APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1996, p. 2875–2882 Vol. 62, No. 8
0099-2240/96/$04.00+0

Keratin Degradation by *Fervidobacterium pennavorans*, a Novel Thermophilic Anaerobic Species of the Order *Thermotogales*

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Received 22 January 1996/Accepted 9 June 1996

From a hot spring of the Azores islands a novel thermophilic bacterium belonging to the *Thermotogales* order was isolated. This strain, which grows optimally at 70°C and pH 6.5, is the first known extreme thermophile that is able to degrade native feathers at high temperatures. The enzyme system converts feather meal to amino acids and peptides. On the basis of physiological, morphological, and 16S rDNA studies the new isolate was found to be a member of the *Thermotogales* order and was identified as *Fervidobacterium pennavorans*. The strain was highly related to *Fervidobacterium islandicum* and *Fervidobacterium pullulanolyticum*. The cell-bound keratinolytic enzyme system was purified 32-fold by detergent treatment with CHAPS (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme was characterized as a serine protease with a molecular mass of 130 kDa and an isoelectric point of 3.8. Optimal activity was measured at 80°C and pH 10.0. Furthermore, 19 anaerobic thermophilic archaea and bacteria belonging to the orders *Thermococcales*, *Thermoproteales*, *Thermotogales*, and *Clostridiales* (growth temperatures between 60 and 105°C) were tested for their abilities to grow on feathers and produce heat-stable keratinolytic enzymes. None of the tested extremophilic microorganisms was able to attack the substrate in a native form.

Keratin is the insoluble structural protein of feathers and wool and is known for its high stability (44, 58). Every year more than 20,000 tons of feathers are produced as waste by poultry farming (60). The feathers, which are hydrolyzed by mechanical or chemical treatment, can be converted to feedstuffs, fertilizers, glues, and foils or used for the production of amino acids and peptides. Because of environmental considerations the use of keratinolytic enzymes in the production of amino acids and peptides is becoming attractive for biotechnological applications. With the help of these enzymes feathers could be converted to defined products such as the rare amino acids serine, cysteine, and proline. This enzymatic process is advantageous over commercial methods, as large amounts of salts, which have to be separated from the end product, would not be produced. The production of keratinases has been a domain of mesophilic fungi and actinomycetes until now (8, 28–30, 50, 61, 67, 68). Recently, two new *Bacillus* species that are able to degrade native feathers and wool were characterized (36, 56, 63). In the last decade several extreme thermophilic and hyperthermophilic microorganisms that are capable of producing extremely thermostable proteases were isolated (11, 12, 26, 34). These belong to the genera *Pyrococcus*, *Thermococcus*, *Staphylothermus*, *Desulforococcus*, and *Sulfolobus*. Except for the enzyme system of a *Sulfolobus* sp., all proteases were found to belong to the serine type (37). So far, only two extremely thermoactive proteases have been studied in detail, namely, the serine proteases from *Thermococcus stetteri* (25) and *Pyrococcus furiosus* (7, 15). No information is, however, available on the production and properties of keratinolytic enzymes from extremely thermophilic microorganisms.

In the present study we report on the screening of anaerobic hyperthermophilic archaea and bacteria for their ability to degrade native feathers and on the isolation and characterization of an extremely thermophilic feather-degrading anaerobe belonging to the *Thermotogales* order.

MATERIALS AND METHODS

Microorganisms and culture conditions. All thermophilic microorganisms were cultivated at temperatures given in parentheses. The hyperthermophilic archaea *Thermococcus stetteri* (DSM 5262; 75°C) (39), *Thermococcus* sp. strain AN1 (DSM 2770; DSM medium 376, 75°C) (40), *Thermococcus celer* (DSM 2476; DSM medium 266, 85°C) (69), *Thermococcus litoralis* (DSM 5473; 85°C) (42), *Staphylothermus marinus* (DSM 3639; DSM medium 377, 90°C) (17), *Pyrococcus furiosus* (DSM 3638; DSM medium 377, 90°C; 16), and *Pyrococcus woesei* (DSM 3773; DSM medium 377, 90°C) (70) and the extreme thermophilic bacteria *Thermoanaerobacter thermohydrosulfuricus* (DSM 567, DSM 569; DSM medium 61, 65°C) (33), *Thermoanaerobacter brockii* (DSM 1457; DSM medium 144, 65°C) (33), *Thermoanaerobacter ethanolicus* (DSM 2246; DSM medium 61, 65°C) (62), *Thermoanaerobacter finnii* (DSM 3389; DSM medium 144, 65°C) (48), *Thermobacteroides proteolyticus* (DSM 5265; DSM medium 481, 60°C) (43), *Fervidobacterium nodosum* (DSM 5306; DSM medium 144, 70°C) (45), *Fervidobacterium islandicum* (DSM 5733; DSM medium 501, 65°C) (21), *Thermosipho africanus* (DSM 5309; DSM medium 483, 70°C) (20), *Thermotoga maritima* (DSM 3109; DSM medium 343, 77°C) (19), *Thermotoga neapolitana* (DSM 4359; DSM medium 343, 77°C) (5, 23), *Thermotoga thermarum* (DSM 5069; DSM medium 489, 70°C) (64), and *Fervidobacterium pullulanolyticum* (DSM 9078; TF medium [see below], 65°C) (1, 27) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany).

For feather degradation experiments carbohydrates such as starch and glucose were omitted and the complex components were reduced to 0.1% (wt/vol). The microorganisms were grown anaerobically by the Hungate culture technique (22). Each Hungate tube contained one native feather (approximately 15 mg) as a source of carbon. Tyndallization was performed at 100°C for 60 min to avoid the destruction of the native structure of the feather.

TF medium. Thermotoga-Fervidobacterium (TF) medium for cultivation of *F. pullulanolyticum* contained the following components (per liter): NH_4Cl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.16 g; K_2HPO_4, 1.6 g; $NaH_2PO_4 \cdot H_2O$, 1 g; yeast extract, 2 g; Trypticase, 2 g; trace element solution (3), 10 ml; vitamin solution (66), 10 ml; resazurin, 1 mg; glucose or starch, 3 g; cysteine, 0.3 g; and $Na_2S \cdot 9H_2O$, 0.3 g. The pH was adjusted to 6.8 with 10 N KOH, and the gas phase was nitrogen.

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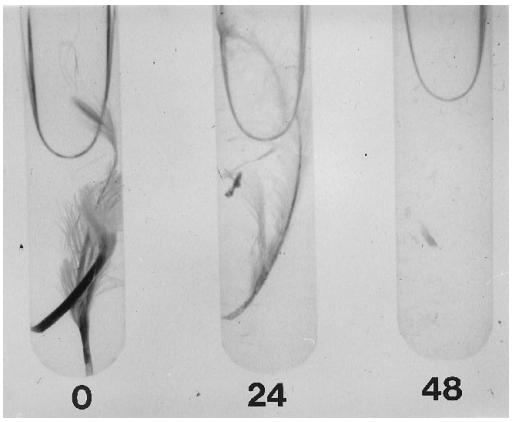

FIG. 1. Complete degradation of native chicken feathers by the new isolate under anaerobic conditions in I medium before and after incubation for 24 and 48 h at 70°C.

Sterilization was performed at 121°C for 20 min. After sterilization 0.06 g of $CaCl_2$ was added aseptically.

I medium. For the isolation of thermophilic anaerobic feather-degrading organisms a medium composed of the following ingredients (per liter) was prepared: K_2HPO_4, 1.6 g; NaH_2PO_4, 1.0 g; $(NH_4)_2SO_4$, 1.5 g; NaCl, 3.0 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; $FeCl_2 \cdot 6H_2O$, 6 mg; $NaHCO_3$, 1 g; yeast extract, 1 g; tryptone, 1 g; trace element solution (see below), 10 ml; vitamin solution (66), 10 ml; resazurin, 1 mg; and cysteine, 0.5 g. The trace element solution contained (per liter) $MnCl_2 \cdot 4H_2O$, 1.0 g; $CoCl_2 \cdot 6H_2O$, 1.0 g; $NiCl_2 \cdot 6H_2O$, 0.5 g; $ZnCl_2$, 0.5 g; $CuSO_4$, 0.5 g; H_3BO_3, 0.2 g; $Na_2MoO_4 \cdot 2H_2O$, 0.1 g; $Na_2SeO_3 \cdot 5H_2O$, 0.1 g; and $VOSO_4 \cdot 5H_2O$, 0.03 g. The pH of the medium (I medium) was adjusted to 6.3 with 5 N HCl; the gas phase was nitrogen. Each Hungate tube contained one native feather (15 mg), and tyndallization was performed at 100°C for 60 min. For the isolation of strains on agar plates 15 g of agar per liter was added and feathers were replaced by 0.5 g of skim milk per liter. Yeast extract and tryptone were reduced to a final concentration of 0.01% (wt/vol).

Enrichment and isolation of bacteria. Samples were taken from various hot springs on San Miguel, an island in the Azores. The new isolate was collected at Caldeira Velha, a hot waterfall with a temperature of 88°C. The samples were incubated anaerobically and transported at 4°C. Enrichment cultures were grown in 100-ml vials containing 50 ml of I medium and 0.1 g of native feathers from chickens, ducks, or geese. Incubation was performed at 70, 80, and 90°C.

Growth determination and analysis of fermentation products. Bacterial growth was monitored by measuring the A_{600} and cell counts using a Neubauer counting chamber. Acids and alcohols were determined in the gas phase by injecting 1-μl samples with a gas chromatograph (HS control 250; Carlo Erba Instruments) equipped with a DB 624 capillary column, a flame ionization detector, and a Shimadzu C-RBA Chromatopac Integrator; the carrier gas was nitrogen, and the flow rate was 1 ml/min. The column temperature was isothermal at 100 and 130°C. Lactate, acetate, and ethanol were determined enzymatically. Fermentation products were measured after 24 and 48 h of growth in the presence of glucose or starch.

Light microscopy. A Zeiss Axioplan light microscope equipped with a Contax Quartz II camera was routinely used to observe the bacteria and to obtain photomicrographs. For documentation Ilford PAN F black-and-white films were used.

Electron microscopy. Carefully washed cell pellets were fixed by addition of the same amount of 1% (wt/vol) agar. Small pieces of 2 mm were cut out and fixed in 3% (vol/vol) glutaraldehyde and 1% (wt/vol) osmium tetraoxide (4, 47). Dehydration was performed in acetone. As embedding resin a mixture described by Spurr (52) was used. The samples were allowed to dry at 70°C for 8 h. Sections were prepared with an ultramicrotome (Ultramicrotome III; LKB, Bromma, Sweden) equipped with a glass knife. The sections were contrasted with 4% (wt/vol) uranyl acetate, pH 4.5, for 5 min (59). Transmission electron micrographs were taken with a Philips EM 301 transmission electron microscope.

Isolation of DNA. For DNA isolation, cells were disrupted with a French pressure cell. The DNA was purified on hydroxyapatite by the procedure of

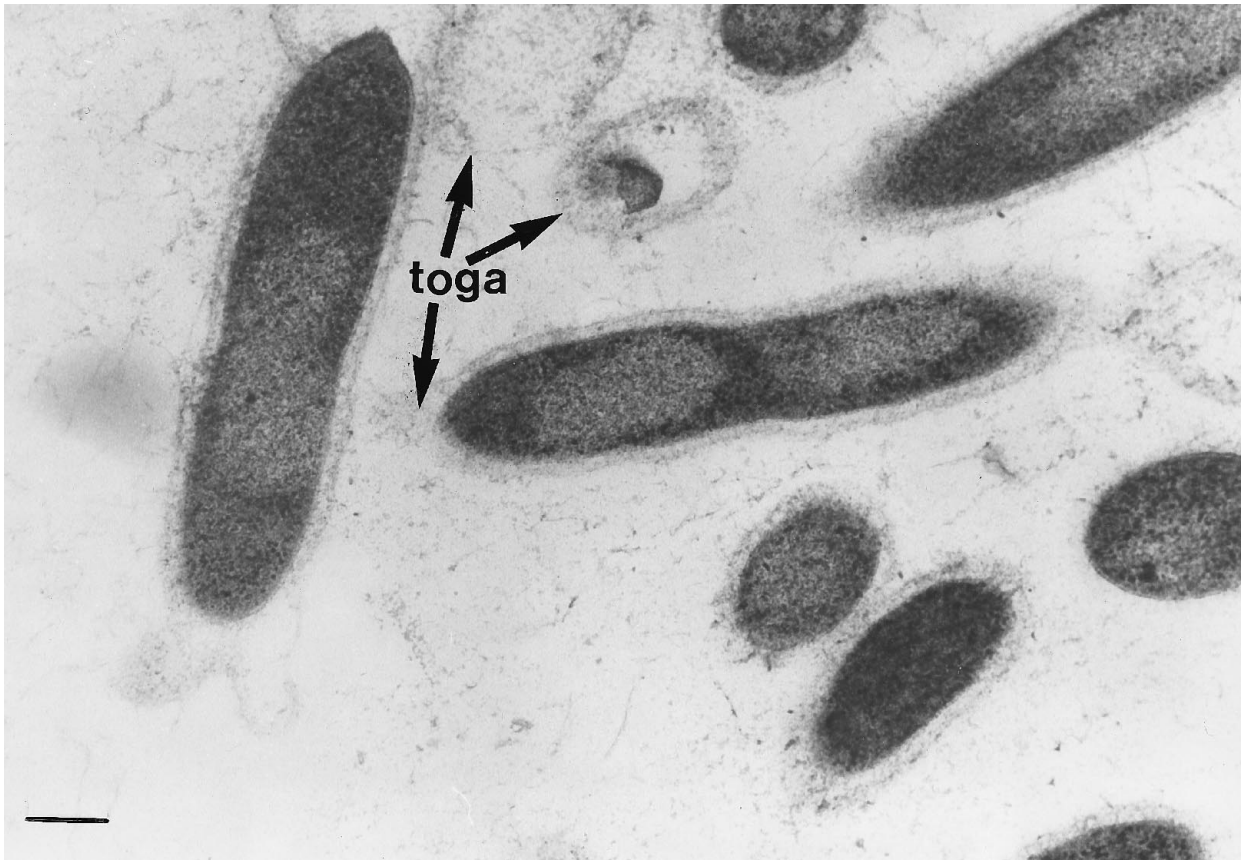

FIG. 2. Transmission electron micrograph of thin sections from the thermophilic isolate. Bar, 0.5 μm.

Cashion et al. (10). The DNA was hydrolyzed with P1 nuclease, and the nucleotides were dephosphorylated with bovine alkaline phosphatase (38). The resulting deoxyribonucleosides were analyzed by high-performance liquid chromatography.

Determination of GC content. The GC content was calculated from the ratio of deoxyguanosine (dG) and deoxythymidine (dT) by the method of Mesbah et al. (38). For the experiments a high-pressure pump, UV detector LKB 2151 connected with a Shimadzu CR-3A integrator, and a Nucleosil 100-5C18 column (250 by 4 mm) equipped with a Nucleosil 100-5C18 precolumn (20 by 4 mm; MELZ VDS Berlin) were used. The chromatographic conditions were 26°C, 0.6 M $(NH_4)H_2PO_4$-acetonitrile, 80/6 (vol/vol) (pH 4.4), as the solvent, and a flow rate of 0.7 ml/min (57). The instrument was calibrated with nonmethylated Lambda-DNA (Sigma) with a GC content of 49.858 mol%.

Sequencing of 16S rDNA. Analysis of 16S rDNA was performed by the method of Rainey et al. (46) using a Taq Dideoxy Terminator Cycle Sequencing kit (Applied Biosystems).

Preparation of cell extracts. For the measurement of protease activity in *Thermotogales* strains, cells were grown anaerobically in 2-liter bottles for 12 to 24 h. The cells were harvested by centrifugation at 26,000 × *g*, and the pellets were washed twice with 50 mM sodium phosphate buffer, pH 6.8. One gram of cells was suspended in 15 ml of the same buffer and sonicated with a Branson sonifier (model 450) in 1-s bursts for 3 min at an output of 20 W, and the cell extracts were centrifuged at 35,000 × *g* for 20 min.

Enzyme assay. Proteolytic activity was detected by a modified method of Kunitz (31). Samples (in duplicates) containing 450 μl of 0.25% (wt/vol) casein (Hammarsten; Merck) in 120 mM universal buffer (9) were incubated in a water bath with a 50-μl enzyme sample (50 to 350 μg of protein per ml) at optimal temperature and pH for 10 to 60 min. The enzyme reaction was stopped by the addition of 500 μl of 10% (wt/vol) trichloroacetic acid. The reaction mixture was centrifuged at 13,000 rpm (Heraeus Sepatech; Osterode) for 10 min, and the absorbance was measured against a blank (nonincubated sample) at 280 nm. One unit of protease was defined as the amount of enzyme yielding the equivalent of 1 μmol of tyrosine per min under the defined assay conditions. Protein concentration was determined by the Bradford method modified by Stoscheck (53) with bovine serum albumin as the standard. The protease inhibitor EDTA, iodoacetate, or phenylmethylsulfonyl fluoride was added to a final concentration of 1 to 5 mM, and 3,4-dichloroisocoumarin was added to a final concentration of 0.2 mM. Ten microliters of the inhibitor solution was incubated with 100 μl of the enzyme sample for 1 h at room temperature. The reaction was started by the addition of 400 μl of the substrate (0.25% [wt/vol] casein). The assay was performed as described above.

SDS-PAGE and zymograms. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 11.5% (wt/vol) uniform gels by the method of Laemmli (32). Silver staining of protein was carried out by the method of Blum et al. (6). Proteolytic activity was detected in situ as described by Heussen and Dowdle (18) with 0.1% (wt/vol) gelatin as the substrate. After electrophoresis the gels were rinsed in 0.25% (vol/vol) Triton X-100 at 4°C for 1 h to remove SDS and incubated (30 min) under optimal assay conditions to detect the proteolytic activity. The gels were stained in a solution of 0.6% (wt/vol) amido black in water-ethanol-acetic acid (60/30/10) for 1 h and destained in water-ethanol-acetic acid (60/30/10). The molecular weight calibration kits from Pharmacia LKB (Freiburg, Germany) were used as standards.

Isoelectric focusing. Isoelectric point determination was performed with Ampholine PAGE plates (pH 3.9 to 9.5) and a Multiphor II Electrophoresis Unit, model 2117 (Pharmacia), according to the manufacturer's instructions. A Pharmacia isoelectric point calibration kit (pH 3.0 to 10.0) was used as the standard. The protein bands were stained with a Phast Gel Blue R staining kit according to the manufacturer's instructions (Pharmacia).

Enzyme purification. Cell extracts were incubated with 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS; 10 mM) overnight at 4°C to solubilize the toga-bound keratinase. The detergent treatment was followed by an ultracentrifugation (Spinco L2 65B; Beckman Instruments, Frankfurt, Germany) at 100,000 × *g* and 15°C for 1 h. Then, 350 ml of 50 mM sodium phosphate buffer, pH 6.8, was added to the supernatant and dialyzed in an ultrafiltration chamber (Amicon, Witten, Germany) using a membrane with a 100-kDa cutoff. The final purification step was performed in a cylindrical SDS-PAGE gel (3.7 by 5 cm; 0.1% [wt/vol] SDS, 6.0% [wt/vol] acrylamide) using a prep-cell unit, model 491 (Biorad, Munich, Germany). The electrophoresis buffer contained 0.3% (wt/vol) Tris-HCl, 1.5% (wt/vol) glycine, and 0.1% (wt/vol) SDS. The samples were treated with Tris-HCl buffer (100 mM; containing 5% [wt/vol] SDS and 2.5% [wt/vol] dithiothreitol) and eluted with sodium phosphate buffer (50 mM, pH 6.8). Fractions of 8.0 ml were collected, pooled, and concentrated in a 10-ml ultrafiltration chamber (Amicon) using a membrane with a 10-kDa cutoff.

Degradation of feather meal. Enzyme samples were incubated with native feather meal (Degussa AG, Hanau, Germany) at 80°C and pH 9.0 in a shaking water bath (model HT; Infors, Einsbach, Germany) at 150 rpm for 7 days. Protease (0.1 U) was added to 40 mg of feather meal by using a glycine-NaOH-NaCl buffer (100 mM). As a control, feather meal was incubated in a sterilized buffer under the same conditions. The dry weight of the remaining feather meal substrate was determined on membrane filters (pore size, 0.2 μm) after drying at 105°C for 12 h.

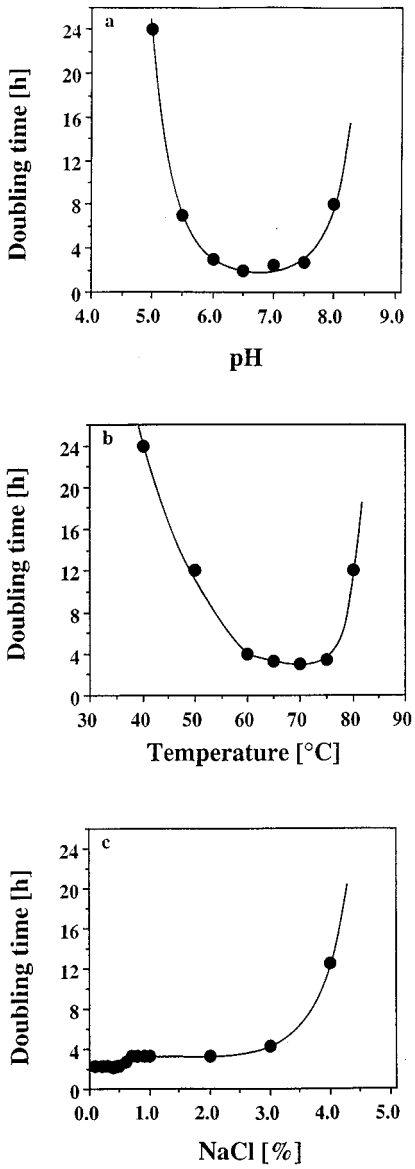

FIG. 3. Influences of pH, temperature, and salt concentrations on growth of the isolated thermophilic bacterium. (a) pH was varied at 70°C; (b) temperature was changed at pH 6.5; (c) NaCl concentration was varied at pH 6.5 and 70°C.

Production of free amino acids and peptides. The production of free amino acids from native feather meal was measured with an amino acid analyzer using ninhydrin as a reagent for postcolumn derivatization. The release and the sizes of peptides from native feather meal were determined by gel permeation chromatography on Superose 12 HR 10/30 and Ultraspherogel columns (Pharmacia). The following proteins were used as standards: thyroglobulin (669 kDa), β-amylase (200 kDa), lactate dehydrogenase (145 kDa), albumin (66 kDa), ovalbumin (45 kDa), carboanhydrase (30 kDa), myoglobin (17 kDa), aprotinin (6.5 kDa), and β-endorphin (3 kDa).

RESULTS

Screening for feather degradation by thermophilic microorganisms. Nineteen strains of extremely thermophilic and hyperthermophilic anaerobic archaea and bacteria were investigated for their ability to grow on native feathers. The investigated strains were *Thermococcus stetteri*, *Thermococcus* sp. strain AN1, *Thermococcus litoralis*, *Thermococcus celer*, *Staphylothermus marinus*, *Pyrococcus furiosus*, *Pyrococcus woe-*

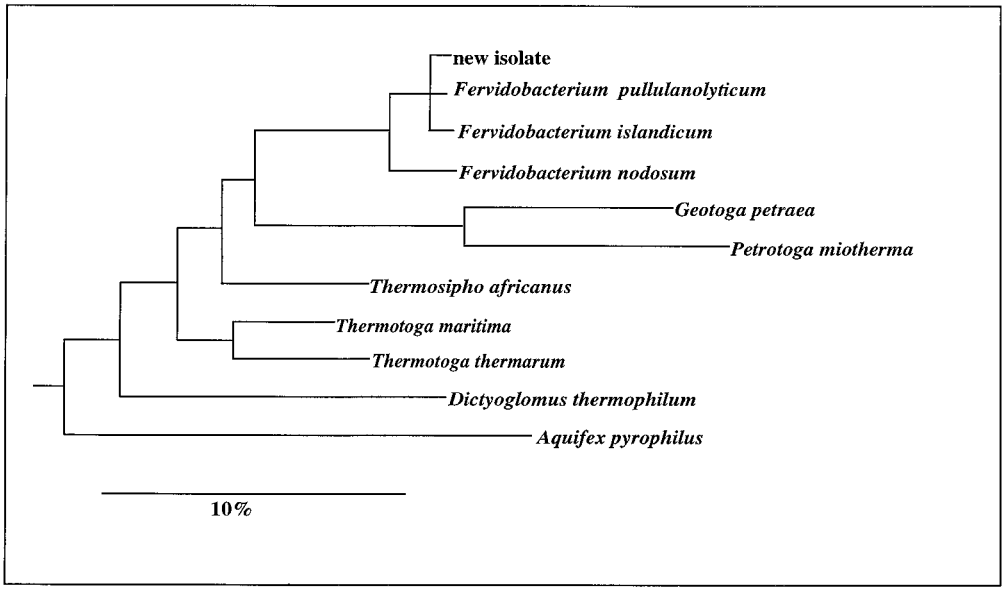

FIG. 4. Phylogenetic tree of members of the *Thermotogales* order as revealed by 16S rDNA analysis.

sei, *Thermoanaerobacter brockii*, *Thermoanaerobacter thermohydrosulfuricus*, *Thermoanaerobacter ethanolicus*, *Thermoanaerobacter finnii*, *Thermobacteroides proteolyticus*, *Thermosipho africanus*, *Thermotoga maritima*, *Thermotoga neapolitana*, *Thermotoga thermarum*, *F. nodosum*, *F. islandicum*, and *F. pullulanolyticum.* Of the 19 investigated extremely thermophilic microorganisms, 18 strains were unable to attack feathers. *F. pullulanolyticum* could attack feathers to a very low extent and only after incubation for 7 days. In vitro experiments with proteases from *Thermococcus stetteri*, *Thermococcus* sp. strain AN1, and *Thermosipho africanus* have also shown that the structure of native feathers was not altered significantly even after incubation at 75°C for 7 days.

Isolation of feather-degrading bacteria. After incubation of samples from hot springs of the island San Miguel (Azores, Portugal) in I medium with native feathers at 70°C under anaerobic conditions enrichments of mixed populations of microorganisms were obtained. The mixed cultures grew to a cell density of 10^7/ml within 12 h. The cultures were then diluted to 10 cells per ml and plated on agar plates, which were incubated under anaerobic conditions. After 3 days milky colonies surrounded by clear substrate lysing zones were picked and transferred into liquid media (I media). After three transfers in liquid cultures and on agar plates a pure culture was obtained. The new isolate was found to degrade native feathers completely within 48 h (Fig. 1). Stock cultures of the newly isolated organism remained viable even after storage for 12 months at 4°C.

Morphology. The feather-degrading isolate was rod shaped with a characteristic outer sheath-like structure. During the exponential growth phase short rods about 2 μm long occurred singly or in pairs, whereas cells grown for 24 h reached a length of 20 μm. At one end of the cell a characteristic bleb was visible (Fig. 2). In stationary-phase cultures spheroids were observed containing up to four cells. The isolate stained gram negative, and no endospores were observed.

Optimal growth conditions. Growth of the newly isolated strain occurred between pH 5.5 and 8.0 (Fig. 3a) and between 50 and 80°C (Fig. 3b) with an optimum at pH 6.5 and 70°C. Growth was detected between 0 and 4% (wt/vol) NaCl; the optimal NaCl concentration was 0.4% (wt/vol) (Fig. 3c). Under optimal growth conditions the doubling time was 2.1 h.

Growth substrates. The new isolate was able to use starch, glycogen, pullulan, glucose, fructose, maltose, and xylose as growth substrates. Starch was the preferred carbon source. The new isolate was not able to utilize lactose, amylose, arabinose, casein, collagen, succinate, or Tween 80. The presence of 0.05% yeast extract was required for growth. Hydrogen gas had an inhibitory effect which could be eliminated by the addition of elemental sulfur or thiosulfate to the medium. The new isolate was a fermentative and strictly anaerobic microorganism. As fermentation products ethanol and acetate were found after 48 h of incubation at 70°C; acetate was the major fermentation product.

DNA base composition. The GC content of the new isolate was 40.0 ± 0.6 mol% (n = 3). This is a typical GC content for members of the *Thermotogales* order.

16S rDNA analysis. Similarities among 16S rDNA sequences of members of the *Thermotogales* family are shown in Fig. 4. The isolated strain showed high levels of homology with *F. pullulanolyticum* (99.0%) and with *F. islandicum* (98.7%). These three strains formed a cluster. Low-level homology was found with *Petrotoga miotherma* (80.2%), *Geotoga petraea* (82.2%), *Dictyoglomus thermophilum* (80.7%), and *Aquifex pyrophilus* (80.2%). On the basis of the physiological, morphological, enzymological, and 16S rDNA studies the new isolate was characterized as a strain of the *Thermotogales* order, named *Fervidobacterium pennavorans*, and deposited at the German Collection for Microorganisms and Cell Cultures (DSMZ) as strain DSM 7003.

Purification and properties of the heat-stable keratinase. The cell-bound keratinase of *F. pennavorans* was purified 32-fold with a yield of 7% (Table 1). Since the enzyme activity was not influenced by 0.1% (wt/vol) SDS, it could be purified by preparative SDS-PAGE (Fig. 5). Analytical SDS-PAGE and zymogram staining showed that the purified native enzyme has a molecular mass of 130 kDa (Fig. 6). The enzyme, which was inhibited by phenylmethylsulfonyl fluoride and 3,4-dichloroisocoumarin, was characterized as a serine protease. EDTA and iodoacetate (1 to 5 mM) had only little effect on the enzyme

TABLE 1. Purification of *F. pennavorans* keratinase by preparative SDS-PAGE

Step[a]	Protein (mg)	Activity (U)	Sp act (U/mg)	Yield (%)	Purification (fold)
Cell extract	130	13	0.1	100	1
CHAPS-treated cell extract	130	13	0.1	100	1
Supernatant after ultracentrifugation	91	9	0.1	69	1
Amicon-washed concentrate	52	5.6	0.11	43	1.1
Prep-cell	0.28	0.9	3.2	7	32

[a] Cells of *F. pennavorans* were cultivated in a 19-liter fermentor (Bioengineering, Wald, Switzerland) and grown for 12 h at 70°C in a nitrogen-CO_2 atmosphere (80:20). After harvesting, cell extracts were prepared and incubated overnight with 10 mM CHAPS at 4°C.

activity. The keratinase was active in a broad pH and temperature range, namely, between pH 6.0 and 10.5 and between 50 and 100°C (Fig. 7 and 8). After isoelectric focusing one single protein band was observed at pH 3.8 (data not shown).

Degradation of native feather meal by *F. pennavorans*. As shown in Table 2, feather meal degradation was observed after incubation with cell extracts, membrane fractions, and the purified keratinase of *F. pennavorans*. In these experiments amino acids and peptides were released when native feather meal was incubated with cell extracts or membrane fractions. After incubation of feather meal with cell extracts all amino acids present in feathers were detected: aspartate, threonine, serine, glutamate, proline, glycine, alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, and arginine. After incubation with membrane fractions no release of aspartate, glutamate, proline, glycine, or lysine was observed. Incubation of feather meal with the purified enzyme, however, resulted in its conversion to peptides but not amino acids. In control experiments containing buffer and feather meal, no amino acids or peptides were detected and the feather meal residual dry weight (total recovery) remained 100%.

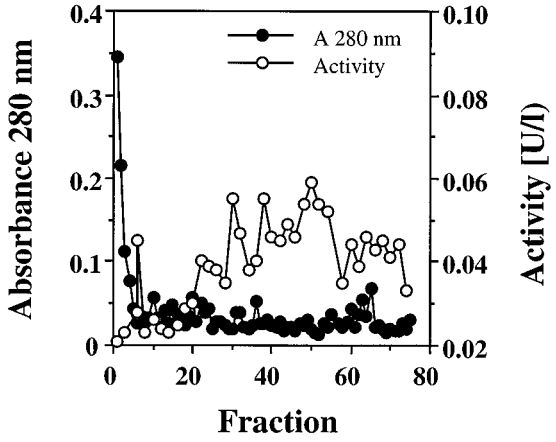

FIG. 5. Elution profile after preparative SDS-PAGE. Cell extracts of *F. pennavorans* were incubated with 10 mM CHAPS overnight at 4°C to solubilize the toga-bound keratinase. After ultracentrifugation at 100,000 × *g* the enzyme solution was dialyzed with 50 mM sodium phosphate buffer, pH 6.8. The final purification step was performed by preparative SDS-PAGE as described in Materials and Methods. Fractions of 8.0 ml were collected, pooled, concentrated, and stored at 4°C for further analysis.

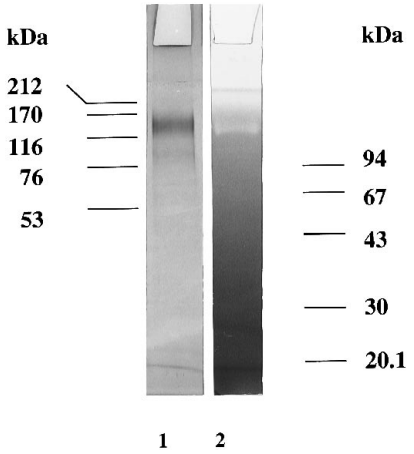

FIG. 6. SDS-PAGE (11.5% polyacrylamide) of the purified keratinase (1 μg of protein per lane) from *F. pennavorans*. Lane 1, detection of protein band by silver staining; lane 2, activity band by zymogram staining.

DISCUSSION

The ability of extremely thermophilic microorganisms to degrade native feathers which are composed of 95% protein seems to be very rare among thermophilic microorganisms and is so far restricted to *F. pennavorans*. The newly isolated strain is, therefore, unique in this respect. The new isolate has been characterized as a member of the *Thermotogales* order. The temperature limits of growth are similar to those of *F. nodosum* and *F. islandicum*. The GC content of 40 mol% is similar to that of *F. islandicum*, *Thermotoga maritima*, and *Petrotoga miotherma* (13). As confirmed by comparative 16S rDNA analysis, the new isolate is highly related to *F. pullulanolyticum* and *F. islandicum*. Unlike the new isolate, both of these strains are, however, unable to degrade native feathers and produce thermostable keratinolytic enzymes. Slight alteration in the structure of feather material, however, has been observed with *F. pullulanolyticum* after cultivation at 70°C for more than 1 week. On the basis of these observations, the physiological and 16S rDNA studies, and the formation of a typical bleb at one end of the cell, we identified the strain as a *Fervidobacterium* species and named it *F. pennavorans*—Friedrich and Antranikian (sp. nov.), pen.′ na.vo.′ rans. L. adj. *penna* feather, *vorans* eating—to describe the first thermophilic anaerobe with a special ability to degrade native feathers.

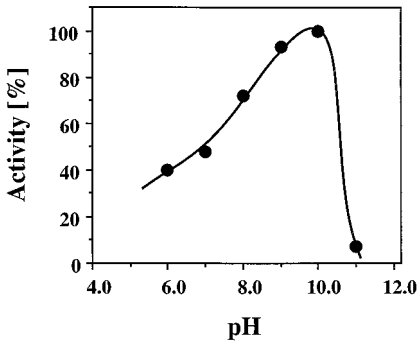

FIG. 7. Influence of pH on the purified keratinase of *F. pennavorans*. Enzyme samples (50 U/liter) were incubated in duplicates for 60 min at different pH values as described in Materials and Methods. The data are averages of three experiments.

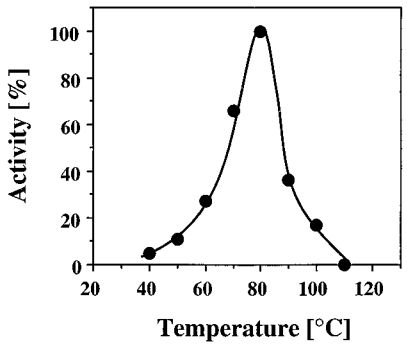

FIG. 8. Effect of temperature on the purified keratinase of *F. pennavorans*. Enzyme samples (50 U/liter) were incubated in duplicates for 60 min at an optimal pH and different temperatures as described in Materials and Methods. The data are averages of those from three experiments.

The resistance of the keratinolytic enzyme system of *F. pennavorans* to SDS was exploited by using SDS-PAGE for enzyme purification. This method has also been successfully employed for the purification of serine protease from the extremely thermophilic archaeon *Thermococcus stetteri* (25). The resistance of these enzymes to SDS seems to be related to the rigid structure of proteins from extremely thermophilic microorganisms. The purified keratinase of *F. pennavorans* has a molecular mass of 130 kDa, which is larger than those of the keratinases of mesophilic microorganisms such as *Bacillus* and *Streptomyces* spp. (20 to 50 kDa) (8, 24, 33, 36, 35, 41, 50, 54, 55). In comparing the sizes of keratinase of *F. pennavorans* before (250 kDa) and after (130 kDa) purification, it can be concluded that the observed large size is probably due to association of the keratinase with the outer cell envelope (toga), which could be solubilized by detergent treatment with CHAPS. A similar phenomenon has been described for amylases of *Thermotoga maritima* and a xylanase of *Thermotoga maritima* MSB8 (49, 65).

Interestingly, the keratinase from *F. pennavorans* is catalytically active at high temperature (80°C) and alkaline pH (pH 10.0). An enzyme with similar properties has been described for *Bacillus* sp. strain AH-101 (54–56). The keratinases of the mesophilic microorganisms *Streptomyces pactum* and *Tritirachium album* Limber are also active under alkaline conditions but are less thermostable (8, 14). The isoelectric point of the keratinase from *F. pennavorans* is pH 3.8. An isoelectric point in the acidic region is rare for keratinolytic enzymes. Most keratinases have isoelectric points ranging from pH 7.0 to 9.0 (2, 14, 36, 35, 41, 54, 55, 67, 68). The keratinase of *F. pennavorans* was classified as serine protease. All keratinases described until now are proteases, mainly of the serine type (2, 8, 14, 24, 36, 35, 41, 54, 55, 68), but not all serine type proteases are able to degrade native keratins (7, 11, 15, 25, 26, 34, 50). The mechanism of hydrolysis of native keratin is still unknown.

TABLE 2. Degradation of native feather meal by *F. pennavorans*

Sample[a]	Total recovery of feather meal (%)	Peptide[b] formation	Release of free amino acids[c]
Control	100	−	0
Cell extract	38	+	25
Membrane fraction	44	+	10
Purified keratinase	85	+	0

[a] Enzyme samples (0.1 U) from *F. pennavorans* were incubated with 40 mg of native feather meal at 80°C and pH 9.0 in a shaking water bath at 150 rpm for 7 days. Membrane fractions were obtained after ultracentrifugation of cell extracts without detergent treatment. Pellets with membrane-bound keratinase were resuspended and incubated with native feather meal as described above. The residual dry weight of the remaining feather meal substrate (total recovery) was determined on membrane filters which were dried at 105°C for 12 h.

[b] ≤3,000 Da.

[c] Percent substrate converted to amino acids.

In vitro experiments with the purified keratinase have shown that endopeptidases are also necessary for the conversion of keratin to amino acids. No cystine or cysteine residues were detected in the hydrolysis products. This might be due to a chemical conversion of cystine to lanthionine under alkaline conditions as described by Spindler and Tanner (51). Although several keratinases were reported to degrade native keratins, no detailed information on the resulting hydrolysis products of these experiments has been provided (2, 8, 14, 36, 35, 41, 55–57, 67, 68).

The keratinase of *F. pennavorans* is a promising enzyme for application in an industrial process. Native feathers from poultry farming could be converted to peptides and rare amino acids like proline and serine. The thermostability of the enzyme is of advantage for these purposes because the process could be performed at around 70°C and the risk of contamination can be minimized. The properties of this enzyme, namely, the thermostability and the ability to degrade native keratin, are also of interest to study structure-function relationships of heat-stable enzymes from extremely thermophilic microorganisms. To the extent that the particular native conformation is governed by the primary structure, additional comparison studies concerning amino acid composition and sequence are necessary for a better understanding of the keratinase thermostability and the properties associated with it. This will be of relevance for special biotechnological applications of this and other thermostable enzymes.

ACKNOWLEDGMENTS

We thank H. Hippe for final characterization of *F. pennavorans*, F. Mayer for electron microscopy, and R. I. L. Eggen and F. A. Rainey for 16S rDNA analysis. Furthermore, we thank K. Drauz and A. Bommarius, Degussa AG, for peptide and amino acid analysis.

We thank the Fonds der Chemischen Industrie for financial support. The work was supported by the Graduiertenkolleg Biotechnologie of the Deutsche Forschungsgemeinschaft.

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