ISSN 0026-2617, Microbiology, 2009, Vol. 78, No. 1, pp. 67–75. © Pleiades Publishing, Ltd., 2009.
Original Russian Text © I.V. Kublanov, K.B. Tsiroulnikov, E.N. Kaliberda, L.D. Rumsh, T. Haertlé, E.A. Bonch-Osmolovskaya, 2009, published in Mikrobiologiya, 2009, Vol. 78, No. 1, pp. 79–88.

EXPERIMENTAL ARTICLES

Keratinase of an Anaerobic Thermophilic Bacterium *Thermoanaerobacter* sp. Strain 1004-09 Isolated from a Hot Spring in the Baikal Rift Zone

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Received September 10, 2007

Abstract—A thermophilic anaerobic bacterial strain 1004-09 belonging to the genus *Thermoanaerobacter* and capable of growth on protein substrates such as albumin, gelatin, casein, and α- and β-keratins was isolated from the Urinskii hot spring (Barguzin river valley, Republic of Buryatia, Russia). A 150-kDa serine proteinase was revealed in the strain supernatant; it exhibited optimal activity at 60°C and pH 9.3 and was capable of keratin hydrolysis. A number of characteristics for the strain 1004-09 keratinase were established including activation by SDS and NaCl and residual activity (15% to the activity of the intact protein) in the presence of 10% ethanol and acetone.

Key words: thermophilic microorganisms, hydrolytic microorganisms, proteinases, protein hydrolysis, keratin.
DOI: 10.1134/S0026261709010093

Thermozymes, the enzymes of thermophilic and hyperthermophilic microorganisms [1], exhibit the catalytic mechanisms similar to those of their mesophilic analogues, yet they possess a number of advantages from the point of view of biotechnology; namely, thermozymes are more easily purified using thermal treatment of the material; often they are more stable in denaturing conditions (for example, in solvents or detergents) [2]; high-temperature enzymatic reactions allow for higher substrate concentrations due to lower viscosity and higher coefficients of diffusion [2, 3]; employing thermozymes eliminates the risk of undesirable bacterial infection because most bacteria, capable of nonspecifically affecting the reaction, fail to survive at temperatures above 70°C [3].

Keratins are insoluble fibrillar proteins found in the skin of mammals, reptiles, and birds. Owing to such structural characteristics as a high content of disulfide and hydrogen bonds, keratins are resistant to both chemical and biological hydrolyzing agents [4]. Keratins are subdivided into significantly differing α- and δ-keratins [5]. α-Keratins, revealed in the epithelium of most vertebrates, are rich in α-helical secondary structures and have a molecular mass of 40 to 70 kDa [6]. The size of β-keratins, found in skin (and its derivatives) of reptiles and birds, is generally smaller than that of α-keratins and varies between 10 and 20 kDa [6]. Molecules of β-keratin are rich in β-sheets (also revealed in some α-keratin molecules), which makes them more stable and hydrolysis-resistant [7].

Although keratins are resistant to hydrolysis, their accumulation does not occur in nature [8] owing to specialized keratinolytic microorganisms; the mesophilic ones are better studied. Each year industries produce significant amounts of keratin-containing wastes [9, 10] thus generating an urgent need for their utilization. Application of microbial keratinases (keratin-hydrolyzing proteins) may be a powerful and environmentally safe method to utilize keratin wastes through biodegradation.

By now, a fairly large number of mesophilic keratinolytic microorganisms have been studied [11, 12]. Some of them produce keratinases active also at higher temperatures [8, 13]. Although a large number of proteases has been discovered in thermophilic and hyperthermophilic bacteria [14, 15], few thermophilic bacteria are known to produce keratinases, including *Fervidobacterium pennivorans* [10], *F. islandicum* [16], *Thermoactinomyces candidus* [17], '*Thermoanaerobacter keratinophilus*' [7], and strains VC15, VC34, and S290 [18].

The aim of the present work is to characterize a thermophilic anaerobic keratinolytic bacterium isolated from a hot spring in the Baikal region as well as the keratinase it produces.

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MATERIALS AND METHODS

Sample collection. The samples used in the present work were collected in the course of the 2002 expedition to the thermal springs Urinskii, Gusikhinskii, and Kulinnyi located in the Barguzin valley in the Baikal rift zone. The springs are characterized by temperatures 40–70°C and pH 8.1 to 9.6. Sterile 15- and 50-ml tubes (Falcon) were filled with water and sediment samples, tightly sealed with screw caps, and delivered to the laboratory for further investigation.

Isolation of the anaerobic thermophilic bacterium possessing keratinolytic activity. Enrichment cultures were obtained by inoculation with 0.1 ml of the sample of a mineral medium [19] containing the following biopolymers of protein nature (2 g/l) as the only source of carbon and energy: gelatin, α-keratin (powdered pig hair, SIFDDA Co., Plouvara, France), and β-keratin (powdered feathers and fluff obtained from V.A. Gorbachuk, Kazan State University); the medium was supplemented with vitamins (V1 vitamins Kit, Sigma, United States, 1 ml/l) and yeast extract (0.1 g/l) as growth factors. The medium (10 ml) was poured into 15-ml Hungate tubes filled with N_2. Initial pH and temperature values of the medium were close to the parameters at the sampling site. Pure cultures were obtained by the end-point dilution method followed by inoculation of a solid medium of the same composition to obtain individual colonies [19].

Determination of the phylogenetic position of the new isolate. DNA was isolated according to Marmur [20]. The sequence of the 16S rRNA gene from the strain 1004-09 was determined according to the technique described in [21] and deposited with the GenBank under accession number EU547802 (http://www.ncbi.nlm.nih.gov/sites/entrez). The phylogenetic position of strain 1004-09 was determined from the phylogenetic dendrogram generated on the basis of 16S rRNA gene sequences of strain 1004-09 and the reference bacteria. The sequences were aligned using the MultAlin software (http://prodes.toulouse.inra.fr/multalin/multalin.html); phylogenetic dendrograms were constructed using the technique of evolutionary distance calculation [22] realized in the TREECON software package [23].

Culturing conditions for keratinase isolation from strain 1004-09. *Thermoanaerobacter* sp. 1004-09 was cultured for 4 days in 1 l of mineral medium [19] containing 100 mg/l sucrose, 200 mg/l yeast extract, 1 ml/l vitamins, 1 g/l peptone, and 2 g/l pig hair (α-keratin). After the incubation, insoluble components of the culture liquid (including cells) were precipitated by centrifugation in a Jouan GR 2022 centrifuge (France) at 10000 *g*, 4°C for 30 min.

Culturing of *Thermoanaerobacteraceae* type strains. Type strains of the *Thermoanaerobacteraceae* family *Thermoanaerobacter siderophilus* (DSM 12299), *T. wiegelii* (DSM 10319), *T. sulfurophilus* (DSM 11584), and *Thermoanaerobacterium aciditolerans* (DSM 16487) were cultured on the same medium containing α-keratin or peptone (2 g/l).

Protein concentration determination. Protein concentration was determined using the Bradford reagent (BioRad). The reagent (200 μl) was added to 800 μl of a protein solution, stirred, and left for 10–15 min for staining; absorbance at 595 nm was then measured. The protein concentration was determined from a calibration curve plotted using standard solutions of albumin (bovine serum albumin, Sigma) of 15–1000 μg/ml.

SDS-PAGE electrophoresis and zymography. Denaturing electrophoresis in polyacrylamide gel (in the presence of sodium dodecyl sulfate) was used to determine the proteins and their molecular weights. 7 ml of a four-fold lysing buffer (200 mM Tris–HCl, pH 6.8, 0.8% SDS, 0.8% bromphenol blue, 10% glycerol, 3% (vol/vol) β-mercaptoethanol) was added to 20 μl of supernatant of strain 1004-09 culture liquid. The mixture was applied to a 4% concentrating polyacrylamide gel [24]. The separating gel concentration was 7.5 or 10%. Electrophoresis was carried out in a Tris–glycine–SDS buffer at 20 mA till the escape of the leading indicator from the separating gel.

The zymogram technique is based on the enzyme activity determination directly in polyacrylamide gel after electrophoretic separation. Gelatin (Sigma) in a final concentration of 0.01 or 0.1% (for gel staining with silver nitrate and Coomassie, respectively) or 0.02% pig hair (SIFDDA Co., Plouvara, France) was added to the concentrating and separating gels as substrates for proteinases. The gels were incubated at 65°C in 50 or 20 mM 3-(N-morpholine)propanesulfonic acid (MOPS) buffer, pH $7.4^{20°C}$ containing 100 mM NaCl and 5 mM $CaCl_2$. After incubation, the gels were stained with silver nitrate [25] or Coomassie (Coomassie Brilliant Blue R250). The zones of proteolytic activity were revealed as blank bands forming at the sites of substrate hydrolysis by keratinase.

Effects of temperature, pH, metal ions, SDS, NaCl, solvents, and inhibitors on the activity of keratinase were determined using synthetic chromogenic substrates, *N*-succinyl-Ala-Ala-Pro-x-*p*-nitroanilides, where x = Phe, Arg, Leu, Lys (Sigma). Ten microliters of 10 mM substrate solution in 100% dimethylsulfoxide (DMSO) were added to 970–980 μl of 20 mM MOPS, pH $7.0^{60°C}$ containing 100 mM NaCl and 5 mM $CaCl_2$. To determine the effect of pH on the activity, 20 mM buffer solutions of various pH values were used, namely acetate buffer, pH $4.0^{60°C}$ and $5.0^{60°C}$, 2-(N-morpholino)ethanesulfonic acid (MES), pH $6.0^{60°C}$, Tris, $7.7^{60°C}$, tricine, pH $8.2^{60°C}$, and glycine buffer, pH $9.0^{60°C}$. In experiments involving SDS or metal ions, $CaCl_2$ was not added. The mixture was put into a cuvette and heated at 60°C (during the studies on temperature dependence, the temperature was varied between 20 and 92°C) in a thermostated spectrophotometric cell. After the temperature settled, 10 μl of the

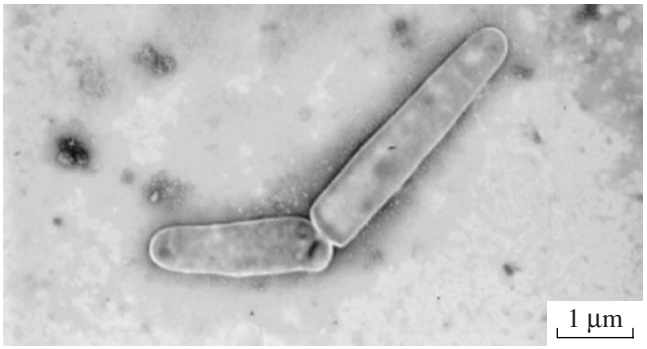

Fig. 1. Electron microphotograph of strain 1004-09 cells. Negative staining with phosphotungstic acid.

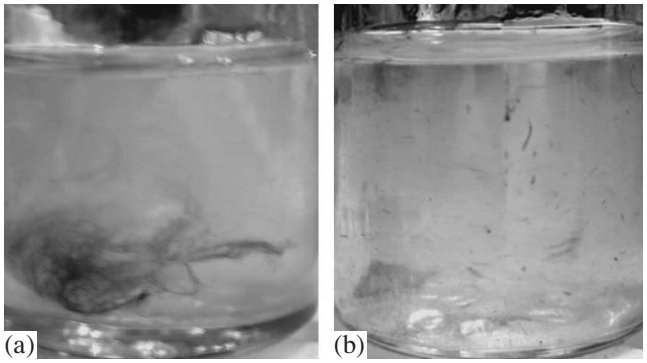

Fig. 2.β-keratin (feathers) and peptone-containing (2 g/l) medium prior to incubation (a) and after 96 h incubation of strain 1004-09 at 658C (b).

supernatant of strain 1004-09 was added, the resulting mixture was incubated for 4–10 min under OD_{405} monitoring. The control solution did not contain keratinase.

The mechanism of catalysis by keratinase was determined by inhibitory analysis. The enzyme was preincubated for 60 min with 10 mM ethylenediaminetetraacetate (EDTA, a metalloproteinase inhibitor), 0.45 mM pepstatin A (an inhibitor of aspartate proteases), 1 mM iodine acetamide (a cysteine protease inhibitor), and 1 mM and 5 mM phenylmethylsulfonyl fluoride (PMSF, a serine protease inhibitor); 10 μl of the collected sample was injected into the reaction mixture. Inhibition efficiency was calculated in relation to the activity of the untreated enzyme.

Insulin hydrolysis and mass spectrometry. The primary specificity of the keratinase was determined on the basis of the analysis of hydrolysis of insulin B-chains (Sigma, cysteine residues oxidized, [26]). The sample (50 μl) was incubated with 950 μl of insulin B-chain (1 mg/ml in 20 mM MOPS buffer solution, pH 7.4) at 65°C for 7 h. The product separation was performed using reverse-phase HPLC (Waters Alliance 2695 chromatograph; a Nucleodur 100-5 C_{18} column, 150 mm long, 4 mm in diameter, Macherey Nagel, France). The column was equilibrated with solution A (0.11% trifluoroacetic acid, TFA, vol/vol) at a flow rate of 0.8 ml/min. The sample (100 μl) was applied to the column and eluted in a linear gradient of solution B (80% acetonitrile, 0.09% TFA, vol/vol) 0–100% for 25 min. Optical density of the eluate was determined at 220 and 280 nm using a Waters 996 photodiode detector.

Molecular masses of the insulin fragments produced by hydrolysis were determined by liquid chromatography followed by electrospray mass spectrometry (LC-ESI/MS) using a Waters 616 pump and a Waters 600 control system integrated with the mass spectrometer (an LCQ Advantage ion trap mass spectrometer, Thermo-Finnigan, San Jose, United States). The ion trap was operated in the positive ionization mode. The data were treated with the X-Calibur (version 1.3, Thermo-Finnigan) software; the *m/z* parameter values ranged between 400 and 2000. Hydrolysis sites were determined using FindPept, a part of the ExPASy software package (Molecular Biology Server, Swiss Institute of Bioinformatics, http://www.expasy.ch/sprot/sprot-top. html).

RESULTS

Isolation of a keratinolytic strain 1004-09. Enrichment culture 1004 obtained from a sample of Urinskii spring sediments (52°C, pH 8.8) was incubated with α- and β-keratins and gelatin. Initially, the incubation was carried out at 55°C and pH 8.8; subsequently, 65°C and pH 7.0 were used as the optimal cultivation conditions. The dominant bacteria were isolated as a pure culture in a medium containing peptone as a substrate. The cells of strain 1004-09 were rods of 3–10 × 0.4–0.6 μm (Fig. 1). The strain 1004-09 exhibited optimal growth at 65°C, pH 6.8. The strain was able to grow on all of the tested substrates (casein, gelatin, albumin, and α- and β-keratins) without any additional substrates or growth factors except for vitamins. After 4 days of incubation, 1.9×10^8 cells per ml were harvested from the β-keratin containing medium and 3.8×10^8 cells per ml, when grown on albumin. Apart from proteins, the strain also grew on peptone and mono- and disaccharides (glucose, fructose, and sucrose). When grown on the medium containing peptone and β-keratin (feathers), complete decomposition of the feathers was observed on day 4 (Fig. 2) in contrast to feathers alone used as a substrate.

Comparison of the 16S rRNA gene sequences of strain 1004-09 (1472 bp) with those available in public databases using BLAST software (http://www.ncbi.nlm.nih.gov/blast) allowed us to identify the strain as a representative of the *Firmicutes*, the group of low-GC gram-positive bacteria. Strain 1004-09 belongs to the class *Clostridia*, order *Thermoanaerobacterales*, family *Thermoanaerobacteraceae*, genus *Thermoanaerobacter* [27]; and the closest relative with 98.4%

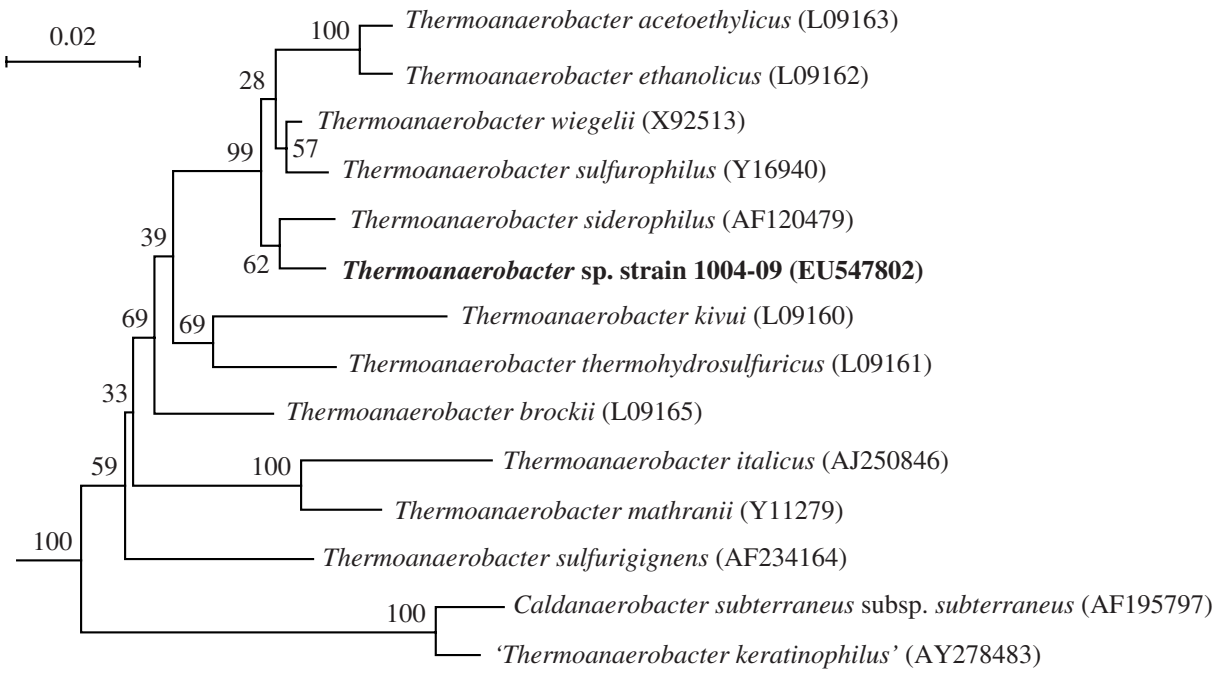

Fig. 3. Phylogenetic position of strain 1004-09 and related bacteria of the family *Thermoanaerobacteraceae* (accession numbers of the sequences deposited in the GenBank http://www.ncbi.nlm.nih.gov/sites/entrez are given in parentheses). The dendrogram was constructed on the basis of 16S rRNA gene sequences using the method of evolutionary distance calculation [22]. The reliability values of the branching order (for 1000 resampling) are presented in percent. *Escherichia coli* strain C2 was used as an outgroup.

homology is *Thermoanaerobacter siderophilus* (DSM 12299).

Characteristics of the keratinase of strain 1004-09. When grown on individual proteins and peptone, strain 1004-09 produced an extracellular proteinase of 150 kDa (Fig. 4). In addition to gelatin, it also hydrolyzed α-keratin thus being a keratinase.

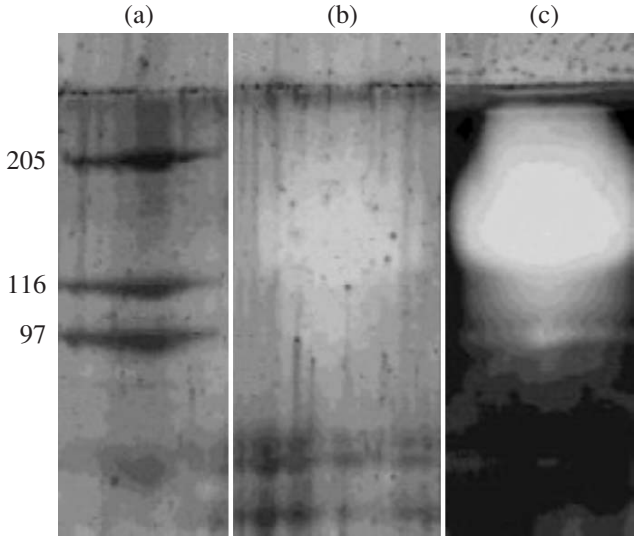

Fig. 4. Zymograms reflecting the activity of the keratinase isolated from strain 1004-09 culture supernatant: protein markers (a); keratin as substrate (b); and gelatin (c) as substrate.

Keratinase of strain 1004-09 was active in the temperature range from 20 to 92°C at pH 6.0–10.5; the activity optimum was at 60°C, pH 9.3 (Fig. 5). Four *p*-nitroanilides were tested as low-molecular synthetic substrates; keratinase of strain 1004-09 exhibited the highest activity towards succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide releasing 0.86 μmol of *p*-nitroaniline per minute per mg protein. A ten times lower activity was observed towards succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide, while succinyl-Ala-Ala-Pro-Lys-*p*-nitroanilide and succinyl-Ala-Ala-Pro-Arg-*p*-nitroanilide were not hydrolyzed by the keratinase. In experiments on insulin B-chain hydrolysis, the keratinase of strain1004-09 displayed a wide specificity, hydrolyzing the bonds between cysteine and glycine, leucine and valine, leucine and tyrosine, and phenylalanine and phenylalanine (Fig. 6).

Keratinase of strain 1004-09 was found to increase its activity in the presence of SDS (by a factor of 1.6–50, at SDS concentrations of 0.002–0.01%, Table 1). Other detergents, such as 0.2 mM [(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propane sulfonate (CHAPSO), 0.5 mM deoxycholic acid, and 0.4 mM Triton X100, as well as reducing (14.3 mM β-mercaptoethanol) and denaturing (0.3 mM urea) agents either inhibited the enzyme activity or did not affect it (Table 1).

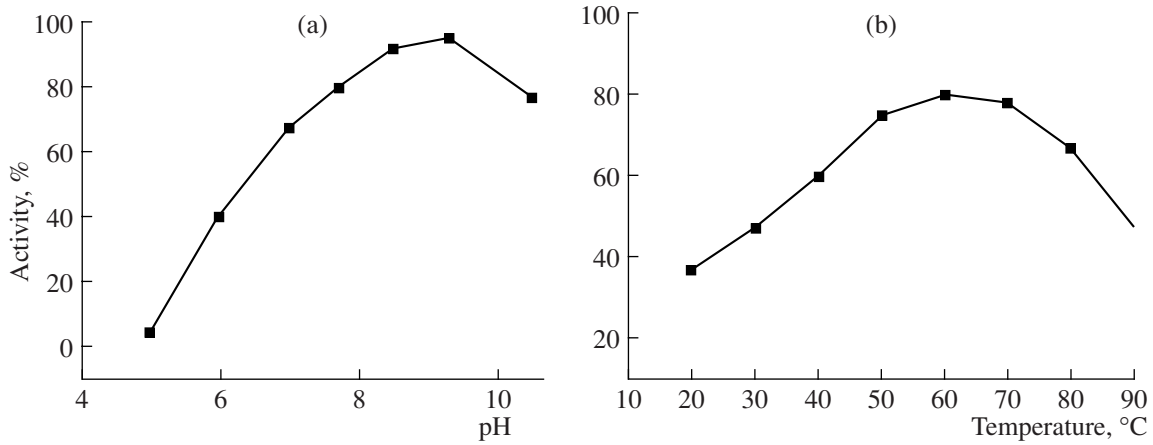

Fig. 5. Activity of strain 1004-09 keratinase as dependent on pH (a, incubation at 60°C), and on temperature (b, incubation at pH 7.0$^{60°C}$ *N*-succinyl-L-Ala-Ala-Pro-Phe-*p*-nitroanilide was used as a substrate.

Pepstatin A, iodine acetamide, and EDTA inhibited the enzyme activity slightly, in contrast to phenylmethylsulfonyl fluoride (PMSF), which at 1 mM completely inhibited the proteolytic activity. These data suggest that the keratinase under study belongs to the serine type, as do all known thermostable keratinases (Table 1).

The experiments on the effect of metal ions on the proteolytic activity revealed that at given concentrations (5 mM) Ca^{2+} displayed no effect on the activity, Mg^{2+} increased it moderately, and Zn^{2+} decreased the activity to the same extent as did EDTA, while Cu^{2+} inhibited the enzyme functioning completely. The effects of metal ions were preserved in the presence of SDS (Table 1). Although calcium did not affect the specific activity of the enzyme, thermal stability of the enzyme (expressed as half life time at 94°C) increased dramatically in the presence of calcium ions, from 10 min to 13 h.

The presence of sodium chloride raised the activity of the keratinase of strain 1004-09; the stimulating effect also grew along with the NaCl concentration up to 5 M NaCl; the activity of the enzyme at this NaCl concentration was 4.4 times higher than in the absence of salt (Table 1).

The addition of 5 and 10% of ethanol or 5 and 10% acetone into the reaction mixture decreased the proteolytic activity, yet did not inhibit it completely. In the presence of 10% ethanol or acetone the activity of the enzyme was 15% of the initial value (Table 1).

Study of the ability of other *Thermoanaerobacteraceae* representatives to grow on keratin and to synthesize keratinase. Some strains of the family *Thermoanaerobacteraceae* were checked for their ability to grow on α-keratin. The ability was found to be characteristic of *T. siderophilus* strain SR4. A keratinase of ~200 kDa was identified in the supernatant of this bacterium. Other strains such as *T. wiegelii*, *T. sulfurophilus*, and *T. aciditolerans* did not exhibit this ability. The same proteinase was detected in *T. siderophilus* grown on peptone, while other bacteria of the family *Thermoanaerobacteraceae* studied in the present work did not synthesize extracellular proteinases when grown on peptone.

DISCUSSION

The family *Thermoanaerobacteraceae* comprises anaerobic thermophilic bacteria capable of fermenting a wide range of organic substrates, including biopolymers [27]. An ability to grow on keratin employing keratinase activity was discovered in '*Thermo-anaerobacter keratinophilus*' [7], which should be assigned to the genus *Caldanaerobacter* [28] according to the 16S rRNA gene sequence analysis (Fig. 3). Another probable representative of the group exhibiting keratinase activity is strain S290 [18], whose phylogenetic position was not determined. Our isolate 1004-09 capable of growth on both α- and β-keratin turned out to be a representative of the genus *Thermoanaerobacter*. The same ability and α-keratinase production were detected in its closest relative *T. siderophilus*, while the type strains of two other *Thermoanaerobacter* species, *T. wiegelii* and *T. sulfurophilus*, were not able to grow on α-keratin or on the even more hydrolysis-stable β-keratin. Earlier, *Thermoanaerobacter brockii* (DSM 1457), *T. thermohydrosulfuricus* (DSM 567), and *T. ethanolicus* (DSM 2246) were shown to lack the ability to grow on β-keratin [10]. Thus, the presence of

Fig. 6. Hydrolysis of insulin B-chains (with oxidized cysteine residues) by keratinase of strain 1004-09. Arrows point at hydrolysis sites, namely peptide bonds between cysteine and glycine, leucine and valine, leucine and tyrosine, phenylalanine and phenylalanine.

Table 1. The effect of detergents, inhibitors, metal ions, NaCl, and solvents on the activity of strain 1004-09 keratinase. *N*-succinyl-L-Ala-Ala-Pro-Phe-*p*-nitroanilide was used as a substrate

Detergents and reducing and hydrolyzing agents, concentration	Activity, %	Metal (5 mM)	Activity, %	
			Without SDS	With SDS
Without a detergent	100	Without metal ions	100	100
SDS, 0.35 mM	5000	Ca^{2+}	105	115
Urea, 300 mM	60	Mg^{2+}	122	126
CHAPSO, 0.2 mM	20	Zn^{2+}	68	64
Deoxycholic acid, 0.5 mM	20	Cu^{2+}	5	1
β-mercaptoethanol, 14.3 mM	80			
Triton X100, 0.4 mM	20			
SDS, mM (%)	Activity, %	NaCl (M)	Activity, %	
0.00	100	0.0	100	
0.07 (0.002)	160	0.1	84	
0.18 (0.005)	360	0.5	110	
0.35 (0.01)	5000	3.0	305	
1.75 (0.05)	4520	5.0	440	
3.50 (0.1)	3600			
7.00 (0.2)	4380			
14.00 (0.4)	2880			
Inhibitors	Activity, %	Solvents	Activity, %	
Without inhibitor	100	Without solvents	100	
PMSF, 1 mM	3	Ethanol, 5%	32	
PMSF, 5 mM	4	Ethanol, 10%	14	
Pepstatin A, 0.45 mM	84	Acetone, 5%	49	
Iodine acetamide, 1 mM	95	Acetone, 10%	16	
EDTA, 10 mM	65			

keratinase may be considered a characteristic of only some representatives of *Thermoanaerobacteraceae*.

All known thermostable keratinases including the one from strain 1004-09 (150 kDa) are high-molecular proteins with molecular masses of 100 kDa and higher, with the exception for *Thermoactinomyces candidus* keratinase, weighing 30 kDa (Table 2), which is close to the molecular masses of mesophilic keratinases [8, 13].

The keratinase isolated from strain 1004-09 is an extracellular enzyme as are other keratinases of the family *Thermoanaerobacteraceae* [7, 18], as well as keratinases from *Thermoactinomyces candidus* and from mesophilic bacteria [11, 29]. In contrast, both species of *Fervidobacterium* synthesize cell-associated keratinases [10, 16].

Inhibition analysis demonstrated that the keratinase from strain 1004-09 is a serine type proteinase as are most of thermostable proteinases (including all known keratinases) [7, 10, 16, 17].

Hydrolysis of both types of substrates, *p*-nitroanilides and B-chain of insulin with oxidized cysteine residues, revealed the keratinase of strain 1004-09 to hydrolyze preferably the peptide bond after hydrophobic amino acids (phenylalanine, tyrosine, leucine, and valine). The enzyme under study is active in wide ranges of temperature and pH as are most of the thermostable keratinases (Table 2), but its optimum activity temperature is somewhat lower (60°C). Variable effects

Table 2. Thermophilic keratinolytic bacteria and characteristics of keratinases

Producer (reference)	Protein substrates for growth	Localization	Size, kDa (according to SDS-PAGE)	Protease subclass	*T*, °C and pH min/opt/max	SDS effect on the activity
Thermoactinomyces candidus [17]	α-keratin (sheep wool)	Extracellular space	30*	Serine proteases	45/70–75/80 8.3/8.6/9.2	ND
Fervidobacterium pennivorans [10]	β-keratin (feathers)	Cell-associated	130	Serine proteases	50/80/100 6/10/10.5	Stable
Fervidobacterium islandicum [16]	β-keratin (feathers)	Membrane-bound	97, 110, >200	Serine proteases	60/100/110 7/9/10	Stable
'*Thermoanaerobacter keratinophilus*' [7]	β-keratin (feathers), α-keratin (wool)	Extracellular space	135*	Serine proteases	40/85/110 6/8/12	ND
Thermoanaerobacter sp. strain 1004-09	β-keratin (feathers) α-keratin (pig cirrus), casein, albumin, gelatin	Extracellular space	~150	Serine proteases	20/60/ 90 5/9.3/10.5	Stimulates the activity

Note: ND, not determined.
* The enzyme was purified to the homogeneous state.

on the keratinase activity were observed in the presence of metal ions: magnesium stimulated the activity moderately, zinc decreased the activity, and copper inhibited it completely, both in the presence and in the absence of SDS (Table 2). The inhibitory effect of copper(II) and zinc ions on the activity of various proteases [12], as well as of other hydrolytic enzymes such as α-amylase from *P. woesei* [30], has been noted previously. The inhibition of α-amylase was shown to be due to copper or zinc ions binding with the active site of the enzyme. Copper ions exhibited a higher inhibitory effect than zinc on all occasions. Riffel et al. [12] reported complete inhibition of the keratinase from a mesophilic bacterium *Chryseobacterium* sp. by copper(II) ions. Although no stimulation of keratinase activity by calcium ions was observed, they did increase the thermal stability of the enzyme significantly.

The activity of strain 1004-09 keratinase increased in the presence of NaCl, up to 5 M. In the presence of 5 M NaCl, the activity increased 4.4-fold. Interestingly, the maximum activity for the protease isolated from the cultural fluid of a haloalkaliphilic archaeon *Natrialba magadii* was observed at only 1.5 M NaCl [31].

The residual activity for the keratinase of strain 1004-09 in the presence of solvents (10% ethanol or acetone) may be of interest for applied studies. There have been no reports on this property of keratinases so far; speaking about thermozymes as a whole, the feature is characteristic of some archaeal hydrolases, for example, the chitinase from *Thermococcus kodakaraensis* [32].

Sodium dodecyl sulfate exhibited an outstanding activation effect towards the keratinase from strain 1004-09. The activity increased sharply (50 times) in the presence of 0.01% (0.35 mM) SDS. Further addition of SDS resulted in a gradual decrease in the activity of the enzyme; at 0.4% SDS, the enzymatic activity was still 30 times higher than the initial level. Thermostable SDS-resistant proteases were presently known [26], including keratinases from *Fervidobacterium pennivorans* [10] and *F. islandicum* [16]. Moreover, a proteinase from *Thermococcus stetteri* was two times more active in the presence of 0.4% SDS [14]; however, the activation of strain 1004-09 keratinase by SDS turned out to be by far more pronounced. The detergents may affect the tertiary and quaternary structure of the protein by causing the subunits to dissociate [33]. Being a strong amphiphilic anion detergent, SDS not only affects the hydrophobic interactions, but also influences electrostatic and hydrogen bonds in the protein molecules due to its negatively charged head [33]. Presumably, the keratinase of strain 1004-09 is excreted in a low-activity form and SDS addition activates it. SDS was also shown to prevent keratin aggregation, thus increasing keratin solubility and availability to the enzyme [34]. Therefore, SDS plays an important role in keratin degradation as a keratinase activator and an agent promoting the substrate solubility and unfolding.

REFERENCES

1. Zeikus, J.G., Vieille, C., and Savchenko, A., Thermozymes: Biotechnology and Structure-Function Relationships, *Extremophiles*, 1998, vol. 2, pp. 179–183.
2. Egorova, K. and Antranikian, G., Industrial Relevance of Thermophilic Archaea, *Curr. Opin. Microbiol.*, 2005, vol. 8, pp. 649–655.
3. Bruins, M., Janssen, A., and Boom, R., Thermozymes and Their Applications, *Appl. Biochem. Biotechnol.*, 2001, vol. 90, pp. 155–186.
4. Arai, K., Naito, S., Dang, V.B., Nagasawa, N., and Hirano, M., Crosslinking Structure of Keratin. VI. Number, Type, and Location of Disulfide Crosslinkages in Low-Sulfur Protein of Wool Fiber and Their Relation to Permanent Set, *J. Appl. Polym. Sci.*, 1996, vol. 60, pp. 169–179.
5. Cregg, K and Rogers, G.E, Feather Keratin: Composition, Structure and Bioenergetics, in *Biology of the Integument, Vertebrates vol. II*, Bereiter-Hahn, J., Maltotsy, A.G., and Richards, K.S., Eds., Berlin: Springer, 1986, pp. 666–694.
6. Shames, R.B., Knapp, L.W., Carver, W.E., Washington, L.D., and Sawyer, R.H., Keratinization of the Outer Surface of the Avian Scutate Scale: Interrelationship of Alpha and Beta Keratin Filaments in a Cornifying Tissue, *Cell Tissue Res.*, 1989, vol. 275, pp. 85–92.
7. Riessen, S. and Antranikian, G., Isolation of *Thermoanaerobacter keratinophilus* sp. nov., a Novel Thermophilic, Anaerobic Bacterium with Keratinolytic Activity, *Extremophiles,* 2001, vol. 5, pp. 399–408.
8. Lin, X., Lee, C.G., Casale, E.S., and Shih, J.C.H., Purification and Characterization of Keratinase from a Feather-Degrading *Bacillus licheniformis* Strain, *Appl. Environ. Microbiol.*, 1992, vol. 58, pp. 3271–3275.
9. Dalev, P.G., Utilisation of Waste Feathers from Poultry Slaughter for Production of a Protein Concentrate, *Biores. Technol.,* 1994, vol. 48, pp. 265–267.
10. Friedrich, A.B. and Antranikian, G., Keratin Degradation by *Fervidobacterium pennivorans,* a Novel Thermophilic Anaerobic Species of the Order *Thermotogales, Appl. Environ. Microbiol.*, 1996, vol. 62-8, pp. 2875–2882.
11. Sangali, S. and Brandelli, A., Feather Hydrolysis by a *Vibrio* sp. Strain kr2, *J. Appl. Microbiol.*, 2000, vol. 89, pp. 735–743.
12. Riffel, A., Lucas, F., Heeb, P., and Brandelli, A., Characterization of a New Keratinolytic Bacterium That Completely Degrades Native Feather Keratin, *Arch. Microbiol.,* 2003, vol. 179, pp. 258–265.
13. De Toni, C.H., Richter, M.F., Chagas, J.R., Henriques, J.A.P., and Termignoni, C., Purification and Characterization of an Alkaline Serine Endopeptidase from Feather-Degrading *Xanthomonas maltophilia* Strain, *Can. J. Microbiol.*, 2002, vol. 48, pp. 342–348.
14. Klingeberg, M., Galunsky, B., Sjoholm, C., Kasche, V., and Antranikian, G., Purification and Properties of High Thermostable, Sodium Dodecyl Sulphate-Resistant and Stereospecific Proteinase from Extremely Thermophilic Archaeon *Thermococcus stetteri, Appl. Environ. Microbiol.*, 1995, vol. 61, no. 8, pp. 3098–3104.
15. Sako, Y., Croocker, P.C., and Ishida, Y., An Extremely Heat-Stable Extracellular Proteinase (Aeropyrolysin) from the Hyperthermophilic Archaeon *Aeropyrum pernix* K1, *FEBS Letters,* 1997, vol. 415, pp. 329–334.
16. Nam, G., Lee, D., Lee, H., Lee, N., Kim, B., Choe, E., Hwang, J., Suhartono, M., and Pyun, Y., Native-Feather Degradation by *Fervidobacterium islandicum* AW-1, a Newly Keratinase-Producing Thermophilic Anaerobe, *Arch. Microbiol.,* 2002, vol. 178, pp. 538–547.
17. Ignatova, Z., Gousterova, A., Spassov, G., and Nedkov, P., Isolation and Partial Characterization of Extracellular Keratinase from a Wool Degrading Thermophilic Actinomycete Strain *Thermoactinomyces candidus, Can. J. Microbiol.*, 1999, vol. 45, pp. 217–222.
18. Tsiroulnikov, K., Rezai, H., Bonch-Osmolovskaya, E., Nedkov, P., Gousterova, A., Cueff, V., Godfroy, A., Barbier, G., Mŭtro, F., Chobert, J.-M., Clayette, P., Dormont, D., Grosclaude, J., and Haertle, T., Hydrolysis of the Amyloid Prion Protein and Nonpathogenic Meat and Bone Meal by Anaerobic Thermophilic Prokaryotes and *Streptomyces* Subspecies, *J. Agric. Food Chem.*, 2004, vol. 52, pp. 6353–6360.
19. Kublanov, I.V., Prokofeva, M.I., Kostrikina, N.A., Kolganova, T.V., Tourova, T.P., Wiegel, J., and Bonch-Osmolovskaya, E.A., *Thermoanaerobacterium aciditolerans* sp. nov., a Moderate Thermoacidophile from a Kamchatka Hot Spring, *Int. J. Syst. Evol. Microbiol.*, 2007, vol. 57, pp. 260–264.
20. Marmur, J., A Procedure for the Isolation of Deoxyribonucleic Acid from Microorganisms, *J. Mol. Biol.*, 1961, vol. 3, pp. 208–218.
21. Sokolova, T.G., Kostrikina, N.A., Chernyh, N.A., Tourova, T.P., Kolganova, T.V., and Bonch-Osmolovskaya, E.A., *Carboxydocella thermautotrophica* gen. nov., sp. nov., a Novel Anaerobic, CO-Utilizing Thermophile from a Kamchatkan Hot Spring, *Int. J. Syst. Evol. Microbiol.*, 2002, vol. 59, pp. 1961–1967.
22. Jukes, T.H and Cantor, C.R, Evolution of Protein Molecules, in *Mammalian Protein Metabolism*, Munro, H.N., Ed., New York: Academic, 1969, pp. 21–32.
23. Van De Peer, Y. and De Wachter, R., TREECON for Windows: a Software Package for the Construction and Drawing of Evolutionary Trees for the Microsoft Windows Environment, *Comput. Applic. Biosci.*, 1994, vol. 10, pp. 569–570.
24. Laemmli, U.K., Cleavage of Structural Protein during the Assemblage of the Head of Bacteriophage T4, *Nature,* 1970, vol. 227 P, pp. 680–685.
25. Nesterenko, M.V., Tilley, M., and Upton, S.J., A Simple Modification of Blum's Silver Stain Method Allows for 30 Minute Detection of Proteins in Polyacrylamide Gels, *J. Biochem. Biophys. Methods,* 1994, vol. 28, pp. 239–242.
26. Peek, K., Daniel, R.M., Monk, C., Parker, L., and Coolbear, T., Purification and Characterization of a Thermostable Proteinase Isolated from *Thermus* sp. Strain Rt41A, *Eur. J. Biochem.*, 1992, vol. 207, pp. 1035–1044.
27. Wiegel, J. and Ljungdahl, L.G., *Thermoanaerobacter ethanolicus* gen. nov., spec. nov., a New, Extreme Thermophilic, Anaerobic Bacterium, *Arch. Microbiol.,* 1981, vol. 128, pp. 343–348.
28. Fardeau, M.-L., Salinas, M.B., L'Haridon, S., Jeanthon, C., Verhe, F., Cayol, J.-L., Patel, B.K.C., Garcia, J.-L., and Olivier, B., Isolation from Oil Reservoirs of Novel

Thermophilic Anaerobes Phylogenetically Related to *Thermoanaerobacter subterraneus:* Reassignment of *T. subterraneus, Thermoanaerobacter yonseiensis, Thermoanaerobacter tengcongensis* and *Carboxydibrachium pacificum* to *Caldanaerobacter subterraneus* gen. nov., sp. nov., comb. nov. as Four Novel Subspecies, *Int. J. Syst. Evol. Microbiol.*, 2004, vol. 54, pp. 467–474.

29. Yamamura, S., Morita, Y., Hasan, Q., Yokoyama, K., and Tamiya, E., Keratin Degradation: a Cooperative Action of Two Enzymes from *Stenotrophomonas* sp., *Biochem. Biophys. Res. Commun.*, 2002, vol. 294, pp. 1138–1143.
30. Linden, A., Mayans, O., Meyer-Klaucke, W., Antranikian, G., and Wilmanns, M., Differential Regulation of a Hyperthermophilic α-Amylase with a Novel (Ca, Zn) Two-Metal Center by Zinc, *J. Biol. Chem.*, 2003, vol. 278, pp. 9875–9884.
31. Gimenez, M.I., Studdert, C.A., Sanchez, J.J., and De Castro, R.E., Extracellular Protease of *Natrialba magadii:* Purification and Biochemical Characterization, *Extremophiles,* 2000, vol. 4, pp. 181–188.
32. Imanaka, T., Fukui, T., and Fujiwara, S., Chitinase from *Thermococcus kodakaraensis* KOD1, *Methods Enzymol.,* 2001, vol. 330, pp. 319–329.
33. Das, T.K., Mazumdar, S., and Mitra, S., Characterization of a Partially Unfolded Structure of Cytochrome *c* Induced by Sodium Dodecyl Sulphate and the Kinetics of Its Refolding, *Eur. J. Biochem.*, 1998, vol. 254, pp. 662–670.
34. Schrooyen, P.M.M., Dijkstra, P.J., Oberthr, R.C., Bantjes, A., and Feijen, J., Stabilization of Solutions of Feather Keratins by Sodium Dodecyl Sulfate, *J. Colloid Interface Sci.,* 2001, vol. 240, pp. 30–39.