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New thermophilic prokaryotes with hydrolytic activities

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Thermophilic microorganisms are capable of growing on polymeric substrates and have been intensively studied for their enzymes, thermostable hydrolases (glycosidases, proteinases, lipases), which have important applications in many fields of bioindustry: production of detergents, food processing, paper and textile industry, biofuel formation

from organic wastes, etc.[1]. The advantages of thermostable enzymes application are in their higher stability not only against temperature, but also against high or low pH, presence of detergents, etc. High temperature increases solubility of substrates[2], thus making them more available, and significantly decreases the contamination risks. Many highly stable hydrolases, produced by thermophilic bacteria and archaea have been discovered[3–6]; however, due to continuous industrial demand and our knowledge that natural environments are a significant reservoir of genetic and hence functional diversity[7], new thermophilic organisms producing hydrolytic enzymes are still of high interest. Here we present our achievements in isolation of novel thermophilic bacteria and archaea with various hydrolytic activities.

The representative of a new bacterial phylum *Ignavibacteriae* – *Melioribacter roseus* was isolated from the microbial biofilm developing at the outlet of an abandoned oil-exploration well in Western Siberia[8]. The well is 2750 m deep, reaching organic-rich Jurassic deposits, the so called Bazhenov Platform. In the water of the well diverse organotrophic thermophiles were found to be present which presumably degrade buried organic matter[9]. When recovered, those capable of growth by aerobic respiration formed a biofilm, degrading dissolved organic matter present in the water. *Melioribacter roseus* was found both in the well water and in biofilms. It is, to our knowledge, the first thermophilic facultatively anaerobic bacterium capable of degrading cellulose and its derivatives. The genome of *M. roseus* contains numerous genes of various glycosidases, glycosyl transferases, carbohydrate esterases and polysaccharide lyases that correlate with its ability to utilise diverse polysaccharides[10]. Some of these genes were heterologously expressed in *Escherichia coli* and characterised[11,12]. Due to the presence of numerous terminal oxidases, *M. roseus* can grow by aerobic or anaerobic respiration with nitrate, ferric iron, or arsenate as electron acceptors[8,13]. It is capable of utilising acetate and performing complete mineralisation of complex organic substrates in anaerobic conditions (for example, by iron respiration). The capacity to use various substrates including insoluble or poorly soluble polysaccharides and a number of electron acceptors including insoluble ones makes *M. roseus* an excellent candidate for utilisation in microbial fuel cells where the energy of organic wastes is converted to electricity.

Planctomycetes is a large and environmentally relevant group of bacteria found in almost all types of ecosystems. It is known that planctomycetes (with uncultivated anammox bacteria as an exception) perform aerobic degradation of organic matter including complex natural compounds such as polysaccharides and proteins, and have complex cell organisation, multistage life cycle and large genomes[14]. Sequences of 16S rRNA genes belonging to planctomycetes were often found in DNA samples from thermal environments, but no thermophilic planctomycetes were described until now. We isolated novel planctomycetes from the hot springs of Kuril Islands and Lake Baikal area, shallow water submarine vents of Italy, and a deep subsurface gold mine in South Africa[15–17]. Three new isolates, *Thermogutta terrifontis, Termogutta hypogea* and *Thermostilla marina*, multiply by budding and belong to class *Planctomycetia*, while the fourth, *Tepidisphaera mucosa*, multiplies by binary fusion and forms a new order *Tepidisphaerales* in class *Phycisphaerae*. Thus, at the moment thermophilic species are known for both classes of cultivated planctomycetes. All isolates are moderate thermophiles (growing optimally at 50–60°C) and facultative anaerobes (capable of growth by fermentation (all), nitrate or sulfur reduction (all excluding *T. mucosa*)). As mesophilic planctomycetes, thermophilic members of this group are able to grow on numerous polysaccharides, including most resistant ones (such as xanthan gum, xylan, etc). The genome of *T. terrifontis* R1 was found to contain genes encoding diverse hydrolases and lyases (our unpublished data). A thermostable esterase from *T. terrifontis* was heterologously expressed in *Escherichia coli* and characterised[3].

Most cultivated representatives of organotrophic hyperthermophilic and thermophilic archaea of two well-studied phyla, *Crenarchaeota* and *Euryarchaeota*, can grow with peptides and proteinaceous substrates[18], but much less of them are able to degrade polysaccharides. 16S rRNA genes analysis in *in situ* enrichments set in Kamchatka hot springs[19], showed that archaea efficiently compete with bacteria for polymeric substrates even at up to 70°C, temperatures considered favourable for bacteria. Similar archaeal 16S rRNA gene sequences were found in numerous Kamchatka hot springs[20] and helped to isolate a themophilic archaeon *Fervidicoccus fontis*, the first representative of a novel order *Fervidicoccales* in phylum *Crenarchaeota*[21,22]. Among terrestrial hyperthermophiles, the ability to grow on polymeric substrates is a specific feature of the representatives of the archaeal genera *Desulfurococcus* and *Thermogladius*, which belong to phylum *Crenarchaeota.* Though preferring peptides, some of these archaea can also utilise polysaccharides: different strains of *Desulfurococcus amylolyticus* grow on starch or cellulose[23–26]. The ability to grow on cellulose was also shown for *Thermogladius calderae*[27]. However, it should be noted that the genome analysis of the cellulotrophic *D. amylolyticus* strain (formerly known as *D. fermentans*) and *T. calderae* (formerly known as *T. cellulolyticus*) did not reveal any exo- or endoglucanases genes of known families[28,29].

Alfa- and beta-keratins are highly resistant proteins of animal fur or bird feathers, respectively. The ability to grow on keratins was found in some thermophilic bacteria[30]. We found that hyperthermophilic archaea of genera *Desulfurococcus* and *Thermogladius* were able to grow on keratins, completely degrading them at 85°C[31]. Zymography of *Desulfurococcus* spp. grown on keratin, showed the presence of the endopeptidases, attached to the cell surface.

A hyperthermophilic representative of the phylum *Euryarchaeota* – *Thermococcus sibiricus* was isolated from a high-temperature oil reservoir in Western Siberia[32,33]. While initially *Thermococcus sibiricus* was described as a peptolytic microorganism, the additional experimental tests showed its ability to grow on numerous polysaccharides. Its genome analysis showed the presence of various glycosidase genes, some of which were located in the gene island, presumably horizontally transferred from a hyperthermophilic bacteria of phylum *Thermotogae*[34]. It was assumed that this organism was buried with the deposits of Jurassic ocean and continued living in a deep subsurface geothermally heated environment slowly degrading energy-rich polymeric substrates of deposited organic residues.

An outstanding representative of the same genus, *Thermococcus* sp. strain 2319x1, was isolated from the hot vent in intertidal zone of Kunashir Island (Southern Kurils). This organism was able to grow efficiently on numerous substrates, including polysaccharides, like amorphous cellulose, carboxymethyl cellulose, xylan, xyloglucan, lichenan, alginate and amorphous chitin. Its genome analysis showed the presence of a unique multidomain glycosidase[5], consisting of three glycoside hydrolase (GH) domains and two carbohydrate-binding modules (CBM) with the domain order GH5-12-12-CBM2-2. The full-length gene, as well as its truncated versions, was heterologously expressed in *E. coli*. The analysis of activity of the complete multidomain glycosidase (MDG) and its truncated versions has shown a vast number of substrates differing in polymerisation degree, type of bond and type of monomers.

Representatives of genus *Thermococcus* are, perhaps, the most easily cultivated hyperthermophilic archaea: the number of species in this genus at present exceeds 30. Most *Thermococcus* species were isolated as peptolytic organisms: they grow on hydrolysed proteins, fermenting peptides with elemental sulfur as the electron acceptor, stimulating the growth by avoiding the inhibitory effect of molecular hydrogen formed in the course of fermentation. The habitats of *T. sibiricus* and *Thermococcus* sp. 2319x1 are characterised by the presence of organic substrates of marine origin, ancient in the first case and modern in the second: dead masses of marine alga. That explains the outstanding hydrolytic capacities of these organisms.

Thermostable enzymes from new thermophilic bacteria and archaea, some of which were represented above, could be used in various fields of biotechnology, such as food and biofuel industry (glycosidases), or poultry wastes recovery (keratinases).

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