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EXPERIMENTAL ARTICLES

Cellulose Decomposition under Nitrogen Deficiency by Bacteria Isolated from the Intestines of Phytophagous Vertebrates

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Abstract—A nitrogen-fixing strain identified as *Klebsiella pneumonia* 402-2 and two endoglucanase-synthesizing *Bacillus* strains were isolated from the intestines of phytophagous animals. One of the *Bacillus* strains was identified as *Bacillus subtilis* GL. *Klebsiella pneumoniae* 402-2 increased the endoglucanase activity of both *Bacillus* strains in mixed cultures.The data on the taxonomic position of strains 402-2 and GL and on the nitrogen-fixing capacity of strain 402-2 were confirmed by sequencing and analyzing their 16S rRNA genes and by amplifying the nitrogenase gene *nifH*.

Key words: bacteria, *Klebsiella, Bacillus,* endoglucanase activity, nitrogen fixation, nitrogenase activity, mixed cultures, identification, 16S rRNA genes, *nifH* genes.

Carbohydrates account for the bulk of the nutrients contained in the plant feed of phytophagous animals. Among these carbohydrates, cellulose represents one of the most important components that undergo anaerobic degradation by the enzymes of the microorganisms inhabiting the alimentary canal. The development of bacteria in the intestines of phytophagous vertebrates is believed to be limited by the availability of utilizable carbon and nitrogen compounds [1]. The carbon requirements of microorganisms are met at the expense of the carbohydrates contained in the feed. The efficiency of the digestion process largely depends on cellulolytic bacteria. The supply of exogenous nitrogen (mainly in the form of proteins of the feed) varies significantly depending on the ration of the animal. Of considerable theoretical interest is the microbial decomposition of cellulose by phytophagous animals that feed on the vegetative parts of plants with a low nitrogen content [2]. Microbial nitrogen fixation is considered a possible mechanism of the formation of nitrogen compounds utilizable by bacteria in the intestines of herbivores. Data have been published on the detection of nitrogenase activity in the bovine rumen. In the moose rumen, nitrogen-fixing activity was detected only in summer, and it disappeared in winter. This activity was attributed to transit nitrogen-fixing microflora that enters the moose rumen in summer with the feed [3]. Based on the data available in the literature, the alimentary canal of some rodents contains bacterial species capable of nitrogen fixation [4]; however, no direct attempts to detect nitrogen fixation have yet been made.

In this work, we isolated a mixed culture possessing nitrogenase and endoglucanase activities and consisting of *Klebsiella* and *Bacillus* representatives (based on the results of preliminary identification) from the cecum of the common vole *Microtus arvalis*, a herbivorous rodent [5]. A monoculture tentatively assigned to the genus *Bacillus* and forming endoglucanase under nitrogen deficiency was isolated from the capercaillie cecum in winter, when capercaillies feed on pine needles.

The goal of this work was to elucidate the physiological and biochemical properties of the isolated cellulolytic and nitrogen-fixing bacteria, identify their strains, and assess the influence of *Klebsiella* sp. on the endoglucanase activity of *Bacillus* representatives.

MATERIALS AND METHODS

Microorganisms and cultivation media. Bacterial strain no. 1 (402-2), tentatively assigned to the genus *Klebsiella,* was isolated from the cecum of the common

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Table 1. Nitrogenase and endoglucanase activities of strains nos. 1–3

Composition of the microbial association	Medium	Nitrogenase activity, nmol $C_2H_2/(10^9$ cells h)	Endoglucanase activity, μmol/(min ml)
Strain no. 1	Ashby + sucrose	99.8	0
Strain no. 2	Ashby + Na-CMC	0	3.31
Strain no. 3	Ashby + Na-CMC	0	4.97
Strain no. 2 + strain no. 1	Ashby + Na-CMC	ND	4.52
Strain no. 3 + strain no. 1	Ashby + Na-CMC	ND	5.41

Note: ND stands for "not determined"; CMC signifies carboxymethylcellulose.

vole *Microtus arvalis*. Strain no. 2, tentatively assigned to the genus *Bacillus* was isolated from the same source. Another *Bacillus*, strain, no. 3, was isolated from the cecum of the capercaillie *Tetrao urogallus* Linnaeus, 1758. The composition of the medium used to isolate a mixed *Bacillus* sp. + *Klebsiella* sp. culture and to cultivate bacteria of the genus *Bacillus* was as follows (g/l tap water): glucose, 1.0; Na-carboxymethylcellulose, 10.0 (or shreded filter paper, 5.0); K_2HPO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; NaCl, 0.1. Bacteria of the genus *Klebsiella* were cultivated on the medium for *Azotobacter* [6] and on the Ashby medium with sucrose [7].

Morphological and physiological properties of the strains. We examined cell morphology and counted cell numbers under a Zeiss Axioskop microscope at a magnification of 1000 × using a KS300 computer-based system of image analysis (Germany). Fixed specimens were stained with carbolic gentian violet.

Endoglucanase activity was determined viscosimetrically [8]. Nitrogenase activity was determined by the acetylene method [9] under anaerobic conditions in liquid Ashby medium. Ethylene was determined using a Chrom-41 gas chromatograph equipped with a flame ionization detector; a 2-m column packed with Spherosil; the carrier gas was argon at a pressure of 2 atmospheres.

A special experiment was conducted to investigate the activities of the tested strains at various nitrogen contents. We prepared medium variants with consecutively increasing nitrogen contents. Liquid medium containing (g/l) tap water Na-carboxymethylcellulose, 10.0 (or shredded filter paper, 5.0); K_2HPO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; and NaCl, 0.1 was supplemented with $(NH_4)_2HPO_4$ at concentrations of 0.0, 0.1, 1.0, and 1.5 g/l. Accordingly, these variants were designated as "0", "0.1", "1.0", and "1.5". The pH value of the medium was adjusted to 7.1 upon sterilization. A thick (35-ml) medium layer was formed in test tubes, and different variants of one-day-old inocula (equilibrated in terms of their cell numbers) were introduced into the test tubes in an amount of 1%. The inoculum variants were either cell suspensions of one of the three isolated strains (nos. 1–3) or their combinations (no. 1 + no. 2 and no. 1 + no. 3). The cultivation was carried out at 37°C for two days. Thereupon, we determined the pH value of the medium, the cell number, and endoglucanase activity.

The statistical treatment of the data obtained was performed using Microsoft Excel 5.0 software package.

Isolation and characterization of total DNA. DNA isolation for amplification in polymerase chain reaction (PCR) was carried out according to Miniprep protocol (Promega, United States) with minor modifications. The preparations obtained had DNA concentrations of 5–7 μg/ml and trace amounts of RNA (below 1% according to electrophoretic data).

Amplification of *nifH* gene fragments. *nifH* gene fragments were amplified using the earlier described [10] primer pair F1 (5'-TAYGGIAARGGIGGIATYGGIAARTC-3') and R6 (5'-TCIGGIGARATGATGGC-

Table 2. Endoglucanase activity of two-day-old cells of strains no. 2 and no. 3 as dependent on the nitrogen content of the medium* and the presence of strain no. 1

Variant*	Number of *Bacillus* cells** per ml, $\times 10^4$				Final pH				Endoglucanase activity, μmol/(min l)			
	Strain				Strain				Strain			
	no. 2	no. 2 + no. 1	no. 3	no. 3 + no. 1	no. 2	no. 2 + no. 1	no. 3	no. 3 + no. 1	no. 2	no. 2	no. 2 + no. 1	no. 3
0	62.4	83.7	71.8	86.3	6.15	5.72	6.02	5.62	1.64	2.21	2.72	3.51
0.1	–	–	94.5	–	–	–	6.01	–	–	–	2.69	–
1.0	210.3	274.1	281.1	328.7	6.08	5.66	5.65	5.57	2.63	3.48	3.27	3.93
1.5	–	–	298.5	–	–	–	5.60	–	–	–	2.88	–

* See the Materials and Methods section for the description of the experiment and the variants of the medium.
** The *Bacillus*/*Klebsiella* cell number ratio was 1 : 1.

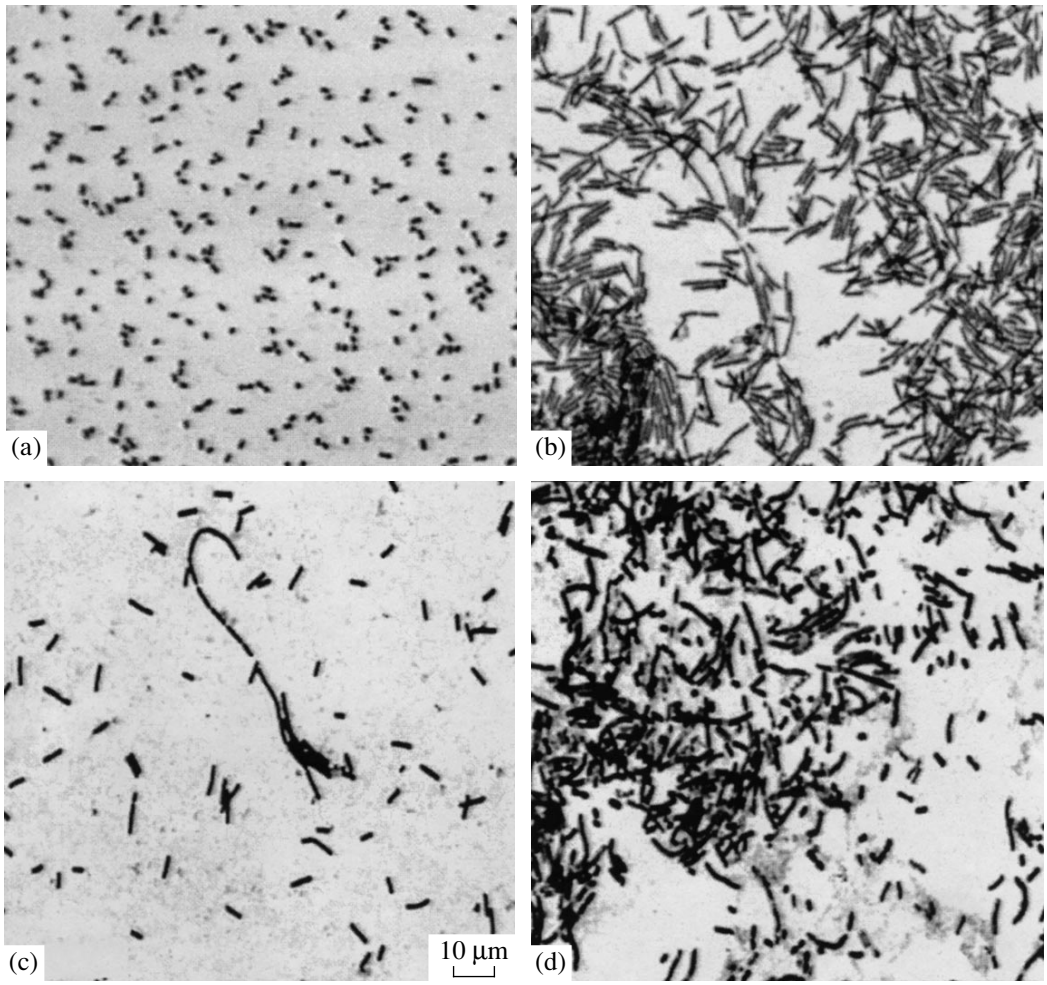

Fig. 1. Computer images of stained preparations:(a) *Klebsiella pneumoniae* 402-2 grown on Ashby medium with sucrose; (b) *Bacillus subtilis* GL grown on glucose–peptone medium; (c) *Bacillus subtilis* GL grown on Ashby medium with Na-carboxymethylcellulose; (d) a mixed culture of *Bacillus subtilis* and *Klebsiella pneumoniae* 402-2 grown on glucose–peptone medium. Fixed smears from one-day-old cultures were stained with carbolic gentian violet. Magnification, 900×.

3'), where I is inositol, R = A or G, Y = T or C. A Cetus 480 device (Perkin Elmers, Sweden) was employed. The volume of the amplification mixture was 20 μl, and its composition was as follows: 200 μM dNTP, 200 ng template DNA, 17 mM $(NH_4)SO_4$, 6 mM Tris–HCl (pH 8.8), 2 mM $MgCl_2$, 5 mM forward primer, 5 mM reverse primer, and 5 units of Biotaq DNA polymerase (Dialat Ltd, Russia). The following temperature regime was used for amplification: 94°C, 3 min, 50°C, 3 min, and 72°C, 3 min for the first cycle; 94°C, 30 s, 50°C, 2 min, and 72°C, 30 s for the five subsequent cycles; and 94°C, 30 s, 40°C, 30 s, and 72°C, 30 s for the last 30 cycles. Thereupon, the mixture was incubated for 7 min at 72°C. The analysis of the PCR products obtained involved their electrophoresis in 1% agarose gel containing ethidium bromide.

Amplification, sequencing, and analysis of the 16S rDNA sequences. The 16S rRNA genes were amplified and sequenced using universal primers efficient for most prokaryotes [11]. The following buffer was used for amplification: 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), and 0.001% gelatine. The volume of the reaction mixtures was 100 μl, and they contained standard dNTP concentrations and equimolar quantities of the pA and pH' primers. PCR involved 30 amplification cycles with the following temperature regime: 94°C, 30 s, for DNA denaturation; 40°C, 1 min, for primer annealing; and 72°C, 2 min 30 s, for elongation. Upon purification on low gelling temperature agarose and Promega columns, the 16S rRNA gene was sequenced in both directions using forward and reverse universal primers and Sequenase (Biochemicals, Cleveland, Ohio, USA).

The primary search for nucleotide sequences similar to those of the 16S rRNA genes of the strains under study was carried out using the BLASTA server. Then the 165 rDNA sequences of our isolates were aligned with those of closely related bacterial species by means of the CLUSALX software. Rootless phylogenetic

trees were constructed using the methods implemented in the TREECONW software package [15].

Depositon of the sequences. The sequenced 16S rRNA genes of the strains *Klebsiella pneumoniae* 402-2 and *Bacillus subtilis* GL were deposited with the GenBank (accession numbers, AY114159 and AY114158, respectively).

RESULTS

Nitrogen fixation and cellulolytic capacity of the isolates. Our research revealed that the bacterial strain of the genus *Klebsiella* exhibited a nitrogenase activity of up to 100 nmol $C_2H_4/(10^9$ cells h) in liquid Ashby medium with sucrose (Table 1).

The bacterial strains assigned to the genus *Bacillus* lacked nitrogenase activity. We observed the growth of these bacteria and detected endoglucanase activity on Ashby medium with Na-carboxymethylcellulose and yeast extract. This activity was enhanced upon cocultivation with *Klebsiella* (Table 1).

Both *Bacillus* strains were capable of weak growth without yeast extract, using the sodium salt of carboxymethylcellulose as the carbon source (Table 2) and forming endoglucanase. The activity of this enzyme in the culture liquid of strain no. 2 was 1.7 times lower than that in the culture liquid of strain no. 3.

Cocultivation of strains no. 2 and no. 3 with strain no. 1 caused a more intense acidification of the medium and an increase in the endoglucanase activity of both *Bacillus* strains. These effects were somewhat more pronounced in the case of strain no. 2: cocultivation with *Klebsiella* resulted in a 25.8 and a 22.5% increase in the endoglucanase activity of strain no. 2 and no. 3, respectively. The pH value of the culture liquid decreased by 0.43 and 0.37 units for strain no. 2 and no. 3, respectively. Both the initial and the final ratio between *Bacillus* and *Klebsiella* cells was 1 : 1 for both *Bacillus* strains. The addition of ammonium nitrogen to the original medium caused an increase in cell growth in strains no. 2 and no. 3 and a decrease in the specific endoglucanase activity. At most, endoglucanase activity increased only 1.6-fold, whereas the cell numbers increased at least 3.5-fold.

From the data of Tables 1 and 2 it follows that of the two endoglucanase-producing strains preliminarily placed in the *Bacillus* genus, strain no. 3 was somewhat more active. Therefore, we used only strains no. 1 and no. 3 in our further studies concerned with the cultural, morphological, and genotypic properties of the isolates.

Description of the cultural and morphological properties of the isolates. Strain no. 1 (402-2) is preliminarily classified under the genus *Klebsiella*: Cells are nonmotile facultatively anaerobic gram-negative rods with capsules, $0.3–0.7 \times 1–2.5$ μm. Cells are solitary or arranged in pairs (Fig. 1a). Growth at 44°C is possible. D-glucose is catabolized with the formation of acid and gas. Cells are oxidase-negative, catalase-positive, lysine decarboxylase–positive, ornithine and arginine decarboxylase–negative. Good growth occurs on nitrogen-free media. Convex mucous colonies are formed on solid Ashby medium. Red glittering colonies are formed on Endo medium. Atmospheric nitrogen is fixed.

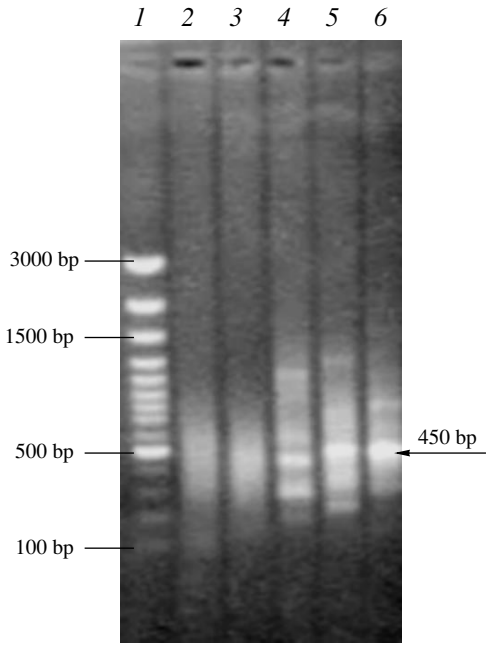

Fig. 2. Amplification of the nitrogenase genes (*nifH*). (*1*) Molecular weight marker GeneRuler 100 bp, MBI Fermentas, #SM0321; (*2*) PCR in the absence of DNA template (a control for the quality of the reaction mixture); (*3*) PCR with the DNA preparation from *E. coli*, strain DH5a (negative control); (*4*) PCR with the DNA preparation from *Klebsiella pneumoniae*, strain 402-2; (*5*) PCR with the DNA preparation from *Bacillus subtilis* GL; (*6*) PCR with the DNA preparation from *Azotobacter vinelandii*, strain 59 (positive control). The arrow on the right indicates the position of the PCR product that corresponds to the *nifH* gene. Arrows on the right indicate components of the DNA molecular weight marker having particular sizes.

Strain no. 3 (GL) is preliminarily classified under the genus *Bacillus*: Facultatively anaerobic spore-forming rods, straight or slightly curved, $0.3–0.5 \times 3.0–7.0$ μm. Filaments of up to 20 μm in length and chains are occasionally formed (Fig. 1b). Cells are gram-positive and catalase-positive. Spores are oval and subterminal. Sporangia are not swollen. Colonies on nutrient agar are dry with wavy edges and occasional creases straw-yellow in color. Endoglucanase is produced, suggesting the involvement of the bacterium in the initial stages of degradation of various plant materials. Growth at 45–50°C is possible. Vitamins are not required. Growth is possible on Ashby medium for *Azotobacter* with sucrose or 1% Na-carboxymethylcellulose (Fig. 1c), on minimum medium for cellulolytic mesophiles with 2% carboxymethylcellulose, and on glucose–peptone medium of the following composition (g/l): glucose, 1.0; peptone, 2.0; yeast extract, 2.0; $CaCO_3$, 3.0; Na-car-

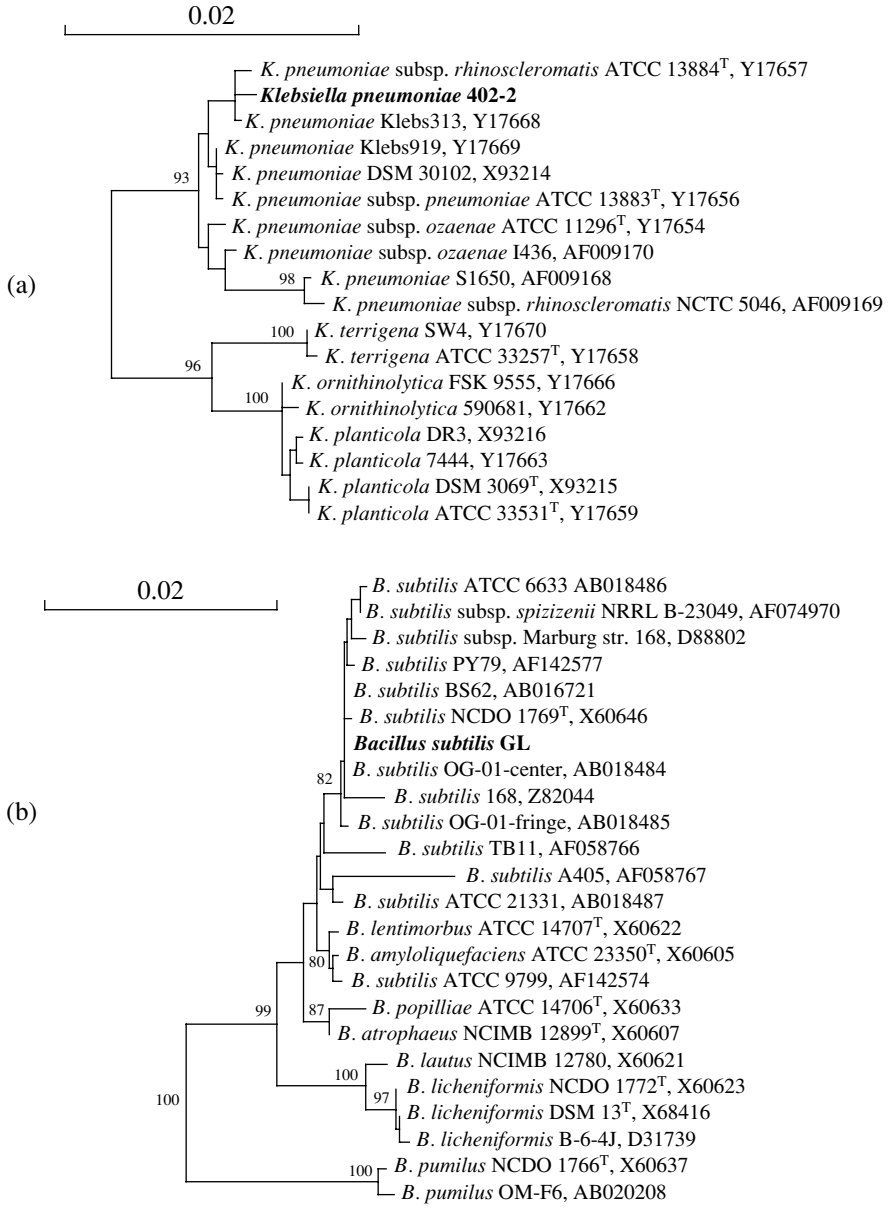

Fig. 3. Phylogenetic trees based on comparative analysis of rRNA genes.(a) Phylogenetic position of the strain *Klebsiella pneumoniae* 402-2; (b) phylogenetic position of the strain *Bacillus subtilis* GL. Bar shows evolutionary distance corresponding to two nucleotide substitutions per 100 nucleotides. The numerals are the estimates of the branching order significance as determined by bootstrap analysis of alternative tree paterns (values over 90 are considered significant).

boxymelthylcellulose, 5.0. Cellulose activity on this medium was at least 2.8 μmol/(min ml) at 45°C on the second day of cultivation.

On glucose-peptone medium (Fig. 1d) and on Ashby medium with Na-carboxymethylcellulose, strains no. 1 and no. 3 form mixed cultures that are balanced in terms of their cell numbers (1 : 1).

Both strains are nonpathogenic, as established at the All-Russia Institute for Veterinary Sanitation, Hygiene, and Ecology of the Russian Academy of Agricultural Sciences in accordance with the regulations adopted by the USSR Ministry of Health. Intraperitoneal administration of 0.5 ml of a suspension containing 10^{10} cells/ml caused no deviations of the physiological variables from their normal values in mice and no dissemination of the microorganisms in their internal organs.

Amplification of the *nifH* genes. To elucidate the genetic factors responsible for nitrogen fixation in our isolates, we amplified *nifH* gene fragments that code for the Fe-protein of the nitrogenase complex. We used *Escherichia coli* and *Azotobacter vinelandii* DNA as the negative and positive controls, respectively. We revealed PCR fragments of the expected length (450 bp, Fig. 2) in strain 402-2 but failed to detect an analogous fragment in strain GL.

Sequence analysis of the 16S rRNA genes. We determined partial sequences (about 1450 nucleotides) of the 16S rDNAs of strains 402-2 and GL. These sequences approximately corresponded to positions 35–1470 in terms of *E. coli* numbering.

Our analysis revealed that strain 402-2 belongs to the phylogenetic group of enterobacteria, specifically, to the genus *Klebsiella* (Fig. 3a). The strain formed part of the phylogenetic cluster composed of *K. pneumoniae* strains that were present in the database (including the type strains of all of the subspecies of this species). The degree of similarity between the 16S rRNAs of strain 402-2 and the strains of *K. pneumoniae* was 99.0–99.8%. This degree corresponded to the intraspecies level of similarity characteristic of this species (99.0–100%).

Analogous studies demonstrated that strain GL belonged to bacilli and, more precisely, to the phylogenetic subgroup of *B. subtilis* (Fig. 3b), containing *B. subtilis* strains, including the type strain of this species. The degree of similarity between the 16S rRNA of strain GL and *B. subtilis* strains contained in this cluster was 98.6–100%. This difference level is comparable with the sequencing error (e.g., replicate sequencing of the 16S rDNA of strain 168 produced sequences differing by 0.2%), and it corresponds to the intraspecies level for *B. subtilis* (98.3–100%).

Based on current taxonomy [13], the similarity degree between 16S rRNA sequences determined in our study enables us to classify strains 402-2 and GL under the species *K. pneumoniae* and *B. subtilis*, respectively.

DISCUSSION

Classification of the active nitrogen-fixing strain 402-2 from the intestines of the common vole under the species *K. pneumoniae* contributes to our understanding of the ecology of this species. Its presence in the intestines is of considerable interest in terms of the nitrogen-fixation process in the intestines of phytophagous vertebrates. The nitrogen-fixing *Klebsiella* strain can enhance the endoglucanase activity of bacilli in mixed cultures. This points to a possible trophic relationship between nitrogen fixation and cellulose hydrolysis in the animal organism.

Initially we suggested that strain GL, a *Bacillus* strain possessing endoglucanase activity and developing on nitrogen-minimized media, is also capable of nitrogen fixation. However, our data on the absence of nitrogenase activity and of the *nifH* genes in this strain disproved this supposition and supported the current idea that, among bacilli, only representatives of the genus *Paenibacillus* are capable of nitrogen fixation [14]. Nevertheless, the capacity of the endoglucanase-possessing *B. subtilis* strain GL to develop at low nitrogen concentrations may promote its survival and active functioning in the alimentary canal of phytophagous animals. The results obtained in this work provide the foundations for the development of a biologically active additive to the feed of agriculturally important animals [15, 16].

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