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EXPERIMENTAL ARTICLES

Capacity of Hyperthermophilic *Crenarchaeota* for Decomposition of Refractory Proteins (α- and β-Keratins)

S. Kh. Bidzhieva[1], K. S. Derbikova, I. V. Kublanov, and E. A. Bonch-Osmolovskaya

Winogradsky Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117312 Russia

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Abstract—Anaerobic thermophilic archaea of the genera *Thermogladius* and *Desulfurococcus* capable of α- and β-keratin decomposition were isolated from hot springs of Kamchatka and Kunashir Island. For two of them (strains 2355k and 3008g), the presence of high-molecular mass, cell-bound endopeptidases active against nonhydrolyzed and partially hydrolyzed proteins at high values of temperature and pH was shown. Capacity for β-keratin decomposition was also found in collection strains (type strains of *Desulfurococcus amylolyticus* subsp. *amylolyticus*, *D. mucosus* subsp. *mobilis*, and *D. fermentans*).

Keywords: hyperthermophilic archaea, hydrolytic microorganisms, keratin, peptidases

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Keratins are the family of insoluble fibrillar proteins that form the skin and its derivatives in mammals, birds, and reptiles. There are two main types of keratins: α-keratins with the molecular mass of 40–68 kDa and β-keratins with the molecular mass of 10–20 kDa; these keratins differ in their structure and physicochemical properties [1]. The molecules of α-keratins are more flexible than those of β-keratins and are contained in keratinocytes of an epidermis layer that mainly contributes to the tensile strength of the epidermis and to water retention in the body. These molecules are also the main constituents of such epidermis derivatives as pelage, hooves, etc. [2]. The molecules of β-keratins are located in keratinocytes of the more rigid outer layer of the epidermis; the package of these molecules is denser and more stable because of the presence of multiple β-layers in the protein structure. That sort of structure makes β-keratins more resistant to the hydrolysis. The molecules of β-keratins participate in the formation of such structures as scales, beaks, feathers, etc. [3].

Keratins are resistant to chemicals (acids and alkalis) because of the strong mechanical stabilization of the proteins by intermolecular/intramolecular disulfide and hydrogen bonds and by hydrophobic interactions. Keratins cannot be hydrolyzed by such proteolytic enzymes as trypsin, pepsin, and papain [4].

Tens of thousands of tons of keratin-containing industrial waste are produced annually. However, they do not accumulate in nature, which indicates that their hydrolysis is efficient [5]. The usage of keratolytic enzymes could be a good solution for bioprocessing of keratin-containing waste of such facilities as poultry farms and the leather industry, while the products of enzymatic hydrolysis could be a rich source of fertilizers and animal feed. Moreover, keratolytic enzymes may be applied in pharmacology and cosmetics [6].

Many keratin-degrading microorganisms growing under various physicochemical environmental conditions are presently known. The vast majority of them are the representatives of the domain *Bacteria* (*Bacillus cereus*, *Fervidobacterium islandicum*, etc.) and the domain *Eucarya* (dermatophytes—*Microsporum*, etc.; nondermatophytes—*Trichoderma*, etc.) [7–11]. However, there are very few reports on the representatives of the *Archaea* domain that are able to hydrolyze keratins [12–14].

The goal of the present work was to isolate pure cultures of new anaerobic thermophilic and hyperthermophilic prokaryotes which have peptidase activity and to study their ability to degrade α- and β-keratins.

MATERIALS AND METHODS

Sources of keratolytic microorganisms. The precipitation and water samples were collected from terrestrial hot springs during the expeditions to Kunashir island (Neskuchensk and Tret'yakov thermal springs) and Kamchatka peninsula (thermal springs of the Uzon volcano caldera) in 2011 and 2012. The temperature of these springs was 71–99°C and the pH varied from 4.0 to 6.8.

Cultivation media. The following media were used to obtain enrichment cultures, to isolate pure cultures, and for the further cultivation of the strains: mineral medium A, containing the following (g/L): $K_2HPO_4 \cdot 2H_2O$, 0.24; KH_2PO_4, 0.24; $(NH_4)_2SO_4$, 0.24; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.1; $CaCl_2 \cdot 2H_2O$, 0.07;

[1] Corresponding author; e-mail: salima.bidjieva@gmail.com

Table 1. New thermophilic and hyperthermophilic archaeal isolates obtained on keratin

Strain no.	Sampling area	Cultivation conditions (T, °C/pH)	Substrates and media	Homology of the 16S rRNA gene sequences to the closest relatives
2319P	Kunashir	92/4.9	α–Keratin, medium A	100%, *D. fermentans* Z-1312^T [20]
2355k	Kunashir	92/6.1	β-Keratin, medium B	99%, *D. amylolyticus* subsp. *kamchatkensis* 1221n^T [20]
2425	Kamchatka	85/6.3	β-Keratin, medium A	99%, *D. amylolyticus* subsp. *amylolyticus* Z-533^T [20]
3008g*	Kamchatka	85/6.1	Gelatin, medium A	100%, *D. amylolyticus* subsp. *kamchatkensis* 1221n^T [20]
2412Fs	Kamchatka	92/6.3	β-Keratin, medium A	99%, "*T. cellulolyticus*" 1633 [22]
2412Pk	Kamchatka	92/6.3	α-Keratin, medium A	99%, "*T. cellulolyticus*" 1633 [22]

* Can grow on α-keratin.

$Ni(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 2; $FeSO_4 \cdot 7H_2O$, 2; and $NaHCO_3$, 0.6 and mineral medium B (modified Viddel medium), containing the following (g/L): Na_2SO_4, 4; KH_2PO_4, 0.2; NH_4Cl, 0.25; NaCl, 1; $MgCl_2 \cdot 4H_2O$, 0.4; KCl, 0.5; $CaCl_2$, 0.1, $Na_2S \cdot 9H_2O$, 0.6; and $NaHCO_3$, 0.15 [15]. The media were additionally supplemented with a vitamin solution (1 mL/L) and a trace elements solution (1 mL/L) [16]. Yeast extract (0.05–0.1 g/L) was added as a source of growth factors. Anaerobically prepared medium containing 0.001 g/L resazurin (Sigma) was reduced by addition of $Na_2S \cdot 9H_2O$. The pH value of the medium was measured at room temperature (~20°C) using a pH-meter (HANNA, Germany, a pH211 model). The pH value was adjusted with oxygen-free 3N HCl solution. Gelatin (Oxoid, England), casein (Sigma, New Zealand), α-keratin (chopped hog bristle, SIFDDA Co., Plouvara, France), or β-keratin (crushed bird feathers and down) were added to the final concentration of 2 g/L as a sole source of carbon and energy. The medium was dispensed (10 mL) into 18-mL Hungate tubes under a flow of 100% nitrogen. In order to clean bird feathers and down of various contaminants, they were pretreated as follows: 20 g feathers (whole feathers with down and spine) were incubated in 1 L 1.2 N HCl solution for 3 h; then, 1 L distilled water was added, and the mixture was filtered. The resulting feather mass was washed copiously with distilled water; the pH value of the resulting solution was controlled until its complete neutralization. The feathers were then dried at 75°C.

Preparation of the enrichment and pure cultures of the anaerobic thermophilic and hyperthermophilic keratolytic archaea. Enrichment cultures of keratin-degrading microorganisms were obtained either in situ [14] or in the laboratory. In the latter case, the Hungate tubes (18 mL) containing 10 mL of anaerobically prepared medium A or B with 2% α-keratin, β-keratin, or gelatin, were inoculated with the sample (1 mL). The cultivation conditions for enrichment cultures and the corresponding new isolates are listed in Table 1.

After three consecutive transfers, the enrichments were used for the preparation of pure cultures. The pure cultures were obtained by tenfold terminal dilutions; in certain cases, the medium was supplied with vancomycin or streptomycin to the final concentration of 0.1 g/L. Microscopic examination of the cultures was performed using an Olympus CX41 phase contrast microscope. To check the purity of the culture, the medium containing peptone and glucose (1 g/L) as substrates was inoculated with a culture sample from the last dilution and incubated at 37, 60, and 85°C. Growth of the isolates (the cultivation conditions and the media are listed in Table 1) was determined by comparison to the biological control (incubation of the isolates in the medium containing 0.05–0.1 g/L yeast extract in the absence of keratins); the degradation of α- and β-keratins by the isolates was studied in comparison with the abiotic control (incubation of the substrate in the absence of the isolates).

Determination of the phylogenetic position of the isolates. DNA was isolated using the method described by Park [17]. The nucleotide sequences of the 16S rRNA genes from the new isolates were determined as described by Sokolova et al. [18]. The resulting sequences of the 16S rRNA genes were compared to those from the public databases using the BLAST algorithm [19].

Strains used in the study. New isolates of *Desulfurococcus* sp. 2319P, *Desulfurococcus* sp. 2355k, *Desulfurococcus* sp. 2425, *Desulfurococcus* sp. 3008g, *Thermogladius* sp. 2412Fs, and *Thermogladius* sp. 2412Pk were used in the study. The following strains of the genus *Desulfurococcus* obtained from the collection of the laboratory of hyperthermophilic microbial communities (Winogradsky Institute of Microbiology, Russian Academy of Sciences) were also used in the study: *D. amylolyticus* subsp. *amylolyticus* Z-533^T (old designation: *D. amylolyticus* Z-533^T), *D. fermentans* Z-1312^T, *D. amylolyticus* subsp. *kamchatkensis* 1221n^T

(old designation: *D. kamchatkensis* 1221n^T), and *D. mucosus* subsp. *mobilis* DSM 2161^T (old designation: *D. mobilis* DSM 2161^T) [20].

Preparation of the samples for biochemical studies. Seven milliliters of the five-day cultures of the strains 2355k and 3008g grown on β-keratin and gelatin were centrifuged for ten minutes at 12100 *g*. The supernatant of the studied cultures was preserved; the pellet was washed with 1.5 mL of 0.05 M MOPS buffer (pH 7.3) and resuspended in 100 μL of the same buffer.

The cells were disrupted by freeze-thawing: the pellet-containing tube was alternately placed in liquid nitrogen and in a water bath at 49°C. The disrupted cells were centrifuged for ten minutes at 12100 *g*; the pellet (the fraction of cell walls containing cell-bound extracellular proteins, including endopeptidases) was washed twice with 500 μL 0.05 M MOPS (pH 7.3) and resuspended in 500 μL of the same buffer. To separate anchored enzymes from the cell walls, 1% Triton X100 was added to the mixture, the mixture was incubated for one hour at 25°C, and supplied with one volume of 0.05 M MOPS, pH 7.3. The resulting mixture was centrifuged for ten minutes at 12100 *g*, and the supernatant containing the enzymes washed out (desorbed) from the cell walls was collected.

Thus, the following samples were prepared for zymography (see below): (1) a supernatant containing the enzymes washed from the cells of strain 2355k grown on gelatin; (2) a supernatant containing the enzymes washed from the cells of strain 2355k grown on β-keratin; (3) a supernatant containing the enzymes washed from the cells of strain 3008g grown on gelatin; (4) a supernatant containing the enzymes washed from the cells of strain 3008g grown on β-keratin; (5) a supernatant of the culture liquid of strain 2355k grown on gelatin; (6) a supernatant of the culture liquid of strain 2355k grown on β-keratin; (7) a supernatant of the culture liquid of strain 3008g grown on gelatin; and (8) a supernatant of the culture liquid of strain 3008g grown on β-keratin.

SDS-PAGE electrophoresis and zymography. Zymography was used for the detection of activity, amount, and molecular mass of the endopeptidases [12]. Gelatin (Sigma, United States) was used as a substrate. Seven microliters of the 4× lysis buffer (200 mM Tris-HCl, pH 6.8; 4% SDS; 0.01% bromophenol blue; and 40% glycerol) were added to 20 μL sample. The resulting mixture was applied to a 4% polyacrylamide stacking gel (4% acrylamide; 0.122 M Tris-HCl, pH 6.8; 3 M urea; 0.075% SDS; and 0.1% gelatin). The concentration of the separating gel was 7.5% (7.5% acrylamide; 0.4 M Tris-HCl, pH 8.8; 3 M urea; 0.1% SDS; and 0.1% gelatin). Electrophoresis was performed in Tris-glycine-SDS buffer at a current of 20–40 mA until the leading bromophenol blue dye was released from the separating gel.

After electrophoresis, the gels were repeatedly washed from SDS with distilled water. For zymography, the gels were incubated for 60 min at 85°C in 0.05 M MOPS buffer, pH 7.3. The gels were stained with the Coomassie solution (Coomassie Brilliant Blue G250, Serva, Germany) of the following composition: 30% ethanol, 5% acetic acid, and 0.2% Coomassie. The dye was washed using the solution of the same composition without Coomassie.

Casein hydrolysis. The substrate was a 0.2% casein solution in 50 mM Tris, pH 8.8. The buffer (100 μL) and the casein solution (200 μL) were added to 100 μL of each sample (the final concentration of casein was 0.1%). The mixture of 200 μL casein solution and 200 μL buffer was used as the control. The reaction mixtures were incubated at 85°C. The samples were taken after two and five hours of incubation. Casein degradation was detected using SDS-PAGE electrophoresis (see above) in the absence of gelatin. Before application to a gel, 7 μL of the 4× lysis buffer (see above) were added to 20 μL of the reaction mixture. The obtained samples were loaded on a 15% stacking gel (15% acrylamide; 0.4 M Tris, pH 8.8, 20°C; 3 M urea; and 0.1% SDS). Concentration of the separating gel was 4% (4% acrylamide; 0.125 M Tris, pH 6.8; 3 M urea; and 0.075% SDS). Electrophoresis was performed in a Tris-glycine-SDS buffer at a current of 30–50 mA until the leading bromophenol blue dye was released from the separating gel. The gel washing and staining methods are described above. The activity was monitored by the disappearance of the casein band [21].

RESULTS

Isolation of anaerobic hyperthermophilic microorganisms that use keratins as growth substrates. Enrichment cultures that retained a steady growth (from 10^7 to 10^8 cells/mL) after 3–4 consecutive transfers on α- and β-keratins or gelatin as the sole source of energy were isolated from terrestrial hydrothermal springs of Kunashir island and Kamchatka peninsula. Enrichment cultures that could grow on keratins and that were obtained from the laboratory samples were no less capable of degrading keratins and grew no less steadily than the in situ enrichment cultures. Six strains of hyperthermophilic microorganisms were isolated from the enrichment cultures (Table 1). The strain 3008g was obtained on the gelatin-containing medium. It was also found to grow on α-keratin and to grow weakly on β-keratin. The strains 2319P and 2412Fs were isolated on α- and β-keratins, respectively. The strains 2412Pk, 2355k, and 2425 were isolated using β-keratin-containing medium. For all the strains, the biomass yield obtained after seven days of incubation on keratins exceeded the control values (incubation in the basal medium without keratins) by a factor of 1.6–11.3, and the utilized substrate (feathers) was almost completely hydrolyzed in comparison with the abiotic control, in which the feather content and the appearance of the feathers were unaltered after incubation under the same conditions (Fig. 1). The

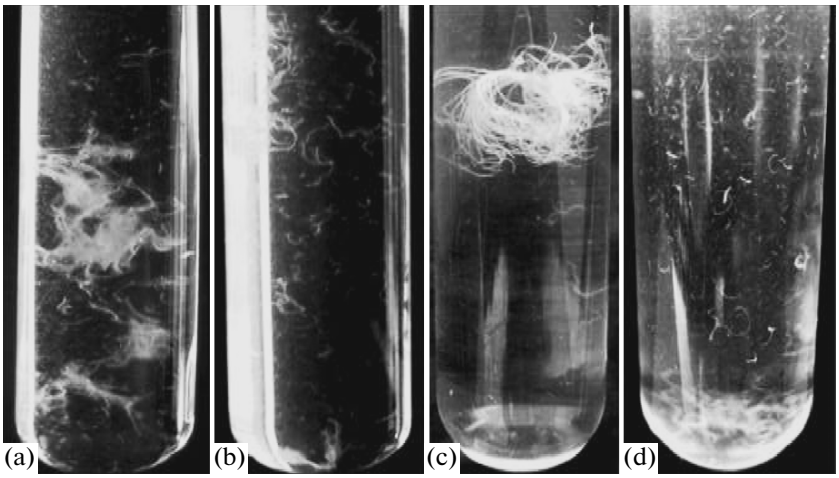

Fig. 1. Feathers (β-keratin) before and after 7-day cultivation of strains 2425 (a and b) and 2355k (c and d).

cells of all the isolates were coccoid, their diameter was of 0.5–1.5 μm. Nucleotide sequence analysis of the 16S rRNA genes from the new isolates showed that they belonged to the genera *Thermogladius* and *Desulfurococcus* (Table 1).

The nucleotide sequences similarity between the 16S rRNA genes of the isolates 2412Pk (389 bp) and 2412Fs (700 bp) and "*Thermogladius cellulolyticus*" 1633 [22] was 99%. A significant degradation of feathers was observed after a 7-day incubation of the strain *Thermogladius* sp. 2412Fs in the medium with β-keratin; the maximum cell concentration was 6.7×10^7 cells/mL. The strain also grew well on α-keratin.

The strains 2319P (734 bp), 2355k (380 bp), 3008g (700 bp), and 2425 (370 bp) belonged to the genus *Desulfurococcus* and had a high level of sequence similarity of the 16S rRNA gene with the known representatives of the genus. The strain 2319P was phylogenetically closest to *D. fermentans* Z-1312^T (100%), while the strains 2355k and 3008g were closest to *D. amylolyticus* subsp. *kamchatkensis* 1221n^T (99 and 100%, respectively); the strain 2425 had 99% similarity with *D. amylolyticus* subsp. *amylolyticus* Z-533^T. All the strains grew on α- and β-keratin. For the strain 2425, the maximum cell yield of $(7.2–9.5) \times 10^7$ cells/mL was observed after 3–4 days of the cultivation (Fig. 2). During further cultivation, cell lysis occurred; however, the degradation of feathers continued. By the seventh day of the incubation of strain 2425, the feathers were almost completely degraded (see Fig. 1). The maximum cell yield was almost twice as high as that of the biological control (growth medium without keratins); in the presence of feathers, the minimum doubling time for the cells of strain 2425 was 12.1 h.

Ability of the collection strains of the genera *Desulfurococcus* and *Thermogladius* to grow on keratins. The ability to grow on β-keratin was demonstrated for the type strains of three subspecies: *D. amylolyticus* subsp. *amylolyticus*, *D. fermentans,* and *D. mucosus* subsp. *mobilis*. The highest cell yield (7.8×10^7 cells/mL) was observed for *D. amylolyticus* subsp. *amylolyticus*; the lowest, $(2.9–3.9) \times 10^7$ cells/mL, for *D. fermentans*. The cell yields in the control experiment (medium without keratins) were 2.6×10^7 and 2.2×10^7 cells/mL, respectively. For *D. mucosus* subsp. *mobilis*, the cell yield was of $(2.9–3.1) \times 10^7$ cells/mL in the control experiment and of $(4.9–5.1) \times 10^7$ cells/mL in the β-keratin-containing medium. After seven days of incubation, the feather degradation was most pronounced in the cultures of *D. amylolyticus* subsp. *amylolyticus* and *D. fermentans*; in the *D. mucosus* subsp. *mobilis* culture, the degradation was somewhat less intense (Fig. 3).

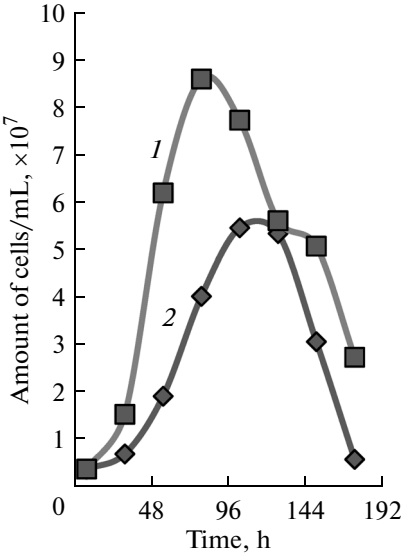

Fig. 2. Growth of strain 2425 in the presence (*1*) and absence (*2*) of β-keratin.

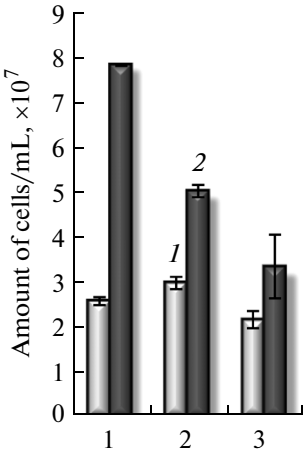

Fig. 3. Growth of *D. amylolyticus* subsp. *amylolyticus* Z-533^T (I), *D. mucosus* subsp. *mobilis* DSM 2161^T (II), and *D. fermentans* Z-1312^T (III) in the control experiment (in the keratin-free medium) (*1*) and in the presence of β-keratin (*2*) after seven days of incubation.

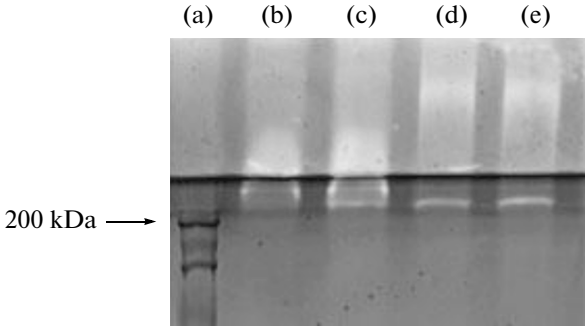

Fig. 4. The zymogram demonstrating proteolytic activity of the endopeptidases produced by strains 3008g and 2355k and washed from the cell walls: (a) marker; (b) strain 3008g grown on gelatin; (c) strain 3008g grown on β-keratin; (d) strain 2355k grown on gelatin; (e) strain 2355k grown on β-keratin. The substrate was gelatin; the incubation time was one hour.

It was shown that the type strain "*T. cellulolyticus*" 1633 was unable to grow on keratins and degrade them.

Endopeptidases from the keratolytic archaeal strains. Proteolytic activity of the endopeptidases from the culture liquid and those bound to the cell surface of the *Desulfurococcus* sp. strains 2355k and 3008g and grown in the media containing β-keratin or gelatin was studied. It was found that, when grown on the proteinaceous substrates (keratin, gelatin), the strains produced extracellular endopeptidases bound to the cell wall and having a molecular mass of over 200 kDa (Fig. 4). The activity could not be detected in the supernatant of the culture liquid. The endopeptidase activity was higher in the samples obtained from the cultures grown on β-keratin than in the samples of the cultures grown on gelatin, while for the biomass yield, the correlation was inverse: 3.4×10^7 cells/mL for 2355k and 1.0×10^7 cells/mL for 3008g were grown on β-keratin; and 5.3×10^7 cells/mL for 2355k and 3.9×10^7 cells/mL for 3008g were grown on gelatin (Fig. 5). Furthermore, the study of the ability of the cultures to hydrolyze casein demonstrated that the cell wall fractions containing cell-bound enzymes of strain 2355k that was grown on gelatin and keratin actively hydrolyzed casein, so that it was almost completely decomposed in five hours (Fig. 5). The enzymes associated with the cell walls of strain 3008g grown on keratin were also highly active against casein, but the samples prepared from the culture of the same strain grown on gelatin were less active.

DISCUSSION

Among organotrophic hyperthermophilic archaea, many representatives that grow on peptides and amino acids are known. However, not all of them can grow on nonhydrolyzed proteins. It is known that some hyperthermophilic archaea grow on partially hydrolyzed casein (*Pyrococcus horikoshii*, *Thermococcus stetteri*, and some others) and on gelatin—the product of partial hydrolysis of collagen (*Ignicoccus pacificus*, *Pyrobaculum aerophilum*, and some others) [23]. Members of the genus *Desulfurococcus* can also grow on casein (*D. amylolyticus* subsp. *amylolyticus* Z-533^T) and gelatin (every member except *D. fermentans* Z-1312^T) [13]. Members of the genus *Desulfurococcus* are the only hyperthermophilic *Crenarchaeota*, for which growth on nonhydrolyzed fibrous protein (α-keratin) was reported: *D. amylolyticus* subsp. *kamchatkensis* 1221n^T and *D. fermentans* Z-1312^T [13, 20].

The stain *Thermococcus* VC13 is the only representative of the phylum *Euryarchaeota* for which growth on keratins was reported; at 80°C, the strain was able to grow on α-keratin and to synthesize several extracellular endopeptidases with molecular masses ranging from 45 to 100 kDa [12]. It was demonstrated that the supernatant of its culture liquid had the ability to hydrolyze α- and β-keratin, which implied that one or several of these extracellular endopeptidases was a keratinase. Under these conditions, hydrolysis of α-keratin proceeds an order of magnitude faster than that of β-keratin.

We obtained new isolates of hyperthermophilic keratolytic archaea; the isolates belonged to the genera *Thermogladius* and *Desulfurococcus* and, during their growth, they were able to completely degrade α- and β-keratins, which are very stable against hydrolysis. Apparently, this property is a generic feature of the genus *Desulfurococcus*.

The ability to degrade keratins and to grow on the products of their degradation was not reported previously for the members of the genus *Thermogladius*.

The organisms studied were selective for α- and β-keratins. The *D. amylolyticus* sp. strains 2425 and

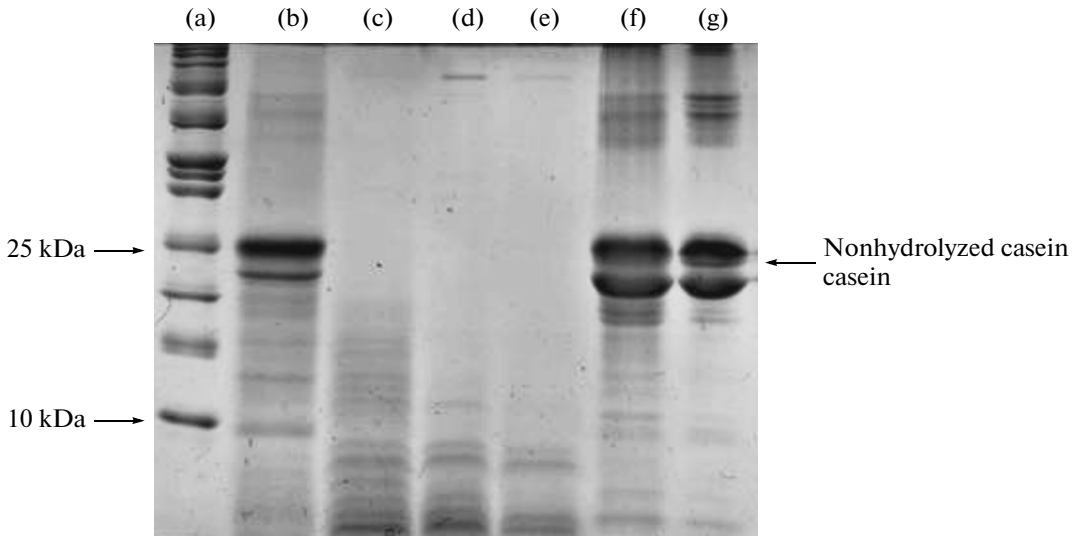

Fig. 5. Casein hydrolysis by the samples of the cell wall fractions from the strains *Desulfurococcus* 3008g and 2355k. Incubation time was five hours; (a) marker; (b) strain 3008g grown on gelatin; (c) strain 3008g grown on β-keratin; (d) strain 2355k grown on gelatin; (e) strain 2355k grown on β-keratin; (f) casein incubated without the enzyme; (g) native casein solution.

2355k grew on both α- and β-keratins and degraded them completely. By contrast, the collection strain *D. amylolyticus* subsp. *amylolyticus* Z-533^T was unable to grow on α-keratin, while *D. amylolyticus* subsp. *kamchatkensis* 1221n could only use α-keratin [13]. As opposed to the strains isolated from the thermal springs of Kamchatka, *D. mucosus* subsp. *mobilis* DSM 2161^T had a weaker ability to grow on keratins and to degrade them. Such selectivity was also noted in the previously described microorganisms with keratolytic activity. For instance, an opposite phenomenon is known in *Doratomyces microsporus* and *Paecilomyces marquandii*: the microorganisms produce enzymes which degrade α-keratins of skin and its derivatives, but these enzymes are incapable of degrading β-keratins of feathers [24]. The peptidase of the strain *Bacillus pseudofirmus* FA30-01 hydrolyzes β-keratin, but is unable hydrolyze α-keratin [25].

Selective keratin hydrolysis may be caused by the structural organization features of α- and β-keratin. The protein molecule of α-keratin is composed of tightly coupled α-helices. Moreover, the α-keratin molecule is rich with cysteine residues, and cysteine is responsible for the formation of the disulfide bonds which are known to stabilize the protein structure and prevent access of the proteases to the peptide bonds. Therefore, in certain cases, not only keratinases, but also disulfide reductases—i.e. disulfide reductase (EC 1.8.1.8)—and other proteins participating in the degradation of disulfide bonds are required to hydrolyze α-keratin [4]. On the other hand, the β-keratin structure is characterized by a high content of β-layers, which makes β-keratin more rigid, and by a fewer amount of disulfide bonds. The ability to hydrolyze α- and/or β-keratin emerges from the specific parameters of the enzyme system of each microorganism. *D. amylolyticus* subsp. *amylolyticus* Z-533^T and *D. mucosus* subsp. *mobilis* DSM 2161^T are probably unable to synthesize disulfide reductases or other enzymes that are necessary for the reduction of disulfide bonds; hence, these organisms are unable to grow on α-keratin. On the contrary, the strain *D. amylolyticus* 2425 that uses α-keratin as its growth substrate is apparently able to synthesize the set of enzymes necessary for this purpose.

In most cases, the peptidases with a putative keratolytic activity and the true keratinases from thermophilic microorganisms are high-molecular mass proteins with an activity optimum at 80–100°C and pH 8–10 (Table 3). The endopeptidases of strains 2355k and 3008g reported in the present work have a molecular mass of over 200 kDa and exhibit high activity against gelatin and, presumably, casein at 85°C and pH 8.8. These properties correspond with the characteristics of the enzymes from other hyperthermophilic keratolytic organisms.

The hydrolytic activity of the endopeptidases from strains 2355k and 3008g grown on keratin with a lower cell yield was higher than that of the endopeptidases from the same strains grown on gelatin. This fact may indicate that growth on a substrate that is harder to hydrolyze (keratin) requires a larger amount of the enzyme per cell.

As is known, keratin-hydrolyzing peptidases have broad substrate specificity [26] and can hydrolyze not only the keratin-containing substrates, but also other high-molecular mass proteinaceous compounds. The endopeptidases identified in our study, which were obtained from the cultures grown on β-keratin and gelatin, exhibited activity against casein; this may also

Table 2. Growth of the *Desulfurococcus* collection strains and the new isolates on keratins

Strains	α-Keratin	β-Keratin
D. amylolyticus subsp. *amylolyticus* Z-533^T	–**	+
D. fermentans Z-1312^T	+**	+
D. mucosus subsp. *mobilis* DSM 2161^T	–**	+
D. amylolyticus subsp. *kamchatkensis* 1221n^T*	+**	–
Desulfurococcus sp. 2319P	+	+
Desulfurococcus sp. 2355k	+	+
Desulfurococcus sp. 3008g	+	+/–
Desulfurococcus sp. 2425	+	+
"*T. cellulolyticus*" 1633	–	–
Thermogladius sp. 2412Fs	+	+
Thermogladius sp. 2412Pk	+	+

* Old designation *D. kamchatkensis* 1221n^T.
** See [13].

Table 3. Characteristics of the endopeptidases from the hyperthermophilic organisms growing on keratins

Producer, reference	Enzyme localization	Molecular mass, kDa	*T*, °C, min/opt/max pH, min/opt/max	Substrate
Fervidobacterium pennavorans [27]	Cell-bound	130	50/80/100 6/10/10.5	β-Keratin
Fervidobacterium islandicum AW-1 [8]	Membrane-bound	97, 110, >200	60/100/110 7/9/10	β-Keratin
'*Thermoanaerobacter keratinophilus*' [28]	Extracellular space	135	40/85/110 6/8/12	α-Keratin, β-Keratin
Thermococcus VC13 [12]	Extracellular space	45–100	80, 7.2	α-Keratin, β-Keratin
Desulfurococcus 2355k	Cell-bound	>200	85, 8.8	Gelatin, casein
Desulfurococcus 3008g	Cell-bound	>200	85, 8.8	Gelatin, casein

be an indication of their ability to hydrolyze a wide range of macromolecular proteinaceous substrates.

Thus, we found that the ability to grow on poorly hydrolysable proteins—keratins—is characteristic of the hyperthermophilic archaea that dwell in terrestrial hot springs. It may be assumed that, in a natural community of thermophiles, the substrates for keratinases are not just keratins, but also other proteins of autochthonous and allochthonous origin. The ability to use a wide range of substrates provides the microorganisms an advantage under the conditions of non-permanent supply of the substrates; it also enables the practical use of archaeal keratinases for disposal of various protein-containing wastes.

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