Journal of Industrial Microbiology & Biotechnology (1999) 23, 214–217
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Gene expression of *Bacillus subtilis* subtilisin E in *Thermus thermophilus*

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The *Bacillus subtilis* subtilisin E gene was cloned into an expression vector of the extreme thermophile, *Thermus thermophilus.* Active subtilisin E was produced in *E. coli*, indicating that the *Thermus* promoter functions in *E. coli*. When the plasmid was further introduced into *T. thermophilus*, the subtilisin E gene was expressed and the gene product accumulated as an inactive pro-form, because the autoprocessing of the wild-type enzyme to the active-form did not occur at 50°C or above.

Keywords: gene expression; subtilisin E; *Thermus thermophilus*

Introduction

One important aim of protein engineering is to enhance the thermostability of enzymes. Recently, a new strategy has been applied in selection of the thermostable proteins utilizing an extreme thermophile, *Thermus thermophilus* [1,5,19]. *T. thermophilus* is a Gram-negative aerobic microorganism that can grow at temperatures ranging from 50 to 80°C [13], and is the only thermophilic bacterium whose host-vector system is now available [4,8,11]. Maseda and Hoshino [10] recently constructed a new expression vector for *T. thermophilus*, in which *T. thermophilus* promoter was added upstream of the kanamycin resistance gene. Thus, the efficient host-vector system would be useful to select heat-resistant mutated proteins in *T. thermophilus* after the introduction of random mutagenesis into the genes [9].

In view of its industrial applications in detergents and food processing, subtilisin, an alkaline serine protease produced by various mesophilic *Bacillus* species has been extensively studied from both basic and applied aspects as a promising target for protein engineering. Using an *Escherichia coli* expression system, we have engineered subtilisin E from mesophilic *Bacillus subtilis* I168 [15–18]. Arnold *et al* [2,7,12,14,20] took a directed evolution approach to screen for laboratory-evolved thermostable enzymes. However, no study has been reported on a mutated protease with increased thermostability using the *T. thermophilus* expression system.

As the first step for this purpose, it would be necessary to establish the efficient expression system of subtilisin E in *T. thermophilus*. In the present paper, we describe the expression of subtilisin E gene under the control of the *Thermus* promoter in *T. thermophilus*.

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Received 17 March 1999; accepted 28 June 1999

Materials and methods

Bacterial strains and plasmids

E. coli JA221 (*hsdM$^+$ trpE5 leuB6 lacY recAl/F′ lacI^q lac$^+$ pro$^+$)* and *T. thermophilus* TH125 (*trpB5*) were used as host cells for subtilisin expression. The DNA fragment containing the *E. coli* OmpA signal peptide gene and the *B. subtilis* I168 subtilisin E gene was obtained from the *E. coli* expression plasmid pHI212 [15]. The plasmid pT8–131 [10] carrying the heat-stable kanamycin resistance (Km^r) gene was used for subtilisin expression in *T. thermophilus*. The pT8–131 also includes the P215 promoter region [9] and the ribosomal binding site sequence derived from *T. thermophilus* HB27 genome.

Media, growth conditions, and transformation procedure

TM medium and minimal medium [6] containing kanamycin (40 μg ml^{-1}) were used for the cultivation of transformants of *T. thermophilus*, and M9-casamino acid medium [15] containing ampicillin (50 μg ml^{-1}) for *E. coli* JA221 transformants. Plasmid marker rescue transformation was performed as previously described [3].

Subtilisin protease assay

After overnight growth on M9-casamino acid, 2 μl of each *E. coli* culture was spotted on M9-casamino acid agar plates containing 1% skim milk. The plates were incubated at 37°C overnight, followed by incubation at room temperature for 2 more days. Cells producing active subtilisin form a clear halo around the colony by hydrolyzing casein. For a synthetic peptide substrate, an *N*-succinyl L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (Sigma, St Louis, MO, USA) assay was performed as previously described [15].

Immunoblotting analysis

The transformants were cultured at 60°C in TM medium containing Km. Cell-free extracts were prepared by sonic oscillation under cooling, and analyzed by SDS-polyacrylamide gel electrophoresis. Recombinant subtilisins E were detected by Western blot analysis using the Vectastain

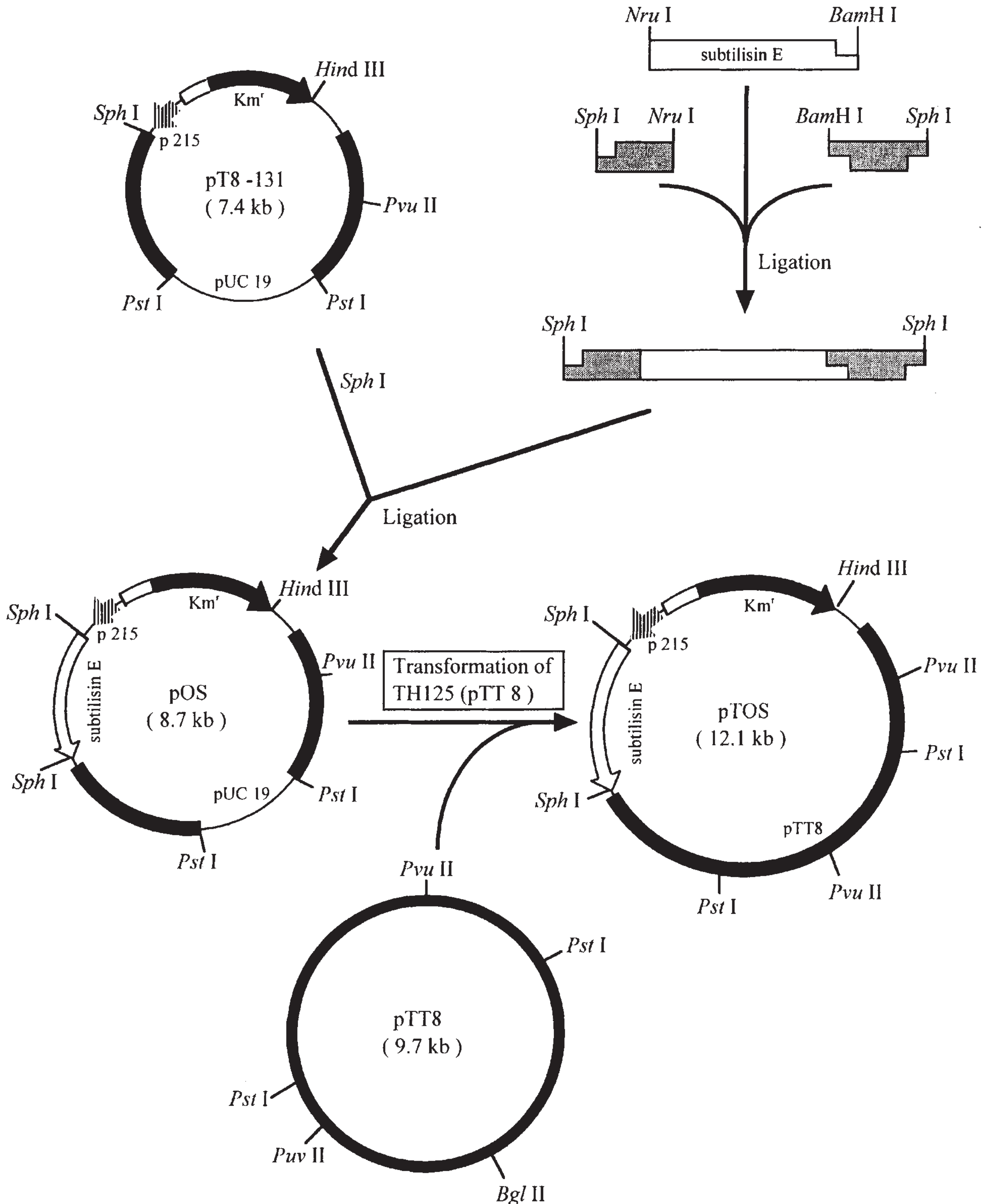

Figure 1 Construction of expression plasmids for the subtilisin E gene in *E. coli* and *T. thermophilus*. pT8–131 possessed a region homologous to the cryptic plasmid pTT8 flanking both sides of the Km^r cassette. The pTOS was then constructed in *T. thermophilus* TH125 harboring pTT8 by homologous recombination between pTT8 and pOS [10]. The hatched arrow represents the P215 promoter derived from the *T. thermophilus* HB27 genome [9]; the open arrow represents the fragment containing the *E. coli* OmpA signal peptide gene and the *B subtilis* subtilisin E gene; the solid arrow represents the Km^r gene; the open box represents the fragment containing the promoter from the HB27 genome for Km^r gene [10]; the solid box represents the pTT8 portion.

ABC kit (Vector Laboratories Inc, Burlingame, CA, USA) and an anti-mature subtilisin E polyclonal antibody.

Results

Construction of the expression plasmids for the subtilisin E gene by the use of Thermus promoter

A *T. thermophilus* expression plasmid (pTOS) for the subtilisin E gene was constructed as described in the legend for Figure 1. The *Bam*HI-*Sph*I and the *Sph*I-*Nru*I adaptors by annealing the oligomers 5′-GATCCGCATGC-3′ plus 5′-GCATGCG-3′, and 5′-CGTAAGACAGCTATCG-3′ plus 5′-CGATAGCTGTCTTACGCATG-3′, respectively, were ligated to the 1.2-kb *Nru*I-*Bam*HI fragment of pHI212 [15] including the *E. coli* OmpA signal peptide gene and the *B. subtilis* wild-type subtilisin E gene. The 1.3-kb *Sph*I fragment was then inserted into the *Sph*I site of pT8–131 located downstream from the P215 promoter region and the ribosomal binding site sequence originated from *T. thermophilus* HB27 [10]. The resultant plasmid was designated pOS.

Expression of the subtilisin E gene in E. coli and T. thermophilus

E. coli JA221 cells were transformed with pOS, and Km^r transformants formed a halo on the skim milk agar plate. When assayed with *N*-succinyl L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide, subtilisin activity was detected in the periplasmic fraction of the cells carrying pOS (Figure 2). These findings indicate that the *Thermus* P215 promoter was functional in *E. coli*, and prosubtilisin E was secreted across the cytoplasmic membrane using OmpA signal peptide to produce active enzyme in the periplasmic space.

The pT8–131 plasmid possessed a region homologous to the cryptic plasmid pTT8 (9.7 kb) flanking both sides of the Km^r cassette [10]. Therefore, introduction of pOS into *T. thermophilus* TH125 harboring pTT8 was performed by homologous recombination between pTT8 and pOS. After incubation at 60°C for 48 h, Km^r colonies were obtained and found to harbor pTOS (Figure 1). The transformants were cultured at 60°C in TM medium containing Km, and the expression of the subtilisin E gene and the enzyme activity were then examined. When total cellular protein of the transformants was analyzed by immunoblotting as shown in Figure 3, the band corresponding to prosubtilisin E was observed in the case of cells with pTOS after 2 h cultivation (lane 3). The amount of the precursor substantially increased with the culture time (lanes 5 and 7). On the other hand, no proteolytic activity was monitored inside and outside the cells harboring pTOS (data not shown). Similar results were obtained when the transformants were cultured at 50–70°C (data not shown). This result suggests that the subtilisin E gene was expressed and the gene product accumulated as an inactive pro-form in *T. thermophilus*.

Discussion

In this study, the DNA fragment containing the *E. coli* OmpA signal peptide gene and the *B. subtilis* subtilisin E gene was cloned into a *T. thermophilus* expression vector, which carries a *T. thermophilus* promoter. Active subtilisin E was produced in the *E. coli* periplasmic space, indicating that the *Thermus* promoter functions in *E. coli*. However, the activity level was relatively low compared with that of *E. coli* expression plasmid pHI212 [15] under the control of the IPTG-inducible *lac* promoter-operator. The −35 and −10 sequences of the P215 promoter are TTGACA and TATCAT, respectively, and the distance between the −35 and −10 regions is 17 nucleotides, both of which showed high similarity with those of *E. coli* consensus sequences [9]. We assumed that the P215 promoter would cause constitutive gene expression in pOS-harboring cells, so that subtilisin E activity might have a harmful influence on the growing cells.

When the plasmid was further introduced into *T. thermophilus* by homologous recombination, an inactive prosubtilisin E resulting from the cleavage of the OmpA signal peptide was observed in the transformants by immunoblotting analysis. These results indicate that: (i) expression of the subtilisin E gene was detected as prosubtilisin E in *T. thermophilus* under the control of the *Thermus* promoter; (ii) at high temperature (50°C or above), prosubtilisin E was unable to be processed autocatalytically and was then not folded to the active-form; and (iii) *E. coli* OmpA signal peptide was presumably cleaved by a signal peptidase of *T. thermophilus*. However, the level of gene expression in this experiment was low because the gene products were not seen when the gel was stained with Coomassie Brilliant Blue (data not shown). The P215 promoter region in pT8–

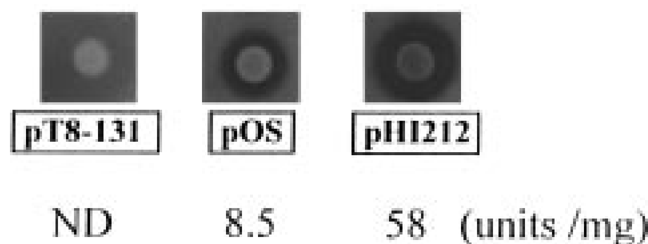

Figure 2 Subtilisin protease assay by *E. coli* cells harboring the recombinant plasmids. Halo formation was detected on a M9-casamino acid agar plate containing 1% skim milk. Specific activity (units mg^{-1}) in the periplasmic fraction from the cells was measured using *N*-succinyl L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide. pHI212 is the *E. coli* expression plasmid under the control of the IPTG-inducible *lac* promoter-operator [15]. ND, not detected.

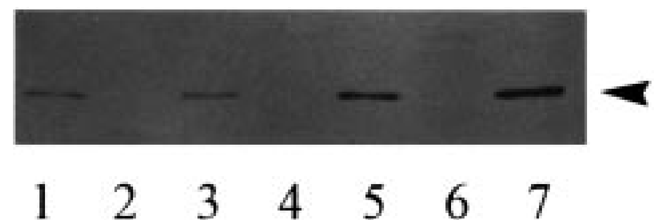

Figure 3 The total cellular protein from *T. thermophilus* cells harboring the recombinant plasmids detected by immunoblotting with anti-subtilisin E antiserum. Cells were cultured in TM medium containing Km at 60°C for 2 h (lanes 2 and 3), 8 h (lanes 4 and 5) and 14 h (lanes 6 and 7), and each 1 μg of protein was then subjected to 15% polyacrylamide gel. An arrowhead shows the position of prosubtilisin E (42 kDa). Lane 1, purified prosubtilisin E; lanes 2, 4 and 6, pT8–131; lanes 3, 5 and 7, pTOS.

131 showed a considerably strong activity relative to other promoters from *T. thermophilus* [9]. The pTOS copy number was supposed to be similar to that of the pTEV131 vector, whose number is eight in *T. thermophilus* [10]. Generally, it is considered that several problems, such as the efficiency of transcription and translation, or the stability of the plasmid, mRNA and its products, are involved in gene expression. We are now analyzing the amount and the stability of subtilisin E mRNA to elucidate these issues.

After introduction of random mutagenesis into the wild-type gene, we will isolate the thermostable mutant subtilisin E in the *T. thermophilus* expression system.

Acknowledgements

We thank H Maseda for his valuable comments and K Hirai for the preparation of figures.

References

1 Akanuma S, A Yamagishi, N Tanaka and T Oshima. 1998. Serial increase in the thermal stability of 3-isopropylmalate dehydrogenase from *Bacillus subtilis* by experimental evolution. Protein Sci 7: 698–705.
2 Arnold F and AA Volkov. 1999. Direct evolution of biocatalysts. Curr Opin Chem Biol 3: 54–59.
3 Hoshino T, H Maseda and T Nakahara. 1993. Plasmid marker transformation in *Thermus thermophilus*. J Ferment Bioeng 76: 276–279.
4 Kim DW, YC Lee and H Matsuzawa. 1997. Role of the COOH-terminal pro-sequence of aqualysin I (a heat-stable serine protease) in its extracellular secretion by *Thermus thermophilus*. FEMS Microbiol Lett 157: 39–45.
5 Kotsuka T, S Akanuma, M Tomuro, A Yamagishi and T Oshima. 1996. Further stabilization of 3-isopropylmalate dehydrogenase of an extreme thermophile, *Thermus thermophilus*, by a suppressor mutation method. J Bacteriol 178: 723–727.
6 Koyama Y, T Hoshino and N Tomizuka. 1986. Genetic transformation of the extreme thermophile *Thermus thermophilus* and other *Thermus* spp. J Bacteriol 166: 338–340.
7 Kuchner O and FH Arnold. 1997. Directed evolution of enzyme catalysts. Trends Biotechnol 15: 523–530.
8 Lee YC, H Koike, H Taguchi, T Ohta and H Matsuzawa. 1994. Requirement of a COOH-terminal pro-sequence for the extracellular secretion of aqualysin I (a thermophilic subtilisin-type protease) in *Thermus thermophilus*. FEMS Microbiol Lett 120: 69–74.
9 Maseda H and T Hoshino. 1995. Screening and analysis of DNA fragments that show promoter activities in *Thermus thermophilus*. FEMS Microbiol Lett 128: 127–134.
10 Maseda H and T Hoshino. 1998. Development of expression vectors for *Thermus thermophilus*. J Ferment Bioeng 86: 121–124.
11 Mather MW and JA Fee. 1992. Development of plasmid cloning vectors for *Thermus thermophilus* HB8: expression of a heterologous, plasmid-borne kanamycin nucleotidyltransferase gene. Appl Environ Microbiol 58: 421–425.
12 Reetz MT and K-E Jaeger. 1999. Superior biocatalysts by directed evolution. Topics in Curr Chem 200: 31–57.
13 Sakaki Y and T Oshima. 1975. Isolation and characterization of a bacteriophage infectious to an extreme thermophile, *Thermus thermophilus* HB8. J Virol 15: 1449–1453.
14 Shao Z, H Zhao, L Giver and FH Arnold. 1998. Random priming *in vitro* recombination: an effective tool for directed evolution. Nucleic Acids Res 26: 681–683.
15 Takagi H, Y Morinaga, H Ikemura and M Inouye. 1988. Mutant subtilisin E with enhanced protease activity obtained by site-directed mutagenesis. J Biol Chem 263: 19592–19596.
16 Takagi H, T Takahashi, H Momose, M Inouye, Y Maeda, H Matsuzawa and T Ohta. 1990. Enhancement of the thermostability of subtilisin E by introduction of a disulfide bond engineered on the basis of structural comparison with a thermophilic serine protease. J Biol Chem 265: 6874–6878.
17 Takagi H. 1993. Protein engineering on subtilisin. Int J Biochem 25: 307–312.
18 Takagi H, I Ohtsu and S Nakamori. 1997. Construction of novel subtilisin E with high specificity, activity and productivity through multiple amino acid substitutions. Protein Engng 10: 985–989.
19 Tamakoshi M, A Yamagishi and T Oshima. 1995. Screening of stable proteins in an extreme thermophile, *Thermus thermophilus*. Mol Microbiol 16: 1031–1036.
20 Zhao H, L Giver, Z Shao, JA Affholter and FH Arnold. 1998. Molecular evolution by staggered extension process (StEP) *in vitro* recombination. Nature Biotechnol 16: 258–261.