Isolation and characterization of *Keratinibaculum paraultunense* gen. nov., sp. nov., a novel thermophilic, anaerobic bacterium with keratinolytic activity

Yan Huang1,2, Yingjie Sun1,2, Shichun Ma1,2, Lu Chen1,2, Hui Zhang1,2 & Yu Deng1,2

1Biogas Institute of Ministry of Agriculture, Chengdu, China; and 2Key Laboratory of Development and Application of Rural Renewable Energy, Ministry of Agriculture, Chengdu, China

**Abstract**

A novel thermophilic, anaerobic, keratinolytic bacterium designated KD-1 was isolated from grassy marshland. Strain KD-1 was a spore-forming rod with a Gram-positive type cell wall, but stained Gram-negative. The temperature, pH, and NaCl concentration range necessary for growth was 30–65 °C (optimum 55 °C), 6.0–10.5 (optimum 8.0–8.5), and 0–6% (optimum 0.2%) (w/v), respectively. Strain KD-1 possessed extracellular keratinase, and the optimum activity of the crude enzyme was pH 8.5 and 70 °C. The enzyme was identified as a thermostable serine-type protease. The strain was sensitive to rifampin, chloramphenicol, kanamycin, and tetracycline and was resistant to erythromycin, neomycin, penicillin, and streptomycin. The main cellular fatty acid was predominantly C15:0 iso (64%), and the G+C content was 28 mol%. Morphological and physiological characterization, together with phylogenetic analysis based on 16S rRNA gene sequencing identified KD-1 as a new species of a novel genus of *Clostridiales* with 95.3%, 93.8% 16S rRNA gene sequence similarity to *Clostridium ultunense* BST (DSM 10521T) and *Tepidimicrobium xylanilyticum* PML14T (= JCM 15035T), respectively. We propose the name *Keratinibaculum paraultunense* gen. nov., sp. nov., with KD-1 (=JCM 18769T =DSM 26752T) as the type strain.

**Introduction**

Keratins are insoluble structural proteins that are extensively cross-linked by disulfide bonds, hydrogen bonds, and hydrophobic interactions. Keratins are abundant in hair, feathers, and horns. The α-helix and β-sheet configurations of keratins are characteristically resistant to common proteases including trypsin, pepsin, and papain (Thys et al., 2004; Riffel et al., 2007). However, keratins can be degraded by keratinase secreted by keratinolytic microorganisms (Lin et al., 1992; Boeckle et al., 1995; Bernal et al., 2006; Ionata et al., 2008). Reflecting the ubiquity of keratinolytic microorganisms in natural environments, keratin accumulation does not naturally occur (Williams et al., 1990; Riffel et al., 2007). Keratinolytic microorganisms include bacteria (mainly actinobacteria and bacteria in the genus *Bacillus*), fungi, and archaea (Brandelli et al., 2010; Korniłowicz-Kowalska & Bohacz, 2011).

We isolated and characterized a thermophilic, anaerobic, feather-degrading bacterium secreting extracellular serine keratinase. Based on the phenotypic, genotypic, and physiological evidence, strain KD-1 is herein identified as a new species of a novel genus of *Clostridiales* and is designated *K. paraultunense* KD-1.

**Materials and methods**

**Enrichment and isolation**

Strain KD-1 was isolated from grassy marshland. The soil was added to an enrichment medium (EM) (L−1: feather 10 g, peptone 10 g, MgSO4·7H2O 0.2 g, L-Cys-HCl 1 g, resazurin 1 mg) in anaerobic bottles sealed with butyl rubber stoppers under a gaseous atmosphere of 100% N2 (Hungate, 1969; Bryant, 1972; Hungate & Macy, 1973). A selective medium (SM) (L−1: feather 10 g, K2HPO4 1 g, KH2PO4 0.4 g, NaCl 3 g, MgSO4·7H2O 0.2 g, vitamin...
solution 10 mL (DSM 141 medium), trace element solution 10 mL (DSM 141 medium), L-Cys-HCl 1 g L⁻¹, and resazurin 1 mg] was used to isolate feather-degrading bacteria. The medium pH was adjusted to 7.0–7.5 with 5 M KOH. The solid version of the SM was of the same composition, with the exceptions of feather meal as replacement for whole feather and inclusion of 2% (w/v) agar. All media were sterilized by autoclaving at 121 °C for 30 min. Feather-degrading bacteria were isolated by serial dilution and the Hungate roll-tube technique (Hungate, 1969). Enrichment culture (0.4 mL) was serially diluted in 4 mL of solid medium in roll tubes using syringes and needles, and incubated at 55 °C. Single colonies were picked and transferred to the SM medium and incubated at 55 °C. Cell morphology was checked using a model 80i phase-contrast microscope (Nikon, Japan). The roll-tube procedure was repeated several times until a pure culture was obtained.

Morphology

Cell morphology were examined using scanning electron microscopy (SEM) with a JSM-7500F microscope (JEOL, Japan) (Cheng et al., 2008) and by transmission electron microscopy (TEM) using a H-600V microscope (Hitachi, Japan) (Mikucki et al., 2003). SEM was also used to observe the surface of chicken feathers. Gram stain reaction was determined by a traditional method (Boone & Whitman, 1988) and KOH technique (Buck, 1982). Spore staining was performed conventionally.

G+C content

Genomic DNA was extracted and purified using a TIANamp bacteria DNA extraction kit (TIANGEN Biotech, China). The G+C content was determined by the thermal denaturation method (Marmur & Doty, 1962; Cheng et al., 2007) using a Lambda35 UV/VIS Spectrometer (Perkin/Elmer) with Escherichia coli K12 (CGMCC 1.365) as the reference bacterial strain.

16S rRNA gene sequencing and analysis

The 16S rRNA gene was amplified from the extracted genomic DNA using a PCR kit (TakaraBio, Japan) with primers 27f (5’AGAGTTTGATCMTGGCTCAG) and 1492r (5’TACGGYTACCTTGTTACGACTT) (Karita et al., 2003). The PCR conditions were 94 °C for 5 min, 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min; and 72 °C for 10 min. The PCR products were purified using a PCR purification kit (TIAGEN Biotech). Sequence data were performed by GeneWiz. The 16S rRNA gene sequence of strain KD-1 was submitted to GenBank to search for similar sequences using the BLAST algorithm. The best matching sequences were retrieved from the database and aligned. The similarity analysis was performed using the ClustalW program within MEGA4 (Tamura et al., 2007). Phylogenetic trees were constructed using the neighbor-joining, maximum-likelihood, and maximum-parsimony methods implemented in MEGA4 (Tamura et al., 2007). The phylogenetic tree was evaluated by bootstrap analysis based on 1000 replications.

Cellular fatty acid content

Fatty acid analyses were carried out by the identification service of the DSMZ, Braunschweig, Germany using the Sherlock MIS (MIDI Inc., Newark) system.

Respiratory quinones

Respiratory quinones of strain KD-1T were extracted, separated, and identified as Minnikin et al. (1984) and analyzed by high-performance liquid chromatography (HPLC) (Collins & Jones, 1980).

Physiology and biochemistry analyses

Potential substrate utilization studies were performed in basal medium (SM culture without feather) containing various substrates. The substrates were proteins and sugars at the final concentration of 1% (w/v) and amino acids at a final concentration of 20 mM. The pH was adjusted to 8.0 with 3% (w/v) Na₂S and was incubated at 55 °C.

To investigate the utilization of electron acceptors, autoclaved anaerobic 9,10-anthraquinone-2,6-disulfonate (AQDS), FeCl₃, Fe₂O₃, citric acid iron; Na₂S₂O₃, NaNO₃, disodium fumarate, Na₂SO₄, NaNO₂, and sodium selenite (final concentration 20 mM) were injected to the SM before the addition of peptone as electron donor (final concentration 1% w/v). Cultivating was carried out at 55 °C. Growth was determined by measuring the optical density at 600 nm (OD₆₀₀ nm) of cultures, and the products were determined as previously described (Slobodkin et al., 1999; Zavarzina et al., 2002).

Volatile fatty acids (VFA) were analyzed by gas chromatography using an Agilent 7820A system equipped with a DB-FFAP column (30 m × 250 μm ×0.5 μm) and a flame ionization detector. Nitrogen was supplied as the carrier gas. The flow rate of nitrogen, hydrogen, and air was 44, 40, and 400 mL min⁻¹, respectively. The injection port and detector temperature were 250 °C. The oven was sequentially maintained at 40 °C for 3 min, 100 °C for 10 min, 180 °C for 5 min, and 200 °C for 15 min. After acidification with 5 M HCl and centrifugation at
12 000 g for 10 min, 1 µL of sample was injected for gas chromatography analysis. A mixture of methanol, ethanol, isobutanol, acetic acid, propionic acid, butyric acid, isobutyric acid, isovaleric acid, and hexanoic acid was used as an external standard.

The growth curve, temperature, pH, and NaCl range for growth were determined by monitoring the OD_{600 nm} of the SM culture. The growth curve test was conducted using optimum conditions of temperature and pH. The effect of temperature was explored using a temperature range of 15–75 °C in 5 °C intervals. The pH range was from 5.0 to 11.0 and was adjusted with HCl or NaOH (1 M). NaCl values ranged from 0 to 10% (w/v).

The antimicrobial susceptibility was tested in SM culture with various antibiotics including neomycin sulfate, ampicillin sodium, streptomycin sulfate, kanamycin sulfate, chloramphenicol, erythromycin, rifampicin, and tetracycline–HCl at a final concentration of 100 µg mL\(^{-1}\). Growth was determined by measuring the OD_{600 nm} of cultures.

All experiments were carried out in triplicate.

**Enzyme assays**

**Keratinase**

Keratinase activity was measured using feather meal as the substrate as described previously (Ramnani et al., 2005) with some modifications. Briefly, 2 mL of 50 mM Tris-HCl buffer (pH 8.5) was mixed with 10 mg of feather meal, 1.0 mL of sterile supernatant was added, and the mixture was incubated at 70 °C for 1 h. The reaction was stopped by the addition of 2 mL of 10% trichloroacetic acid (TCA), and the mixture was stored on ice for 10 min prior to centrifugation at 11 963 g for 10 min. The absorbance of the supernatant was measured at 280 nm in a model DU730 spectrophotometer (Beckman). The control was prepared by adding TCA to the reaction mixture before adding the enzyme solution. One unit of keratinase activity was defined as the amount of enzyme that produced an increased absorbance of 0.01.

**Thiol group and disulfide reductase**

Free thiol content in KD-1 cultures was determined as described previously (Ellman, 1959) with only minor modification (use of reduced glutathione as the standard). A 50 mM working solution of 5,5-dithiobis, 2-nitrobenzoic acid (DTNB) was prepared in a phosphate buffer (pH 8.0) in a 1 : 50 ratio. Two hundred microliters of the working solution was mixed with 20 µL extracellular broth. Absorbance was measured at 412 nm after 15 min at room temperature. Phosphate buffer was used to replace the sample in the control preparations. Disulfide reductase activity was measured as previously described (Ramnani et al., 2005). Extracellular, lyzed cell, and lyzed broth were tested. One milliliter of enzyme mixture (sample, phosphate buffer (100 mM, pH 8.0), and distilled water in a 3 : 2 : 5 ratio) was incubated with 1 mL of 2 mM oxidized glutathione (dissolved in 100 mM, pH 7.0 phosphate buffer) for 30 min at 55 °C with 5 mM phenylmethylsulfonyl fluoride. The mixture was centrifuged at 1000 g for 10 min, and the supernatant was analyzed for the content of the thiol group. Phosphate buffer (100 mM, pH 7.0) was used instead of oxidized glutathione as the control.

**Keratinolytic protease**

Crude enzyme was used to study the characteristics of keratinolytic protease. The pH optimum was determined in the pH range 7.0–9.0 with 50 mM Tris-HCl buffer. To determine the optimal temperature for keratinolysis, enzyme reactions were carried out at different temperatures from 45 to 80 °C in Tris-HCl buffer (50 mM, pH 8.5) for 1 h. To investigate thermostability, the crude enzyme was preincubated for 0–210 min at 70, 80, and 90 °C. The residual activity was measured as described above. The effects of metal ions, enzyme inhibitors, detergents, and organic solvents on keratinase activity were ascertained as described above.

**Results and discussion**

Strain KD-1 was a Gram-negative, spore-forming anaerobe. Colonies were white, round, and smooth. Cells were rod shaped (0.3–0.5 × 1.5–2.7 µm) and were arranged alone or linked together end-to-end (Fig. 1a). TEM of ultrathin sections showed a Gram-positive cell wall structure (Fig. 1b). Growth was observed at 30–65 °C (optimum 55 °C), pH 6.0–10.5 (optimum 8.0–8.5), and NaCl concentration of 0–6% (optimum 0.2%). The mean generation time of strain KD-1 was 3.02 h at optimum condition. The strain was sensitive to rifampin, chloramphenicol, tetracycline, and kanamycin and was resistant to erythromycin, neomycin, penicillin, and streptomycin.

Strain KD-1 could degrade native feather within 24 h, and the cells adhered to the surface of feathers (Fig. 2). These findings were similar to those for *Bacillus subtilis* SLC (Cedrola et al., 2012), *B. subtilis* S8 (Jeong et al., 2010), and *B. licheniformis* RG1 (Ramnani et al., 2005). Gelatin, soybean protein, peptone, beef extract, yeast extract, skimmed milk, collagen, casein, chicken feather, duck hair, hair, ox hair, pig hair, and nails could be used as sources of carbon and energy. Strain KD-1 could also use xylan, glycogen, succinate, disodium fumarate,
proline + alanine, proline + valine, and proline + serine.

Weak growth was observed in the presence of arabinose, maltose, rhamnose, melibiose, raffinose, fructose, sucrose, glucose, xylose, cellobiose, and trehalose. Mannose, amylose, amylpectin, chitin, sodium citrate, sodium hydroxyacetate, sodium carboxymethyl cellulose, 3-hydroxybutyrate, sodium formate, sodium acetate, sodium pyruvate, sodium butyrate, sodium benzoate, sodium malate, 1,2-propylene glycol, methanol, alcohol, 2,3-butanediol, glycerol, olive oil, pectin, D-sorbitol, and betaine were negative. Acetic acid, propionic acid, isobutyric acid, butyric acid, and isovaleric acid were the predominant VFAs produced, with trace amounts of methanol, ethanol, isobutanol, and hexanoic acid produced using peptone or chicken feather as the substrate.

The cellular fatty acids of strain KD-1 were C15:0 iso (64.5%), C15:0 iso dma (22.5%), C16:0 dma (2.1%), C16:0 (2.0%), C14:0 dma (1.5%), C11:0 dma (1.5%), C14:0 (1.5%), C17:0 iso dma (1.3%), C17:0 iso (0.7%), C18:0 (0.6%), C18:1o9c (0.6%), C13:0 iso (0.5%), C18:0 dma (0.5%), and C11:0 iso (0.4%). The G+C content of strain KD-1 was 28 mol% based on Tm. No respiratory quinone was detected in the cells. The near-complete 16S rRNA gene sequence (1491 bp) of strain KD-1 was compared with the most similar sequences retrieved from GenBank. Blast analysis revealed 95.3% and 93.8% similarity with Clostridium ultunense BST and Tepidimicrobium xylanilyticum PML14T, respectively (Fig. 3). Table 1 summarizes some similarities and differences of strain KD-1 with some related taxa. Strain KD-1T had no flagella, while C. ultunense BST and T. xylanilyticum PML14T did. Strain KD-1T produced indole and liquefied gelatin and degraded keratin, while
Fig. 3. Unrooted phylogenetic tree based on 16S rRNA gene sequences of strain KD-1 and related species using neighbor-joining methods in the program MEGA4. The bar denotes 1% sequence divergence.

Table 1. Differential characteristics of strain KD-1 and species most closely related phylogenetically.

<table>
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<th>1</th>
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<tbody>
<tr>
<td>1. Gram staining</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2. Spore forming</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>3. Cell shape</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
</tr>
<tr>
<td>4. Colonies</td>
<td>Whitish round, 0.5 to 1 mm in diameter</td>
<td>Whitish, nearly transparent, disc-shaped, 0.5–1 mm in diameter</td>
<td>ND</td>
<td>Round, whitish</td>
</tr>
<tr>
<td>5. Cell width x length (µm)</td>
<td>0.3–0.5 x 1.5–2.7</td>
<td>0.5–0.7 x 0.5–7</td>
<td>0.5–0.6 x 3.0–7.0</td>
<td>0.4–0.5 x 4.0–10.0</td>
</tr>
<tr>
<td>7. pH range (optimum)</td>
<td>6.0–10.5 (8.0–8.5)</td>
<td>5.0–10.0 (7)</td>
<td>5.5–9.5 (7.5–8.0)</td>
<td>5.8–9.3 (8.5)</td>
</tr>
<tr>
<td>8. Motility</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>9. Flagella</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>10. Indole production</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>11. Gelatin hydrolysis</td>
<td>+</td>
<td>–</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>12. NaCl range (optimum)/ (%;w/v)</td>
<td>0–6.0% (0.2)</td>
<td>0.8M</td>
<td>0–3.5%</td>
<td>0–4.5% (3.0%)</td>
</tr>
<tr>
<td>13. Keratinolytic</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>14. DNA G + C content/ (mol%)-Tm</td>
<td>28</td>
<td>32</td>
<td>33</td>
<td>36.2 ± 0.8</td>
</tr>
<tr>
<td>15. Predominant fatty acids</td>
<td>C15:0 iso (64%)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>16. Main substrate utilization</td>
<td>Some rigid keratins, peptone, casein, yeast extract</td>
<td>Formate, ethylene, glycol, pyruvate, betaine, cysteine, glucose</td>
<td>Peptone, tryptone, Casamino acids, yeast extract, beef extract, casein hydrolysate, valine, alanine plus glycine, alanine plus proline, n-propanol</td>
<td>Number of proteinaceous compounds and carbohydrates</td>
</tr>
<tr>
<td>Reference</td>
<td>This study</td>
<td>Schnüer et al. (1996)</td>
<td>Slobodkin et al. (2006)</td>
<td>Niu et al. (2009)</td>
</tr>
</tbody>
</table>

Species: 1, Strain KD-1; 2, *Clostridium ultunense* (DSM 10521T); 3, *Tepidimicrobium ferrophilum* (DSM 16624T); 4, *Tepidimicrobium xylanilyticum* (PML14T). ND, Not determined.
**C. ultunense** BST and *T. xylanilyticum* PML14T did not. Further, there had almost 20 °C difference between strain KD-1T and *C. ultunense* BST. Furthermore, *C. ultunense* BST could oxidize acetate in syntrophic association with hydrogenotrophic methanogenic bacteria, while strain KD-1T did not. The G+C content of strain KD-1, *C. ultunense* BST and *T. xylanilyticum* PML14T was 28 mol%, 32 mol%, and 36.2 mol%, respectively, which were significantly different. Therefore, based on the phenotypic, genotypic, and physiological evidence, strain KD-1 was identified as a new species of a novel genus of Clostridiales and named *K. paraultunense* gen. nov., sp. nov.

**Determination of thiol group and disulfide reductase**

Free thiol groups were detected in the extracellular broth. The content of free thiols increased with cultivation time (Supporting information, Fig. S1). No disulfide reductase activity was evident in vitro, suggesting another mechanism of disulfide bond reduction. It is conceivable that KD-1-mediated disulfide bond reduction is important in the adhesion of KD-1 to the feather surface.

**Effect of pH and temperature on crude keratinase enzyme activity**

The keratinase of strain KD-1 was active at neutral and alkaline pH and exhibited optimum activity at pH 8.5 (Fig. S2a). The enzyme was stable at high temperature (70–90 °C) (Fig. S2b), but keratinase activity declined precipitously at 90 °C. The optimum temperature for keratinase activity of strain KD-1 was 70 °C (Fig. S2c).

**Effect of metal ions, enzyme inhibitors, detergents, and organic solvents on keratinase activity**

The effects of metal ions, enzyme inhibitors, detergents, and organic solvents on keratinase activity are summarized in Table 2. This keratinase was strongly inhibited by phenylmethanesulfonyl fluoride and weakly inhibited by EDTA, which confirmed this keratinase as a serine alkaline protease. The reducing agents β-mercaptoethanol and dithiothreitol enhanced enzyme activity by reducing disulfide bonds. Sodium dodecyl sulfate strongly decreased keratinase activity, similar to *Bacillus* sp. MKR5 (Ghasemi et al., 2012). Zn²⁺ (20 mM), Ca²⁺ (5 mM and 20 mM), Mg²⁺ (5 mM and 20 mM), dimethylsulfoxide, and acetone decreased keratinase activity. Na⁺, urea, Triton X-100, ethanol, and isopropanol had no significant effects.

**Description of the genus Keratinibaculum**

*Keratinibaculum* [Ke.ra.ti.ni.ba’cu.lum. N.L. n. *keratinum*, keratin; L. neut. n. *baculum*, stick, rod; N.L. neut. *Keratinibaculum*, keratin (degrading) rod].

Anaerobic rod-shaped cells with a Gram-positive type cell wall; may stain Gram-negative. Moderately thermophilic and alkaliphilic. The major fatty acids are C₁₅:₀ iso, C₁₅:₀ iso dma. Low G+C content. Grow on a number of proteinaceous and some saccharides. Proteolytic.

The type species is *K. paraultunense*.

**Description of *K. paraultunense* gen. nov., sp. nov**

*Keratinibaculum paraultunense* [paraultunense: pa.ra.ul.tun.en’se. Gr. prep. para, beside, near; N.L. neut. adj. *ultunense*, a bacterial-specific epithet; N.L. *paraultunense*, near (*Clostridium*) *ultunense*, phylogenetically related to *C. ultunense*].

The morphological, chemotaxonomic, and general characteristics are as described for the genus. Cells are...
produced. AQDS, FeCl₃, Fe₂O₃, and ferric citrate are produced on peptone medium. Indole and thiol are positive, acetic acid, isobutyric acid, butyric acid, and isovaleric acid negative. D-sorbitol, and betaine are negative. Acetic acid, propionic acid, isovaleric acid, and isovaleric acid are produced on peptone medium. Indole and thiol are produced. AQDS, FeCl₃, Fe₂O₃, and ferric citrate are reduced, Na₂SO₄, NaNO₂, and sodium selenite are not. The cellular fatty acids are C₁₅:0 iso, C₁₅:0 iso dma, C₁₇:0 iso, C₁₈:0, C₁₈:1, C₁₇:0, C₁₆:0, C₁₆:0, C₁₄:0, C₁₁:0, C₁₂:0, C₁₀:0, C₁₄:0, C₁₃:0, C₁₁:0, and C₁₀:0. The G+C content is 28% (Tₘ). No respiratory quinone was detected. Excretes thermophilic alkaline keratinase, degrades keratin. The type strain is KD-1ᵀ (= JCM 18769ᵀ = DSM 26752ᵀ).

Acknowledgments

We thank Anna Schnirrer for supplying the genome of C. ultunense and J.P. Euzéby for the etymology of the novel taxon. This study was supported by the Science Infrastructure Platform of Sichuan province and National High Technology Research and Development Program of China (863 Program) (grants 2013AA102805).

References


Moorella pennavorans strain F21.


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Free sulfydryl groups in the extracellular broth measured over time.

**Fig. S2.** The effect of pH (a) and temperature (b and c) on crude enzyme activity.