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Highly efficient production of enzymes of an extreme thermophile, *Thermus thermophilus*: A practical method to overexpress GC-rich genes in *Escherichia coli*

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Abstract The GC-rich *leuB* gene (coding for 3-isopropylmalate dehydrogenase) of *Thermus thermophilus* is scarcely expressed in *Escherichia coli*, unless a leader open reading frame (ORF) is provided. We conducted experiments on nonexpressible plasmids and obtained a modified plasmid showing greatly enhanced expression: the degree of expression from the plasmid was higher than that from any other plasmid so far constructed. Sequence analysis of the plasmid showed that a 258-bp leader ORF overlapped with the initiation codon of *leuB* was newly formed as a consequence of the insertion of a 0.5-kb *Bam*HI fragment derived from the *E. coli* chromosome. The degree of expression from the plasmid was further improved by shortening the leader ORF to 36 bp without changing the overlapping portion, and the flanking sequence between the promoter and the leader ORF was removed. The expression in *E. coli* of the *pfk1* gene (coding for phosphofructokinase) of *T. thermophilus* was improved by the construction of a structure similar to that which enhanced the expression of the *leuB* gene. Based on the results, a practical method for the overexpression of GC-rich genes in *E. coli* is proposed.

Key words *Thermus thermophilus* · GC-rich gene · 3-Isopropylmalate dehydrogenase · Phosphofructokinase · Overproduction · Leader open reading frame (ORF) · Gene overlapping

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Introduction

Enzymes from the extreme thermophile *Thermus thermophilus* are useful experimental materials in the laboratory for studying protein structural stability, as well as for materials in industrial applications. For the studies in which the thermophile enzymes are used as experimental materials, it is essential to overexpress their genes in *Escherichia coli*. Many genes have been cloned from *T. thermophilus*; however, most of them were scarcely or poorly expressed in *E. coli*. In order to establish a general way to overproduce the thermophile enzymes, we investigated the effects of the upstream noncoding regions of the thermophile genes on the efficiency of their expression.

We previously reported that the translation of the *T. thermophilus leuB* gene, coding for 3-isopropylmalate dehydrogenase (EC 1.1.1.85), was inhibited by secondary structure(s) in the translational initiation region of the mRNA (G + C content of the thermophile DNA is about 70%), and that the inhibition was relieved by the introduction of a leader open reading frame (ORF) (Ishida and Oshima 1994). The use of a potent promoter alone, such as the *tac* promoter, did not significantly improve the expression efficiency in *E. coli* of the thermophile *leuB* without a leader ORF (Ishida and Oshima 1996). The plasmid used, ptacI12 (4.5 kb), has a *tac* promoter and no leader ORF. Although *leuB*-deficient *E. coli* grow weakly in a leucine-free medium when the plasmid is introduced, there is no detectable activity of the *T. thermophilus* enzyme in its heat-treated cell-free extract (Ishida and Oshima 1996). We conducted experiments on the insertion of several *Bam*HI-fragments derived from the upstream noncoding region of *T. thermophilus leuB* into a *Bam*HI site in front of the *leuB* coding region on ptacI12. As a result, it was confirmed that the insertion of the thermophile *Bam*HI-fragments somewhat inhibited the expression (Ishida and Oshima 1996). During the experiments, however, a modified plasmid, named ptacXI16, with highly increased expression was unexpectedly obtained. Based on the upstream structures of the thermophile *leuB* on ptacXI16 and its derivative

plasmid with further increased expression, in this paper we propose a practical method for greatly enhanced production of enzymes in *E. coli* from GC-rich genes of *T. thermophilus*.

Materials and methods

Reagents, plasmids, and bacterial strains

3-Isopropylmalate was purchased from Wako Pure Chemical (Tokyo, Japan). Isopropyl-β-D-thiogalactopyranoside was purchased from Takara Shuzo (Shiga, Japan). The recombinant plasmid ptacI12, carrying *T. thermophilus leuB*, was described in our previous papers (Ishida and Oshima 1994, 1996). The *pfk1* gene of *T. thermophilus* was cloned into a pUC vector plasmid by Xu et al. (1991), and in this paper, the recombinant plasmid is named pTPFK. The *E. coli* strains JA221 (F^-, *hstR*, Δ*trpE5*, *leuB6*, *lacY*, *recA1*) (Almond et al. 1985) and JM109 [*recA1*, *supE44*, *endA1*, *hsdR17*, *gyrA96*, *relA1*, *thi*, Δ(*lac-proAB*), F′(*traD36*, $proAB^+$, $lacI^qZ'\Delta M15$)] (Yanisch-Perron et al. 1985) were used as hosts to produce 3-isopropylmalate dehydrogenase and phosphofructokinase, respectively. *E. coli* JM109 was also used to measure the gene expression under lactose repression.

Molecular manipulations of recombinant plasmids and DNA sequencing

Plasmid preparation and bacterial transformation were carried out according to the procedures described by Sambrook et al. (1989). The nucleotide sequence was determined by the dideoxy chain termination method using M13 mp18 and mp19 (Sanger et al. 1977). Other common methods used for DNA manipulations were as described by Owen (1984). DNA sequences were analyzed by using the GENETYX program (Software Development, Tokyo).

Oligonucleotide-directed mutagenesis

Mutation for the shortening of the leader ORF in front of *leuB* was carried out by using the polymerase chain reaction (PCR) (Saiki et al. 1988). Oligonucleotides were chemically synthesized by a DNA synthesizer Model 394 (Applied Biosystems, Perkin-Elmer, Foster City, CA, USA). The first 44-mer primer, 5′-AAGGATCCTATGACCATGATTACG-TACCCCAGGAGGACGGAATG-3′, was designed to add a new *Bam*HI restriction site and an identical 15-bp sequence to the N-terminal portion of *E. coli lacZ* at 18-bp upstream of the initiation codon of *leuB* on ptacI12. The underline indicates the 5′ add-on sequence. The second 18-mer primer, 5′-CCGGTGAGCTCCCGGACG-3′, is complementary to the template around the unique *Sac*I restriction site located at 399-bp from the initiation codon of *leuB*. PCR was carried out for 30 cycles of 94°C for 45 s, 59°C for 45 s, and 72°C for 20 s in a Zymoreactor II-Model 1820 (Atto, Tokyo, Japan). The product was cleaved with *Bam*HI and *Sac*I, then ligated between *Bam*HI (partial digestion) and *Sac*I sites of ptacI12. As a result of this mutation, a short leader ORF (36 bp), overlapped with the *leuB* coding region, was constructed by the read-through of the *lacZ* sequence.

Bacterial growth and enzyme extraction

For Leu^+ phenotype screening, recombinant *E. coli* JA221 was grown in M9 solid medium (Sambrook et al. 1989) with 0.2% of glucose and ampicillin sodium salt (50 μg/ml) at 37°C for 24 h. For the production of the thermophile enzymes, *E. coli* JA221 or JM109 harboring a recombinant plasmid was grown in 200 ml of YT medium (1% Bacto tryptone, 0.5% Bacto yeast extract, and 0.5% NaCl) containing ampicillin sodium salt (50 μg/ml). The bacterial cultivation, the enzyme extraction, and the heat treatment were as described in our previous paper (Ishida and Oshima 1996).

Enzyme assays

Measurement of the 3-isopropylmalate dehydrogenase activity in the heat-treated extract was as described in our previous paper (Ishida and Oshima 1996). One unit of activity was defined as 1 μmol of NADH produced per min at 30°C. The phosphofructokinase activity in the heat-treated extract was measured according to the method of Xu et al. (1990). One unit of activity was defined as 1 μmol of NADH reduced per min at 25°C.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for the *T. thermophilus* enzyme in the heat-treated extract was performed according to the method of Laemmli (1970) using a 7.5% gel. The heat-treated extracts corresponding to each 2.5 mg of *E. coli* cells were loaded. The gel was stained with Coomassie Brilliant Blue-R 250. M_r standards—myosin heavy chain (M_r 200000), phosphorylase B (M_r 97400), bovine serum albumin (M_r 68000), ovalbumin (M_r 43000), and carbonic anhydrase (M_r 29000)—were purchased from Bethesda Research Laboratories (Gaithersburg, MD, USA).

Results

High-level expression of *T. thermophilus leuB* from a plasmid, ptacXI16

E. coli JA221 harboring ptacXI16 (5.0 kb) was obtained as a Leu^+ colony with increased growth rate on the leucine-free medium. Restriction-map analysis of ptacXI16 showed that the plasmid was formed by insertion of an unknown *Bam*HI

fragment (0.5kb), which might be derived from *E. coli* chromosome DNA, into the *Bam*HI site between the *tac* promoter and the *leuB* coding region of ptacI12 (4.5kb) (Fig. 1). A much higher activity of the thermophile enzyme was detected in the heat-treated extract of *E. coli* JA221 (ptacXI16) than that of *E. coli* JA221 (ptacI12) (Fig. 2). This increase was also confirmed by electrophoretic analysis (Fig. 3). Such high-level production reappeared when the isolated ptacXI16 was newly introduced into *E. coli* JA221 cells.

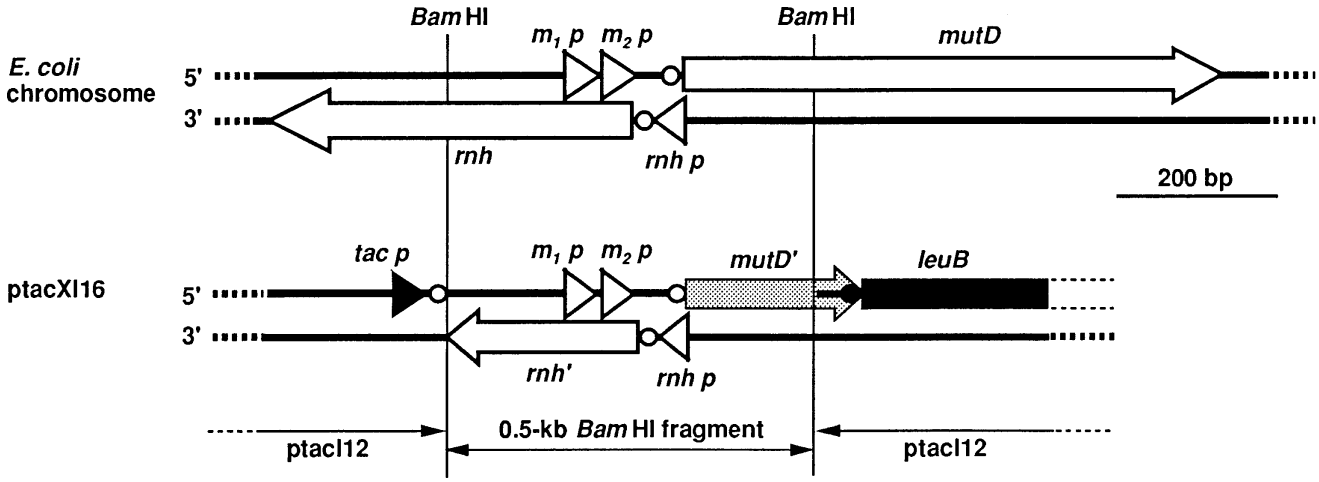

Fig. 1. Upstream structure of *T. thermophilus leuB* on a plasmid, ptacXI16. ptacXI16 was formed from patcI12 by the insertion of a 0.5-kb *Bam*HI fragment that is part of the *mutD–rnh* region of *E. coli* chromosome DNA. *Closed box*, the *leuB* coding region; *open arrows*, *mutD* and *rnh* genes and a truncated *rnh* (*rnh'*); *shaded arrow*, *mutD'*-open reading frame (ORF); *closed triangle*, the *tac* promoter; *open triangles*, the promoters of *mutD* and *rnh*; and *open* and *closed circles*, ribosome binding site

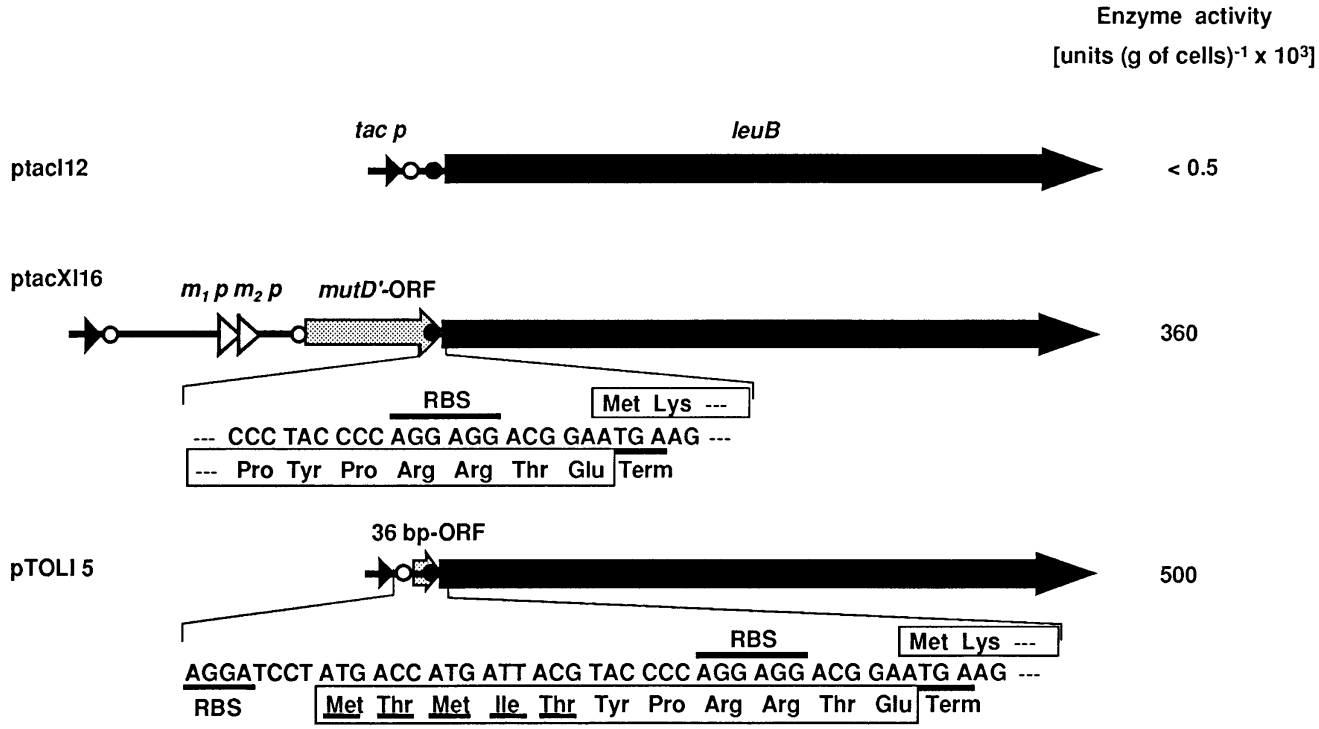

Fig. 2. The upstream structures of the thermophile *leuB* on ptacI12, ptacXI16, and pTOL15, and the activity of 3-isopropylmalate dehydrogenase produced from each plasmid. *Boxed* amino acids represent predicted amino acid sequences of leader ORFs and the *leuB* coding regions. The first five amino acids (*underlined*) coded by the 36 bp-ORF are identical to those coded by *E. coli lacZ*. The enzyme activity was measured with the cell-free extract after heating at 70°C for 30 min. *RBS* and *Term* indicate the ribosome binding site and the termination codon, respectively. *Closed arrows* represent the *leuB* coding region. Other symbols are the same as those in Fig. 1

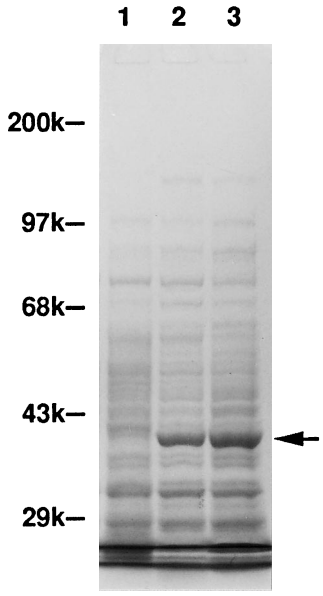

Fig. 3. Sodium dodecylsulfate-polyacrylamide gel electraphoresis (SDS-PAGE) analysis of *T. thermophilus* 3-isopropylmalate dehydrogenase in the heat-treated extracts of recombinant *E. coli* JA221 cells. Plasmids used were: *lane 1*, ptacI12; *lane 2*, patcXI16; and *lane 3*, pTOLI5. The protein band corresponding to the 3-isopropylmalate dehydrogenase is indicate by an *arrow*. The positions of standard proteins are indicated

Upstream structure of the thermophile *leuB* on ptacXI16

The *Bam*HI fragment (483 bp) on ptacXI16 was sequenced and database analysis revealed that the fragment was derived from the *mutD–rnh* region of the *E. coli* chromosome (5.2′–5.3′ in the map of *E. coli*) (Cox and Horner 1986) (Fig. 1). On ptacXI16, a new leader ORF, named *mutD′*-ORF (255 bp), was formed as a result of the read-through of a part of the *mutD* coding region on the inserted sequence (Fig. 1). Moreover, the termination codon of *mutD′*-ORF was overlapped with the initiation codon of *leuB*: the two ORFs overlapped by 4 bp, ATGA (Fig. 2).

Since two promoters, m_1 and m_2, of *mutD* were in the 0.5-kb fragment, the *leuB* gene in ptacXI16 was possibly transcribed under not only the *tac* promoter but also the promoters, m_1 and m_2. To measure the expression of *leuB* under the m_1m_2 promoters, ptacXI16 was transferred into a lactose-repressible *E. coli*, JM109, in which the transcription from the *tac* promoter with the lactose operator is repressed. The level of expression in *E. coli* JM109 decreased to about 40% of that in the nonrepressible *E. coli*, JA221, although the production was not completely repressed. When an inducer, isopropyl-β-D-thiogalactopyranoside (1 mM), of the *lac* operon was added to the medium, the production level in *E. coli* JM109 was restored to the same level as that in *E. coli* JA221. In contrast, the *leuB* gene under only the *tac* promoter on pTOLI5, on which the m_1m_2 promoters had been removed (see later), was completely repressed in *E. coli* JM109. Therefore, the *leuB* gene on ptacXI16 is transcribed under both promoters, *tac* and m_1m_2, and the expression under the m_1m_2 promoters is probably constitutive.

Optimization of the overlapping leader ORF

Although the upstream structure of the *leuB* coding region on ptacXI16 was complex (Figs. 1, 2), our previous studies (Ishida and Oshima 1994, 1996) strongly suggested that the main structure required to enhance the expression of the thermophile gene was the leader ORF, and that the expression was higher when the leader ORF was shorter and placed near the downstream gene. Moreover, to overexpress broader kinds of GC-rich genes in *E. coli*, it is desirable to use a promoter that can be regulated, such as the *tac* promoter. Thus, to test whether or not *T. thermophilus leuB* preceded by the strong *tac* promoter plus a short leader ORF is expressed as strongly as that from ptacXI16, *mutD*-ORF was shortened and its 5′ flanking region, containing the m_1m_2 promoters, was deleted by oligonucleotide-directed mutation. On the resulting plasmid, pTOLI5, the leader ORF, which overlapped with the *leuB* coding region, was shortened to 36 bp, and the size of the upstream region for the expression of *leuB* was kept within about 110 bp, containing the *tac* promoter (Fig. 2). The expression of the thermophile enzyme in *E. coli* JA221 (pTOLI5) was about 0.4-fold higher than that in *E. coli* JA221 (ptacXI16) (Figs. 2, 3). These results indicate that the essential structure for high expression of the thermophile *leuB* gene is a potent promoter such as the *tac* promoter and a short leader ORF overlapping with the initiation codon of the *leuB* gene, and suggest that the coding and 5′ regulator regions of *E. coli mutD* are unnecessary for the high-level expression.

Improvement of the production of *T. thermophilus* phosphofructokinase

A method similar to that used for the thermophile *leuB* gene was used to improve the expression of the *T. thermophilus pfk1* gene (coding for phosphofructokinase) in *E. coli*. Since the second codon of the thermophile *pfk1* gene is AAA, the same 4-bp motif, ATGA, as the *leuB* gene occurred naturally in the translational initiation site of *pfk1* when the gene was placed in the *lacZ′* coding region under the *lac* promoter on a pUC vector plasmid. The read-through of the upstream *lacZ′* was terminated at the TGA codon in the motif using an oligonucleotide-directed mutation (detailed procedure is omitted). On the resulting

Table 1. Expression of *Thermus thermophilus pfk1* in *Escherichia coli* JM109

Plasmid	IPTG[a]	Enzyme activity[b]
pTPFK	−	10
	+	90
pTPFK-L2	−	12
	+	300

[a] IPTG (isopropyl β-D-thiogalactopyranoside) was added to 1 mM.
[b] Enzyme activity was measured with the cell-free extract after heating at 70°C for 30 min and is expressed in units/g of recombinant *E. coli* cells.

plasmid, pTPFK-L2, a 39-bp leader ORF, overlapped with the *pfk1* coding region by the 4-bp overlapping motif, was constructed. The enzyme activity in the heat-treated cell-free extract of *E. coli* JM109 harboring pTPFK-L2 was about 2.3-fold higher than in the JM109 harboring the original plasmid pTPFK (Table 1). The improvement of the enzyme production was also confirmed by electrophoretic analysis (Fig. 4).

Discussion

In this study, *E. coli* JA221 harboring ptacXI16 was found by using an index of the increased growth rate on leucine-free medium. This suggests that the activity of the thermophile enzyme in *E. coli* harboring the original plasmid ptacI12 is so low that the level of activity is rate-limiting for *E. coli* growth. Such a low level of activity might correlate with not only a much lower efficiency of expression but also the environmental temperature of 37°C: the specific activity of the enzyme at the mesophile-growing temperature is only about one-tenth that at the thermophile-growing temperature (Yamada et al. 1990).

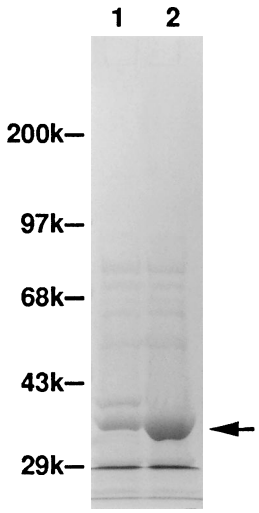

Fig. 4. SDS-PAGE analysis of *T. thermophilus* phosphofructokinase in the heat-treated extracts. Plasmids used were: *lane 1*, pTPFK; and *lane 2*, pTPFK-L2. *E. coli* JM109 cells were cultured in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside. The protein band corresponding to the phosphofructokinase is indicated by an *arrow*

On ptacXI16, a part of the coding and 5′ regulator regions of *E. coli mutD* were inserted upstream of the thermophile *leuB* (Fig. 1). Since the *E. coli mutD* gene codes for the ε subunit of the DNA polymerase III holoenzyme (Cox and Horner 1986), it can be speculated that the expression efficiency of the downstream *leuB* gene was increased by some specific effect of the *mutD* sequence or the product of the *mutD′-ORF*. Nevertheless, the high-level expression was not lost when the sequence derived from *E. coli mutD* was deleted from ptacXI16 (pTOLI5, Fig. 2), suggesting that there is no specific effect of the *mutD* sequence or its product on the high-level expression.

The present results, together with our previous studies (Ishida and Oshima 1994, 1996), show that the essential structure for overexpression of the thermophile genes in *E. coli* is a leader ORF, that would activate the translation of a downstream gene by translational coupling (Berkhout et al. 1987; Gold and Stormo 1987; Yanofsky and Crawford 1987). Although *T. thermophilus leuB* is expressed in *E. coli* whenever a leader ORF is in front of the gene, the level of the product varies greatly depending upon the structure of the leader ORF (Table 2). A large and distant leader ORF on ptacI16-1CAT is weakly effective in enhancing the enzyme production, even if the gene is transcribed under the strong *tac* promoter. On the other hand, a large leader ORF, but placed close to the *leuB* coding region with 4-bp overlapping, on pHB2 is more effective than that on ptacIl6-1CAT, despite a weak *tet* promoter. Short leader ORFs on pTCTHI and pTCTHI2 are more effective than that on pHB2. Among all the plasmids (Table 2), the highest production level is achieved by the present plasmid, pTOLI5, in which the promoter is strong, *tac*, and the size of the leader ORF is very short (36 bp) and placed close (4-bp overlapping) to the *leuB* coding region. Consequently, these results provide a practical method for overproduction from GC-rich genes in *E. coli*: (1) use of a strong promoter; (2) introduction of the translation initiation region of a gene

Table 2. Expression of *T. thermophilus leuB* in *E. coli*

Plasmid	Promoter	Leader ORF			Enzyme activity[b]	Reference
		Origin and structure	Size (bp)[a]	Position from *leuB*		
ptacI12 (control)	*tac*	None			<0.5	Ishida and Oshima 1996
patcI16-1CAT	*tac*	*cat*[c]	660	0.6-kb distant	12	Ishida and Oshima 1996
pHB2	*tet*	Read-through of Tet[r,d]	825	Overlapping by 4 bp	45	Ishida and Oshima 1994
pTCTHI	*tet*	Truncated Tet[r,d]	27	347-bp distant	88	Ishida and Oshima 1994
pTCTHI2	*tet*	Truncated Tet[r,d]	27	83-bp distant	163	Ishida and Oshima 1994
patcXI16	*tac*, m_1m_2	Read-throught of *mutD′*	258	Overlapping by 4 bp	360	This study
pTOLI5	*tac*	Read-through of *lacZ′*	36	Overlapping by 4 bp	500	This study

ORF, open reading frame.
[a] Termination codon is included in the size estimation.
[b] Enzyme activity is expressed in (units/g of recombinant *E. coli* JA221 cells) $\times 10^3$.
[c] The *cat* gene codes for chloramphenicol acetyltransferase.
[d] Tet[r] represents tetracycline resistance gene product.

such as *lacZ* that can be efficiently translated, in order to construct a very short leader ORF; (3) placing the leader ORF immediately after the promoter; and (4) formation of a short overlap, such as the ATGA motif, with the gene to be overexpressed.

The overlapping motif, ATGA, was also found in *E. coli* genes, *ompR-envZ* (Comeau et al. 1985; Lijestroem et al. 1988) and *motA-motB* (Dean et al. 1984; Stader et al. 1986). In cases of introduction of a short upstream ORF for overexpressing mammalian genes in *E. coli*, it was proposed that the termination codon of an upstream ORF be positioned between the ribosome binding site and the initiation codon of the gene to be overexpressed (Schoner et al. 1986, 1991). In *T. thermophilus leuB*, it is difficult to test whether or not the introduction of a leader ORF terminating at such a position was more effective for the overexpression, because a new termination codon could not be introduced in the position without destroying the inhibitory secondary structure (Ishida and Oshima 1994). However, in a mutation study on *Salmonella typhimurium ompR-envZ* with a 4-bp overlap (Lijestroem et al. 1988), an upstream shift of only 5 bp in *ompR* termination resulted in a significant decrease of the expression of *envZ*. Thus, it is presumed that an overlapping leader ORF, like that on pTOLI5, improves the gene expression more effectively than a nonoverlapping one.

Using the present method, we improved the production of phosphofructokinase from *T. thermophilus* in *E. coli* (Table 1 and Fig. 4). No such stable secondary structure as that in the thermophile *leuB* gene (Ishida and Oshima 1994) was found around the translational initiation site of the thermophile *pfk1* gene. However, the production of the thermophile phosphofructokinase in *E. coli* was increased by the introduction of an overlapping leader ORF (39 bp). This suggests that the introduction of an overlapping leader ORF can improve the expression efficiency in *E. coli* of a GC-rich gene, even if no stable secondary structure is found in the translational initiation site of the gene. It is speculated that the efficiency of the translational initiation in *E. coli* of such a GC-rich gene without a secondary structure is lower than that of a non-GC-rich gene. Thus, the method can potentially be used for the overproduction in *E. coli* from any GC-rich gene of not only the extreme thermophile but also other important organisms in biotechnology, such as *Pseudomonas* species.

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References

Almond J, Beecroft L, Blair E, Meacock P, Windass J (1985) Transformation of competent *E. coli* cells by plasmid DNA. In: Pritchard RH, Holland IB (eds) *Basic cloning techniques: A manual of experimental procedures*. Blackwell Scientific, London

Berkhout B, Schmidt BF, van Strien A, van Boom J, van Westrenen J, van Duin J (1987) Lysis gene of bacteriophage MS2 is activated by translation termination at the overlapping coat gene. J Mol Biol 195:517–524

Comeau DE, Ikenaka K, Tsung K, Inouye M (1985) Primary characterization of the protein products of the *Escherichia coli ompB* locus: Structure and regulation of synthesis of the OmpR and EnvZ proteins. J Bacteriol 164:578–584

Cox EC, Horner DL (1986) DNA sequence and coding properties of *mutD* (*dnaQ*) a dominant *Escherichia coli* mutator gene. J Mol Biol 190:113–117

Dean GE, Macnab RM, Stader J, Matsumura P, Burks C (1984) Gene sequence and predicted amino acid sequence of the *motA* protein, a membrane-associated protein required for flagellar rotation in *Escherichia coli*. J Bacteriol 159:991–999

Gold L, Stormo G (1987) Translational initiation. In: Neidhardt FC, Ingraham JL, Low KB, Magasanik B, Umbarger HE (eds) Escher*ichia coli and Salmonella typhimurium cellular and molecular biology*. American Society for Microbiology, Washington, DC

Ishida M, Oshima T (1994) Overexpression of genes of an extreme thermophile, *Thermus thermophilus*, in *Escherichia coli* cells. J Bacteriol 176:2767–2770

Ishida M, Oshima T (1996) A leader open reading frame is essential for the expression in *Escherichia coli* of GC-rich *leuB* gene of an extreme thermophile, *Thermus thermophilus*. FEMS Microbiol Lett 135:137–142

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lijestroem P, Laamanen I, Palva ET (1988) Structure and expression of the *ompB* operon, the regulatory locus for the outer membrane porin regulon in *Salmonella typhimurium*. J Mol Biol 201:663–673

Owen MJ (1984) DNA sequence determination using dideoxy analogs. In: Walker JM (ed) Methods in molecular biology. Humana, Clifton, NJ

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239: 487–491

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: A laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Schoner BE, Belagaje RM, Schoner RG (1986) Translation of a synthetic two-cistron mRNA in *Escherichia coli*. Proc Natl Acad Sci USA 83:8506–8510

Schoner BE, Belagaje RM, Schoner RG (1991) Enhanced translational efficiency with two-cistron expression system. Methods Enzymol 185:94–103

Stader J, Matsumura P, Vacante D, Dean GE, Macnab RM (1986) Nucleotide sequence of the *Escherichia coli motB* gene and site-limited incorporation of its product into the cytoplasmic membrane. J Bacteriol 166:244–252

Xu J, Oshima T, Yoshida M (1990) Tetramer–dimer conversion of phosphofructokinase from *Thermus thermophilus* induced by its allosteric effectors. J Mol Biol 215:597–606

Xu J, Seki M, Denda K, Yoshida M (1991) Molecular cloning of phosphofructokinase 1 gene from a thermophilic bacterium, *Thermus thermophilus*. Biochem Biophys Res Commun 176:1313–1318

Yamada T, Akutsu N, Miyazaki K, Kakinuma K, Yoshida M, Oshima T (1990) Purification, catalytic properties, and thermal stability of *threo*-Ds-3-isopropylmalate dehydrogenase coded by *leuB* gene from an extreme thermophile, *Thermus thermophilus* strain HB8. J Biochem 108:449–456

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103–119

Yanofsky C, Crawford IP (1987) The tryptophan operon. In: Neidhardt FC, Ingraham JL, Low KB, Magasanik B, Umbarger HE (eds) Escherichia coli and Salmonella typhimurium cellular and molecular biology. American Society for Microbiology, Washington, DC