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ORIGINAL PAPER

# Characterization of three putative Lon proteases of *Thermus thermophilus* HB27 and use of their defective mutants as hosts for production of heterologous proteins

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**Abstract** In the genome of a thermophilic bacterium, *Thermus thermophilus* HB27, three genes, *TTC0418*, *TTC0746* and *TTC1975*, were annotated as ATP-dependent protease La (Lon). Sequence comparisons indicated that TTC0418 and TTC0746 showed significant similarities to bacterial LonA-type proteases, such as *Escherichia coli* Lon protease, especially in regions corresponding to domains for ATP-binding and hydrolysis, and for proteolysis, but TTC1975 exhibited a similarity only at the C-terminal proteolytic domain. The enzymatic analyses, using purified recombinant proteins produced by *E. coli*, revealed that TTC0418 and TTC0746 exhibited peptidase and protease activities against two synthetic peptides and casein, respectively, in an ATP-dependent manner, and at the same time, both the enzymes had significant ATPase activities in the presence of substrates. On the other hand, TTC1975 possessed a protease activity against casein, but addition of ATP did not enhance this activity. Moreover, a *T. thermophilus* mutant deficient in both TTC0418 and TTC0746 showed a similar growth characteristic to an *E. coli lon* mutant, i.e., a growth defect lag after a nutritional downshift. These results indicate that TTC0418 and TTC0746 are actually members of bacterial LonA-type proteases with different substrate specificities, whereas TTC1975 should not be classified as a Lon protease. Finally, the effects of mutations deficient in these proteases were assessed on production of several heterologous gene products from *Pyrococcus horikoshii* and *Geobacillus stearothermophilus*. It was shown that *TTC0746* mutation was more effective in improving production than the other two mutations, especially for production of *P. horikoshii* α-mannosidase and *G. stearothermophilus* α-amylase, indicating that the *TTC0746* mutant of *T. thermophilus* HB27 may be useful for production of heterologous proteins from thermophiles and hyperthermophiles.

**Keywords** *Thermus thermophilus* · Lon protease · Protease/peptidase · ATPase · Heterologous gene expression

**Abbreviations**

| | |
|---|---|
| AAA+ | ATPase associated with various cellular activities |
| ORF | Open reading frame |
| IPTG | Isopropyl-β-D-thiogalactopyranoside |
| *Ec*Lon | Lon protease of *E. coli* |
| *Tt*Lon | Lon protease of *T. thermophilus* |
| SDS-PAGE | SDS-polyacrylamide gel electrophoresis |
| 5-FOA | 5-Fluoroorotic acid |
| Glt-AAF-MNA | Glutaryl-Ala-Ala-Phe-methoxynaphthylamide |
| Suc-FLF-MNA | Succinyl-Phe-Leu-Phe-methoxynaphthylamide |
| Thr-DH | Threonine dehydrogenase |
| α-Man | α-Mannosidase |
| Glu-DH | Glutamate dehydrogenase |
| α-amy | α-Amylase |

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## Introduction

An ATP-dependent protease, Lon, was first identified in *Escherichia coli* (Swamy and Goldberg 1981), and later in many organisms, from bacteria to eukaryotic mitochondria. The Lon protease family can be divided into two subfamilies, LonA and LonB, mainly based on the sources and the domain structures of the proteins (Rotanova et al. 2004). LonA is a bacterial and eukaryotic enzyme composed of three conserved domains: the N-terminal "N-domain," possibly related to substrate recognition (Ebel et al. 1999), the "AAA+ domain" at the center of the protein, responsible for ATPase activities (Fischer and Glockshuber 1994), and the C-terminal "P-domain" responsible for protease activities (Goldberg et al. 1994). LonB is an archaeal protein and consists of only the latter two domains. Of the Lon proteases, LonA of *E. coli* and LonB of *Thermoplasma acidophilum* have been enzymatically well characterized, e.g., catalytic residues for proteolysis (Starkova et al. 1998; Besche and Zwickl 2004; Rotanova et al. 2004), residues for ATP binding and hydrolysis, and three-dimensional structures (Besche et al. 2004; Botos et al. 2004; Rotanova et al. 2006). Physiologically, they are known to contribute to protein quality control in the cells, as well as regulating many cellular processes, by determining the fates of short-lived proteins (Goldberg 1992; Gottesman and Maurizi 1992; Gottesman 1996; Wicker et al. 1999). The Lon protease was also reported to degrade heterologous gene-products in *E. coli* (Buell et al. 1985; Miyamoto et al. 1985; Brodin et al. 1986; Walker et al. 1990; Alexander et al. 1992; Singh et al. 1992; Hutter and Singh 1998; Philibert and Martineau 2004).

In addition to these two subfamilies of Lon proteases, there are ORFs in some bacterial genomes showing certain similarities to Lon proteases, such as b0955 (YcbZ) of *E. coli* K-12 MG1655, which were annotated as "putative ATP-dependent proteases" or "Lon-like ATP-dependent proteases". These were classified as "bacterial LonB" by Maupin-Furlow et al. (2000), but enzymatic characterization of these ORFs, with respect to protease and ATPase activities, has not yet been reported.

We have recently developed a host-vector system for *Thermus thermophilus* HB27 (Maseda and Hoshino 1998; Takayama et al. 2004; Nakamura et al. 2005), an extremely thermophilic, Gram-negative bacterium growing between 50 and 82°C (Oshima and Imahori 1974), and used it to test for expression of heterologous genes from a hyperthermophilic archaeon, *Pyrococcus horikoshii*. We succeeded in detecting corresponding activities of *PH0655*, *PH0835* and *PH1593* genes, encoding threonine dehydrogenase, α-mannosidase, and glutamate dehydrogenase, respectively, when these genes were expressed under the control of strong promoters of *T. thermophilus*, P31 (Maseda and Hoshino 1995) and P*slp* (Faraldo et al. 1992); however, the amounts of the products were low: no overproduced proteins corresponding to their gene products were detected on an SDS-PAGE gel by Coomassie blue staining when total cellular proteins from the transformants were loaded. This is contrary to the result when an intrinsic gene, such as *Tth* DNA polymerase gene, was overexpressed in *T. thermophilus* (Moreno et al. 2005).

To improve the production of heterologous gene products, we have focused on genes encoding Lon proteases in *T. thermophilus* HB27: one of the Lon proteases may act to degrade the heterologous gene products, and therefore the production may be improved by mutations deficient in this protease. There are three genes annotated to "ATP-dependent proteases (Lon)" (putative *Tt*Lon) in the genome of *T. thermophilus* HB27: *TTC0418*, *TTC0746* and *TTC1975*. Watanabe et al. (1999) reported that TTHA0770 of *T. thermophilus* HB8, whose amino acid sequence was 100% identical to that of TTC0418, proved to exhibit ATP-dependent peptidase activity that is characteristic of Lon proteases, indicating that TTC0418 was a Lon protease. However, no biochemical analysis of TTC0746 and TTC1975, or physiological characterization of these three proteins was conducted. Moreover, it is not known whether some of these proteins are responsible for degradation of heterologous gene products.

In this paper, we describe the biochemical and physiological characterization of these three proteins, and also show the effects of gene disruption on the production of several heterologous gene products in *T. thermophilus* HB27.

## Materials and methods

### Bacterial strains, plasmids and media

The bacterial strains used in this study are listed in Table 1. Plasmids pUC19 and pET21a were used for construction of putative *Ttlon* mutants in *T. thermophilus*, and for the production of the putative *Tt*Lon proteases in *E. coli*, respectively. Plasmids pT8s-P31-hph5 (Nakamura et al. 2005) and pINV (Tamakoshi et al. 1997) were sources of selection markers in *T. thermophilus*, *hph5* encoding the thermostable hygromycin B phosphotransferase and *pyrE* gene of *T. thermophilus* encoding orotate phosphoribosyltransferase (Yamagishi et al. 1996), respectively. pT8s-P*slp* (Takayama et al. 2004) was used for cloning an α-amylase gene of *Geobacillus stearothermophilus* DY-5 in *E. coli*, and expression of the gene in *T. thermophilus*. pTEV-P31-PH0655, pTEV-P31-PH0835 and pTEV-P31-PH1593 were expression plasmids of *PH0655*, *PH0835* and *PH1593* genes of *P. horikoshii* in *T. thermophilus* (Takayama et al. 2004).

**Table 1** Bacterial strains used in this study

| Strains | Genotype | Reference or source |
|---|---|---|
| *T. thermophilus* | | |
| TH104 (pTT8) | *proC4* mutant of HB27 harboring pTT8 | Takayama et al. (2004) |
| MT111 | Δ*pyrE* mutant of HB27 | Tamakoshi et al. (1999) |
| TM104 | *proC4* Δ*pyrE* | This study |
| TL101 | *proC4* Δ*pyrE TTC0418*::*hph5* | This study |
| TL102 | *proC4* Δ*pyrE TTC0746*::*hph5* | This study |
| TL103 | *proC4* Δ*pyrE TTC1975*::*hph5* | This study |
| TL104 | *proC4* Δ*pyrE TTC0418*::*pyrE* | This study |
| TL105 | *proC4* Δ*pyrE TTC0746*::*pyrE* | This study |
| TL012 | *proC4* Δ*pyrE TTC0418*::*hph5 TTC0746*::*pyrE* | This study |
| TL023 | *proC4* Δ*pyrE TTC0746*::*pyrE TTC1975*::*hph5* | This study |
| TL013 | *proC4* Δ*pyrE TTC0418*::*pyrE TTC1975*::*hph5* | This study |
| *E. coli* | | |
| JM109 | *recA1 endA1 gyrA96 thi supE44 relA1 hsdR17*Δ(*lac-proAB*) F′ (*traD36 proAB lacI*$^{q}$ *lacZ* ΔM15) | Yanisch-Perron et al. (1985) |
| BL21(DE3) | F$^{-}$ *ompT hsdSB* $_{(rB^{-}mB^{-})}$ *gal dcm* (DE3) | Novagen, Madison, WI, USA |
| BL21(DE3) pLysS | BL21(DE3) harboring pLysS | Novagen, Madison, WI, USA |

*T. thermophilus* strains were grown at 70°C in a rich medium (TM) or a minimal medium (MM), as described earlier (Koyama et al. 1986). When necessary, proline and/or uracil (50 μg/ml each) were added to MM.

*E. coli* strains were grown in Luria–Bertani (LB) medium. Ampicillin (100 μg/ml), kanamycin (20 μg/ml) and hygromycin (200 μg/ml) were added to the medium when necessary.

## Expression and protein-purification of putative *Ttlon* genes in *E. coli*

The ORFs of *TTC0418*, *TTC0746* and *TTC1975* genes were amplified by PCR with Pyrobest DNA polymerase (Takara), using genome DNA of *T. thermophilus* HB27 as a template and each of the following primer pairs: *TTC0418*-F, 5′-agttccatatgaaggactttctgcgcctag-3′, and *TTC0418*-R, 5′-agttcgcggccgcagcgcccacgccggggct-3′; *TTC0746*-F, 5′-ggaattccatatgctaccggaaaccatgcccg-3′, and *TTC0746*-R, 5′-agttcgcggccgcggcatgggccacgagct-3′; *TTC1975*-F, 5′-ggaattccatatgttccccatgcgggtcgc-3′, and *TTC1975*-R, 5′-agttcgcggccgccttctcctccccgttctc-3′, respectively. In the forward primers, the ATG sequences of the *Nde*I sites introduced correspond to the initiation codons of the ORFs, and in the reverse primers, the *Not*I sites were introduced instead of the termination codons. The PCR fragments were digested with *Nde*I and *Not*I and cloned in the respective sites of pET21a (Novagen, Madison, WI, USA), giving rise to plasmids pET21a-*TTC0418*, pET21a-*TTC0746* and pET21a-*TTC1975*. In these constructs, the 6 × His-tag sequences were fused in-frame with the ORFs at their C-termini. After correct construction was confirmed by sequencing with a CEQ2000XL DNA sequencer (Beckman), plasmids pET21a-*TTC0746* and pET21a-*TTC1975* were introduced into *E. coli* BL21(DE3), and pET21a-*TTC0418* was introduced into strain BL21(DE3) pLysS (Novagen, Madison, WI, USA).

*E. coli* strains harboring pET21a-*TTC0418* and pET21a-*TTC1975* were cultivated in LB medium until the early logarithmic growth phase, then 0.1 mM IPTG was added to the cultures and cultivation was further continued for 4 h in the case of strains carrying pET21a-*TTC0418* or for 20 h for pET21a-*TTC1975*. For the strain harboring pET21a-*TTC0746*, cultivation was conducted in the same way as that for the strain harboring pET21a-*TTC1975*, but without IPTG addition. The cells cultured in this way were collected by centrifugation at 2,500×*g* for 5 min, washed with TE buffer, and suspended in 10 mM Tris-HCl buffer, pH 7.5. The cells were then disrupted by sonication, and the cell debris was removed by centrifugation at 4,000×*g* for 20 min. The crude extracts thus obtained were incubated at 70°C for 20 min to heat-denature the *E. coli* proteins. After centrifugation at 11,100×*g* for 30 min, the resultant supernatants were loaded onto a column of HiTrap Chelating HP (0.7 × 2.5 cm; GE healthcare) pre-equilibrated with 200 mM $NiCl_2$, and the column was washed with 0.02 M sodium phosphate buffer containing 0.5 M NaCl, pH 7.4. The recombinant protein was eluted with the above buffer containing 0.5 M imidazole, and again loaded onto a HiTrap Desalting column (1.6 × 2.5 cm; GE healthcare) to remove imidazole, using a buffer of 50 mM Tris-HCl,

pH 7.5. Purification was monitored by SDS-PAGE stained with Coomassie brilliant blue.

### Assay of Lon protease activity

Protease activity was determined by the method of Kunitz (1947) with casein as the substrate. Three hundred microliters of purified enzyme in 50 mM Tris-HCl, pH 7.5, was added to 700 µl of 2% casein in 50 mM Tris-HCl buffer containing 10.7 mM $MgCl_2$, pH 7.5, and incubated at 70°C for 20 min. The reaction was then stopped by adding 1 ml of 0.4 M trichloroacetic acid (TCA). After 30 min-incubation at room temperature, the supernatant was separated by centrifugation at 11,100×*g* for 20 min and its absorbance at 280 nm was measured. One unit of activity was defined as the amount of enzyme which catalyzed an increase at $A_{280}$ of 0.001 per minute under the above conditions. The reaction was conducted with or without 1 mM ATP in the reaction mixture.

Peptidase activity was assayed using two fluorogenic peptides, succinyl-Phe-Leu-Phe-methoxynaphthylamide (Suc-FLF-MNA; Backem) and glutaryl-Ala-Ala-Phe-MNA (Glu-AAF-MNA; Sigma) (Waxman and Goldberg 1985; Watanabe et al. 1999). The purified enzymes (1 µg) were mixed with 0.3 mM fluorogenic peptides in a buffer of 50 mM Tris-HCl, 7.5 mM $MgCl_2$, pH 7.5, with or without 1 mM ATP and 0.1 mg/ml casein in a total volume of 200 µl. Reaction mixtures were incubated for 1 h at 70°C, after which the reaction was stopped by placing on ice, and the hydrolysis of the peptides was monitored by measuring the increase in fluorescence (excitation at 370 nm, emission at 535 nm), using a microplate reader (DTX880; Beckman Coulter). One unit of the activity was defined as the amount of the enzyme which released 1 nmol of MNA per h under the assay conditions.

For the determination of ATPase activity, the amounts of ATP in the reaction mixtures were measured before and after the reactions described above, using the luciferin-luciferase assay with a CheckLite 250 Plus kit (Kikkoman). One unit of activity was defined as the amount of enzyme, which consumed 1 nmol of ATP per hour under the assay conditions.

### Genetic manipulation of *T. thermophilus*

Transformation of *T. thermophilus* cells was performed as previously described (Koyama et al. 1986; Hoshino et al. 1993), and transformants were selected by 40 µg/ml of kanamycin at 60°C, 40 µg/ml of hygromycin at 65°C and 200 µg/ml of 5-FOA at 70°C. Plasmid isolation was conducted by the alkali-lysis method (Sambrook and Russell 2001).

### Construction of putative *Ttlon* mutants

*TTC0418*, *TTC0746* and *TTC1975* genes were PCR-amplified with primer pairs of *TTC0418*-Fh, 5′-agttcgtcgactgaaggactttctgcgc-3′, and *TTC0418*-Rh, 5′-agttcgaattcctaagcgcccacgccgg-3′; *TTC0746*-Fh, 5′-agttcgtcgactgctaccggaaaccatg-3′, and *TTC0746*-Rh, 5′-agttcgaattcctaggcatgggccacga-3′; *TTC1975*-Fh, 5′-agttcgtcgactgttccccatgcgggtc-3′, and *TTC1975*-Rh, 5′-agttcgaattcttacttctctccccgt-3′, in which *Sal*I and *Eco*RI sites were added to the forward and reverse primers, respectively. The resultant PCR fragments were digested with *Sal*I and *Eco*RI, and cloned into the respective sites of pUC19. After correct construction was confirmed by sequencing, the plasmids thus obtained were designated pUC19LON1, pUC19LON2 and pUC19LON3, respectively.

Next, each of the plasmids was digested with *Msc*I, located in the middle of each ORF, and ligated with the *hph5* cassette, obtained by PCR amplification from pT8s-P31-hph5 (Nakamura et al. 2005) with primers hph5-f, 5′-attcggcccaaggtttacaaaatcc-3′, and hph5-r, 5′-aacgcatgctattcctttgccctcggacga-3′. The resultant plasmids were designated pULH1, pULH2 and pULH3, respectively. In the cases of pUC19LON1 and pUC19LON2, a DNA fragment containing the *pyrE* gene, which was obtained by PCR amplification from pINV (Tamakoshi et al. 1997) with primers pyrE-f, 5′-atggacgtcctggagcttta-3′, and pyrE-r, 5′-ctagacctcctccaagggca-3′, was also inserted at the same *Msc*I site, giving rise to pULE1 and pULE2, respectively. The plasmids thus constructed were used to transform *T. thermophilus* strain TM104, a *pyrE* mutant of TH104, which was obtained by transformation of TH104 with genomic DNA of MT111 (Tamakoshi et al. 1999) and selection by 5-FOA resistance. After the transformants were selected on TM containing hygromycin B or MM without uracil for insertions of *hph5* or *pyrE*, respectively, correct integration was confirmed by Southern hybridization (Sambrook and Russell 2001), using digoxigenin-labeled PCR fragments of *TTC0418*, *TTC0746*, or *TTC1975* gene, and *hph5* or *pyrE* gene as probes. Strains were designated as TL101, TL102, TL103, TL104 and TL105 for those carrying *TTC0418::hph5*, *TTC0746::hph5*, *TTC1975::hph5*, *TTC0418::pyrE* and *TTC0746::pyrE* mutations, respectively. Double mutants TL012, TL023 and TL013, were obtained by transformation of TL105, TL105 and TL104 with genomic DNA of TL101, TL103 and TL103, respectively (Table 1).

### Growth tests of the mutants

The strain TM104 and its putative *lon* mutants were cultured in liquid TM at 65°C overnight, inoculated into fresh TM or MM supplemented with proline and uracil (MM-PU), and the cultures were continued at 65°C. Growth of the strains was monitored by following the optical density at 600 nm.

For a nutritional downshift experiment, the strains were cultivated in liquid TM until the mid-logarithmic phase of growth, after which the cells were washed once with 0.85% NaCl, and inoculated in MM-PU, either supplemented with or without 0.2% casamino acid, to give a final O.D. at 600 nm of 0.4. Culturing was continued at 65°C and the growth of the strains was monitored.

### Construction of plasmid pTEV-P*slp*-α-amy

An α-amylase gene of *Geobacillus stearothermophilus* DY-5 (Tsukagoshi et al. 1984) was amplified by PCR with Ex-Taq DNA polymerase (Takara), using the primer pair of Amy-sigf, 5′-tacatgcatatgctaacgtttcaccgcatcat-3′, and Amy-matr, 5′-tacatggcatgccgcaggcatcaaggccatgc-3′. In the forward primer, the ATG sequence of the *Nde*I site introduced corresponds to the initiation codon, and in the reverse primer, the *Sph*I site was introduced just downstream of the termination codon. The PCR fragment was digested with *Nde*I and *Sph*I, and cloned into the respective sites of pT8s-P*slp* (Takayama et al. 2004), giving rise to plasmid pT8s-P*slp*-α-amy. After the nucleotide sequence had been confirmed by DNA sequencing, the plasmid was used to transform *T. thermophilus* TH104 (pTT8). Through a homologous recombination event between pTT8 and pT8s-P*slp*-α-amy in the cells, the plasmid pTEV-P*slp*-α-amy, replicable in *T. thermophilus*, was obtained (Hoshino et al. 1993).

### Expression of heterologous genes in *T. thermophilus* mutants

Strain TM104 and its putative *Ttlon* mutants described above were transformed with pTEV-P31 plasmids harboring *PH0655*, *PH0835* and *PH1593* genes of *P. horikoshii* (Takayama et al. 2004), and pTEV-P*slp*-α-amy, and cultured in liquid TM containing 40 μg/ml of kanamycin at 60°C for 18 h. Cells were collected by centrifugation at 5,800×*g* for 10 min and washed with 0.85% NaCl. The cells were then suspended in 50 mM Tris-HCl, pH 7.0, and subjected to sonication. After cell debris had been removed by centrifugation (11,100×*g*, 25 min, 4°C), the supernatants obtained were used for corresponding enzyme assays.

### Enzyme assays of heterologous gene products

Thr-DH, α-Man and Glu-DH activities in the crude extracts of strains harboring the respective *P. horikoshii* genes were assayed as described earlier (Takayama et al. 2004), and the enzyme activities were expressed as units per milligram of total protein. Protein concentration of crude extracts was estimated using a protein assay kit (Bio-Rad, CA, USA) with bovine serum albumin as the standard.

For measurement of α-amylase activity, crude extracts (200 μl) of strains harboring pTEV-P*slp*-α-amy were mixed with 500 μl of 50 mM Tris-HCl, pH 7.0, containing 0.25% starch. After a 10-min incubation at 65°C, total released reducing sugar was determined by the dinitrosalicylic acid method (Miller 1959). One unit of the activity was defined as the amount of enzyme which liberated 1 μmol of reducing sugar estimated as glucose per min. under the above conditions.

## Results and discussion

### Comparison of amino acid sequences of three putative *Tt*Lons of strain HB27 with the related sequences

In the genome sequence of *T. thermophilus* HB27, three genes, *TTC0418*, *TTC0746* and *TTC1975*, were annotated as ATP-dependent protease La (Lon). Comparison of amino acid sequences of these ORFs with that of *E. coli* Lon protease (*Ec*Lon) revealed that TTC0418 and TTC0746 showed moderate similarities to *Ec*Lon in overall length, the identities of which were 51.4 and 48.2%, respectively (Fig. 1a). Of the three domains conserved among Lon protease family proteins, regions corresponding to the AAA+ and P-domains were highly conserved in TTC0418 and TTC0746. Moreover, motifs and residues required for substrate binding or catalysis in the AAA+ domain, such as the Walker A and B motifs, the Sensor-1 and 2 motifs, and the R-finger (Iyer et al. 2004), as well as the catalytic residues in the P-domain, Ser679 and Lys722 in *Ec*Lon numbering, were specifically conserved, indicating that these regions might have similar functions to those of *Ec*Lon.

Contrary to the above, the amino acid sequence of TTC1975 showed only a limited similarity to that of *Ec*Lon; the overall identity was 19.3% and significant conservation was observed only in the P-domain (Fig. 1a). In a phylogenetic analysis based on the amino acid sequence of TTC1975 and those of proteins showing similarities to TTC0418 or TTC1975 sequence from the KEGG genome database, TTC1975 was included in a monophyletic cluster of bacterial proteins annotated to "putative ATP-dependent proteases" or "Lon-like

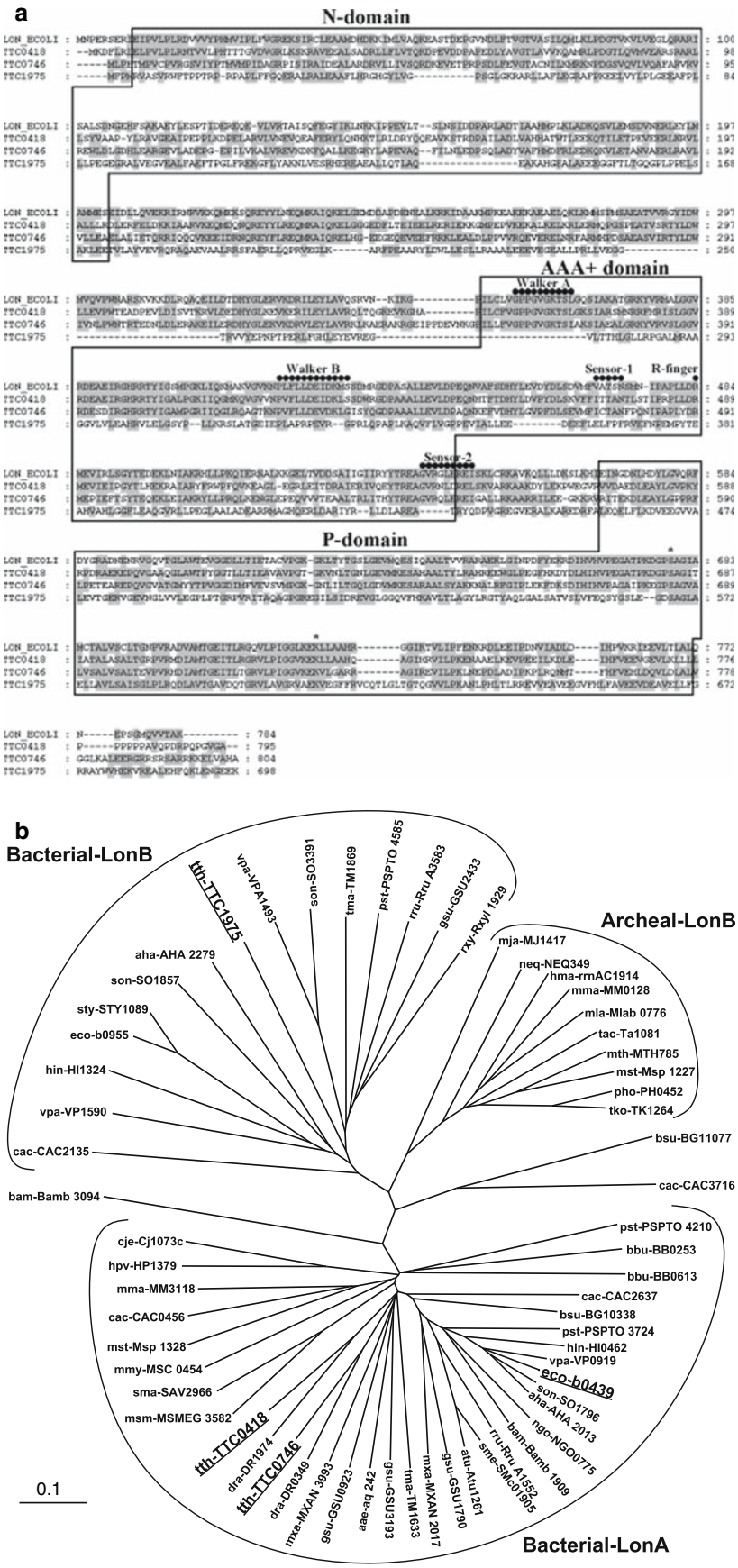


**Fig. 1** Comparison of amino acid sequences of putative Lon proteases of *T. thermophilus* HB27 with related sequences. **a** Amino acid sequences of *Ec*Lon (LON_ECOLI) and three putative Lon proteases of *T. thermophilus* HB27 (TTC0418, TTC0746 and TTC1975) were aligned using ClustalX software (Thompson et al. 1997). Conserved amino acids are *shaded*, and regions corresponding to three domains in *Ec*Lon, according to a conserved domain database in the NCBI homepage (http://www.ncbi.nlm.nih.gov/Structure/cdd/; Marchler-Bauer et al. 2007), are *boxed*. *Circles* indicate regions and a residue responsible for ATP binding and hydrolysis in the AAA+ domain. *Asterisks* in the P-domain indicate the catalytic residues for proteolysis. **b** Phylogenetic relationships of Lon proteases or Lon-like proteases from Bacteria and Archaea. Several amino acid sequences showing similarities to that of TTC0418 or TTC1975 were selected from the KEGG genome database (http://www.genome.ad.jp/en/gn_kegg.html), and the tree was constructed by neighbor-joining method using Clustal X software and visualized by Treeview software (Page 1996). The names of organisms and accession numbers were indicated as KEGG organisms and accession numbers. *Ec*Lon, TTC0418, TTC0746 and TTC1975 were indicated by *larger fonts* with *underlines*

proteases", or the so-called "bacterial LonB" subfamily (Maupin-Furlow et al. 2000), a representative of which was *E. coli* b0955 (YcbZ) (Fig. 1b). All of these proteins in this cluster also shared a similarity in the P-domain to the Lon proteases; notably, the regions around the catalytic residues of Lon proteases were highly conserved. On the other hand, regions other than the P-domain of these proteins did not show any similarities to the Lon proteases, including the AAA+ domain.

From these observations, it was suggested that, in addition to TTC0418, which was previously identified as a Lon protease in strain HB8 (Watanabe et al. 1999), TTC0746 also functions as a Lon protease in *T. thermophilus*, i.e., an ATP-dependent protease, but TTC1975 may not require ATP for its proteolytic activity if the protein possesses such activity. To investigate the function of these proteins in more detail, enzymatic and functional analyses were conducted.

## Enzymatic characterization of the putative *Tt*Lon proteases

The three putative *Ttlon* genes were expressed in *E. coli*, using pET21a as a vector, and the protein products were purified by heat treatment at 70°C for 20 min and affinity chromatography against the 6xHis-tag attached to the C-termini of the proteins. The purified proteins showed single bands on SDS-PAGE, with expected molecular weights of 89, 90 and 77 kDa for TTC0418, TTC0746 and TTC1975, respectively (Fig. 2).

Protease activities of these three proteins were first examined at 70°C with casein as a substrate, in either the presence or the absence of 1 mM ATP. At the same time, ATPase activities of the proteins were also measured in the presence or the absence of 2% casein. As shown in Table 2, all three proteins showed some proteolytic activity in the absence of ATP, with TTC1975 showing fairly strong activity. When ATP was added to the reaction mixtures, the activity of TTC0746 was enhanced about tenfold, but those of TTC0418 and TTC1975 were not affected. In addition to the above observation, apparent ATPase activity was detected only when TTC0746 was incubated with the protease substrate. These results clearly indicate that TTC0746 exhibits protease activity with concomitant hydrolysis of ATP. On the other hand, TTC0418 and TTC1975 did not show ATP-dependency of their protease activities. The results of TTC0418 are contrary to the previous results of Watanabe et al. (1999); they demonstrate that the same protein to TTC0418 from the strain HB8, TTHA0770, exhibited ATP-dependent peptidase activity against two synthetic peptides.

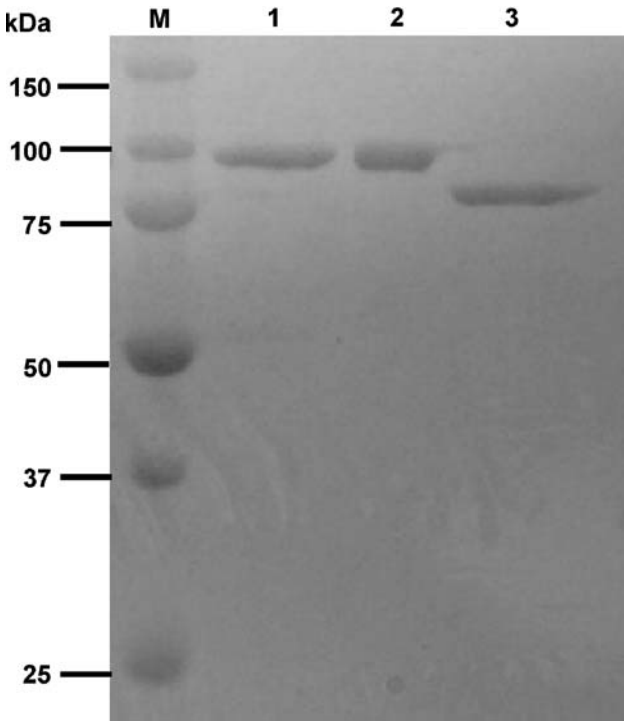


**Fig. 2** SDS-PAGE of the purified recombinant proteins of three putative Lon proteases. The purified proteins after affinity chromatography were loaded onto a 12.5% SDS-polyacrylamide gel. *Lane M*, molecular mass markers (values in *left*); *1* TTC0418, *2* TTC0746, *3* TTC1975

We thought that this discrepancy might be derived from the substrates used: casein and the synthetic peptides. To test this possibility, and also to analyze the properties of the activities of TTC0746 and TTC1975 in more detail, we next measured the peptidase activities of the three proteins, using two synthetic peptides similar to those Watanabe et al. used. It should be noted that one of the peptides we used, Glt-AAF-MNA, was different from that Watanabe et al. used in its protecting group at the $NH_2$-terminus. As expected, TTC0418 clearly showed ATP-dependent peptidase activity; the activities toward Suc-FLF-MNA and Glt-AAF-MNA were enhanced by the addition of ATP by about 30- and 15-fold, respectively. At the same time, TTC0418 exhibited ATPase activity only when the peptide substrate was present. Watanabe et al. reported that TTHA0770 exhibited stronger activity toward Suc-FLF-MNA than Suc-AAF-MNA about 14-fold, but in our result, no such substrate specificity was observed. The precise reason for this discrepancy is not known, but it may be derived from differences in the assay conditions, or that the difference in the protecting groups of the substrates used affected substrate recognition of the enzyme.

When compared to TTC0418, TTC0746 showed similar peptidase activities toward both substrates in the absence of

**Table 2** Protease/peptidase and ATPase activities of the putative *Tt*Lon proteases

| Substrate | Enzyme | Protease or peptidase activity (U/mg)[a] | | ATPase activity (U/mg)[a] | |
|---|---|---|---|---|---|
| | | −ATP[b] | +ATP[b] | −Substrate[c] | +Substrate[c] |
| Casein | TTC0418 | 14.3 ± 5.72 | 10.0 ± 4.04 | <50[d] | <50 |
| | TTC0746 | 4.02 ± 2.00 | 47.2 ± 11.1 | <50 | $2.25 \times 10^3 \pm 113$ |
| | TTC1975 | 48.9 ± 13.4 | 57.6 ± 21.3 | <50 | <50 |
| Suc-FLF-MNA | TTC0418 | 34.5 ± 8.07 | $1.07 \times 10^3 \pm 71.1$ | $<2 \times 10^2$ [d] | $1.12 \times 10^3 \pm 211$ |
| | TTC0746 | 57.3 ± 1.91 | 136 ± 37.6 | $<2 \times 10^2$ | $7.84 \times 10^2 \pm 75.2$ |
| | TTC1975 | 37.1 ± 10.9 | 50.8 ± 10.5 | $<2 \times 10^2$ | $<2 \times 10^2$ |
| Glt-AAF-MNA | TTC0418 | 71.7 ± 9.50 | $1.17 \times 10^3 \pm 112$ | n.a.[e] | n.a. |
| | TTC0746 | 65.4 ± 18.3 | 157 ± 21.1 | n.a. | n.a. |
| | TTC1975 | 9.54 ± 0.313 | 10.6 ± 2.76 | n.a. | n.a. |

[a] Enzyme activities were assayed in triplicate, and expressed as mean ± S.D.

[b] The activities were assayed in the absence (−ATP) or the presence (+ATP) of 1 mM ATP

[c] The activities were assayed in the absence (−substrate) or the presence (+substrate) of protease/peptidase substrates

[d] Below the levels of detection under these assay conditions

[e] Not analyzed

ATP, and a weak but distinct activation of about 2.4-fold by the addition of ATP was detected on both substrates. On the other hand, TTC1975 exhibited a rather weak peptidase activity toward the substrates that was not affected by the addition of ATP. As for ATPase activity, TTC0746 exhibited the activity under the same conditions as TTC0418 did, but TTC1975 did not, in either the presence or absence of the substrate.

In *Ec*Lon and TTHA0770, it was reported that addition of 0.1 mg/ml casein to the reaction mixtures enhanced their peptidase and ATPase activities (Waxman and Goldberg 1986; Watanabe et al. 1999). We observed similar enhancement in TTC0418 reactions using Suc-FLF-MNA as a substrate. In the case of TTC0746, its peptidase activity toward Suc-FLF-MNA was not affected by addition in the absence of ATP, but in the presence of ATP, a twofold enhancement was observed. At the same time, the ATPase activity was also enhanced about twofold (data not shown). On the other hand, the peptidase activity of TTC1975 was not affected by the addition.

From these observations, we concluded that, in addition to TTC0418, TTC0746 was apparently an ATP-dependent protease and could be classified as a Lon protease, as suggested by its sequence similarity to *Ec*Lon. Therefore, we hereinafter renamed TTC0418 as *Tt*LonA1 and TTC0746 as *Tt*LonA2. The difference in protease and peptidase activities that *Tt*LonA1 and *Tt*LonA2 showed may reflect differences in the substrate specificity of the enzymes. On the other hand, TTC1975 possessed apparent protease activity, but this activity neither was affected by the addition of ATP nor was ATPase activity detected. Taken together with the loss of the sequence similarity of TTC1975 at the region corresponding to the AAA+ domain, we concluded that this protein was an ATP-independent protease, and therefore should not be classified as a Lon protease.

## Physiological characterization of *Ttlon* mutants

It has been reported that an *Eclon* mutant shows a characteristic growth phenotype; it grows normally in a rich medium, but shows an extended growth lag after transition to a minimal medium, possibly due to low availability of free amino acids caused by less efficient protein turnover (Kuroda et al. 2001). To test whether mutants deficient in *Tt*LonA1, *Tt*LonA2, or TTC1975, or their double mutant show a similar phenotype to the *Eclon* mutant, we constructed knock-out mutants as described in Materials and methods, and observed their growth in TM and MM, and after transition from TM to MM (nutritional downshift). As shown in Fig. 3, the growth of all single mutants deficient in either *Tt*LonA1, *Tt*LonA2 or TTC1975, and two double mutants deficient in either *Tt*LonA1 or *Tt*LonA2, and TTC1975, was indistinguishable from that of their parental strain, both in TM and in MM, whereas a *TtlonA1 TtlonA2* double mutant, TL102, showed a slight delay in growth in TM (Fig. 3a), and severe growth defects in MM (Fig. 3b). Also, this strain showed the same growth defects after nutritional downshift (Fig. 3c); the growth did not restore even after the cultivation was continued for overnight at 65°C (data not shown). An addition of 0.2% casamino acid

to MM restored the growth of the mutant, indicating that the growth defect of the mutant was due to low availability of free amino acids, like that observed in the *Eclon* mutant.

These results suggest that *Tt*LonA1 and *Tt*LonA2 function in the same way as *Ec*Lon in vivo, i.e., regulation of intracellular protein turnover upon nutritional downshift. In spite of the difference in the substrate specificities observed above, these two enzymes seem likely to have the same function, or a redundant function, since neither a *TtlonA1* nor *TtlonA2* single mutation affected the growth of the mutants, at least under these conditions. Moreover, the *TTC1975* mutation, either in combination with the *TtlonA1* or *TtlonA2* mutation, did not show any effects on growth, indicating that this enzyme's function is not the same as that of Lon proteases.

## Effect of *lon* mutations on the production of heterologous gene products

Finally, to investigate whether the introduction of the *TtlonA1*, *TtlonA2* or *TTC1975* mutation can improve production of heterologous gene products, we introduced three reference genes from *Pyrococcus horikoshii*, *PH0655*, *PH0835* and *PH1593* genes encoding threonine dehydrogenase (Thr-DH), α-mannosidase (α-Man) and glutamate

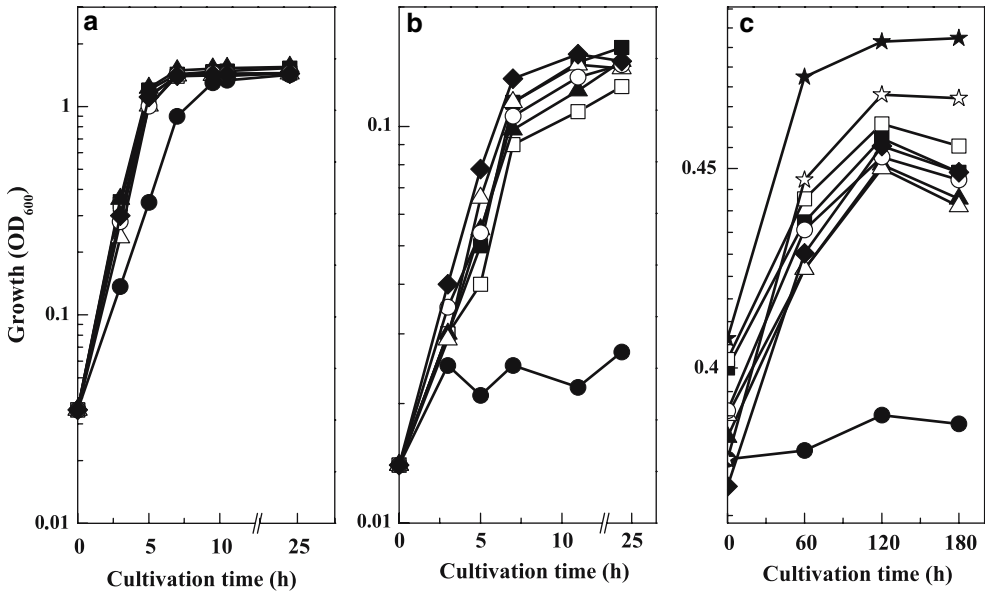


**Fig. 3** Growth of *T. thermophilus* HB27 wild type and *lon* mutants in TM (**a**), MM (**b**) and in MM after nutritional downshift (**c**). Culture conditions are described in Materials and methods. Symbols: *filled squares* TM104; *open squares* TL101; *filled triangles* TL102; *open triangles* TL103; *filled circles* TL012; *open circles* TL023; *diamonds* TL013; *open and filled asterisks* TM104 and TL012 in MM supplemented with 0.2% casamino acids, respectively

**Table 3** Comparison of heterologous enzyme activities expressed in *T. thermophilus*

| Host strain | Enzyme activity (mU/mg protein)[a] | | | |
|---|---|---|---|---|
| | Thr-DH (PH0655) | α-Man (PH0835) | Glu-DH (PH1593) | α-amy |
| TM104 | 11.4 ± 3.90 | 9.72 ± 3.97 | 322 ± 41.5 | 263 ± 43.6 |
| TL101 | 6.82 ± 1.67 | 6.81 ± 2.87 | 267 ± 31.5 | 88.6 ± 21.6 |
| TL102 | 12.7 ± 5.31 | 19.6 ± 4.25 | 418 ± 73.0 | 426 ± 70.5 |
| TL103 | 6.27 ± 1.45 | 7.93 ± 3.33 | 361 ± 122 | 237 ± 73.3 |
| TL012 | 7.17 ± 2.14 | 5.13 ± 0.87 | 213 ± 77.5 | 867 ± 59.6 |
| TL023 | 7.91 ± 2.11 | 20.0 ± 4.09 | 262 ± 68.0 | 735 ± 149 |
| TL013 | 5.00 ± 0.46 | 9.23 ± 3.81 | 233 ± 47.5 | 403 ± 100 |
| TM104/control[b] | 0.98 ± 0.11 | 0.354 ± 0.037 | 12.1 ± 3.00 | 13.4 ± 0.29 |

[a] Enzyme activities were measured in triplicate, and expressed as mean ± S.D.

[b] Enzyme activities were assayed with crude extracts of *T. thermophilus* TM104 harboring vector plasmid pTEV-P31 for Thr-DH, α-Man and Glu-DH activities, or pTEV-P*slp* for α-amy activity

dehydrogenase (Glu-DH), respectively, and an α-amylase (α-amy) gene from *Geobacillus stearothermophilus*, to strain TM104 and its mutants described above, using a pTEV plasmid replicable in *T. thermophilus*. The introduced genes were expressed under the control of P31 or P*slp* promoter, and the corresponding enzyme activities were measured in cell extracts of the transformants, as described in Materials and methods. It is notable that the α-amy is an extracellular enzyme in *G. stearothermophilus*, but its activity was not detected in the cultural broth when this gene was expressed in *T. thermophilus* (data not shown), indicating that the enzyme was produced in the intracellular fraction or in the periplasmic space of the cells. Also, it should be noted that each of the four enzyme activities detected in the cell extracts of the strain TM104 containing the vector plasmid, pTEV-P31 or pTEV-P*slp*, was less than 5% of that detected in the extracts of the same strain expressing the respective heterologous gene (Table 3), indicating that most of the activities in strains expressing the heterologous genes were derived from the heterologous proteins produced, not from proteins of the host strains.

As shown in Table 3, introduction of the *TtlonA2* mutation somewhat improved the production of all four proteins; notably, about a twofold increase was observed in the production of the α-Man. On the other hand, *TtlonA1* or *TTC1975* mutation had no, or somewhat negative, effects on the production of all the enzymes tested. In particular, the production of Thr-DH in both mutants and that of α-Man in the *TtlonA1* mutant sharply reduced to about half of that in the wild type strain. The precise reason why the production of the heterologous proteins was reduced in these mutants is not known, but from these results we conclude that only the *TtlonA2* mutation is effective for production of heterologous proteins. The difference observed between *TtlonA1* and *TtlonA2* mutations may reflect the difference in the substrate specificities of the enzymes, since *Tt*LonA2 preferred larger substrates such as casein (Table 2).

When *TtlonA1* or *TTC1975* mutation was combined with *TtlonA2* mutation, the production of α-amy was specifically improved, reaching about double the amount produced in the *TtlonA2* mutant. Also, the production of this enzyme slightly increased in the *TtlonA1 TTC1975* double mutant to about 1.5-fold of that in the wild type strain. It may thus be possible that *TtlonA1* or *TTC1975* mutation was effective for production of a specific heterologous protein when it was combined with *TtlonA2* mutation, or less efficiently, with each other.

In *E. coli*, strains deficient in *Ec*Lon were used to direct overproduction of several heterologous gene products (Buell et al. 1985; Miyamoto et al. 1985; Brodin et al. 1986; Walker et al. 1990; Alexander et al. 1992; Singh et al. 1992; Hutter and Singh 1998; Philibert and Martineau 2004). In some cases, the production was observed only in *Eclon* mutants, not in wild type strains. In the cases of bovine intestinal calcium binding protein and human somatomedin-C, it was reported that the production was enhanced about two- to threefold by the introduction of *Eclon* mutation (Buell et al. 1985; Brodin et al. 1986). Our results described, especially those with *TtlonA2* mutants, were almost comparable with those with *Eclon* mutant. However, as any overproduced bands corresponding to the expressed gene products were not detected on an SDS-PAGE gel when the whole-cell extracts of the transformants described above were loaded (data not shown), it may be possible that other protease(s) produced by the host cells is responsible for degradation of the products.

In conclusion, we analyzed enzymatically the three putative *lon* genes in *T. thermophilus* HB27. The results indicated that, in addition to TTC0418 (*Tt*LonA1), TTC0746 also exhibited an ATP-dependent protease activity with different substrate specificity from *Tt*LonA1, and therefore should be classified as a Lon protease (*Tt*LonA2). On the other hand, TTC1975 did not show ATPase activity or ATP-dependent activation of protease activity, but demonstrated significant protease activity, indicating that this enzyme should be classified as another type of protease, distinct from Lon protease. In addition, the *TtlonA1 TtlonA2* double mutant showed a characteristic growth feature similar to an *Eclon* mutant, indicating that these two enzymes have a similar function to *Ec*Lon in vivo. Finally, we discovered that the *TtlonA2* mutation is effective for the improvement of production of several heterologous proteins in *T. thermophilus*.

We are now conducting experiments to identify other proteases responsible for degradation of heterologous proteins by constructing mutant strains in which genes annotated to proteases or peptidases are disrupted, then using them as hosts for the expression of heterologous genes.

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