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BIOCHEMISTRY, BIOPHYSICS AND MOLECULAR BIOLOGY

Molecular Analysis of the Intestinal Microbiome Composition of Mammoth and Woolly Rhinoceros

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The community of microorganisms (microbiome) of the gastrointestinal tract plays the key role in digestion and, thus, is an important factor that determines the health of the animal as a whole. In particular, a microbial community is supported in the gastrointestinal tract of herbivores that provides a rapid digestion of plant polysaccharides under anaerobic conditions [1]. The coevolution of herbivorous mammals and their gut microbiome was aimed at increasing the volume of the stomach and/or various parts of the intestine, which ensured an increased time of digestion and the selection of corresponding microbial communities [2]. The comparison of the intestinal microbe of modern animals and their extinct ancestors can provide information regarding their food resources and the pathways of evolution of microbial communities. We investigated the intestinal microbiome composition of two extinct animals—mammoths (*Mammuthus primigenius*) and the woolly rhinoceros (*Coelodonta antiquitatis*). The findings of samples preserved in the permafrost are extremely rare and the molecular analysis of the microbe has not yet been performed. The results show that the microbiome of the woolly rhinoceros was dominated by cellulolytic clostridia, and the microbiome of the mammoth was dominated by the members of the family Pseudomonadaceae, which reflects the nutritional habits of these animals.

The young mammoth, named Lyuba, were discovered on the Yamal peninsula in Western Siberia in 2007 [3]. Although the geological age of the finding is about 40 thousand years, this is the most well-preserved sample of a mammoth. Luba was a milk calf, her intestinal tract contained milk and fecal material, probably of her mother. [4] The sample of an adult female woolly rhinoceros was found in 2007 near the village Cherskii in Eastern Siberia [5]. Her intestine contained partially digested plant material of a complex composition. DNA samples were isolated from the intestinal contents by the method for isolating DNA from soil, which was developed by us earlier [6].

To analyze the composition of the microbial communities, we used the method based on pyrosequencing the fragments of 16S ribosomal RNA genes [7]. PCR fragments of 16S rRNA genes were obtained using the "universal" primers 11F (5'-GTTTGATCMTGGCTCAG-3') and 519R (5'-GWATTACCGCGGCKGCTG-3') and sequenced in the GS FLX instrument (Roche) using the Titanium protocol. As a result, we obtained 7242 sequences over 300 bp long for the mammoth microbiome sample and 11301 sequences for the woolly rhinoceros microbiome sample.

Data were analyzed using the RPD Classifier [8] and MOTHUR [9] software packages. The initial taxonomic classification of the sequences was performed using the RDP classifier (http://rdp.cme.msu.edu/classifier/ classifier.jsp) (table). Then, cluster analysis of sequences was performed using the MOTHUR software. The taxonomic identification of the sequences representing the cluster was performed by comparing them with the nucleotide sequences deposited in the GenBank database using the BLASTN software. The taxonomic classification of the sequences representing the clostridia, was refined by constructing and analyzing the phylogenetic trees, including the representative sequences of clusters and the set of 16S rRNA gene sequences of the known Clostridiales members.

In the intestinal microbiome of the woolly rhinoceros, we identified representatives of the five types of bacteria (Fig. 1): Firmicutes (68% of all sequences), Proteobacteria (19.2%), Actinobacteria (5.7%), TM7 (4.3%), and Bacteroidetes (0.2%). Archaea were not detected. The most numerous type, Firmicutes, was mainly represented by various *Clostridium* lines (Fig. 2). The most numerous group, which includes about half

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The composition of intestinal microbe of woolly rhinoceros and mammoth

Taxonomic classification of sequences[1]			Number of sequences	
type	class	family	woolly rhinoceros	mammoth
Bacteroidetes			25	33
Actinobacteria	Actinobacteria	Micrococcaceae	104	1024
		Microbacteriaceae	411	185
		Other[2]	132	101
TM7			483	1
Proteobacteria	Alpha-proteobacteria	Bradyrhizobiaceae	28	198
		Phyllobacteriaceae	38	122
		Brucellaceae	893	1
		Other[2]	93	86
	Beta-proteobacteria		12	41
	Gamma-proteobacteria	Pseudomonadaceae	912	5083
		Moraxellaceae	80	24
		Other[2]	70	108
	Other		40	102
Firmicutes	Bacilli	Leuconostocaceae	334	0
		Streptococcaceae	196	0
		Other[2]	19	3
	Clostridia	Lachnospiraceae	125	0
		Peptostreptococcaceae	253	0
		Clostridiaceae	6488	13
		Other[2]	211	1
	Other[2]		64	4
Other[2]			290	112
Total			**11301**	**7242**

[1] Taxonomic classification using the RDP Classifier, accuracy over 70%.
[2] Other—unclassified.

of all sequences referred to the family Clostridiaceae (cluster *1* in Fig. 2), is close to the cellulolytic bacterium *Clostridium longisporum*, which was previously detected in the stomach of herbivores [10]. Another "saccharolytic" line, which is close to *Clostridium beijerinckii*, includes about 8% of the sequences (cluster *3* in Fig. 2). About one-forth of the *Clostridium* sequences were clustered with the 16S rRNA of

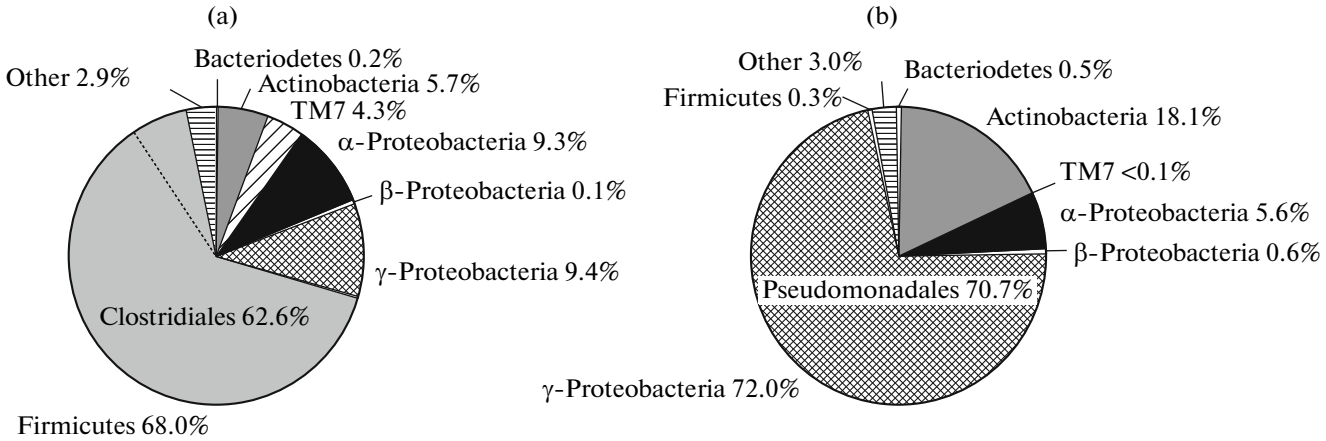

Fig. 1. The composition of the intestinal microbial communities of (a) woolly rhinoceros and (b) mammoth Lyuba.

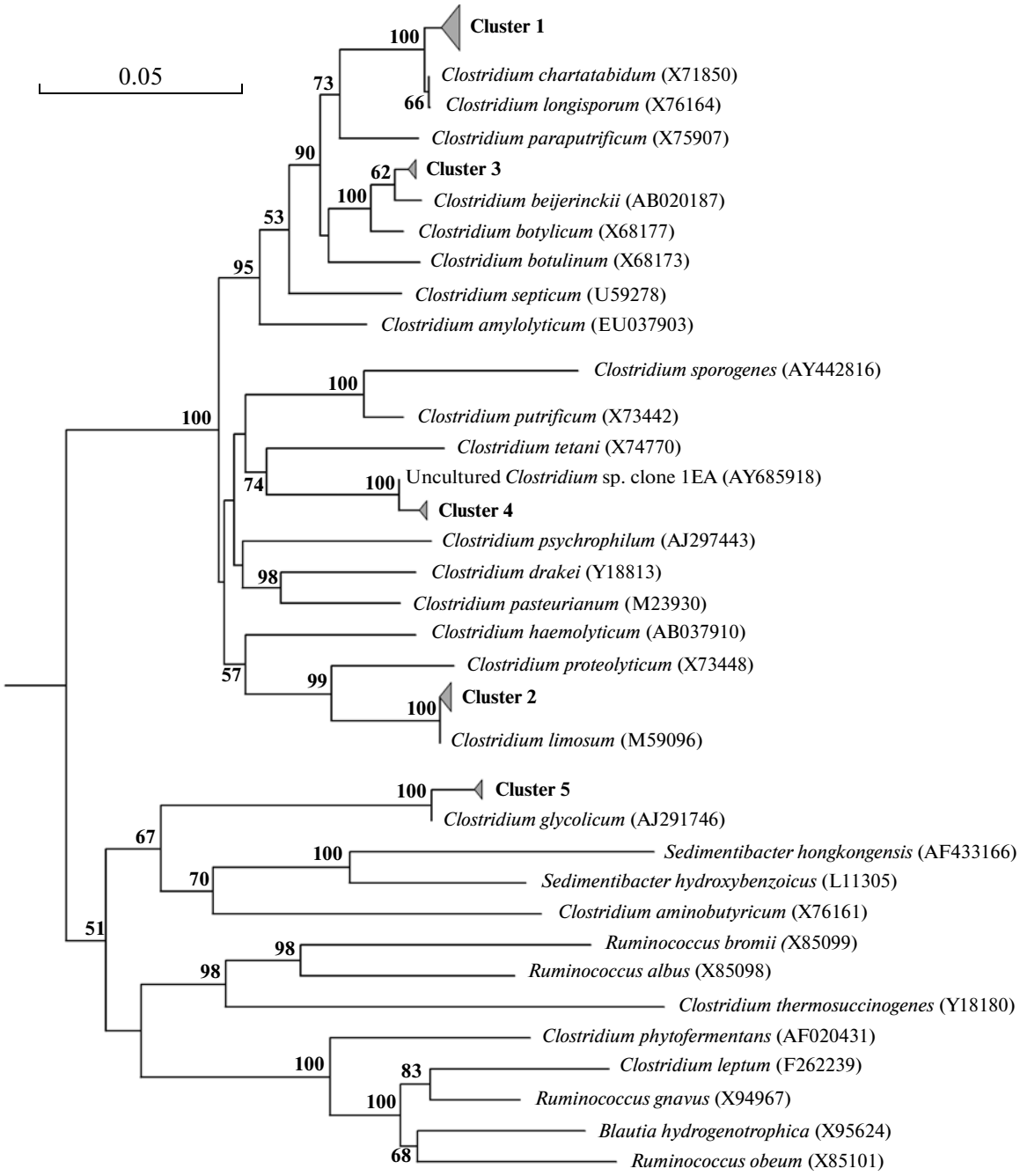

Fig. 2. Position of the main phylotypes of bacteria of families Clostridiaceae (designated as clusters *1*–*5*) on the phylogenetic tree of 16S rRNA sequences of representatives of Clostridia. The sequences were aligned using the CLUSTALX software; the phylogenetic tree was constructed by the neighbor-joining method using the TREECON software. The 16S rRNA sequence of *Aquifex pyrophilus* was used as an outgroup. The numerals on the branches indicate the bootstrap support (100 replicas), only the values not less than 50% are shown. The scale corresponds to 0.05 nucleotide substitutions per position.

Clostridium limosum [11]. This proteolytic microorganism is found in soil but has been also isolated from tissues of animals suffering from various infections. It should be emphasized that the *Ruminicoccus* and *Selenomonas* genera, which are most often isolated from the gastrointestinal tract of ruminant and non-ruminant herbivores [12], were not detected. Another feature of the microbiome of the woolly rhinoceros was the low abundance of representatives of *Bacteriodetes*, which usually ranks second after *Firmicutes* in the intestinal microbiome of herbivores [13], including the modern black and Indian rhinoceros [2]. The suf-

ficiently high proportion of bacteria of the phylum TM7 (4.3%) is also unusual. Clones with similar 16S RNA sequences were found in cellulose-containing wastes [14], suggesting that TM7-type bacteria may play an important role in the degradation of polysaccharides in the gut of animals.

The intestinal microbiome of mammoth Lyuba had a completely different composition. The vast majority of bacteria were representatives of two types: Proteobacteria (81% of all clones) and Actinobacteria (18%), while the proportion of Firmicutes, TM7, and Bacteriodetes accounted for less than 0.5% of all sequences (Fig. 1). The majority of microorganisms represented the family Pseudomonadaceae—a widespread group of gamma-Proteobacteria with diverse metabolism. Although the representatives of *Pseudomonas* are not the dominant group in the intestinal microbiome of animals, some species cause souring of dairy products [15]. The presence of undigested milk in Luba's stomach and intestines can be explained by the dominance of *Pseudomonas* in the microbiome. Another feature of Luba's microbiome was an almost complete absence of representatives of *Firmicutes*, constituting the bulk of intestinal microbiome of the woolly rhinoceros, which may also reflect the differences in the diet of these animals (milk vs. plant biomass).

In general, our data provide initial idea on the composition of the intestinal microbiome of the "megafauna" of Pleistocene. Some groups of bacteria that are characteristic of modern herbivores were also found in the microbiome of the woolly rhinoceros, whereas some important groups (e.g., *Ruminicoccus* spp.) were missing. Probably, the occurrence and widespread distribution of such specialized bacteria took place at the later stages of evolution of herbivorous and/or was associated with human activities. It is also possible that these differences reflect the specific characteristics of the diet of the woolly rhinoceros. For example, the fraction of *Ruminicoccus* spp. in the intestines of the sheep that consumed food rich in starch was higher than in the sheep that fed on grass [12]. Analysis of a larger number of microbe samples of animal preserved in permafrost will help to answer these questions. Another factor that should be taken into account in the analysis of such microbes is the possibility of selective growth of certain groups of microorganisms after the death of animals, which might explain the high proportion of *Pseudomonas* spp. in the intestinal microbiome of mammoth Lyuba.

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