	+
L-glucuronate	+	+	+	nd	+	nd	–	nd	–

Note: +, intense growth of bacteria on the carbon source; ±, weak growth; – , growth was not detected; nd stands for "no data"; !, the data presented in the literature contradict the results of the experiment; ?, the data presented in the literature contradict each other.

the studied characteristic traits of the strains B601 and *H. huttiense* DSM 10281^T (Table 1), which suggested that strain B601 belonged to the species *H. huttiense*. It should be noted that there is a discrepancy between the characteristics of *H. huttiense, H. putei*, and *H. frisingense* published by different authors who described the species *H. putei* [21] and *H. hiltneri* [22] (Table 1). This makes all attempts for phenotypic differentiation of any newly isolated strains belonging to this group useless without the involvement of the relevant type strains. The situation becomes more complicated due to the scarcity of the differentiating characteristics of *H. huttiense* and *H. putei* [21], as well as due to the fact that the species *H. putei* was proposed based on the description of the only strain, making it impossible to assess, even approximately, their variability within this species.

Strain B416, affiliated to the species *H. frisingense* on the basis of the results of phylogenetic analysis, is capable of utilizing a wider range of carbon sources than the strains B601 and *H. huttiense* DSM 10281^T (Table 1). On the whole, this finding corresponds to the published data on the physiological and biochemical properties of *H. frisingense* and *H. huttiense* [19, 22], although it should be noted that some characteristics of the *H. frisingense* type strain described in [21] and [22] contradict each other (Table 1). The capability of nitrogen fixation, which distinguishes *H. frisingense* from *H. huttiense, H. putei*, and *H. chlorophenolicum* [23], is an important trait. We have previously demonstrated that strain B416 was capable of nitrogen fixation [2], whereas, in the case of strain B601, we failed to detect the nitrogen-fixing activity [3].

The phenotypic characteristics of the strains 418 and *B. xenovorans* DSM 17367^T were similar. Both strains utilized α-D-glucose, D-galactose, D-xylose, D-mannose, D-fructose, citrate, tartrate, pyruvate, malate, Twin-80, L-leucine, L-threonine, *i*-inositol, DL-norleucine, benzoate, and *para*-oxybenzoate as the only carbon sources. The strains did not utilize L-arabinose, L-lactose, D-trehalose, L-serine, malonate, oxalate, dulcite, *meta*-oxybenzoate, salicylate, *para*-aminobenzoate, and 2,4D. Both strains were cetavlon-sensitive and did not grow at 42°C, but grew at 40°C.

The strains 418 and *B. xenovorans* DSM 17367^T differed only in their capacity to utilize glucuronic acid and maltose (Table 2). However, these character-

Table 2. Differentiation of *B. xenovorans, B. fungorum*, and *B. phytofirmans* based on their phenotypic characteristics

Differentiating characteristics	Bacterial species and strains							
	418	*B. xenovorans* DSM 17367^T		*B. fungorum* LMG 16225^T		*B. phytofirmans* PsJN^T	
	Data source							
	Experiment		[23]	[26]	[24]	[26]	[25]	[26]
Utilization of carbon sources:								
L-arabinose	–	–	–	nd	+ (v)	nd	+	nd
D-xylose	+	+	+	+	+ ? (v)	w ?	+	nd
D-fructose	+	+	+	nd	+ (v)	nd	+	nd
Citrate	+	+	+	+	+	+	nd	–
D-glucuronate	–	+	nd	nd	nd	nd	nd	nd
D-maltose	+	–	–	–	– (v)	–	– (v)	–
Growth on medium with 0.3% cetavlon	–	–	–	–	+ (v)	+	+ ? (v)	– ?

Note: (v), the characteristics varies in other strains of this species; w, the characteristic is weak; ?, the data presented in the literature contradict each other.

istics were not mentioned as differentiating ones upon description of the species *B. fungorum, B. xenovorans*, and *B. phytofirmans*. The inability of *B. xenovorans* to utilize L-arabinose distinguishes this microorganism from other closely related species of the genus *Burkholderia* [24], except for some *B. fungorum* strains [25]. However, it should be kept in mind that the description of *B. fungorum* was based on the results of characterization of nine strains [25], while only three strains were characterized in *B. xenovorans*, which is hardly enough to determine the variability of characteristics. A similar pattern was observed when comparing *B. xenovorans* with *B. fungorum* and *B. phytofirmans* with respect to their resistance to cetavlon (Table 2). It should be noted that there a mismatch between the descriptions of this characteristic of the *B. phytofirmans* type strain presented in [26] and [27]. Both *B. xenovorans* DSM 17367^T and strain 418 differed from the *B. phytofirmans* type strain in their ability to utilize citrate; however, in the case of *B. phytofirmans*, the variability of this characteristic was not studied. Hence, the differentiating phenotypic characteristics of the species *B. fungorum, B. xenovorans*, and *B. phytofirmans* cannot be considered fully reliable, and, due to their scarcity, the species identification of new strains of this group is hardly possible.

Characterization of the 16S–23S intergenic spacer region. The 16S–23S intergenic spacer regions of all the studied strains, as well as of the type strains, were amplified as the only main fragments of about 920 bp (*B. xenovorans* DSM 17367^T and strain 418) and 880 bp (strains B416, B601 and *H. huttiense* DSM 10281^T) (Fig. 4a). In addition to the main fragments, minor amplicons were detected, which, however, did not allow reliable differentiation. Treatment of the obtained amplification products with a mixture of restriction endonucleases revealed significant differences between all the studied strains, except for the pair B601–*H. huttiense* DSM 10281^T (Fig. 4b).

Rep-PCR genotyping. To determine the level of relatedness between the strains within the pairs B601–*H. huttiense* DSM 10281^T and 418–*B. xenovorans* DSM 17367^T, Rep-PCR genotyping allowing for intraspecific differentiation was performed [12]. The similarity between the patterns of the amplified fragments, irrespective of some differences, may be considered as supporting evidence that the strains belong to different clones of one species. Figures 5b and 6 demonstrate that the similarity between the products of amplification of the genomic DNA of the strains B601 and *H. huttiense* DSM 10281^T was observed when using the primer ERIC1R; the amplification

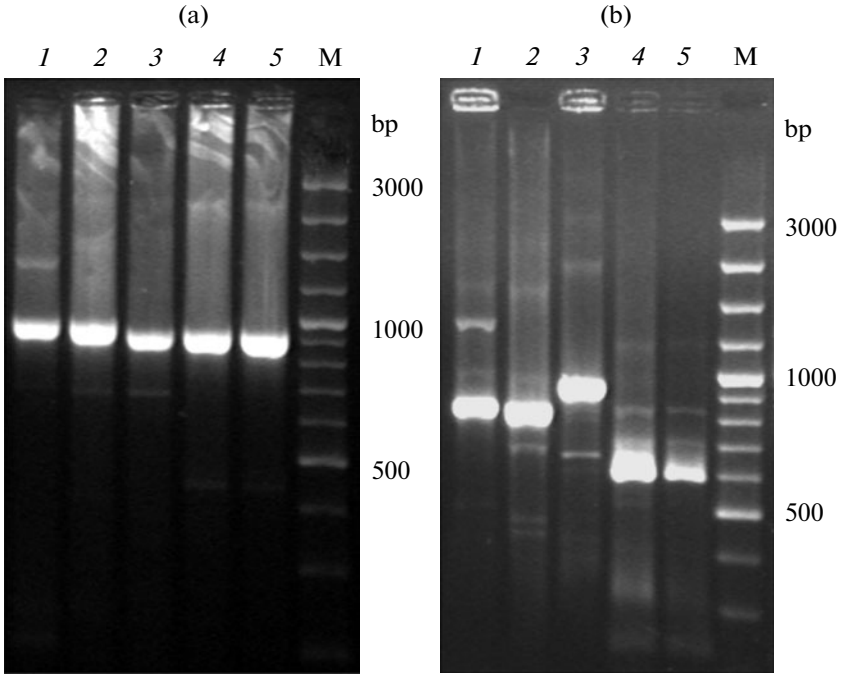

Fig. 4. Amplification of the 16S–23S intergenic spacer region (a) and differentiation of strains after the treatment of the obtained amplification products with the mixture of restriction endonucleases (b): M, molecular weight marker, Gene Ruler^{TM} 100 bp Plus DNA Ladder (Fermentas, Lithuania).

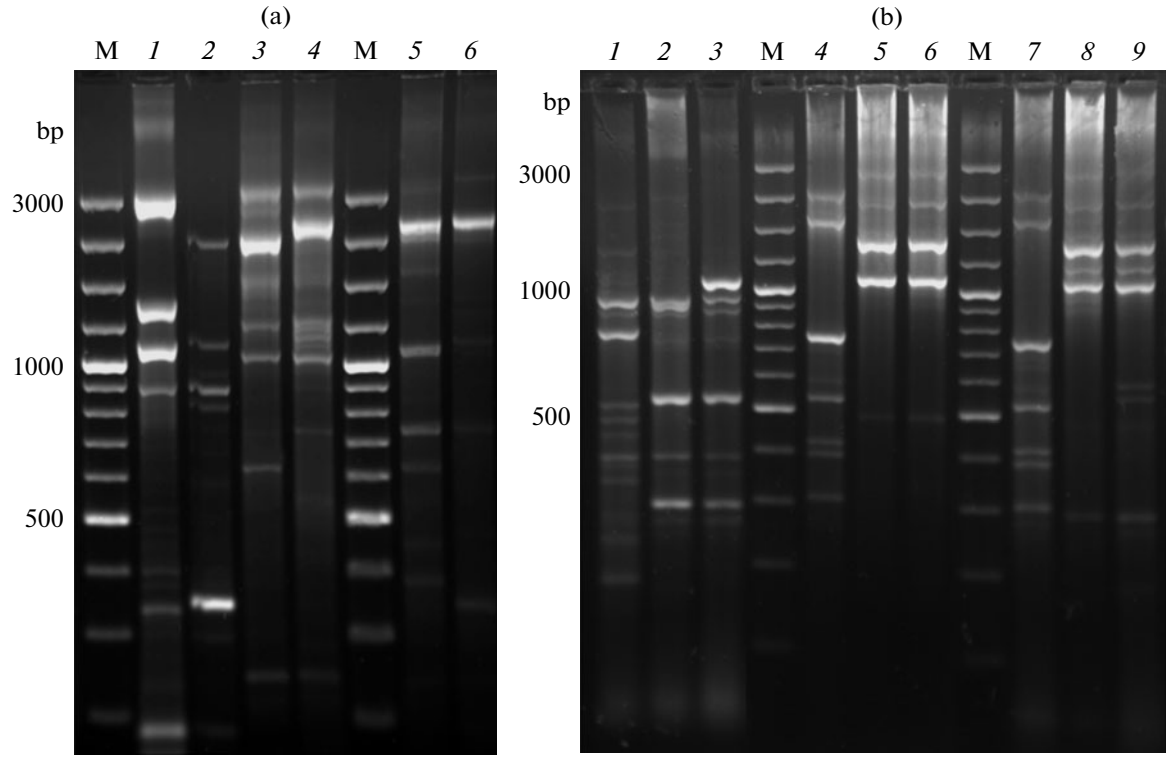

Fig. 5. (a) Genetic typing of strain 418 (lanes *1, 3*, and *5*) and *B. xenovorans* DSM 17367^{T} (lanes *2, 4*, and *6*) by ERIC-PCR. The primers ERIC1R (lanes *1* and *2*), ERIC2 (lane *3* and *4*), and the mixture of ERIC1R and ERIC2 (lanes *5* and *6*) were used to obtain the fingerprints. (b) Genetic typing of the strains B416 (lanes *1, 4*, and *7*), B601 (lanes *2, 5*, and *8*), and *H. huttiense* DSM 10281^{T} (lanes *3, 6*, and *9*) by ERIC-PCR. The primers ERIC1R (lanes *1–3*), ERIC2 (lanes *4–6*), and the mixture of ERIC1R and ERIC2 (lanes *7, 8*) were used to obtain the fingerprints. M, molecular weight marker, Gene Ruler^{TM} 100 bp Plus DNA Ladder (Fermentas, Lithuania).

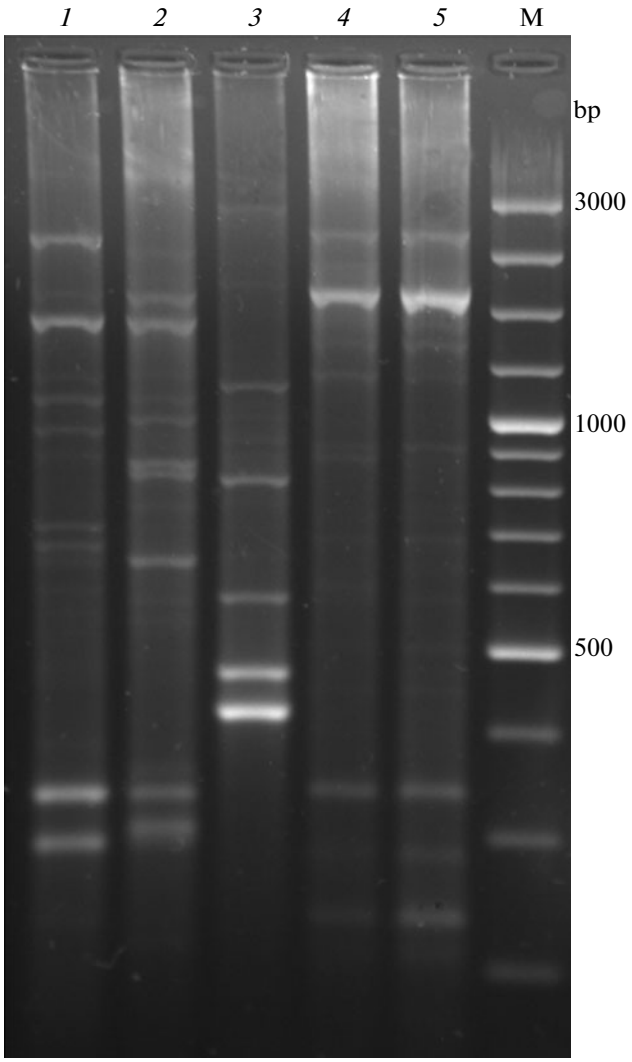

Fig. 6. Genetic typing of strain 418 (lane *1*), *B. xenovorans* DSM 17367^T (lane *2*), B416 (lane *3*), B601 (lane *4*), and *H. huttiense* DSM 10281^T (lane *5*) by BOX-PCR. M, molecular weight marker, Gene Ruler^{TM} 100 bp Plus DNA Ladder (Fermentas, Lithuania).

products were virtually the same when using the primers ERIC2 and BOXA1R. The similarity between the Rep-PCR fingerprints obtained using different primers indicated a high level of genome homology, which supported affiliation of strain B601 to the species *H. huttiense*, known as *Pseudomonas huttiensis* before the taxonomic revision [21].

The fingerprints of the strains 418 and *B. xenovorans* DSM 17367^T obtained using any of the genotyping primers differed significantly (Figs. 5a and 5b). Only some fragments were of the same size, which can be attributed to an accidental coincidence. Hence, the results of Rep–PCR do not unambiguously indicate a high level of relatedness between the genomes of these bacteria.

Various authors considered the 98–99.9% similarity between the 16S rRNA gene sequences as a threshold for species affiliation [28]. It is obvious that this value is not universal and should be specified, to the extent possible, for each case. Phylogenetic analysis with the addition of the 16S rRNA gene sequences of the type strains and other precisely identified strains made it possible to assess the ratio between their intra- and interspecific variability and to confirm the validity of the species affiliation of the studied strains. The situation is possible in which interspecies differences between the 16S rRNA gene sequences are insignificant and do not exceed the level of intraspecific variability, as in the case of *H. huttiense* and *H. putei*.

On the other hand, the intraspecific variability of 16S rRNA gene sequences may be quite significant, as in the case of *H. frisingense*. In this case, the suggestion that strain B416 belongs to *H. frisingense* is based on the fact that this strain is the closest relative (among other known bacteria of this species) of the *H. frisingense* type strain. The similarity between the phenotypic characteristics of strain B416 and those of *H. frisingense* confirmed that the identification was correct.

It should be kept in mind that physiological and biochemical characteristics are often unsuitable as a decisive criterion for determination of the species affiliation due to the scarcity of the differentiating traits of "new" species, their intraspecific variability being poorly studied (and cannot be studied if the species is represented by a single strain), and that the data from different articles published in prestigious scientific journals often contradict each other.

The latter problem can be solved by experimental comparison of the identified and type strains. If the phenotypic differences are scarce or absent, the results of genetic typing can serve as a decisive criterion for determination of the species affiliation. The same ribotypes and almost identical fingerprints of strain B601 and *H. huttiense* DSM 10281^T obtained using different Rep primers provided direct evidence that these microorganisms are closely related and that strain B601 belongs to the species *H. huttiense*.

Genetic typing of strain 418 and *B. xenovorans* DSM 17367^T confirmed our doubts about the validity of identification of strain 418 as *B. xenovorans*, which had been raised by the results of phylogenetic analysis of the 16S rRNA gene sequences. Importantly, the type strain and the reliably identified *B. xenovorans* strains isolated from geographically distant ecological niches had the same ribotypes and insignificant differences in their BOX-PCR patterns [24]. At the same time, strains G44-5 and G6-5 of the closely related species *B. phytofirmans*, identification of which was not subject to doubt, belonged to different (although closely related) DGGE-BOX groups [29]. Therefore, significant differences between the Rep-PCR fingerprints of strain 418 and *B. xenovorans* DSM 17367^T are possibly due to intraspecific variability; thus, the results of genotyping of these strains cannot be inter-

preted unambiguously. According to phylogenetic analysis of the 16S rRNA gene sequences, as well as on the basis of its phenotypic characteristics, strain 418 is most closely related to *B. xenovorans*. To determine more precisely its taxonomic position, further analyses are required, including DNA–DNA hybridization.

REFERENCES

1. Stackebrandt, E., Frederiksen, W., Garrity, G.M., Grimont, P.A.D., Kampfer, P., Maiden, M.C.J., Nesme, X., Rossello-Mora, R., Swings, J., Trüper, H.G., Vauterin, L., Ward, A.C., and Whitman, W.B., Report of the ad hoc Committee for the Re-Evaluation of the Species Definition in Bacteriology, *Int. J. Syst. Evol. Microbiol.*, 2002, vol. 52, pp. 1042–1047.
2. Bazhanov, D.P., Rudova, E.V., and Troitskii, N.A., Isolation and Characterization of Aerobic Nitrogen-Fixing Bacteria from the Rhizoplane of Wild and Cultivated Monocotyledonous Plants, *Izvestiya AN BSSR*, 1991, no. 5, pp. 8–12.
3. Bazhanov, D.P. and Novitskii, V.F., Isolation and Characterization of a Simazine-Utilizing Rhizosphere Bacterium, in *Mikrobiologiya i biotekhnologiya na rubezhe XXI stoletiya. Mat. mezhd. konf.* (Microbiology and Biotechnology at the Border of the 21st Century. Proc. Int. Conf), Minsk, 2000, pp. 21–23.
4. Bazhanov, D.P. and Yatsevich, K.K., Mobilization of Genes for Simazine Degradation by the pSa Plasmid, *Tsitol. Genet.,* 2007, vol. 41, no. 1, pp. 16–22 [*Cytol. Genet.* (Engl. Transl.), vol. 41, no. 1, pp. 12–17].
5. Yang, H., Li, J., Chen, K., and Bazhanov, D., Beneficial Effects of Multifunctional Biocontrol Agents on Tomato, *Proc. Int. Conf. on Environmentally Sustainable Agriculture for Dry Areas for the 3rd Millennium, Shijiazhuang, Hebei, P.R. China*, 2002, pp. 406–408.
6. Bazhanov, D.P. and Troitskii, N.A., Expression of the *Klebsiella pneumoniae nifA* gene in Diazotrophic Rhizobacteria, *Genetika*, 1992, vol. 28, no. 10, pp. 40–47 [*Sov. Genet.,* vol. 28, no. 10, pp. 1275–1281].
7. Bazhanov, D.P., Bazhanova, A.A., Rudova, E.V., and Troitskii, N.A., Transposon-Induced Mutagenesis in Rhizobacteria, *Genetika*, 1993, vol. 29, no. 10, pp. 1630–1639 [*Sov. Genet.*, vol. 29, no. 10, pp. 1197–1205].
8. Bazhanov, D.P., Bazhanova, A.A., Urbanovich, O.Yu., and Trukhanovets, N.L., Mutants of Nitrogen-Fixing Rhizospheric Bacterium *Pseudomonas* sp. 418 with Loss of the Ability to Colonize Roots, *Genetika*, 2000, vol. 136, no. 2, pp. 159–164 [*Russ. J. Genetics*, vol. 36, no. 2, pp. 106–110].
9. Kersters, K., Ludwig, W., Vancanneyt, M., De Vos, P., Gillis, M., and Shleifer, K.-H., Recent Change in the Classifcation of the Pseudomonads: An Overveiw, *Syst. Appl. Microbiol.*, 1996, vol. 19, pp. 465–477.
10. Anzai, Y., Kim, H., Park, J.-Y., Wakabayashi, H., and Oyaizu, H., Phylogenetic Affiliation of the Pseudomonads Based on 16S rRNA Sequence, *Int. J. Syst. Evol. Microbiol.*, 2000, vol. 50, pp. 1563–1589.
11. Olsen, G.J. and Woese, C.R., Ribosormal RNA: A Key to Phylogeny, *FASEB J.*, 1993, vol. 7, pp. 113–123.
12. Olive, D.M. and Bean, P., Principles and Applications of Methods for DNA-Based Typing of Microbial Organisms, *J. Clin. Microbiol.*, 1999, vol. 37, pp. 1661–1669.
13. Weisburg, W.G., Barns, S.M., Pelletier, D.A., and Lane, D.J., 16S Ribosomal DNA Amplification for Phylogenetic Study, *J. Bacteriol.*, 1991, vol. 173, pp. 697–703.
14. Reed, D.L. and Hafner, M.S., Phylogenetic Analysis of Bacterial Communities Associated with Ectoparasitic Chewing Lice of Pocket Gophers: A Culture-Independent Approach, *Microbial. Ecol.*, 2002, vol. 44, pp. 78–93.
15. Tamura, K., Dudley, J., Nei, M., and Kumar, S., MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0, *Mol. Biol. Evolut.*, 2007, vol. 24, pp. 1596–1599.
16. Versalovic, J., Koeuth, T., and Lupski, J.R., Distribution of Repetitive DNA Sequences in Eubacteria and Application to Fingerprinting of Bacterial Genomes, *Nucleic Acids Res.*, 1991, vol. 19, pp. 6823–6831.
17. Koeuth, T., Versalovic, J., and Lupski, J.R., Differential Subsequence Conservation of Interspersed Repetitive *Streptococcus pneumoniae* BOX Elements in Diverse Bacteria, *Genome Res*, 1995, vol. 5, pp. 408–418.
18. Kostman, J.R., Edlind, T.D., LiPuma, J.J., and Stull, T.L., Molecular Epidemiology of *Pseudomonas cepacia* Determined by Polymerase Chain Reaction Ribotyping, *J. Clin. Microbiol.,* 1992, vol. 30, pp. 2084–2087.
19. Kirchhof, G., Eckert, B., Stoffels, M., Baldani, J.I., Reis, V.M., and Hartmann, A., *Herbaspirillum frisingense* sp. nov., a New Nitrogen-Fixing Bacterial Species That Occur in C4-Fibre Plants, *Int. J. Syst. Microbiol.*, 2001, vol. 51, pp. 157–168.
20. Coenye, T. and Vandamme, P., Diversity and Significance of *Burkholderia* Species Occupying Diverse Ecological Niches, *Environ. Microbiol.*, 2003, vol. 5, pp. 719–729.
21. Ding, L. and Yokota, A., Proposals of *Curvibacter gracilis* gen. nov., sp. nov. and *Herbaspirillum putei* sp. nov. for Bacterial Strains Isolated from Well Water and Reclassification of [*Pseudomonas*] *huttiensis,* [*Pseudomonas*] *lanceolata*, [*Aquaspirillum*] *delicatum* and [*Aquaspirillum*] *autotrophicum* as *Herbaspirillum huttiense* comb. nov. and *Herbaspirillum autotrophicum* comb. nov, *Int. J. Syst. Evol. Microbiol.*, 2004, vol. 54, pp. 2223–2230.
22. Rothballer, M., Schmid, M., Klein, I., Gattinger, A., Grundmann, S., and Hartmann, A., *Herbaspirillum hiltneri* sp. nov., Isolated from Surface-Sterilized Wheat Roots, *Int. J. Syst. Evol. Microbiol.*, 2006, vol. 56, pp. 1341–1348.
23. Im, W.-T., Bae, H.-S., Yokota, A., and Lee, S.T., *Herbaspirillum chlorophenolicum* sp. nov., a 4-Chlorophenol-Degrading Bacterium, *Int. J. Syst. Evol. Microbiol.*, 2004, vol. 54, pp. 851–855.
24. Goris, J., De Vos, P., Caballero-Mellado, J., Park, J., Falsen, E., Quensen, III, J.F., Tiedje, J.M., and Vandamme, P., Classification of the Biphenyl- and Polychlorinated Biphenyl-Degrading Strain LB400T and Relatives as *Burkholderia xenovorans* sp. nov., *Int. J. Syst. Evol. Microbiol.*, 2004, vol. 54, pp. 1677–1681.

25. Coenye, T., Laevens, S., Willems, A., Ohlen, M., Hannant, W., Govan, J.R.W., Gillis, M., Falsen, E., and Vandamme, P., *Burkholderia fungorum* sp. nov. and *Burkholderia caledonica* sp. nov., Two New Species Isolated from the Environment, Animals and Human Clinical Samples, *Int. J. Syst. Evol. Microbiol.*, 2001, vol. 51, pp. 1099–1107.

26. Sessitsch, A., Coenye, T., Sturz, A.V., Vandamme, P., Ait Barka, E., Salles, J.F., Van Elsas, J.D., Faure, D., Reiter, B., Glick, B.R., Wang-Pruski, G., and Nowak, J. *Burkholderia phytofirmans* sp. nov., a Novel Plant-Associated Bacterium with Plant-Beneficial Properties, *Int. J. Syst. Evol. Microbiol.*, 2005, vol. 55, pp. 1187–1192.

27. Vandamme, P., Opelt, K., Knöchel, N., Berg, C., Schönmann, S., De Brandt, E., Eberl, L., Falsen, E., and Berg, G., *Burkholderia briophyla* sp. nov. and *Burkholderia megapolitana* sp. nov., Moss-Associated Species with Antifungal and Plant-Growth-Promoting Properties, *Int. J. Syst. Evol. Microbiol.*, 2007, vol. 57, pp. 2228–2235.

28. Clarridge, III, J.E., Impact of 16S rRNA Gene Sequence Analysis for Identification of Bacteria on Clinical Microbiology and Infection Diseases, *Clin. Microbiol. Rev.*, 2004, vol. 17, pp. 840–862.

29. Salles, J.F., Samyn, E., Vandamme, P., Van Veen, J.A., and Van Elsas, J.D., Changes in Agricultural Management Drive the Diversity of *Burkholderia* Species Isolated from Soil on PCAT Medium, *Soil Boil. Biochem.*, 2006, vol. 38, pp. 661–673.