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# Gene expression and activity analysis of the first thermophilic U32 peptidase

**Research Article**

Andrius Jasilionis[1], Algirdas Kaupinis[2], Marija Ger[2], Mindaugas Valius[2], Donaldas Chitavichius[1], Nomeda Kuisiene[1,*]

[1]*Department of Microbiology and Biotechnology, Vilnius University, Vilnius LT-03101, Lithuania*

[2]*Proteomics Centre, Vilnius University Institute of Biochemistry, Vilnius LT-08662, Lithuania*

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**Abstract:** Peptidase family U32 is one of the few whose catalytic type and structure has not yet been described. It is generally accepted that U32 peptidases represent putative collagenases and contribute to the pathogenicity of some bacteria. Meanwhile, U32 peptidases are also found in nonpathogenic bacteria including thermophiles and hyperthermophiles. Here we report cloning of the U32.002 peptidase gene from thermophilic *Geobacillus thermoleovorans* DSM 15325 and demonstrate expression and characterization of the recombinant protein. It has been determined that U32.002 peptidase is constitutively expressed in the cells of thermophilic *G. thermoleovorans* DSM 15325. The recombinant oligomeric enzyme showed its activity only against heat-treated collagen. It was unable to degrade albumin, casein, elastin, gelatine and keratin. In contrast to this, the monomeric recombinant protein showed no activity at all. This paper is the first report about the thermophilic U32 peptidase. As the thermophilic bacteria are non-pathogenic, the role of constitutively expressed extracellular collagenolytic U32 peptidase in these bacteria is unclear.

**Keywords:** *Constitutive expression • Collagenolytic activity • Thermophilic collagenase • Geobacillus thermoleovorans • Geobacillus lituanicus*



## Abbreviations

IPTG - isopropyl-β-D-thiogalactopyranoside;
ACN - acetonitrile.

## 1. Introduction

Peptidase family U32 is one of the few whose catalytic type and structure has not yet been described [1; http://merops.sanger.ac.uk/index.shtml]. Although generally U32 peptidases (U32s) are recognized as bacterial enzymes [1], their distribution is not restricted only to this domain of life. U32s are also found in *Archaea* and *Eukarya* (protozoa, fungi, plants and animals) and even in bacteriophages. Five holotypes of U32s are known: U32.001 (*Porphyromonas*-type), U32.002 (*Helicobacter*-type), U32.003 (*Salmonella*-type), U32.004 (putative peptidase of *Clostridium beijerinckii*) and U32.A01 (YhbV protein of *Escherichia coli*).

To our knowledge, only U32s of pathogenic bacteria have been analyzed until now. These pathogenic bacteria are *Porphyromonas gingivalis* [2], *Proteus mirabilis* [3], *Helicobacter pylori* [4], *Salmonella enterica* [5] and *Aeromonas veronii* [6]. It is generally accepted that U32s represent putative collagenases and contribute to the pathogenicity of these bacteria [2,4,7]. These collagenases are supposed to play a role in tissue destruction and progression of periodontitis [2], wound infections [6], gastritis and gastric cancer [4] in humans as well as encephalopathies in calves [7]. It was shown that U32.003 of *Salmonella* is expressed inducibly [7].

Genes of U32s are also found in the genomes of nonpathogenic bacteria, [8] including thermophiles and hyperthermophiles, however nothing is known about the expression, characteristics and functions of these enzymes in nonpathogenic bacteria [9]. Thermophiles can serve as excellent models for U32s investigations in nonpathogenic bacteria. Collagen, the substrate of collagenases, is composed of three parallel polypeptide

* *E-mail: nomeda.kuisiene@gf.vu.lt*

chains rich in the triplet sequence Gly-X-Y, where X and Y are often proline and hydroxyproline, respectively. The triple helix is stabilized by interchain hydrogen bonding and inter- and intramolecular cross-linking that make the molecule highly resistant to hydrolysis by proteolytic enzymes [10]. True collagenases may cleave the triple helix of collagen [11], however collagen is not stable at high temperature, *i.e.* it cannot maintain the triple-helical structure at this temperature [12]. Consequently, thermophilic U32s cannot be interpreted as true collagenases.

The genus *Geobacillus* is one of the most commonly studied thermophilic genera of bacteria. A lot of information is available on both extracellular and intracellular proteins of these bacteria. U32s have never been studied in geobacilli despite the fact that U32 genes have been identified in the completely sequenced genomes. It is not known whether U32s are expressed, or if the genes of U32s are only pseudogenes in these microorganisms. In order to answer this question, we have chosen to carry out an analysis of U32 of thermophilic *Geobacillus thermoleovorans* DSM 15325 (previously *Geobacillus lituanicus* DSM 15325$^T$ [13]). In this paper we report expression analysis of the first thermophilic U32, cloning of U32.002 gene and some biochemical characteristics of the recombinant U32.002 protein.

# 2. Experimental Procedures

## 2.1 Growth conditions of *G. thermoleovorans* DSM 15325

*G. thermoleovorans* DSM 15325 was grown in mM9 medium containing (ml $l^{-1}$) 5x salts solution, 200.0; 1M $MgSO_4$, 2.0 (final concentration 1 mM); 1M $CaCl_2$, 0.1 (final concentration 0.1 mM); 1000x vitamin solution, 1.0; 10x amino acid solution, 100.0. The medium was prepared in MES buffer, pH 6.5/60ºC. Glucose (2.5 g $l^{-1}$) was also added to the medium. 5x salts solution contained: 22 mM $KH_2PO_4$; 8.6 mM NaCl; 18.7 mM $NH_4Cl$. 1000x vitamin solution contained (mg $l^{-1}$) D-biotin, 10.0; inositol, 100.0; niacin, 100.0; pyridoxine, 100.0; riboflavin, 100.0; thiamine, 100.0. 10x amino acid solution was prepared according to Leejeerajumnean *et al.* [14]. Bacteria were cultivated in 250 ml Erlenmeyer flasks at 55, 60 and 70°C at 180 rpm. Growth was determined by measuring optical density at 600 nm.

## 2.2 Construction of primers

In order to characterize U32.002 of *G. thermoleovorans* DSM 15325, the primers GEOCOL and LITCOL were constructed (Table 1). U32.002 sequences extracted from the genomic sequences of *Geobacillus thermodenitrificans* NG80-2 (CP000557, 2585077...2586348), *Geobacillus kaustophilus* HTA426 (BA000043, 2561422...2562690) and *Geobacillus sp.* WCH70 (CP001638, 2509134...2510402) were used for the construction of these primers. Sequences were aligned and analyzed using the MEGA 4.0 program [15]. The primers were designed using the PRIMERSELECT component of LASERGENE 6 (DNASTAR).

## 2.3 Transcriptional analysis of U32.002 peptidase of *G. thermoleovorans* DSM 15325

In order to determine whether the U32.002 gene is transcribed in thermophilic *G. thermoleovorans* DSM 15325, RT-PCR was performed. For total cell RNA extraction, the strain DSM 15325 was grown in the medium mM9 for 2, 4, 6 and 7 hours. Cells were removed by centrifugation (7000×*g*, 10 min, 4°C) and treated with lysozyme. Total RNA was extracted using the GeneJET™ RNA Purification Kit (Thermo Fisher Scientific). Eluted RNA was treated with RiboLock™ RNase Inhibitor and DNase I (Thermo Fisher Scientific) and analysed by electrophoresis through 1% agarose gels. RT-PCR was performed using the RevertAid™ Premium First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). PCR reactions were carried out on both cDNA and RNA to ensure that there was no genomic DNA amplification. Primers used for amplification are listed in Table 1. PCR was carried out in 50 µl reaction mixtures containing PCR buffer with 20 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.2 mM

| Primer | Sequence (5'-3') | Target site | Restriction site incorporated (underlined) |
|---|---|---|---|
| GEOCOL-F | CGGGCCAACGCTGACAACTTTACG | 163-186 | - |
| GEOCOL-R | GAACCTTGACGATTTGCAGCGGATGG | 1194-1219 | - |
| LITCOL-F | GGTCGGATCCCCATGCTTTTAAAAAATG | 1-16 | *Bam*HI |
| LITCOL-R | TTTGCGGCCGCTTAATTCTCCTTTCTC | 1254-1269 | *Not*I |
| LITCOL-45Sal | GCTCCGTCGACGATGGTCTTTATCGGAG | 133-145 | *Sal*I |

**Table 1.** Primers designed and used in this study.

each dNTP, 0.25 mM each primer, 1.25 U recombinant *Taq* DNA Polymerase and 10 ng of cDNA or RNA. PCR was conducted under the following conditions: initial denaturation at 95°C for 2 min followed by 29 cycles each consisting of 95°C for 1 min, 60°C for 2 min and 72°C for 3 min with a final extension step at 72°C for 7 min in an Eppendorf thermal cycler. Products of amplification were analysed by electrophoresis through 1% agarose gels. All experiments of transcriptional analysis were repeated twice. Results of these completely independent experiments were identical.

## 2.4 Mass spectrometric analysis of U32.002 peptidase expression

For sample preparation, *G. thermoleovorans* DSM 15325 cells were grown in mM9 for 3 hours (exponential growth phase, Figure 1a) at a temperature of 60°C at 180 rpm. Cells were collected by centrifugation (7000×*g*, 10 min, 4°C), resuspended in 50 mM Tris-HCl (pH 7.4/50°C) and disrupted by sonication. Cell debris was removed by centrifugation at 12000×*g* for 20 min at 4°C. The lysate was dialysed against 50 mM Tris-HCl (pH 7.4/50°C). SDS-PAGE of lysate and recombinant U32.002 protein was performed by the method of Laemmli [16] on a 12% running gel, and proteins were visualized with Coomassie blue staining. Protein slices corresponding to the 50 kDa marker in the cell lysate line and recombinant U32.002 line were excised from stained SDS-PAGE gel and trypsin-digested as described by Hellman *et al.* [17] with minor modifications. In short, gel slices were destained with 25 mM ammonium bicarbonate in 50% acetonitrile (ACN) and the gel pieces were vacuum-dried and rehydrated in 100 µl of 20 µg ml$^{-1}$ trypsin (EMP Biotech) in 25 mM ammonium bicarbonate in 9% ACN and covered with an additional 75 µl of 25 mM ammonium bicarbonate in 9% ACN. The digestion was performed for 18 h at 37°C. The peptides were extracted from the gel using 100 µl 5% trifluoracetic acid in 50% ACN for 30 min. This extraction procedure was repeated two times. The peptides extracted in two steps were combined together, concentrated by vacuum drying to 40 µl and subjected to LC-MS/MS analysis.

Reversed-phase (RP) nano-liquid chromatography directly coupled with mass spectrometry (LC-MS/MS) was performed using an Ultimate 3000 nano-flow LC system (LC Packings, Dionex) connected to QTRAP 4000 (AB/MDS Sciex). Peptides were loaded on a RP trap column (PepSwift PS-DVB, 200 µm, LC Packings) with a flow-rate of 20 µl min$^{-1}$ (loading buffer: 2% ACN and 0.1% trifluoracetic acid) and subsequently separated with analytical 75-µm-diameter $C_{18}$ 3 µm bead size and 15 cm length column (Acclaim PepMap 100, Dionex) in 50 min linear gradient (A: 0.05% trifluoracetic acid, B: 80% ACN and 0.04% trifluoracetic acid) at a flow rate of 300 nl min$^{-1}$.

Initially recombinant U32.002 peptidase tryptic-digest was analyzed by QTRAP 4000 (AB/Sciex) mass spectrometry using data-dependent acquisition and dynamic exclusion. Dynamic ion exclusion was set for exclusion of precursor ions after 2 occurrences for 60 s in liquid chromatogram. The acquisition cycle consisted of a survey MS scan with a set of mass range from 400 m/z to 1400 m/z followed by enhanced resolution and 6 data-dependent MS/MS scans acquired in the linear ion trap (LIT).

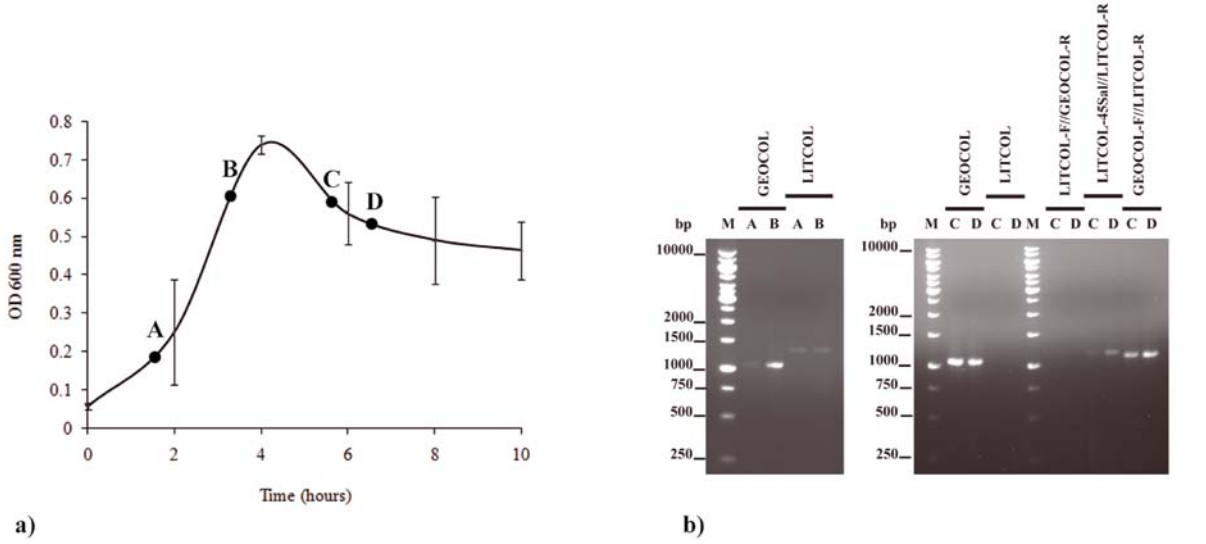


**Figure 1.** Transcriptional analysis of the thermophilic U32.002 peptidase at the optimum growth temperature. a) Growth curve of *G. thermoleovorans* DSM 15325. The culture was grown in mM9 medium at 60°C. The circles on the curve indicate the sampling points for the isolation of total RNA. b) RT-PCR products, obtained using different combinations of GEOCOL and LITCOL primers.

This led to the identification of 23 peptides (data not shown); the 18 most prominent peptides from this list were used as a referee to identify U32.002 peptidase in cell lysate samples using QTRAP 4000 in triple quadrupole (QqQ) mode by applying multiple reaction monitoring (MRM) protocol. For the MRM method the first quadrupole of QTRAP 4000, Q1, was set to transmit ions matching the *m*/*z* value of a protonated peptide (parental ion); these ions then were fragmented in Q2 by collision-induced dissociation (CID). In turn, Q3 was set to transmit only those product ions of a particular *m*/*z* value that were deemed to be diagnostic for the parental ion monitored. The signal of the fragment ion was then monitored over the chromatographic elution time as described by Anderson and Hunter [18].

Peak lists were generated using Analyst software 1.4.2 (AB/Sciex) and Mascot search script 1.6b9 and searched with Mascot v2.2.07 (Matrix Science) against a TrEMBL database (3-23-10 release). Taxonomy filter was *Geobacillus* sp. Y412MC52. Each of the peak lists were searched using the Mascot algorithm (maximum p-value of 0.05) for full tryptic peptides using a precursor ion tolerance window set at ±0.5 Da, variable methionine oxidation and NQ deamidation and maximally one missed cleavage were allowed. The maximum fragment ion tolerance (MS/MS) was ±0.35 Da. A peptide false discovery rate was 0% for MRM experiment and ions expectation value for peptide mach was set to 0.01.

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry was performed using 4800 MALDI TOF/TOF mass spectrometer (AB/Sciex). Peptide mass spectra were acquired in reflector positive ion mode. M/z range was set from 800 to 4000 Da, 400 laser shots were summed for each sample with mass accuracy ±50 ppm. MS/MS spectra for dominating peptides were acquired in MS/MS positive mode with ion collision energy set to 1 keV, 500 laser shots were accumulated for each spectrum with mass accuracy ±0.1 Da. Proteins were identified in the TrEMBL database (3-23-10 release) using the Mascot algorithm with taxonomy filter for *Geobacillus* sp. Y412MC52, unless indicated otherwise.

## 2.5 Cloning and analysis of the U32.002 peptidase gene

In order to characterize thermophilic U32.002, PCR amplicons containing the complete U32.002 coding sequence were prepared using the primers LITCOL-F and LITCOL-R (Table 1). Restriction sites were incorporated into the primers for cloning into pET-28c(+). LITCOL-PCR conditions were the same as stated for RT-PCR, except that native *Pfu* DNA Polymerase (Thermo Fisher Scientific) was used instead of the aforementioned recombinant *Taq* DNA Polymerase. PCR products were sequenced using the ABI Prism 3130xl Genetic Analyzer system (Applied Biosystems). LITCOL-PCR products were cloned into *E. coli* DH5α using the CloneJET™ PCR Cloning Kit (Thermo Fisher Scientific). Plasmid DNA was isolated from clones with inserts using the GeneJET™ Plasmid Miniprep Kit (Thermo Fisher Scientific) and digested with *Bam*HI and *Not*I (Thermo Fisher Scientific). The U32.002 gene of *G. thermoleovorans* DSM 15325 was ligated into the pET-28c(+) vector, and the products were transformed into *E. coli* BL21 (DE3). The sequences were aligned and analysed using the MEGA 4.0 program [15]. Signal sequence prediction was performed using both SignalP 3.0 and SIG-Pred servers.

## 2.6 Expression and purification of the recombinant U32.002 peptidase

Transformants were grown in Luria-Bertani medium containing 30 μg kanamycin $ml^{-1}$. Protein expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when the OD600 reached 0.6, and the incubation was continued for another 4 h at 37°C. Cultures were harvested by centrifugation (7000×*g*, 20 min, 4°C) and resuspended in 50 mM sodium phosphate buffer (pH 7.7/20°C), containing 300 mM NaCl and 10 mM imidazole. Cells were disrupted by sonication, and the cell debris was removed by centrifugation at 12000×*g* for 20 min at 4°C. The His-Spin Protein Miniprep™ kit (Zymo Research) was used for the purification of His-tagged recombinant U32.002 peptidase. Purified recombinant U32.002 peptidase was dialysed overnight at room temperature against 50 mM Tris-HCl (pH 7.4/50°C). Protein concentration was determined by Bradford's assay using bovine serum albumin as a standard [19].

## 2.7 Effect of calcium and zinc ions on the thermal stability of U32.002

Effect of calcium and zinc ions was tested using the purified recombinant U32.002. For sample preparation, the purified recombinant U32.002 was dialysed overnight at room temperature against one of following buffers: 50 mM Tris-HCl (pH 7.4), 10 mM 2-mercaptoethanol (control buffer); 50 mM Tris-HCl (pH 7.4), 10 mM 2-mercaptoethanol containing 1 mM $CaCl_2$ or 1 mM $ZnCl_2$ or both salts. Protein concentration was determined by Bradford's assay using bovine serum albumin as a standard [19]. The samples containing equal amounts (0.01 mg) of recombinant U32.002, were incubated at 37, 45, 50, 55, 60, 65, 70, 80 and 95°C. The denatured U32.002 was removed by centrifugation at 12000x*g* for 15 min at 4°C prior to the final concentration measurement. Effect of the tested ions was analysed by comparison of calculated half-lifes.

### 2.8 Zymographic analysis of the recombinant U32.002 peptidase

Zymographic analysis of the purified recombinant U32.002 was performed according to Gogly *et al.* [20] in 7.5% SDS/polyacrylamide gels containing 0.5% (w/v) of one of the following substrates: bovine serum albumin, casein, elastin, gelatine and keratin. Type I collagen from rat tail (0.05%; Fluka) was also used as the substrate in zymographic analysis. Collagen was dissolved in 10 mM acetic acid at 4°C and then dialysed overnight against sterile distilled water at 4°C. Electrophoresis was run under non-reducing conditions. After electrophoresis, zymograms were rinsed for two hours at room temperature with 0.1% (w/v) Triton X-100 in order to remove SDS. Triton X-100 was washed out using the incubation buffer prior to incubation. Zymograms were incubated for 20 h at 50°C in 50 mM Tris-HCl (pH 7.4/50°C) containing 20 mM $CaCl_2$, 10 µM $ZnCl_2$, 200 mM NaCl, 40 µM Brij 35, 3 mM $NaN_3$. The active bands were visualized by staining with the PageBlue™ Protein Staining Solution (Thermo Fisher Scientific).

## 3. Results and Discussion

### 3.1 U32.002 expression analysis in thermophilic *G. thermoleovorans* DSM 15325

In order to determine whether U32.002 is indeed expressed in thermophilic bacteria, transcriptional as well as proteomic analyses of U32.002 of *G. thermoleovorans* DSM 15325 were carried out. Strain DSM 15325 was cultivated at 60°C (optimum growth temperature), 55°C (minimum growth temperature) and 70°C (maximum growth temperature) [21].

RT-PCR was performed on total RNA isolated from cells in exponential growth phase and in death phase (only from cells grown at 60°C) (Figure 1a; Suppl. Figure S1a). For exponential growth phase, the RT-PCR products were obtained using both GEOCOL and LITCOL primers (Figure 1b; Suppl. Figure S1b), *i.e.* full-length transcripts of U32.002 gene were present in these cells. It should be noted, that U32.002 expression did not depend on the cultivation temperature. In contrast, for death phase cells only GEOCOL products were obtained (Figure 1). In order to reveal the reason for the unsuccessful PCR with LITCOL primers, PCR reactions using primer pairs LITCOL-F//GEOCOL-R, LITCOL-45Sal//LITCOL-R and GEOCOL-F//LITCOL-R were performed. PCR using LITCOL-F//GEOCOL-R was also unsuccessful, but products were obtained for the other two primer pairs (Figure 1b). These results showed that for the death phase cells the full-length transcript of U32.002 gene can not be obtained – most likely, due to its degradation at the 5‘ end.

Proteomics analysis was carried out to detect U32.002 in lysates of *G. thermoleovorans* DSM 15325. Expression of U32.002 peptidase in *G. thermoleovorans* lysates was assayed by a linear ion trap survey mass spectrometry approach and a more sensitive multiple reaction monitoring (MRM) method using triple quadruple option of a QTRAP 4000 mass spectrometer. The initial approach, using survey mass spectrometry, did not lead to identification of any U32.002 tryptic peptides in bacterial cell lysates, although a number of other lysate proteins were identified (data not shown). Therefore, a more sensitive and direct MRM method [18], designed to find selected U32.002 tryptic peptides in complex cell lysate mixtures, was applied. This led to the identification of three tryptic peptides, out of eighteen peptides analyzed, that correspond to U32.002 peptidase: KPELLAPAGNLEK (Mascot score 32, P=0.01), ANADNFTIEEIR (Mascot score 31, P=0.01) and AIPVMIELGVDSLK (Mascot score 35, P=0.01) (Figure 2). Thus, the data show that U32.002 is translated in *G. thermoleovorans*, albeit the expression level is apparently low.

In summary, our results show that U32.002 is transcribed and translated in thermophilic strain DSM 15325. It should be noted, that the strain DSM 15325 was isolated from crude oil of the oilfield [21], *i.e.* an environment without collagen. Medium mM9, used for the cultivation of this strain for the expression analysis, also contains no collagen. Therefore, neither a native nor heat-treated inductor of collagenase was present in the environment of *G. thermoleovorans* DSM 15325. Nevertheless, transcription and translation of U32.002 continued in the exponential growth phase suggesting constitutive expression of U32.002 in these thermophilic bacteria.

### 3.2 Cloning, expression and activity assay of the recombinant U32.002 of G. thermoleovorans DSM 15325

The U32.002 gene of *G. thermoleovorans* (GenBank acc. No. JN628020) is 1269 bp in length encoding a protein of 422 amino acids. Its molecular mass was calculated to be 47.9 kDa. U32.002 gene sequence was the most similar to the respective sequences of *G. kaustophilus* HTA426, *Geobacillus* sp. C56-T3 and *Geobacillus* sp. Y412MC61. Those three sequences were completely identical to each other (data not shown) and differed in seven nucleotides from that of *G. thermoleovorans*. Characteristic consensus sequence of U32 (E-x-F-$x_{(2)}$-G-[SA]-[LIVM]-C-$x_{(4)}$-G-x-C-x-[LIVM]-S; [22]) was found in the sequence of *G. thermoleovorans*. No Zn-binding motif H-E-$x_{(2)}$-H [11], collagen-binding motif F-A-x-W-$x_{(2)}$-T [23] or cell surface anchoring motif L-P-x-T-G [24] were

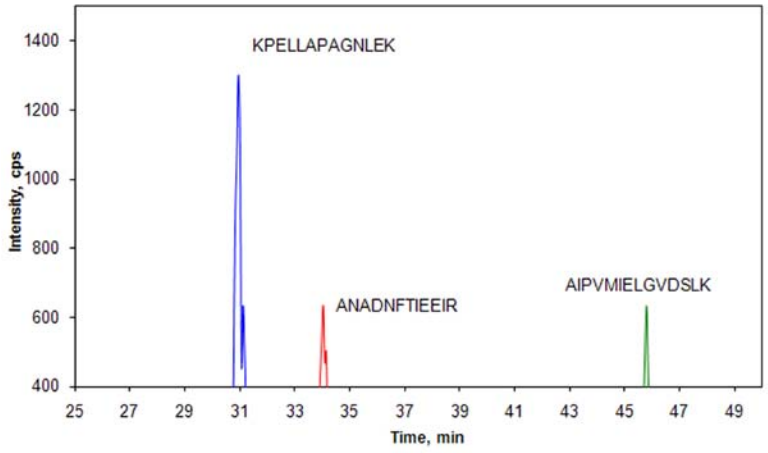


**Figure 2.** Identification of U32.002 peptidase expression in a *G. thermoleovorans* DSM 15325 cell lysate by the selected peptidase-specific peptide monitoring using triple quadrupole mass spectrometer. Extracted ion chromatogram shows the corresponding three tryptic peptides elution retention times detected by MRM m/z transitions of 690.4/728.4, 698.4/760.4 and 742.9/1104.6 ions. Peptide sequences corresponding to ion current profile indicated near the MS peak.

found in the sequence of *G. thermoleovorans*. Signal sequence prediction was also performed. Both SignalP 3.0 and SIG-Pred could not find any signal sequence in U32.002 peptidase of *G. thermoleovorans*. These results do not preclude the possibility that U32.002 could be secreted by another mechanism. Yang *et al.* [25] have recently shown that the secretion of several cytoplasmic proteins without signal peptides, in another endospore-forming bacteria *Bacillus subtilis,* is a general phenomenon during late stationary phase. Kavermann *et al.* [4] also reported the lack of a typical hydrophobic signal sequence in U32.002 of *H. pylori* and hypothesized this protein to be secreted *via* a specific secretion mechanism.

In order to characterize U32.002 peptidase of *G. thermoleovorans*, the U32.002 gene was subcloned into pET-28c(+) and expressed in *E. coli* BL21 (DE3). After induction with IPTG, the lysate of recombinant *E. coli* was analysed by SDS-PAGE (Figure 3). His-tagged peptidase U32.002 was observed only in the induced recombinant bacterium. It should be noted that the recombinant U32.002 was observed both as monomer and oligomer. The mass of the detected oligomers was determined to be ~190 kDa. This suggests that this form was a tetramer. Remarkably, oligomers were detected only when SDS-PAGE was run under non-reducing conditions. Consequently, disulfide bonds were suggested to play a key role in the oligomerization of U32.002 of *G. thermoleovorans.* It should be noted that the U32.002 of *G. thermoleovorans* has 8 cysteine residues, and some of them could be involved in disulfide bond formation. Up until now, only dimerization of U32.001 from *P. gingivalis* has been demonstrated, although the mechanisms underlying this process remain unknown [2]. To our knowledge, oligomerization of U32.002 has not yet been examined.

In order to investigate the catalytic activity of U32.002 from DSM 15325, zymography analysis, using different globular and fibrillar proteins, was carried out. Our data revealed that recombinant U32.002 of *G. thermoleovorans* was highly specific and was able to degrade only type I collagen (Suppl. Figure S2). It was unable to degrade albumin, casein, elastin, gelatine or keratin. Considering the temperature at which the zymographic analysis was carried out (50ºC), these results can be interpreted as activity against heat-treated collagen. Only oligomeric but not monomeric recombinant U32.002 showed collagenolytic activity. It should be noted that activity of U32.001 peptidase against heat-treated collagen as well as lack of activity against gelatine were previously demonstrated for the *P. gingivalis* enzyme [2], however for U32.002, only the collagenolytic activity has been assayed until now [4]. Our results allowed us to conclude that, unlike most bacterial collagenolytic enzymes, both U32.001 and U32.002 had substrate specificity against two types of denatured collagen – heat-treated and gelatine. The reason for this difference in substrate specificity is presently unknown [2].

Proteomic analysis was also applied to confirm the presence of recombinant U32.002 in the zymogram and to correlate it with enzymatic activity. The transparent band from the zymogram gel was excised and subjected to proteomic analysis. The proteins in the gel slice were digested with trypsin and analysed with MALDI TOF/TOF mass spectrometer. U32.002 protein was identified with

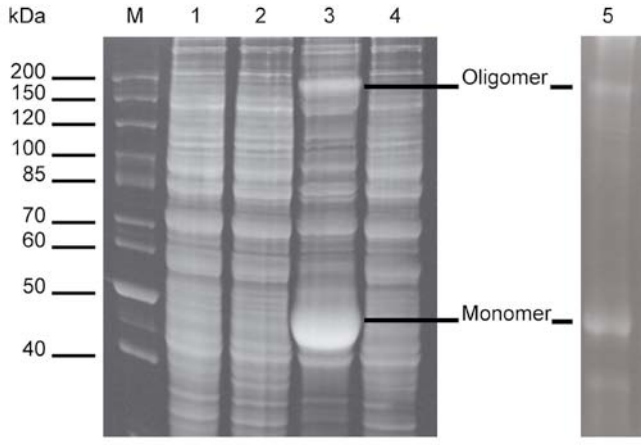


**Figure 3.** SDS-PAGE of expressed recombinant U32.002 peptidase of *G. thermoleovorans* DSM 15325. Lanes: 1 – control (uninduced recombinant *E. coli* BL21(DE3)); 2 – control (lysate of *E. coli* BL21(DE3)); 3 – induced recombinant *E. coli* BL21(DE3); 4 – control (*E. coli* BL21(DE3) with empty vector); 5 – purified His-tagged recombinant U32.002 peptidase; M – PageRuler™ Unstained Protein Ladder (Thermo Fisher Scientific).

high Mascot protein score 412, total ion score 374; eight peptides of U32.002 peptidase were detected. No other proteases were identified in the same zymogram slice in analysis without taxonomy filter (data not shown).

Kato *et al.* [2] reported the slight stimulating effect of $Ca^{2+}$ as well as the inhibitory effect of $Zn^{2+}$ on U32.001 activity. Effect of divalent metal ions on the activity and/or stability of other U32 proteins was not examined [4,6]. Incubation of U32.002 of *G. thermoleovorans* without divalent metal ions resulted in denaturation of this protein at temperatures above 55°C. The half-life at 55°C was determined to be 1.2 h. Calcium ions (1 mM) had no enhancing effect on the thermal stability of U32.002 - the half-life of U32.002 at 55°C was 1.1 h. The presence of zinc ions (1 mM) increased U32.002 stability – the half-life at 55°C raised to 1.7 h.

In conclusion, we have showed that U32.002 peptidase is constitutively expressed in the cells of thermophilic *G. thermoleovorans* DSM 15325. To our knowledge, this is the first report on the constitutive expression of U32 in bacteria. Generally, bacterial collagenases as well as other degradative enzymes are inducible but not constitutive. Up until now the only expression analysis carried out on a collagenolytic U32.003 peptidase was performed in *Salmonella* where it was shown that U32.003 is indeed expressed inducibly [7]. The role of constitutively expressed collagenolytic U32.002 peptidase in thermophilic bacteria is unclear.

Despite some similarities with previously characterized U32s (catalytic activity against heat-treated collagen, absence of activity against gelatine, the lack of a typical hydrophobic signal sequence, oligomerization), U32.002 of *G. thermoleovorans* DSM 15325 showed some unique characteristics: tetramerization *vs.* dimerization of U32 of *P. gingivalis;* effect of zinc ions on the thermal stability; catalytic activity of the recombinant oligomeric but not the monomeric enzyme. These biochemical characteristics of the constitutively expressed U32.002 of *Geobacillus* let us to hypothesize about the unique role of this protein in the metabolism of these bacteria. Analysis of the *in vivo* role of U32.002 in thermophilic bacteria is in progress in our laboratory.

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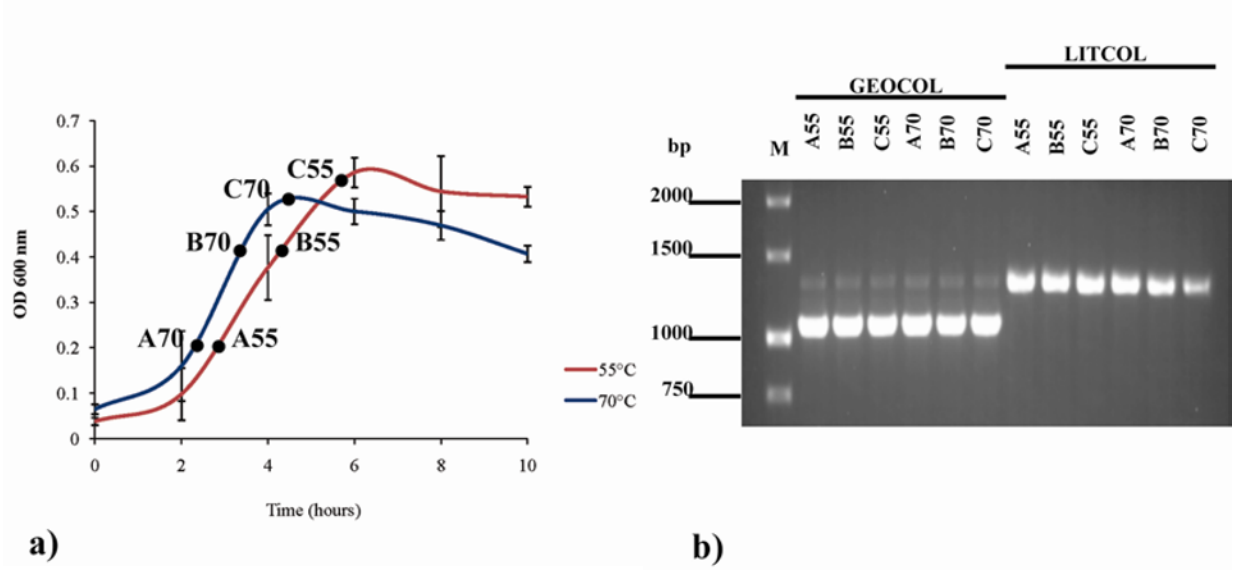
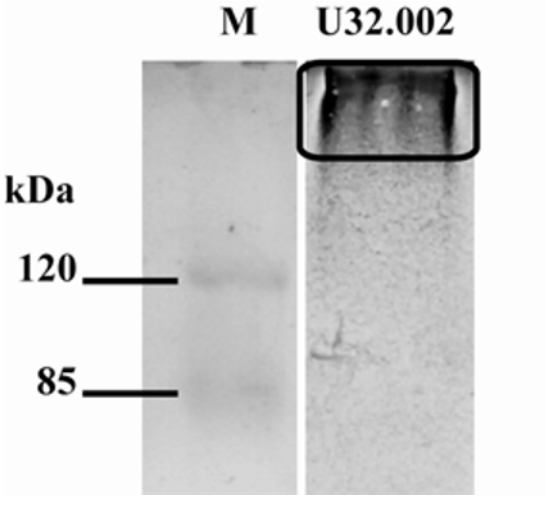
# Appendix




**Supplementary Figure S1.** Transcriptional analysis of the thermophilic U32.002 peptidase at the minimum (55°C) and maximum (70°C) growth temperature. a) growth curves of *G. thermoleovorans* DSM 15325. The cultures were grown in mM9 medium. The circles on the curves indicate the sampling points for the isolation of total RNA. b) RT-PCR products, obtained using GEOCOL and LITCOL primers.M - GeneRuler™ 1 kb DNA ladder (Thermo Fisher Scientific).




**Supplementary Figure S2.** Collagen zymography of the purified recombinant U32.002 of *G. thermoleovorans* DSM 15325. The active band of tetrameric U32.002 is marked by a rectangular box. M - Prestained Protein Molecular Weight Marker (Thermo Fisher Scientific).