Bacteria revived from an ancient bison gut

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Prokaryotic organisms are able to survive extreme conditions, which can be by all means considered as unsuitable for living or even deadly, and microbes from the permafrost are of special interest (Gilichinsky et al., 2007). Thousands of years of conservation in an unfavorable ambience at subzero temperatures not only allow for the microbial viability, but also form a unique background for an original "bank" of prehistoric genomes. The icy environment promotes nearly a total abolishment of cell division in most types of microbes, which is perfect for maintenance of the genomic sequence. The analysis of such genomes involving the tools and approaches of modern genomics enriches our knowledge on the life potential of the cell, helping to clarify the questions of phylogeny.

Cryobilogy of microorganisms from permafrost soils, including their characterization, is a well-developed direction of research (Gilichinsky et al., 2007; Hinsa-Leasure et al., 2010). However, the microflora of ancient animals is poorly studied, since in most cases the retrieved animals have poor preservation, making it nearly impossible to discriminate between the intrinsic and environmental microbes. Moreover, the chance to reveal the microbes naturally existing in animal organisms in soil samples is quite low, due to non-optimal environment and the competition for resources with other, more fitted species.

That is why the discovery of a mummified *Bison priscus* Bojanus with well-preserved organs in the permafrost of the Siberia in 2009 opened a great possibility for studying representatives of the fossil intestinal microbiome as a part of the ANBIS project (Nikolskiy & Shidlovsky, 2014). The specimen is approximately 50000 YBP, and being kept unthawed (Nikolskiy & Shidlovsky, 2014) for the whole storage period, is a promising source of a variety of revivable single-cell organisms. Considering the results of the ongoing experiment of Richard Lenski for maintaining some *E.coli* cultures (Raeside et al., 2014) for already more than 25 years, we have a good possibility to register multiple changes accumulated

during this time period and assess their functional significance, when comparing an ancient genome with its modern relative from a living bacterium. Thus the general task of the study is to make a comparative analysis of permafrost-derived bacterial genomes.

Excrement samples from the small intestine were taken at -21^{o}C and kept frozen in sterile flasks. Fecal pieces were placed in different nutritional media (LB, laurel tryptose broth or R2A), incubated at various temperatures (4, 22 and 37^{o}C) and plated on agar. The 16S rRNA gene sequences of the purified cultures of the obtained bacteria were amplified from the genomic DNA using standard primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACTT-3') on a thermal cycler DT-322 (DNA Technology, Russia) in the following mode: 3 minutes of preheating at 95°C and 30 cycles of 95°C 45 sec (DNA melting), 55°C 60 sec (primer annealing), 72°C 90 sec (DNA synthesis). The synthesized DNA fragments were visualized in 1.5% agarose and purified using Cleanup Mini kit from Evrogen according to the protocol of the manufacturer. Consequently they were sequenced with the same 27F and 1492R primers and the resulting nucleotide sequences assembled from two complementary parts were analyzed by BLAST. The nucleotide sequences of related bacteria were picked out from the database to build a phylogenetic tree with MEGA 5.22 (Tamura et al., 2011) using neighbor-joining statistical method.

To date we were able to revive 15 individual microbial strains, according to the results of the 16S rRNA gene sequencing. They could have been part of the native gut flora of the animal, but not all of them are expected to be constant residents of the small intestine, and could have come with food and water. Among those are *Okibacterium* sp., *Paenibacillus* sp., *Leucobacter* sp., *Acinetobacter* sp., *Carnobacterium* sp., *Bacillus* spp. etc. The homology of the 16S rRNA gene sequence was estimated to be 99-100%. In the last decades the significance threshold for the homology level was re-estimated, and the identity of as high as 99% can reflect a novel species (Kim et al., 2014). For some genera the conservation of this reference gene is so high, that it can be up to 99.8 or even 100% identical between different species, as in the case of the *rrs* gene of the *Edwardsiella* or *Bacillus* bacteria (Janda & Abbott, 2007; Fox et al., 1992).

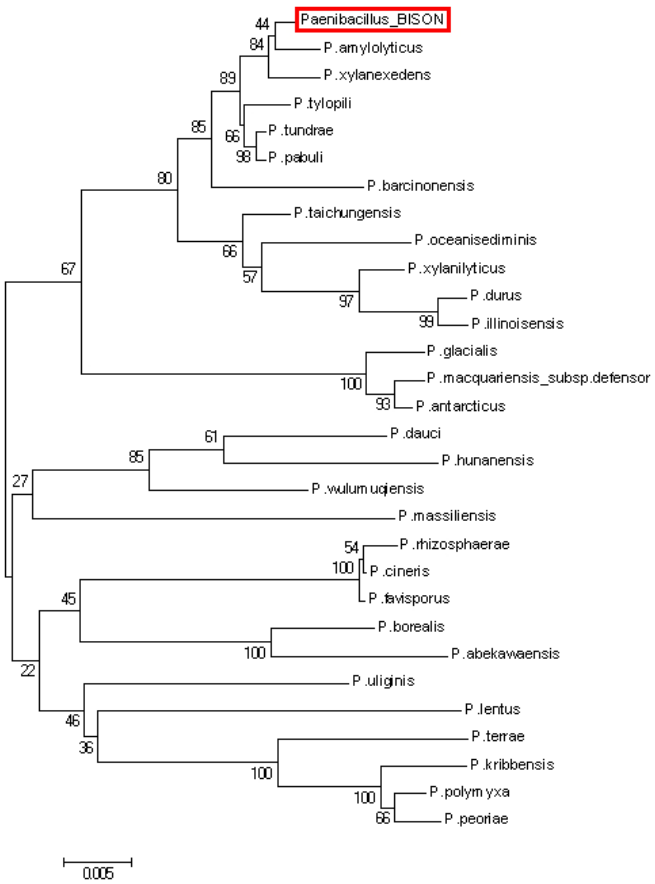

Figure 1. Bootstrapped phylogenetic 16S rRNA gene tree representing the isolated *Paenibacillus* bacterium and the relative species; constructed by neighbor-joining.

The results of sequencing assume that one of the most promising of the recovered bacteria belongs to the *Paenibacillus* genus, having 12 substitutions as compared to the most related sequence of *P.xylanexedens* (1529/1541 nucleotides). However, preliminary phylogenetic assessment using a considerable set composed of the available 16S rRNA gene sequences from the *Paenibacillus* species does not appear to be very promising (Fig. 1). At the same time, another bacterium belonging to the *Leucobacter* genus, with only 8 substitutions against the closest *L.aridicollis* (1429/1437) occupies a separate branch, denoting that these two homologous sequences are of different ancestral origin (Fig. 2).

This observation testifies the necessity of supporting results for a thorough analysis. It is expected that the phylogeny will be improved upon consideration of conservation for specific housekeeping genes (Volokhov et al., 2007). Analysis of whole-genome DNA

polymorphism might be also helpful in establishing most suitable reference sequences (Bohle and Gabaldon, 2012). For this purpose we are currently obtaining the complete genome sequences for several revived bacteria, which are to be analyzed in the near future, hopefully providing an insight into the bacterial genome evolution.

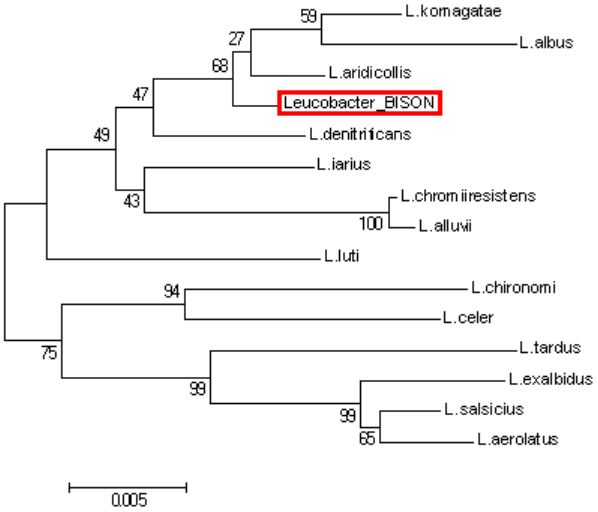

Figure 2. Bootstrapped phylogenetic 16S rRNA gene tree representing the isolated *Leucobacter* bacterium and the relative species; constructed by neighbor-joining.

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