Use of a *Thermus thermophilus* host-vector system for expression of genes from the hyperthermophilic archaeon *Pyrococcus horikoshii*

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Abstract Genes annotated to threonine dehydrogenase, α -mannosidase, and glutamate dehydrogenase of the hyperthermophilic archaeon *Pyrococcus horikoshii* OT3 were used to test the expression system of the thermophilic eubacterium *Thermus thermophilus* HB27. Using the previously described P215 promoter, the three genes were successfully expressed, but at significantly lower levels than in the *Escherichia coli* expression system with the T7 promoter. Replacement of the promoter region with P31 or Pslp promoter improved the expression to a level comparable to or exceeding that in the *E. coli* system. Notably, α -mannosidase activity was clearly detected with the P31 and Pslp promoters, which could not be detected in the *E. coli* system. Moreover, with 6 x His-tag fusions of these enzymes at their COOH- or NH₂-termini, these proteins could be detected in the crude extracts of *T. thermophilus* by Western blotting, indicating that the increment of each enzyme activity was actually the result of enzyme production. These results demonstrate that the host-vector system of *T. thermophilus* is useful for expression of genes from hyperthermophiles.

Keywords *Thermus thermophilus*, *Pyrococcus horikoshii*, expression vector.

Abbreviations used: Km^r, kanamycin nucleotidyltransferase; ORF, open reading frame; IPTG, isopropyl- β -D-thiogalactoside; PVDF, polyvinylidene difluoride; NBT, nitro blue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolylphosphate; Thr-DH, threonine dehydrogenase; a-Man, α -mannosidase; Glu-DH, glutamate dehydrogenase

Introduction

Proteins from thermophilic microorganisms are of interest in the industrial and biochemical fields. Enzymes from thermophiles show very high stability and enhanced activity in spite of the presence of protein denaturants such as heat, detergents, organic solvents, and extreme pH. Hyperthermophiles, which can grow preferentially at extremely high temperatures around the boiling point of water, produce highly thermostable enzymes. Most of the hyperthermophiles belong to the phylogenetical domain Archaea¹⁶⁾, which have been isolated in the vicinity of geothermally heated environments¹³⁾. The complete genome sequences of several hyperthermophilic archaea have been determined, including *Pyrococcus horikoshii* OT3⁵⁾, but most of their genes have been classified as “unidentified open reading frames”. Since a gene manipulation system for these microorganisms has not been fully developed, it is necessary to express those unknown genes using other host-vector systems to elucidate their functions.

In general, the *Escherichia coli* host-vector system has been used to elucidate the function of unknown genes; however, not all the genes from hyperthermophiles can be successfully expressed in *E. coli*. For example, glutamate dehydrogenase from *P. furiosus* is inactive at temperatures below 40 °C, but undergoes heat activation above 40°C, accompanied by a conformational change⁶⁾, suggesting that some hyperthermophilic proteins are not folded accurately when expressed in a mesophilic host. We have assumed that high temperatures are needed for the correct folding of some gene products from hyperthermophiles. It is therefore preferable to use a high-temperature gene expression system, using a

thermophilic microorganism as host.

The extreme thermophile *Thermus thermophilus* HB27¹²⁾, an aerobic, rod-shaped, Gram-negative bacterium that grows at temperatures between 50 and 82 °C, and is known to exhibit high frequencies of natural transformation. Based on the cryptic plasmid pTT8, we have already constructed an expression vector designated pTEV131¹¹⁾. This plasmid contains a promoter sequence from the *T. thermophilus* genome, and a kanamycin nucleotidyltransferase (Km^r) gene as a selection marker which functions in *T. thermophilus*^{9, 10)}. Using derivatives of pTEV131, we have succeeded in inducing expression of the *T. thermophilus crtB* gene¹¹⁾ and the *Bacillus subtilis* subtilisin gene¹⁴⁾.

To demonstrate that the expression system of *T. thermophilus* is useful for the analysis of genes from hyperthermophiles, some typical genes from a hyperthermophilic archaeon, *P. horikoshii* OT3, were introduced into *T. thermophilus* HB27. By replacing the promoter regions for the expression with stronger ones, we have achieved an expression level comparable to that seen in the *E. coli* system. Moreover, a gene not expressed in *E. coli* could be expressed in our system. The present results clearly indicate the potential for use of the *T. thermophilus* expression system to analyze genes from hyperthermophiles.

Materials and methods

Bacterial strains, media and transformation procedures

DNA manipulation was conducted in *E. coli* JM109¹⁷⁾. *E. coli* BL21 (DE3) pLysS (Novagen, Madison, WI) and *T. thermophilus* HB27 TH104 (*proC4*)²⁾ harboring the plasmid pTT8 were used for the expression of *P. horikoshii* genes. *E. coli* and *T. thermophilus* were grown in LB medium and TM medium⁷⁾, respectively. For positive selection, 40 µg/ml of kanamycin, 100 µg/ml of ampicillin or 34 µg/ml of chloramphenicol was added to liquid medium or agar (1.5% w/v) plates. Transformation was performed by the same method as described previously³⁾.

Cloning and expression of the *P. horikoshii* genes in *E. coli*

The PH0835 gene of *P. horikoshii* was amplified by PCR with primers PH0835f and PH0835r (Table 1). In the forward primer, the ATG sequence of the *NdeI* site, CATATG, corresponds to the initiation codon of the open reading frame (ORF), and in the reverse primer, the *XhoI* site is located just downstream of the termination codon. The fragment was digested with *NdeI* and *XhoI*, cloned into the *NdeI*-*XhoI* sites of pET15b, and transferred to pET11a, using the *NdeI* and *Bpu1102I* sites.

Table 1 Primers used in this study.

Primer	Sequence (5'-3') ^{a)}
PH0655f	GGAATTCC <u>CATATG</u> TCAGAAAAGATGGTAGCTATCATGAAG
PH0655r	GCCCTAGGGATCC <u>TTAG</u> TAAAGGTATCATTTAAGCATAA
PH0655a	GGTCTTGCAACCAGTATATGTGGAAGTATCTTCA
PH0655b	TGAAGATCAGTTCCACATAT <u>ACT</u> GGTTGCAAGAACC
PH0655Hr	CGGGATCCCGTTTAAGCATAAAAAACAACCTTACCCG
PH0835f	GGAATTCC <u>CATATG</u> ACTAACCCCTGGAAAATTTTCCTTG
PH0835r	ATAAGAACTCGAGTCACAACACGGTGATGAGTAAGGTT
PH0835Hr	ATAAGAAATGCGGCCGCCAACACGGTGATGAGTAAGGTTCTCAC
PH1593f	TCGACATATGGATATGGTTGAGCAG
PH1593r	TCGAAAGCTCGAGTCAGTGCTTAACCCATCCGCG
PH1593a	ATAAGTGAAGAAGCTCTAGAGTTCTTGAAGAGGCCT
PH1593b	AGGCCTCTTCAAGAACTCTAGAGCTTCTTCACTTAT
PH1593Hr	TCGAGCGGCCCGCGTGCTTAACCCATCCGCG
MCS-f	AATTAATACGACTCACTATAG
MCS-r	CGGCATGCGATATCACCTCCTAGTTATTGCTCAGCGGTGGC
SLP-f	CGTCTAGACGCCTCCACCTCCCCCGG
SLP-r	TTCTTCATATGCCTCACACCT
NH-f	GAAGGAGATATACCATGGGCAGCAG
NH-r	GCCCATGGTATATCTCCTTCTTAAAG
Km-r	CACCTGAGATGCATAATCTAGTAGAA

^{a)} Restriction sites are underlined, and the start and stop codons of native ORFs are shaded in gray. Bold-face letters show base-substitutions introduced by the overlap extension PCR.

To eliminate the *NdeI* and *XhoI* sites in the PH0655 and PH1593 ORFs, respectively, the overlap extension PCR method⁴⁾ was used. The 5' and 3' portions of the PH0655 gene were amplified with the primer pairs of PH0655f and PH0655b, and PH0655r and PH0655a, respectively, and joined by PCR with primers PH0655f and PH0655r. In this construct, the *NdeI* site (CATATG) in the ORF was converted to TATATG without amino acid substitution. The resultant fragment was digested with *NdeI* and *BamHI*, and cloned into the *NdeI*-*BamHI* sites of pET11a. Likewise, the 5' and 3' portions of PH1593 gene were amplified with the primer pairs of PH1593f and PH1593b, and PH1593r and PH1593a, respectively, joined by PCR with primers PH1593f and PH1593r. In this construct, the *XhoI* site (CTCGAG) in the ORF was converted to CTAGAG without amino acid substitution. The resultant fragment was digested with *NdeI* and *XhoI*, first cloned into pET15b, and then transferred to pET11a, using the *NdeI* and *Bpu1102I* sites. The nucleotide sequences of the cloned fragments were verified by DNA sequence analysis (Big Dye cycle sequencing, ABI, CA, USA). Each recombinant plasmid was used to transform *E. coli* BL21 (DE3) pLysS. The transformants were cultured at 37 °C in 10 ml of LB medium containing ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml). When the cell density at OD₆₀₀ reached 0.8, 0.4 mM isopropyl-β-D-thiogalactoside (IPTG) was added and the cells grown for an additional 4 h.

Construction of expression vectors for *T. thermophilus*

Based on the plasmid pT8-131¹⁴⁾, the following procedures were conducted to construct several expression vectors (Fig. 1). First, a 2.3-kb *BstEII*-*DraI* fragment and a 0.8-kb *PvuII* fragment, each containing a part of the pT8 sequence and a portion of pUC19, were removed from pT8-131, giving rise to plasmid pT8-131S. Next, the fragment containing the multiple-cloning sites of pET-23a (+) (Novagen) was amplified by PCR with primers MCS-f and MCS-r, and digested with *XbaI*, internal to the fragment, and *SphI*. The *XbaI*-*SphI* fragment of pT8-131S containing the P215 promoter was replaced with the above fragment, and the resultant plasmid was designated pT8-131MCS. Then the *EcoRI* fragments containing the P215, P214 and P31 promoters, previously obtained from *T. thermophilus* genome by the promoter-screening experiment⁹⁾, were inserted into the *XbaI* site of pT8-131MCS, after converting both fragments to blunt ends by T4 DNA polymerase, giving rise to plasmids pT8S-P215, pT8S-P214 and pT8S-P31, respectively. For construction of pT8S-Pslp, a fragment containing the

promoter and ribosome binding site of the S-layer protein gene of *T. thermophilus* HB8¹⁾ was amplified with primers SLP-f and SLP-r, and the resultant fragment was inserted into the *XbaI*-*NdeI* sites of pT8-131MCS.

The direction of each promoter in the constructed plasmid was confirmed by DNA sequencing.

Cloning and expression of the *P. horikoshii* genes in *T. thermophilus*

The cloned PH0655 gene in pET11a was excised by digestion with *NdeI* and *BamHI*, and the cloned PH0835 and PH1593 genes in pET11a were excised by digestion with *NdeI* and *Bpu1102I*, and re-inserted into the same restriction sites of expression vectors pT8S-P215, pT8S-P214, pT8S-P31, and pT8S-Pslp. In these constructs, the ORFs were not fused with the 6 x His-tag sequence in the plasmids.

To check the expression by Western blotting, the ORFs were fused with the 6 x His-tag on the basis of plasmid pT8S-P31. Using the cloned genes in pT8S-P31 as templates, PH0655, PH0835 and PH1593 genes were amplified by PCR with primer pairs of PH0655f and PH0655Hr, PH0835f and PH0835Hr, and PH1593f and PH1593Hr, respectively. The PCR products were digested with *NdeI* and *BamHI* for PH0655 gene, or *NdeI* and *NotI* for PH0835 and PH1593 genes, and again inserted to the respective sites of pT8S-P31. In these constructs, the original termination codons were eliminated and these ORFs were fused in-frame with the 6 x His-tag in the plasmid.

For PH0835 and PH0655 genes, the NH₂-terminal fusions with the 6 x His-tag were also constructed. As the cloned PH0835 ORF in pET15b was already fused with the 6 x His-tag sequence in the multiple cloning sites, this fragment was amplified with primers NH-f and MCS-r, and a fragment containing the P31 promoter and a part of the Km^r gene was amplified from pT8S-P31 with primers NH-r and Km-r. These two fragments were then joined by PCR with primers Km-r and MCS-r. The resultant fragment was digested with *BssHII*, located in the promoter region of Km^r gene, and *Bpu1102I*, and cloned into the same restriction sites of pT8S-P31. The resultant plasmid was designated pT8S-P31-His-PH0835. Next, the PH0835 ORF in pT8S-P31-His-PH0835 was replaced with PH0655 ORF in pT8S-Pslp-PH0655, by digestion of both the plasmids with *NdeI* and *SphI*, giving rise to plasmid pT8S-P31-His-PH0655.

Plasmid construction was confirmed by restriction analysis and DNA sequencing. Each recombinant plasmid was used to transform the *T. thermophilus* HB27 TH104 harboring pTT8.

The transformants were cultured at 70 °C in 10 ml of TM medium containing kanamycin (40 µg/ml) until the cell density at OD₆₀₀ reached 1.0, and used for preparation of cell extracts.

Preparation of cell extracts from *E. coli* and *T. thermophilus* cells

Cells were collected by centrifugation at 8,000 rpm for 10 min and washed with 0.85% NaCl. The cells were suspended in 5 ml of 50 mM Tris-HCl buffer (pH 7.0) containing 1 mM DTT, and subjected to sonication (200 W for 10 min). After cell debris had been removed by centrifugation (15,000 rpm, 15 min, 4 °C), the supernatant obtained was used for each enzyme assay.

Enzyme assays

The enzyme activities were determined spectrophotometrically, using a spectrophotometer equipped with a thermostat. For all assays, specific activities are expressed as units per mg of total protein. The protein concentration of crude extracts was estimated using a protein assay kit (Bio-Rad, CA, USA) with bovine serum albumin as the standard.

Threonine dehydrogenase activity was determined by NADH formation. Crude extracts (20 µl) from strains harboring plasmids expressing the PH0655 gene, or control plasmids, were incubated at 90 °C with 180 µl of 100 mM L-threonine in 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM β-NAD⁺. The reaction was monitored by following the reduction at 340 nm. One unit (U) of the activity was defined as the amount of enzyme that catalyzed the formation of 1.0 µmol of NADH per min.

The α-mannosidase activity was determined with *p*-nitrophenyl-α-D-mannopyranoside as substrate. Crude extracts (20 µl) from strains harboring plasmids expressing the PH0835 gene, or control plasmids, were incubated at 70 °C with 180 µl of 1.0 mM substrate in 50 mM HEPES buffer (pH 7.0). The reaction was monitored by following the reduction at 405 nm. One unit of enzyme activity was defined as the amount of enzyme that liberated 1.0 µmol *p*-nitrophenol per minute under the assay conditions.

The glutamate dehydrogenase activity of crude extracts was determined using NADPH formation. Crude extracts (20 µl) from strains harboring plasmids expressing the PH1593 gene, or control plasmids, were incubated at 70 °C with 180 µl of 10 mM L-glutamic acid in 100 mM sodium phosphate buffer (pH 7.5) containing 1.0 mM β-NADP⁺. One unit (U) of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1.0 µmol of NADPH per min.

Western blot analysis

Ten µg of the crude extracts from *T. thermophilus* strains harboring plasmids expressing the 6 x His-tagged genes, or pTEV-P31, were loaded onto a 12.5% SDS-polyacrylamide gel and resolved by SDS-PAGE according to the method of Laemmli⁸⁾. Electrophoresed proteins were transblotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore) for 1 h at 200 mA in a transfer buffer containing 192 mM glycine, 25 mM Tris-HCl (pH 8.0) and 20% methanol. The membrane was incubated in blocking buffer (20 mM Tris-HCl, 150 mM NaCl, 3%(w/v) BSA, 0.1% Tween 20, pH 8.0) for 1 h at room temperature, and then incubated with mouse anti-tetra His IgG (Qiagen; 1:1000 in blocking buffer) for 1 h at room temperature. The membrane was washed with blocking buffer without BSA (TBS/Tween), and incubated with alkaline phosphatase-conjugated anti-mouse IgG (Pierce; 1:2000 in blocking buffer) for 1 h at room temperature. Then the membrane was again washed in TBS/Tween, and treated with nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolylphosphate (BCIP) detection reagents (Roche), according to the manufacturer's instructions.

Results and discussion

Comparison of expression of *P. horikoshii* OT3 genes between *T. thermophilus* and *E. coli*

Earlier, we developed an expression system for *T. thermophilus* and succeeded in the production of *B. subtilis* subtilisin E¹⁴⁾. To assess this system for the expression of genes from hyperthermophiles, we selected PH0655, PH0835 and PH1593 genes from *P. horikoshii* OT3, and compared the expression level with an *E. coli* expression system. Genome sequencing analysis revealed these genes to have annotated to threonine dehydrogenase (Thr-DH), α-mannosidase (α-Man) and glutamate dehydrogenase (Glu-DH), respectively, of which the PH1593 gene had been successfully expressed in *E. coli*, and the corresponding enzyme activity had been detected¹⁵⁾.

Based on the previously constructed plasmid pT8-131, two homologous regions to plasmid pTT8, necessary for the plasmid marker-rescue transformation³⁾, were truncated, and the region containing the P215 promoter functional in *T. thermophilus*⁹⁾ was replaced by the multiple cloning sites from pET23a (+), to reduce the size of the plasmid and enable easy cloning of heterologous genes, respectively. The resultant plasmid, pT8-131MCS, contains a thermostable Km^r cassette functional both in *E. coli* and *T. thermophilus*, and two regions of 961 bp and 661 bp homologous to plasmid pTT8. The plasmid could replicate in *E. coli* but not in *T. thermophilus*;

however, a reconstitution process based on homologous recombination with the cryptic plasmid pTT8 in *T. thermophilus* cells would give rise to a pTEV131-type plasmid replicable in *T. thermophilus*, as was the case of the parental plasmid pT8-131¹⁴⁾ (Fig. 1). As expected, transformation of

pT8-131MCS into *T. thermophilus* harboring pTT8 gave Km^r colonies after incubation at 65 °C for 36 h at an efficiency of about 8×10^5 /μg DNA, which was about 100-fold lower than that of pT8-131 (data not shown).

Figure 1 Schematic representation of the construction of expression plasmids in *T. thermophilus*.

pT8-131S was constructed by the deletion of *Bst*EII-*Dra*I and *Pvu*II-*Pvu*II regions of pT8-131. pT8-131MCS was constructed by replacing the P215 region of pT8-131S with the multiple cloning sites from pET23a. Plasmids pT8S-P215, pT8S-P214 and pT8S-P31 were constructed by addition of the promoter sequences (P215, P214 or P31) at the *Xba*I site of pT8-131MCS. Plasmid pT8S-Pslp was constructed by addition of the promoter sequence (*Pslp*) and its Shine Dalgarno region at the *Xba*I-*Nde*I site of pT8-131MCS. After transformation of these plasmids into *T. thermophilus* HB27 TH104 harboring pTT8, the pTEV-derivatives were then constructed by homologous recombination between pT8-derivatives and pTT8. Thin and bold lines respectively show the pTT8 and pUC19 regions. Seven restriction sites (*Nde*I, *Nhe*I, *Bam*HI, *Not*I, *Bpu*1102I, *Eco*RV, and *Sph*I) are available for cloning of foreign genes into these plasmids.

Next, to express the *P. horikoshii* genes in *T. thermophilus*, the P215 promoter was again inserted into the *Xba*I site of pT8-

131MCS, giving rise to pT8S-P215, and PH0655, PH0835 and PH1593 genes were individually inserted into this plasmid,

using *NdeI* site at the translational initiation codon of each ORF and a restriction site located downstream of each ORF, giving rise to pT8S-P215-PH0655, pT8S-P215-PH0835 and pT8S-P215-PH1593, respectively. These plasmids were used to transform *T. thermophilus* harboring pTT8. After selection of transformants by 40 µg/ml of kanamycin, plasmids were recovered from the cells and checked by restriction analysis. The plasmids obtained through these procedures were designated pTEV-P215-PH0655, pTEV-P215-PH0835 and pTEV-P215-PH1593, respectively.

For the expression of these genes in *E. coli*, a strong T7 promoter of pET11a was used. The structural genes were inserted into pET11a, using the restriction sites described above, and the resultant plasmids were used to transform *E. coli* BL21 (DE3) pLysS strain.

The *T. thermophilus* and *E. coli* transformants were cultivated in TM containing 40 µg/ml of kanamycin, and LB broth containing ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml), respectively. Then the crude extracts were prepared as described in Materials & Methods.

Each enzyme activity was measured with the extracts, and the enzyme production in *T. thermophilus* was compared with that in *E. coli* (Table. 2). In *E. coli*, the enzyme activities of the PH0655 and PH1593 gene products, Thr-DH and Glu-DH, respectively, could be detected, indicating that these genes had been successfully expressed via the pET expression system (Table 2). However, the α -Man activity of the PH0835 gene product was not detected, and this result was also confirmed by

SDS-PAGE analysis (data not shown). On the other hand, in *T. thermophilus*, all the enzyme activities of these gene products increased, compared with the control strain harboring the vector pTEV131. This result strongly indicates that *P. horikoshii* genes were successfully expressed in *T. thermophilus* HB27, using pTEV-P215-type plasmids as expression vectors. However, compared with the *E. coli* expression system, the expression level of the PH0655 and PH1593 genes were significantly low in *T. thermophilus*.

Replacement of promoter regions improved the production of *P. horikoshii* proteins in *T. thermophilus*

To improve the expression level of these genes in *T. thermophilus*, at least at the transcriptional level, the P215 promoter region in pT8S-P215 was replaced with a fragment containing a strong promoter, P214 or P31, obtained previously by our promoter-screening experiment ⁹⁾. In the previous experiment with the *Km^r* gene as a reporter, P214 and P31 promoters showed transcriptional activities that were 2.7- and 2.3-fold higher than the P215 promoter, respectively ⁹⁾. The resultant plasmids were designated pT8S-P214 and pT8S-P31, respectively (Fig. 1).

We also used a promoter region of the S-layer protein gene, *Pslp*, which is also known as one of the strongest promoters in *T. thermophilus* ¹⁾. The promoter region containing the ribosome-binding site was amplified by PCR and inserted into the *XbaI-NdeI* sites of pT8-131MCS, giving rise to pT8S-Pslp (Fig. 1).

Table 2 Comparison of *P. horikoshii* enzyme activities expressed in *E. coli* and *T. thermophilus*.

Host/Promoter	Enzyme activity ^{a)} (mU/mg protein)		
	Thr-DH (PH0655)	α -Man (PH0835)	Glu-DH (PH1593)
<i>T. thermophilus</i> /P215	6.6	<1.0	32.5
<i>T. thermophilus</i> /P31	19.9	9.4	129.7
<i>T. thermophilus</i> /P214	3.6	<1.0	24.5
<i>T. thermophilus</i> /Pslp	15.0	2.7	56.2
<i>T. thermophilus</i> /control ^{b)}	N.D. ^{c)}	N.D.	1.2
<i>E. coli</i> /T7	88.2	N.D.	69.8
<i>E. coli</i> /control ^{b)}	N.D.	N.D.	N.D.

^{a)} Enzyme activities were measured in triplicate, and the average values are shown. The maximum variations from the values were within 10%.

^{b)} Enzyme activities were assayed with the crude extracts of the *T. thermophilus* and *E. coli* strains harboring vectors pTEV131 and pET11a, respectively.

^{c)} N.D., not detected.

Each *P. horikoshii* gene was cloned into pT8S-P214, pT8S-P31 and pT8S-Pslp, and the resultant plasmids were again used to transform TH104 harboring pTT8 as described above. Cell-free extracts were prepared from the Km^r transformants of *T. thermophilus*, and each of the enzyme activities was examined. As shown in Table 2, increased enzyme activity, compared with that of the control, was detected in all the constructs, indicating that all three genes were also expressed in *T. thermophilus* under the control of the P214, P31 and Pslp promoters. A significant increase was observed in these enzyme activities when they were expressed under the control of P31 and Pslp promoters. Notably, in the case of PH0835 gene with P31 and Pslp promoters, the corresponding activity of α -Man was clearly detected, unlike in the *E. coli* system. In the case of the PH0655 and PH1593 genes, the corresponding enzyme activities, Thr-DH and Glu-DH, increased about 3.5-fold under the control of the P31 promoter, compared with those with the P215 promoter, which coincides with our previous results obtained for the Km^r gene¹⁰⁾. On the other hand, the use of P214 promoter did not lead to overexpression of these genes, which was in disagreement with our previous results. One possible reason for this discrepancy is the differences in the sequences around the promoter regions between the promoter-probe vector pPP11 and the expression vectors constructed in this study. It is notable that in spite of the overexpression achieved by the use of P31 promoter, growth of the recombinants in TM medium was almost the same as that of the strain harboring the vector pTEV131 (data not shown), indicating that this level of overexpression did not affect the growth or viability of the recombinants.

Immunodetection of *P. horikoshii* proteins fused with 6 x His-tag

When we analyzed the crude extracts expressing the *P. horikoshii* genes by SDS-PAGE, we could not detect any overproduced proteins by Coomassie blue staining. To confirm that the increment in the enzyme activities described above was the result of the production of *P. horikoshii* proteins, we appended the 6 x His sequence in the multiple cloning site to the COOH-termini of the *P. horikoshii* genes by adjusting the frames as described in Materials and Methods, and the resultant genes were expressed under the control of the strongest P31 promoter. The resultant plasmids were designated pTEV-P31-PH0655-His, pTEV-P31-PH0835-His and pTEV-P31-PH1593-His for those expressing the COOH-terminally-fused PH0655, PH0835 and PH1593 genes, respectively.

The enzyme activity of the crude extracts of *T. thermophilus*

harboring pTEV-P31-PH1593-His was 121.3 mU/mg protein, which was almost the same as that without the addition of 6 x His-tag. In the Western blot analysis with an anti-tetra-His antibody, we detected a specific band of 46 kDa in the extract, which coincided with the estimated molecular weight from the nucleotide sequence of PH1593 of 47.3 kDa (Fig. 2, lane 4).

Figure 2 Western blots of crude extracts.

Crude extracts were prepared from *T. thermophilus* HB27 TH104 harboring each plasmid, and 10 μ g of protein was loaded in each lane of the SDS-12.5% polyacrylamide gel. Positions of molecular mass markers (in kDa) are indicated. Bands corresponding to PH0655, PH0835 and PH1593 gene products are indicated by arrowheads. Lanes 1, crude extracts from strain harboring pTEV-P31-His-PH0655; 2, pTEV-P31-PH0835-His; 3, pTEV-P31-His-PH0835; 4, pTEV-P31-PH1593-His; and 5, pTEV-P31. 6 x His-tagged proteins were detected with anti-tetra His antibody followed by chromogenic detection with AP-conjugated rabbit anti-mouse IgG and NBT/BCIP detection reagent.

Contrary to the above, the Thr-DH and α -Man activities of those harboring pTEV-P31-PH0655-His and pTEV-P31-PH0835-His, respectively, could not be detected. Similarly, in the case of pTEV-P31-PH0835-His, the corresponding band to the estimated molecular weight of the enzyme, 100.4 kDa was also not detected in the Western blot (Fig. 2, lane 2). The addition of 6 x His-tag to the COOH-terminus of the α -Man might lead to incorrect folding of the protein and result in degradation by proteases intrinsic to the host strain. Therefore, we constructed new plasmids, pT8S-P31-His-PH0655 and pT8S-P31-His-PH0835, in which 6 x His-tag was added in-

frame to the NH₂-termini of the PH0655 and PH0835 ORFs, respectively, and tested them using Western blot analysis and for enzyme activity. As a result, we were able to detect specific bands of 40 and 100 kDa in the extracts, which coincided with the estimated molecular weights from the nucleotide sequences of the PH0655 and PH0835 genes of 37.8 and 100.4 kDa (Fig. 2, lane 1 and lane 3). In addition, the enzyme activities in the crude extracts of *T. thermophilus* harboring pTEV-P31-His-PH0655 and pTEV-P31-His-PH1593 were 22.8 and 12.2 mU/mg protein, almost the same as those obtained without the addition of 6 x His-tag.

In mesophilic expression systems like the *E. coli* system, the expressed thermophilic proteins can be readily purified by heat treatment and subsequent centrifugation. However, in the *Thermus* expression system, heat treatment is not applicable, since proteins from the host are also heat-stable. Instead, a peptide-tag like 6 x His-tag in this study may be useful for purification of products. Since few immunoreactive bands were detected in *T. thermophilus* extracts (Fig. 2), His-tagged products can be easily purified to high quality using affinity chromatography to 6 x His-tag in *T. thermophilus*.

In conclusion, using the expression system developed in this study, we succeeded in expressing three genes from *P. horikoshii*. The use of a stronger promoter such as P31 or *Pslp* was able to improve the expression to a level comparable to, or more than, that in the *E. coli* system. Moreover, we achieved the successful expression of a gene that was not expressible using the conventional *E. coli* host. The host-vector system of *T. thermophilus* has potential as an efficient tool for the expression of genes from thermophiles and hyperthermophiles.

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