Journal of Microbiology (2013) Vol. 51, No. 6, pp. 783–790
DOI 10.1007/s12275-013-2619-6

Influence of *Acetobacter pasteurianus* SKU1108 *aspS* Gene Expression on *Escherichia coli* Morphology

Kannipa Tasanapak[1#], Uraiwan Masud-Tippayasak[1,2], Kazunobu Matsushita[3], Wichien Yongmanitchai[1], and Gunjana Theeragool[1*]

[1]*Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand*
[2]*Center for Advanced Studies in Tropical Natural Resources, NRU-KU, Kasetsart University, Bangkok 10900, Thailand*
[3]*Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-8515, Japan*
[#]*Present address: Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, Phitsanulok 65000, Thailand*

(Received November 13, 2012 / Accepted June 3, 2013)

The *aspS* gene encoding Aspartyl-tRNA synthetase (AspRS) from a thermotolerant acetic acid bacterium, *Acetobacter pasteurianus* SKU1108, has been cloned and characterized. The open reading frame (ORF) of the *aspS* gene consists of 1,788 bp, encoding 595 amino acid residues. The highly conserved Gly-Val-Asp-Arg ATP binding motif (motif 3) is located at the position 537-540 in the C-terminus. Deletion analysis of the *aspS* gene upstream region suggested that the promoter is around 173 bp upstream from the ATG initiation codon. Interestingly, transformation with the plasmids pGEM-T138, pUC138, and pCM138 synthesizing 138 amino acid C-terminal fragments of AspRS, that carry the ATP binding domain, caused *E. coli* cell lengthening at 37 and 42°C. Moreover, *E. coli* harboring pUC595 (synthesizing all 595 amino acids) and a disordered *aspS* gene in pGEM-T138 had normal rod shapes. The normal rod shape was observed in *E. coli* harboring pD539V following site-directed mutagenesis of the ATP binding domain. We propose that overproduction of truncated C-terminal peptides of AspRS may cause sequestration of intracellular ATP in *E. coli*, leaving less ATP for cell division or shaping cell morphology.

Keywords: *Acetobacter pasteurianus*, aspartyl-tRNA synthetase, *aspS* gene, ATP-binding domain, filamentous cell

Introduction

Aminoacyl-tRNA synthetase plays an essential role in the accurate interaction of an amino acid with its cognate tRNA, which is a crucial step in protein synthesis. Aminoacylation consists of two sequential reactions: (i) formation of aminoacyl-adenylate by activation of an amino acid with ATP; (ii) transfer of the activated amino acid to tRNA followed by the release of the aminoacyl-tRNA (Ibba and Söll, 2000). For aspartyl-tRNA synthetase (AspRS), the co-substrate ATP preferentially binds three Mg^{2+} ions, which raises the binding free energy of Asp compared to Asn and this improves amino acid discrimination (Thompson and Simonson, 2006). The *aspS* gene encoding AspRS from *Escherichia coli* has been cloned and characterized (Eriani *et al*., 1990). It consists of 590 residues and showed homology with LysRS and AsnRS, each of which contains the C-terminal tetrapeptide sequence (Gly-Leu-Asp-Arg), a potential ATP-binding site. A single point mutation within motif 3, Leu535Pro of AspRS, leads to conformational disorganization and inactivation of the catalytic site (Eriani *et al*., 1990).

Sharples and Lloyd (1991), described a *tls-1* mutant of *E. coli* K12 exhibiting a temperature-sensitive growth phenotype in low salt media and showed that the DNA fragment encoding the C-terminal of AspRS was sufficient to allow its growth. Another mutation of *aspS* at Pro555Ser in motif 3 displays reduced stability of both the acylation and amino acid activation sites (Martin *et al*., 1997). For yeast AspRS, it was proposed that a mutation in the C-terminal sequence reduced ATP binding and thus aspartic acid binding to AspRS fails (Cavearelli *et al*., 1994; Ador *et al*., 1999). ATP is essential in aminoacylation and also important for FtsH, an ATP-dependent protease in *Caulobacter crescentus*. Mutants lacking FtsH protease can grow under normal conditions but are highly sensitive to elevated temperature and increased salt concentration. Over-expression of the normal *ftsH* gene resulted in an increased salt tolerance but it still showed thermo-sensitivity (Fischer *et al*., 2002).

Acetobacter pasteurianus SKU1108, isolated from grapes in Thailand, is a promising thermotolerant acetic acid bacterium for industrial vinegar fermentation at high temperature (Saeki *et al*., 1997). We cloned various constructs of the *aspS* gene from *A. pasteurianus* SKU1108, which included motif 3, and transferred these into *E. coli* and *A. pasteurianus* SKU1108, both of which then show reduced temperature tolerance. We propose that the temperature sensitive strain over produces a dysfunctional AspRS protein that still maintains ATP binding, thus reducing cytosolic ATP to a level insufficient for supporting cell division at elevated temperatures.

Materials and Methods

Bacterial strains, culture media, and culture conditions

The bacterial strains and plasmids used in this study are described in Table 1. *A. pasteurianus* SKU1108 was main-

*For correspondence. E-mail: fscignt@ku.ac.th; Tel.: +66-29428445-9; Fax: +66-29428512

tained on potato medium (5 g glucose, 20 g glycerol, 10 g yeast extract, 10 g polypeptone, and 15 ml potato extract, made up to 1,000 ml with tap water) (Matsutani *et al*., 2011). Cultivation was performed at 30°C on a rotary shaker at 200 rpm. *E. coli* DH5α harboring recombinant plasmids was cultured in Luria-Bertani medium (10 g polypeptone, 5 g yeast extract, 5 g NaCl, made up to 1,000 ml) containing appropriate antibiotic and incubated at 37°C with vigorous shaking for 18–24 h.

Cloning and nucleotide sequencing of the *aspS* gene

Molecular cloning of the *aspS* gene was performed by Random Amplified Polymorphic DNA (RAPD) and shotgun cloning into pGEM®-T Easy and pUC18 vectors, respectively. The random 10-mer oligonucleotide primer, AD01 (CAAA GGGCGG) was used as a random primer to amplify DNA fragments from several strains of acetic acid bacteria; including *A. pasteurianus* SKU1108. The recombinant plasmid pGEM-T138 containing a 1.3 kb RAPD-PCR product was digested with *Eco*RI and a 1.3 kb DNA fragment encoding the C-terminal sequence of AspRS was purified from it. This was used as a DNA probe for shotgun cloning. The aspartyl-tRNA synthetase gene was isolated from a plasmid pool containing completely digested *Hin*dIII DNA fragments of *A. pasteurianus* SKU1108 chromosomal DNA inserted in the appropriate *Hin*dIII site of the pUC18 vector. These plasmids were transformed into *E. coli* DH5α competent cells. Among these transformants, a colony showing a positive hybridization signal was confirmed by PCR and its nucleotide sequence was determined. The plasmid carried an entire copy of the *aspS* gene and was designated as pUC595.

Deletion analysis of the *aspS* gene for identification of a possible promoter

To analyze the *aspS* gene for a possible promoter, five constructs of the *aspS* gene were obtained by PCR amplification using specific oligonucleotide primers. These five DNA fragments had different lengths, beginning in the upstream region of the *aspS* gene and were obtained by PCR amplification using 5 forward primers and 1 reverse primer that incorporated *Pst*I and *Bam*HI restriction sites, respectively. The resultant PCR products were digested with *Pst*I and *Bam*HI and separately introduced into the appropriate sites of the promoter probe vector, pGSA, with the promoterless *lacZ* gene (Masud *et al*., 2011). This vector was used for the construction of the transcriptional *aspS-lacZ* gene fusion. The resulting plasmids are described in Table 1. Each of the *aspS-lacZ* fusion plasmids was separately transferred to *E. coli* DH5α by heat shock and selected on LB agar containing 10 μg/ml gentamicin and 20 μg/ml X-gal. *E. coli* DH5α cells harboring the transcriptional *aspS-lacZ* plasmids, inclusive of the promoter probe vector pGSA, were cultured in 100 ml of LB medium in a 500-ml Erlenmeyer flask and cultivated at 37°C on a rotary shaker at 200 rpm for 24 h. A 5 ml aliquot of each culture was separately harvested by centrifugation at 12,000 rpm for 1 min and the supernatant was discarded. The precipitates were collected and stored at -20°C to assay β-galactosidase activity according to the standard method (Miller, 1972).

Construction of pGEM-T138 derivatives

The cell morphology of *E. coli* DH5α harboring pGEM-T138 changed from short rod to long filamentous chains when

Table 1. Bacterial strains and plasmids used in this study

Bactrial strain and Plasmid	Relevant characteristics	Reference
A. pasteurianus SKU1108	Source of *aspS* gene	Saeki *et al.* (1997)
E. coli DH5α	*supE*44 Δ*lacU*169 (ϕ80*lacZ*Δ*M*15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Toyobo
pGEM-T® Easy	Ap^r, *lacZ*	Promega
pGEM-T138[a]	Ap^r, 1.3 kb DNA fragment carrying truncated C-terminal AspRS	This study
spGEM-T138[a]	Ap^r, 0.7 kb *Eco*RI fragment DNA fragment carrying truncated C-terminal AspRS	This study
pGEM-T138R[a]	Ap^r, constructed for blocking C-terminal AspRS synthesis by inserted at opposite direction from promoter	This study
pGEM-T138FP[a]	Ap^r, constructed for blocking C-terminal AspRS synthesis by created frame-shift mutation	This study
pGEM-T138C::Km^r	Ap^r, insertion of Km^r cassette at amino acid 535 in truncated *aspS* gene on pGEM-T138	This study
pD539V[a]	Ap^r, 0.8 kb DNA fragment carrying truncated C-terminal AspRS; 539Asp of AspS is changed to Val	This study
pUC18	Ap^r, *lacZ*', *lacI*	Biolab
pUC138[a]	Ap^r, 0.8 kb DNA fragment carrying truncated C-terminal AspRS	This study
pCM62	*Acetobacter-E. coli* shuttle vector, Tc^r, *lacZ*	Marx and Lidstrom (2001)
pCM138[a]	Tc^r, 0.8 kb DNA fragment carrying truncated C-terminal AspRS	This study
pUC595[b]	Ap^r, 2.7 kb DNA fragment carrying entire AspRS	This study
pUC595N::Km^r	Ap^r, insertion of Km^r cassette at amino acid 44 in *aspS* gene on pUC595	This study
pUC595C::Km^r	Ap^r, insertion of Km^r cassette at amino acid 508 in *aspS* gene on pUC595	This study
pGSA	Gm^r, promoter probe vector carrying the promoterless *lacZ* gene	Masud *et al.* (2011)
pGSAF1	Gm^r, carrying a 563 bp promoter region of *aspS* gene[c]	This study
pGSAF2	Gm^r, carrying a 283 bp promoter region of *aspS* gene[c]	This study
pGSAF3	Gm^r, carrying a 173 bp promoter region of *aspS* gene[c]	This study

[a] The indicates DNA fragment carrying 138 amino acid truncated C-terminal AspRS
[b] The indicates DNA fragment carrying entire 595 amino acid AspRS
[c] The indicates length excluded some part of structural gene, *aspS* (22 bp)

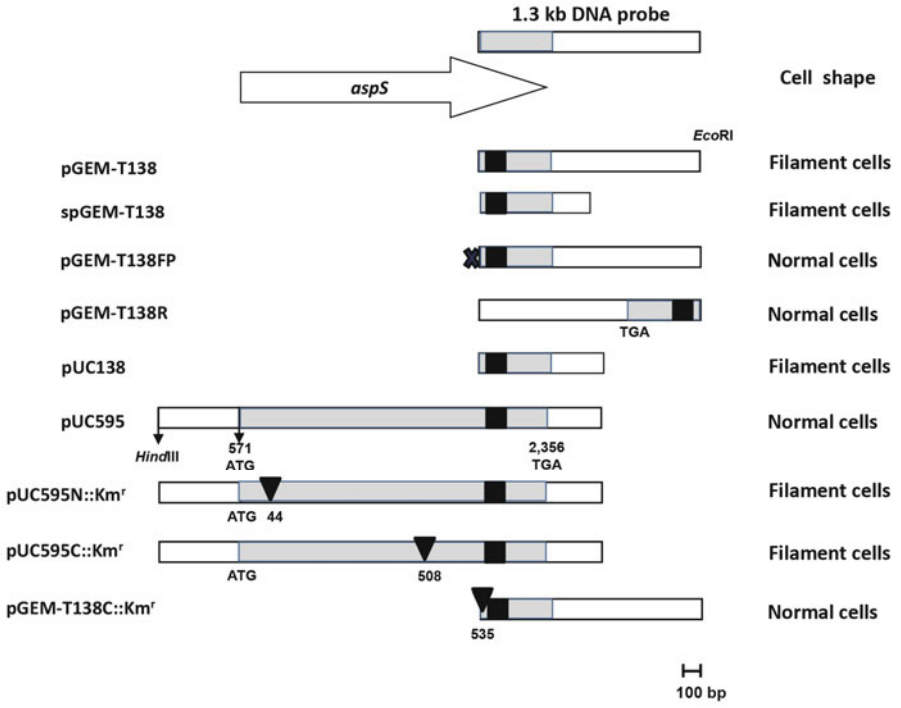

Fig. 1. Schematic representation of plasmids pGEM-T138, spGEM-T138, pUC138 (harboring a truncated aspartyl-tRNA synthetase comprised of 138 C-terminal residues), and pUC595 (harboring a complete *aspS* gene encoding 595 residues of the same protein) carrying a 2.7 kb *Hin*dIII DNA fragment. The ATP binding motif (motif 3) and Km^r cassette are indicated by black boxes and triangles, respectively. Plasmids pGEM-T138 and spGEM-T138 allow the synthesis of 138 C-terminal amino acid residues of AspRS, whereas pGEM-T138FP and pGEM-T138R were constructed to prevent AspRS synthesis.

grown at 37 and 42°C. Such morphological changes might be caused by N-terminal β-galactosidase fusing with the 138 amino acid C-terminal residues of aspartyl-tRNA synthetase. To test this, three pGEM-T138 derivatives were constructed (also shown in Fig. 1) by (i) removing the non-coding AspRS segment, (ii) a frame shift mutation and (iii) sequence reversal. In detail, plasmid spGEM-T138 was constructed by removing the non-coding AspRS segment in order to allow the synthesis of the 138 residues of C-terminal fragments of AspRS. Plasmids pGEM-T138FP and pGEM-T138R were constructed for blocking AspRS synthesis. To construct pGEM-T138FP, pGEM-T138 was digested with *Pst*I to linearize the DNA, followed by end-blunting and self-ligation. This end-blunting limited the synthesis to 39 amino acids of the β-galactosidase N-terminal. Plasmid pGEM-T138R was constructed by digesting pGEM-T138 with *Eco*RI to linearize followed by re-ligation. The sequence of the insertion fragment in pGEM-T138R was the reverse of pGEM-T138.

The DNA fragment encoding 138 amino acid residues of the C-terminal fragment of AspRS from pGEM-T138 was subcloned into pUC18, designated pUC138 and was used to transfer into *E. coli* DH5α competent cells. *E. coli* DH5α harboring pGEM-T138 and its derivatives were grown on LB medium containing ampicillin at 30, 37, and 42°C for 24 h. The overnight cultures were smeared onto glass slides, Gram stained, and the cell morphology was observed by light microscopy (1,000x).

Construction of *aspS* disruptant

The plasmid pUC595 and pGEM-T138 (containing the entire 595 or the truncated 138 residues of AspRS, respectively) were separately digested with the selected restriction enzymes to create the appropriate site for Kanamycin resistance (Km^r) cassette insertion, this insertion causes a frame-shift in the DNA sequence (Fig. 1). The resultant plasmids carried an insert of the 900 bp *Eco*RV Km^r cassette from *Eco*RV digested pTKm (Yoshida *et al*., 2003) at either the region encoding the N-terminus or the C-terminus, and were designated pUC595N::Km^r and pUC595C::Km^r, respectively. In addition, the plasmid pGEM-T138 was digested with *Sma*I, then the 900 bp *Eco*RV Km^r cassette was inserted at the *Sma*I site located in the coding sequence of the C-terminal *aspS* gene (adjacent to motif 3), designated pGEM-T138C::Km^r (Fig. 1). Finally, three plasmids containing the *aspS* disruptants, *aspS* -595N, *aspS* -595C or *aspS* -138C, were separately transferred into *E. coli* DH5α by heat shock and these are designated pUC595N::Km^r, pUC595C::Km^r, and pGEM-T138C::Km^r. *E. coli* DH5α cells harboring those plasmids were grown at 30, 37, and 42°C for 24 h. The overnight cultures were observed microscopically as Gram-stained smears.

Site-directed mutagenesis of *aspS* gene

To determine the role of the ATP-binding motif in the truncated C-terminal AspS, site-directed mutagenesis based on the PCR fusion method was performed. F-SDaspS and R-SDaspS were designed to cover the sequence between 1,388 nucleotides (nt) downstream of the ATG codon to 410 nt downstream of the TGA stop codon of the *aspS* gene. These primers generate an 811-bp PCR product coding for 132 amino acid residues of truncated C-terminal AspRS. A mutagenic primer set was designed in order to generate a one amino acid change in a conserved ATP-binding motif. A 237-bp DNA fragment containing the upstream region of the *aspS* gene was amplified by F-SDaspS and R-D539V using pGEM-T138 as a template. In addition, a 592-bp downstream fragment was amplified by F-D539V and R-SDaspS. These

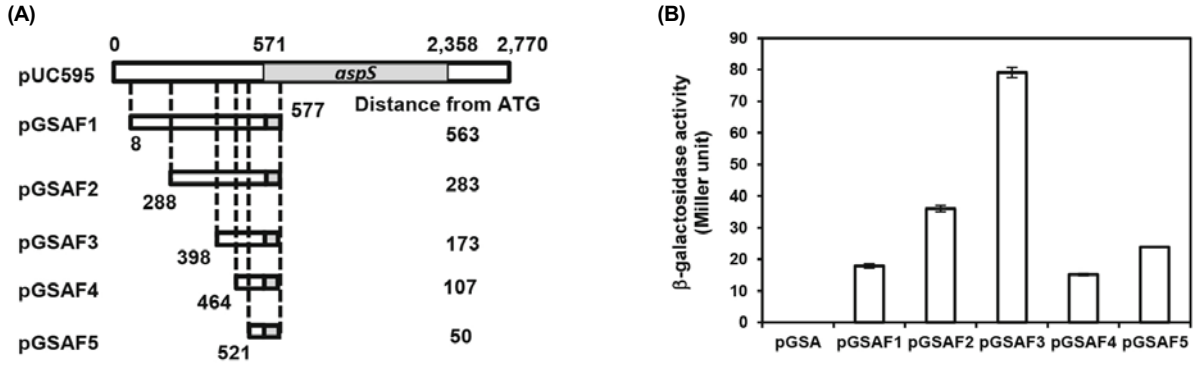

Fig. 2. (A) Structure of DNA fragments that are carrying sequences around the tentative *aspS* promoter and (B) comparison of β-galactosidase activity in *E. coli* DH5α harboring the various transcriptional *aspS-lacZ* fusion plasmids shown.

two PCR products were purified using a MagExtractor purification kit (Toyobo, Japan). The second PCR amplification was performed to fuse the two DNA fragments using F-SDaspS and R-SDaspS as PCR primers. Fifty nanogram of each 237-bp and 592-bp DNA fragment carrying the *aspS* gene were used as DNA templates. An 811-bp PCR product was obtained and cloned into pGEM-T® Easy Vector. A recombinant plasmid with the insert in the same direction as the *lac* promoter was selected. Alteration of the sequence at the ATP-binding motif was confirmed by nucleotide sequencing.

Truncated *aspS* gene and cell morphology at high temperature

A 1.3 kb *Eco*RI DNA fragment carrying a partial *aspS* gene encoding truncated C-terminal fragments of AspRS was constructed from the *Eco*RI-digested pGEM-T138 and then inserted into the broad host range vector pCM62 (Marx and Lidstrom, 2001) at the *Eco*RI site. The resultant plasmid was designated pCM138 which was transferred into *E. coli* DH5α cells while the pCM62 vector was transferred to control cells. These *E. coli* DH5α cells were grown at 30, 37, 39, and 42°C for 24 h and then the morphology was examined microscopically in Gram-stained smears. The plasmids pCM-138 or pCM62 were also transferred via conjugation into *A. pasteurianus* SKU1108 and grown at 30, 37, 39, and 42°C for 24 h and again cell morphology was assessed.

The complete sequence of the *aspS* gene from *A. pasteurianus* SKU1108 has been submitted to the GenBank databases under accession no. JQ837915.

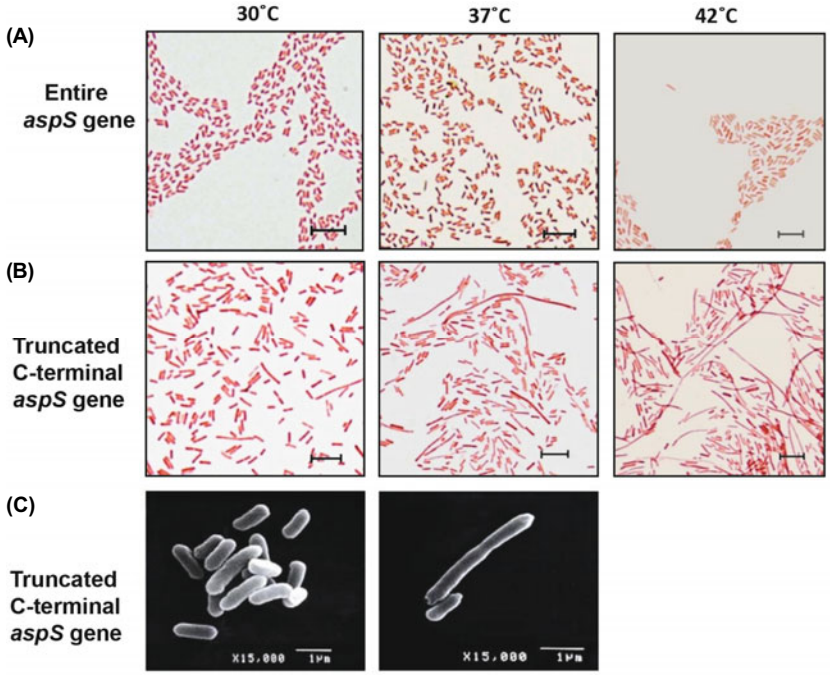

Fig. 3. Microscopic appearance of *E. coli* DH5α harboring pUC595 (A), pUC138 or pGEM-T138 (B), and scanning electron microscopic appearance of the cells from B (C) grown on LB medium containing 50 μg/ml ampicillin at 30, 37, and 42°C. The bacterial cells were observed under light and scanning electron microscopes with magnifying power of 1,000x (scale bars = 10 μm) and 15,000x (scale bars = 1 μm).

Results

Cloning, nucleotide sequencing, and putative promoter region of *aspS*

The chromosomal DNA from *A. pasteurianus* SKU1108 was used as the template for RAPD amplification using the random 10-mer AD01 as described in 'Materials and Methods'. Three typical RAPD-PCR amplified DNA fragments (0.6, 1.0, and 1.3 kb) could be clearly observed in 13 thermotolerant strains; including *A. pasteurianus* SKU1108 (unpublished data). Each of them was cloned into pGEM®-T Easy vector. Of these, pGEM-T138, containing the 1.3 kb RAPD product, caused a morphological change in *E. coli* DH5α at high temperature. The 1.3 kb *Eco*RI DNA fragment was used as a DNA probe for Southern hybridization of the chromosomal DNA from *A. pasteurianus* SKU1108, and then for colony hybridization for shotgun cloning, and the resultant plasmids are shown in Fig. 1. The *Hin*dIII DNA fragment in pUC595 consisted of 2,770 nucleotides. The open reading frame (ORF) show high sequence similarity to the *aspS* gene, this ORF consisted of 1,788 nucleotides, started with an ATG at nucleotide 571–573 and terminated with TGA at 2,356–2,358. The highly conserved tetrapeptide Gly-Val-Asp-Arg motif (motif 3) was located at amino acids 537-540 of the AspRS C-terminus.

The upstream region(s) of the *aspS* gene was analyzed by deletion analysis as described in 'Materials and Methods'. The resulting plasmids labeled pGSAF1, pGSAF2, pGSAF3, pGSAF4, and pGSAF5 carried 50, 107, 173, 283, and 563 bp of the upstream region of the *aspS* gene, respectively (Table 1). The structures of the resultant DNA fragments are shown in Fig. 2A. Among the cultured transformants, the one harboring plasmid pGSAF3 (173 bp upstream from ATG) showed the highest β-galactosidase activity (Fig. 2B).

Effect of truncated *aspS* gene from *A. pasteurianus* SKU1108 on cell morphology

Figure 3 shows *E. coli* DH5α cells grown at 30, 37, and 42°C, while harboring either pUC595 (synthesizing complete 595 residues), pUC138 or pGEM-T138 (synthesizing 138 residues of the C-terminus of AspRS). The pUC595 cells showed normal rod shapes at all temperatures (Fig. 3A). Those cells harboring pUC138 or pGEM-T138 were also normal rods at 30°C; however, cells incubated at 37 and 42°C displayed abnormal, short and long filamentous shapes (Fig. 3B). Figure 3C shows normal cells incubated at 30°C and abnormal filamentous pGEM-T138 cells from 37°C as viewed by scanning electron microscopy. Plasmid pGEM-T138 contained a C-terminal fragment of AspRS with the same orientation as the SP6 promoter and in-frame fusion with N-terminal β-galactosidase. Several derivatives of pGEM-T138 were constructed (Fig. 1). The plasmid spGEM-T138 was constructed by deletion of 0.6 kb of the region downstream from the stop codon of the *aspS* gene. This plasmid caused the cells to become long and filamentous at 37 and 42°C because the deletion had no effect on the fusion peptide. Two plasmids were constructed to create a frame-shift mutation (pGEM-T138FP) and reversed sequence from the promoter (pGEM-T138R). These two plasmids prevented the synthesis of the β-gal C-terminal fragments of AspRS fusion peptides. *E. coli* DH5α cells harboring either of these two plasmids grew normally at 37 or 42°C.

Disruption of the *aspS* gene and cell morphology

The recombinant plasmids pUC595N::Km^r, pUC595C::Km^r, pGEM-T138C::Km^r, which coded the AspRS protein with a

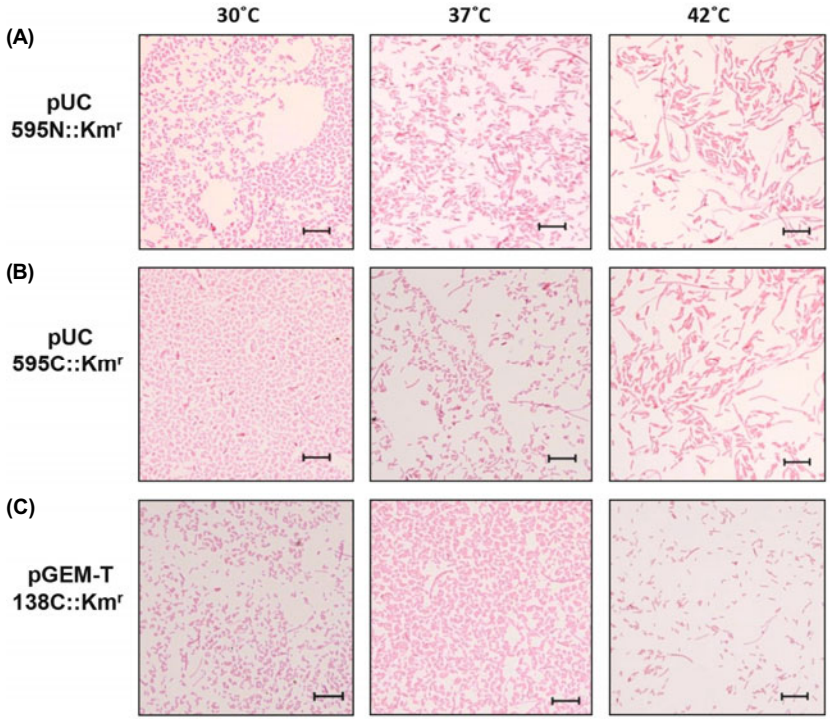

Fig. 4. Microscopic appearance of *E. coli* DH5α harboring recombinant plasmids carrying a disrupted *aspS* gene. Plasmids pUC595N::Km^r (A), pUC595C::Km^r (B), and pGEM-T138C::Km^r (C) carrying Km^r cassettes inserted at amino acid 44, 508, and 535, respectively. Then cultures were grown on LB medium at 30, 37, and 42°C for 24 h. Magnification 1,000x (scale bars = 10 μm).

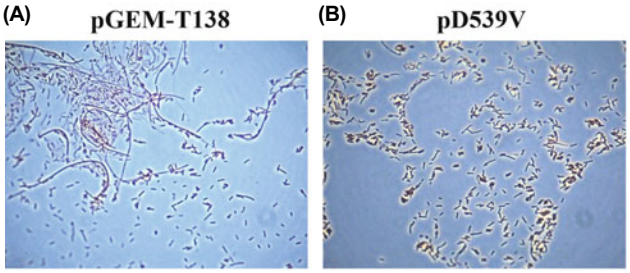

Fig. 5. Microscopic appearance of *E. coli* DH5α harboring pGEM-T138 (A) and pD539V (B) grown on LB medium containing 50 μg/ml ampicillin at 37°C. The bacterial cells were observed under light microscope, Axio Imager, A1, ZEISS, with magnifying power of 1,000x.

Km^r insertion at positions 44, 508, and 535 downstream from the first amino acid (methionine), were introduced into *E. coli* DH5α. The morphology of *E. coli* DH5α harboring pUC595N::Km^r was changed from a short rod to a few long filaments when grown in LB medium at 37 and 42°C (Fig. 4A). Nevertheless, cells harboring pUC595C::Km^r in which the AspRS protein was disrupted at the C-terminus, but carrying the normal motif 3, exhibited the same morphological change (Fig. 4B) as those cells harboring pUC595N::Km^r, but there was no change in *E. coli* harboring pUC595. Moreover, Km^r insertion into pGEM-T138C::Km^r at amino acid position 535 adjacent to the motif 3 ATP binding domain produced short rod morphology (Fig. 4C).

Effect of altered amino acid residue within the ATP-binding motif

Site-directed mutagenesis based on the PCR fusion method was used to determine an essential role of the ATP-binding motif in the truncated C-terminal AspS protein. A plasmid encoding a mutated ATP-binding motif, was prepared and designated as pD539V. In pD539V, a single amino acid alteration was present in the conserved motif 3 (Gly-Val-Asp-Arg), where 539Asp was changed to Val. *E. coli* DH5α harboring the mutated plasmid was grown on LB agar at 37°C for 18 h, and the cell morphology was compared with cells containing the original pGEM-T138 plasmid (Fig. 5). The cells harboring pGEM-T138 displayed abnormal short and long filamentous shapes at 37°C (Fig. 5A). In contrast, *E. coli* DH5α harboring the pD539V plasmid with a mutated ATP-binding site had normal shape at 37°C (Fig. 5B).

Effects of overexpression of the truncated *aspS* gene on cell morphology

The 1.3 kb DNA fragment carrying the partial *aspS* gene encoding 138 amino acids of the C-terminus of AspRS was inserted into a broad host range vector, pCM62. *A. pasteurianus* SKU1108 harboring each of pCM138 and pCM62 are illustrated in Fig. 5. Normally, *A. pasteurianus* SKU1108 grows as short rods at 30, 37, and 39°C but with different morphology at 42°C, and the two conjugants, pCM138 and pCM62, exhibited the same cell shape as the parental strain. Minor abnormalities were only observed when *A. pasteurianus* SKU1108 harboring the truncated C-terminal fragments of AspRS was grown at 39°C (Fig. 6A). Thus, the truncated C-terminal fragments of AspRS cause less severe cell structural changes in *A. pasteurianus* SKU1108 at this temperature than they do in *E. coli*. The truncated C-terminal fragment of AspRS generated from pCM138 inhibited division of *E. coli* DH5α cells at high temperature, an effect that was more severe than that seen in *A. pasteurianus* SKU1108 (Fig. 6).

Discussion

In this study, we cloned recombinant plasmids that contain different length constructs of the *aspS* gene encoding Aspartyl-tRNA synthetase (AspRS) from *A. pasteurianus* SKU1108, a thermotolerant, acetic acid bacterium. All the shortened plasmids, carrying the truncated C-terminal sequence containing the functional ATP binding site at motif 3, were unable to support cell division. The full length gene can support cell division, but not when a Km^r cassette was inserted at amino acid position 44 and 508. A Km^r cassette attached directly to motif 3 restored normal cell morphology, presumably by interfering with ATP binding so the amount of cytosolic ATP was adequate to support normal cell morphology.

The amino acid sequence of AspRS from *A. pasteurianus* SKU1108 showed 99% sequence similarity with *A. pasteurianus* NBRC 3283. These two strains have led to a physiological understanding of the relationships between thermotolerance and phylogeny via genome-wide phylogenetic analysis (Matsutani *et al*., 2012).

Deletion analysis of upstream regions of the *aspS* gene suggested that the *aspS* gene promoter might be located at around 173 bp upstream from the start codon. However, the enzyme activity is reduced with incorporation of additional upstream sequences, which might be due to the additional sequences containing a repressor binding site.

Excess of intact *aspS* in *E. coli* DH5α harboring pUC595, overexpressing the complete gene sequence, preserved nor-

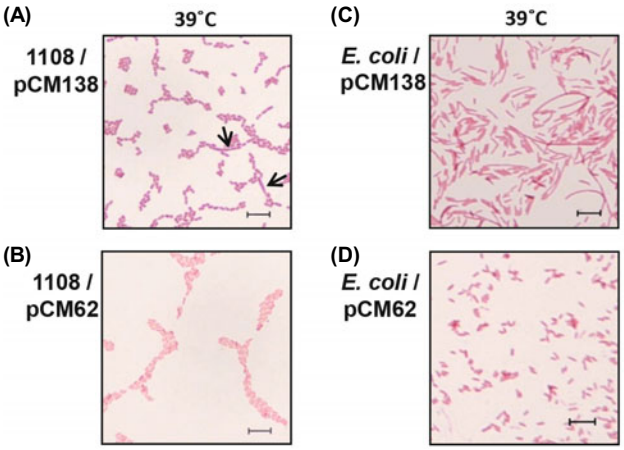

Fig. 6. Microscopic appearance of *A. pasteurianus* SKU1108 harboring pCM138 (A), pCM62 (B) that were grown on potato medium and *E. coli* DH5α harboring pCM138 (C), pCM 62 (D) that were grown on LB medium at 39°C for 24 h. Magnification 1,000x (scale bars = 10 μm). The black arrows indicate the minor abnormal cells of *A. pasteurianus* SKU1108 harboring pCM138 when grown at higher temperature (39°C).

mal cell morphology which may reflect the presence of a feedback regulation of gene expression, perhaps thereby preventing excessive binding of ATP. Such feedback regulation has been observed in yeast by Frugier *et al.* (2005), who cloned the *aspS* gene into a yeast strain that was null for AspRS.

Insertion of the Km^r cassette into pUC595N::Km^r and pUC595C::Km^r created abnormal *E. coli* cells implying excessive ATP binding at motif 3. This ATP binding domain plays an important role in amino acid activation in aminoacylation during protein synthesis (Martin *et al.*, 1997; Ibba and Söll, 2000; Metlitskaya *et al.*, 2006). Sequence analysis of yeast *aspS* mutants shows that charged residues at the C-terminus are necessary for ATP binding and stabilizing the reaction transition states (Cavarelli *et al.*, 1994). Eriani *et al.* (1990) showed that all residues in motif 3 (Gly-Leu-Asp-Arg) are required for ATP binding. Thus, a single amino acid change causes a dramatic loss of activity and reduced ATP affinity (Ador *et al.*, 1999). In this study, we have confirmed by site-directed mutagenesis that alteration of Asp to Val at residue 539 in the ATP binding domain reduces ATP binding affinity. Here, truncated, C-terminal fragments of AspRS only affect cell function at higher temperatures (for *A. pasteurianus*) as judged by the effect on cell morphology.

In this study, we predicted that excessive synthesis of truncated C-terminal fragments of AspRS may cause excessive sequestration of intracellular ATP at high temperature (37°C), so that the cell enters into an ATP starvation state. This would have inevitable consequences on cell growth. For example, Fischer *et al.* (2002) reported that one of the cell division genes in *C. crescentus*, namely *ftsH*, encodes the ATP-dependent protease, FtsH. This gene is also found in *E. coli* so a shortage of intracellular ATP may fail to support cell division via FtsH.

Many truncated aa-RS paralogs have been found in genomes of several bacteria and are considered to be pseudogenes. The *E. coli yadB* gene encodes a truncated glutamyl-tRNA synthetase (GluRS) that enables activation and transfer of glutamate on to $tRNA^{Asp}$ (Salazar *et al.*, 2004). The function of the shorter aa-RS paralogs is not restricted to amino acid biosynthesis: some of them have been shown to have specific esterase function by hydrolysis of misacylated tRNA (Campanacci *et al.*, 2004). In the present study the truncated gene caused short filament formation in *A. pasteurianus* SKU1108 but at different temperatures compared to *E. coli*. This implies that the truncated *aspS* gene caused less severe effects at restrictive temperature in *A. pasteurianus* SKU1108 than in *E. coli*.

Our results suggest that the ATP-binding motif 3 of the *aspS* gene has a permissive effect on cell growth in *A. pasteurianus* SKU1108, including at the higher temperature (39°C). While *E. coli* can normally grow at higher temperatures, this fails when expressing the truncated *aspS* gene. Thus this gene might be involved in cell growth and heat stress response in *A. pasteurianus* SKU1108 as well as the response for cell survival at high temperature.

There are several limitations to our work, as well as further studies that need to be undertaken to determine the hypothesized role of ATP binding in the morphological changes we observed. It would be valuable in future work to assess the extent of over-expression of the various gene fragments and constructs. Furthermore, determination of cellular ATP concentrations would be valuable in testing our hypothesis of ATP starvation associated with over-expression of motif 3.

Acknowledgements

This work was financial supported by Thailand Research Fund (TRF) under project code RSA4780013, Kasetsart University Research and Development Institute (KURDI), the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, Department of Microbiology and The Graduate School, Kasetsart University. Sincere thanks are also expressed to Dr. Wasu Pathom-Aree and Mr. Worapot Jinda for their skillful experiments. Special thanks to Dr. C. Norman Scholfield and Professor Gavin P. Reynolds Naresuan University, Thailand, for their help with the manuscript. Part of this work was carried out through the Asian Core Program collaboration supported by the Japan Society for the Promotion of Science (JSPS) and the National Research Council of Thailand (NRCT).

References

Ador, L., Camasses, A., Erbs, P., Cavarelli, J., Moras, D., Gangloff, J., and Eriani, G. 1999. Active site mapping of yeast aspartyl-tRNA synthetase by *in vivo* selection of enzyme mutations lethal for cell growth. *J. Mol. Biol.* **288**, 231–242.

Campanacci, V., Dubois, Y.D., Becker, D.H., Kern, D., Spinelli, S., Valencia, C., Pagot, F., Salomoni, A., Grisel, S., Vincentelli, R., and *et al.* 2004. The *Escherichia coli yadB* gene product reveals a novel aminoacyl-tRNA synthetase like activity. *J. Mol. Biol.* **337**, 273–283.

Cavarelli, J., Eriani, G., Rees, B., Ruff, M., Boeglin, M., Mitschler, A., Martin, F., Gangloff, J., Thierry, J.C., and Moras, D. 1994. The active site of yeast aspartyl-tRNA synthetase: structural and functional aspects of the aminoacylation reaction. *EMBO J.* **15**, 327–337.

Eriani, G., Dirheimer, G., and Gangloff, J. 1990. Aspartyl-tRNA synthetase from *Escherichia coli*: cloning and characterisation of the gene, homologies of its translated amino acid sequence with asparaginyl- and lysyl-tRNA synthetases. *Nucleic Acids Res.* **18**, 7109–7118.

Fischer, B., Rummel, G., Aldridge, P., and Jenal, U. 2002. The FtsH protease is involved in development, stress response and heat shock control in *Caulobacter crescentus*. *Mol. Microbiol.* **44**, 461–478.

Frugier, M., Ryckelynck, M., and Giegé, R. 2005. tRNA-balanced expression of a eukaryal aminoacyl-tRNA synthetase by an mRNA-mediated pathway. *EMBO Rep.* **6**, 860–865.

Ibba, M. and Söll, D. 2000. Aminoacyl-tRNA synthesis. *Annu. Rev. Biochem.* **69**, 617–650.

Martin, F., Sharples, G.J., Lloyd, R.G., Eiler, S., Moras, D., Gangloff, J., and Eriani, G. 1997. Characterization of a thermosensitive *Escherichia coli* aspartyl-tRNA synthetase mutant. *J. Bacteriol.* **179**, 3691–3696.

Marx, C.J. and Lidstrom, M.E. 2001. Development of improved versatile broad-host-range vectors for use in methylotrophs and other Gram-negative bacteria. *Microbiology* **147**, 2065–2075.

Masud, U., Matsushita, K., and Theeragool, G. 2011. Molecular cloning and characterization of two inducible NAD^+-*adh* genes

encoding NAD^+-dependent alcohol dehydrogenases from *Acetobacter pasteurianus* SKU1108. *J. Biosci. Bioeng.* **5**, 422–431.

Matsutani, M., Hirakawa, H., Nishikura, M., Soemphol, W., Ibnaof Ali, I.A., Yakushi, T., and Matsushita, K. 2011. Increased number of Arginine-based salt bridges contributes to the thermotolerance of thermotolerant acetic acid bacteria, *Acetobacter tropicalis* SKU1100. *Biochem. Biophys. Res. Commun.* **409**, 120–124.

Matsutani, M., Hirakawa, H., Saichana, N., Soemphol, W., Yakushi, T., and Matsushita, K. 2012. Genome-wide phylogenetic analysis of differences in thermotolerance among closely related *Acetobacter pasteurianus* strains. *Microbiology* **158**, 229–239.

Metlitskaya, A., Kazakov, T., Kommer, A., Pavlova, O., Praetorius-Ibba, M., Ibba, M., Krasheninnikov, I., Klob, V., Khmel, I., and Severinov, K. 2006. Aspartly-tRNA synthetase is the target of peptide nucleotide antibiotic microcin C. *J. Biol. Chem.* **281**, 18033–18042.

Miller, J.H. 1972. Experiments in molecular genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, N.Y., USA.

Saeki, A., Theeragool, G., Matsushita, K., Toyama, H., Lothong, N., and Adachi, O. 1997. Development of thermotolerant acetic acid bacteria useful for vinegar fermentation at higher temperatures. *Biosci. Biotechnol. Biochem.* **61**, 138–145.

Salazar, C.J., Ambrogelly, A., Crain, F.P., McCloskey, A.J., and Söll, D. 2004. A truncated aminoacyl-tRNA synthetase modifies RNA. *Proc. Natl. Acad. Sci. USA* **101**, 7536–7541.

Sharples, G.J. and Lloyd, R.G. 1991. Location of a mutation in the aspartyl-tRNA synthetase gene of *E. coli* K-12. *Mutat. Res.* **264**, 93–96.

Thompson, D. and Simonson, T. 2006. Molecular dynamics simulations show that bound Mg^{2+} contributes to amino acid and aminoacyl adenylate binding specificity in aspartyl-tRNA synthetase through long-range electrostatic interactions. *J. Biol. Chem.* **281**, 23792–23803.

Yoshida, T., Ayabe, Y., Yasunaga, M., Usami, Y., Habe, H., Nojiri, H., and Omori, T. 2003. Genes involved in the synthesis of the exopolysaccharide methanolan by the obligate methylotroph *Methylobacillus* sp. strain 12S. *Microbiology* **149**, 431–444.