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Influence of the aspS Gene Sequence from Acetobacter pasteurianus SKU1108 on Escherichia coli Morphology

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Abstract:	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 aspS gene consists of 1,788 bp and coding 595 amino acid residues. The highly conserved of Gly-Val-Asp-Arg (motif 3), the ATP binding motif, is located at the position 537-540 in the C-terminus. Deletion analysis of the aspS gene upstream region suggested that the tentative promoter is around 173 bp upstream from ATG initiation codon. Interestingly, transformation with the plasmid pGEM-T138, pUC138 and pCM138 synthesizing 138 amino acids of C-terminal fragments of AspRS harboring the ATP binding domain caused E. coli cell lengthening at 37 and 42˚C. Moreover, E. coli harboring pUC595 (synthesizing 595 amino acids) and 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 over-production of truncated C-terminal sequence of aspS may cause sequestration of intracellular ATP of E. coli leaving less ATP for cell division or shaping cell morphology.
Response to Reviewers:	Dear Dr. Beom Sik Kang and Reviewers Thank you for the opportunity to revise the manuscript entitled “Clones of aspS Gene Encoding Aspartyl-tRNA Synthetase from Acetobacter pasteurianus SKU1108 Affect Escherichia coli Morphology”. We have substantially revised the paper in accordance with the reviewers’ comments and in addition have had it checked carefully by a native English speaker who is willing to confirm this (email contact: gavin.reynolds@hotmail.com).

	We note that both referees feel the study is interesting; one (2nd reviewer) recommends to concentrate more on the cloning and characterisation of the gene and its promoter, while the other wishes to de-emphasise the promoter studies and undertake more work on the ATP-binding hypothesis. We have tried to finalise the paper that addresses as much as possible of each reviewer's concerns. However, we recognise that we cannot do this completely for both reviewers. We should be grateful if the manuscript can be assessed on the basis of the work reported without further studies. Reviewer comments: (line number refer to original comments) 1: The title has been changed to address this. 28: "coding" add as reviewer suggestion 29: "highly conserved" as reviewer suggestion 30: change from "domain" to "motif" as reviewer comment 36-37: We change from "conclusion" to "propose" 50: rewrite for clearer understanding 57: change from "3" to "three" as reviewer suggestion 61: rewrite for clearer understanding 62: Leu535Pro "of AspRS" as reviewer comment 72: rewrite for clearer understanding 82: change from "retains" to "maintains" 194: high is that mean "high temperature" already present in the text 195-197: rewrite for clearer understanding 201: "high sequence homology" was add as reviewer suggestion 205: change from "regulatory region" to "upstream region" as reviewer suggestion 211: already move to discussion section on line 309 246: This section of the result has been removed. 253: Site-directed mutagenesis was performed as reviewer suggestion 256: change from "division" to "morphology" as reviewer suggestion 261: "not at 42oC" change to "but with different morphology at 42oC" as reviewer suggestion 264: why 39oC: already explain on line 249 272: "several constructs" was change to "recombinant plasmids that contain different length constructs of the aspS gene" as reviewer suggestion 280: change from "homology" to "similarity" as reviewer comment 216/239/283: we have mentioned the value of quantifying protein expression in the final section of the discussion. 292/299: This section of the discussion has been removed 300: 37oC is high temperature for A. pasteurianus 305: rewrite for clearer understanding

Influence of the *aspS* Gene Sequence from *Acetobacter pasteurianus* SKU1108 on *Escherichia coli* Morphology

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Running title: Aspartyl-tRNA synthetase from *A. pasteurianus* SKU1108

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ABSTRACT

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 *aspS* gene consists of 1,788 bp and coding 595 amino acid residues. The highly conserved of Gly-Val-Asp-Arg (motif 3), the ATP binding motif, is located at the position 537-540 in the C-terminus. Deletion analysis of the *aspS* gene upstream region suggested that the tentative promoter is around 173 bp upstream from ATG initiation codon. Interestingly, transformation with the plasmid pGEM-T138, pUC138 and pCM138 synthesizing 138 amino acids of C-terminal fragments of AspRS harboring the ATP binding domain caused *E. coli* cell lengthening at 37 and 42˚C. Moreover, *E. coli* harboring pUC595 (synthesizing 595 amino acids) and 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 over-production of truncated C-terminal sequence of *aspS* may cause sequestration of intracellular ATP of *E. coli* leaving less ATP for cell division or shaping cell morphology.

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

1. 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 contain the C-terminal tetrapeptide sequence (Gly-Leu-Asp-Arg), a potential ATP-binding site. A single point mutation at Leu535Pro of AspRS in motif 3 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). In 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. Nevertheless, over-expression of the normal

ftsH gene resulted in an increased salt tolerance but still showed thermo-intolerance (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 but still maintains ATP binding thus reducing cytosolic ATP to a level which is not able to support cell division at elevated temperatures.

2. Materials and methods

2.1 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 maintained on potato medium (5 g glucose, 20 g glycerol, 10 g yeast extract, 10 g polypeptone, and 15 ml potato extract, made up to 1000 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 were cultured in Luria-Bertani medium (10 g polypeptone, 5 g yeast extract, 5 g NaCl, made up to 1000 ml) containing appropriate antibiotic and incubated at 37˚C with vigorous shaking for 18-24 h.

2.2 Cloning and nucleotide sequencing of the *aspS* gene

Molecular cloning of *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 (CAAAGGGCGG) 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 contains 1.3 kb RAPD-PCR product was digested with *Eco*RI and purified a 1.3 kb DNA fragment that contained the C-terminus sequence of AspRS. This was used as 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, the colony showing a positive hybridization signal was confirmed by PCR and its nucleotide sequence was determined. The plasmid carrying an entire *aspS* gene was designated as pUC595.

2.3 Deletion analysis of *aspS* gene for tentative promoter

In order to analyze the tentative *aspS* gene promoter, five constructs of the *aspS* gene were obtained by PCR amplification using specific oligonucleotide primers. These five DNA fragments had different lengths beginning at the upstream region of the *aspS* gene and were obtained by PCR amplification using 5 forward primers and 1 reverse primer that were composed of *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 plasmid was

separately transferred to *E. coli* DH5α by heat shock and selected on a LB agar containing 10 μg/ml gentamicin and 20μg/ml X-gal. *E. coli* DH5α 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).

2.4 Construction of pGEM-T138 derivatives

The cell morphology of *E. coli* DH5α harboring pGEM-T138 changed from short rod to long filamentous chains when grown at 37 and 42˚C. Such morphological changes might be caused by N-terminal β-galactosidase fusing with 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 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 DNA, 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 fragments 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 on glass slides, stained by Gram staining and the cell morphology was observed by light microscopy (1,000x).

2.5 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 cause 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 the region encoding N-terminal and C-terminal, 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 C-terminal *aspS* gene (adjacent 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. The *E. coli* DH5α harboring those plasmids were grown at 30, 37 and 42˚C for 24 h. The overnight cultures were observed microscopically as Gram stained smears.

2.6 Site-directed mutagenesis of *aspS* gene

In order 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 *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 one amino acid changed 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 two PCR products were purified by MagExtractor-Purified Kit (Toyobo, Osaka, Japan). The second PCR amplification was performed to fuse the two DNA fragments using F-SDaspS and R-SDaspS as PCR primers. Fifty ng of each 237-bp and 592-bp DNA fragments 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 same direction as the *lac* promoter was selected. Alteration of the sequence at the ATP-binding motif was confirmed by nucleotide sequencing.

2.7 Truncated *aspS* gene and cell morphology at high temperature

A 1.3 kb *Eco*RI DNA fragment carrying partial *aspS* gene encoding truncated C-terminal fragments of AspRS was constructed from the *Eco*RI-digested pGEM-T138 and further 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 examined

199 microscopically in Gram stained smears. The plasmids pCM138 or pCM62 were also
200 transferred via conjugation into *A. pasteurianus* SKU1108 and grown at 30, 37, 39 and 42˚C
201 for 24 h and again cell morphology assessed.
202
203 The complete sequence data of the *aspS* gene from *A. pasteurianus* SKU1108 have
204 been submitted to the GenBank databases under accession no. JQ837915.
205
206 **3. Results**
207
208 3.1 Cloning, nucleotide sequencing and putative promoter region of *aspS*
209
210 The chromosomal DNA from *A. pasteurianus* SKU1108 was used as the template for
211 RAPD amplification using the random 10-mer AD01 as described in materials and methods.
212 Three typical RAPD-PCR amplified DNA fragments (0.6, 1.0 and 1.3 kb) could be clearly
213 observed in 13 thermotolerant strains; including *A. pasteurianus* SKU1108 (unpublished
214 data). Each of them was cloned into pGEM®-T Easy vector. Of these, only pGEM-T138
215 containing the 1.3 kb RAPD product caused a morphological change in *E. coli* DH5α at high
216 temperature. The 1.3 kb *Eco*RI DNA fragment was used as a DNA probe for Southern
217 hybridization of the chromosomal DNA from *A. pasteurianus* SKU1108 and then colony
218 hybridization for shotgun cloning and the resultant plasmids are shown in Fig. 1. The *Hin*dIII
219 DNA fragment in pUC595 consisted of 2,770 nucleotides. The open reading frame (ORF)
220 show high sequence homology to the *aspS* gene, this ORF consisted of 1,788 nucleotides,
221 started with an ATG at nucleotide 571-573 and terminated with TGA at 2,356-2,358. The
222 highly conserved tetrapeptide Gly-Val-Asp-Arg motif (motif 3) was located at amino acids
223 537-540 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 labelled pGSAF1, pGSAF2, pGSAF3, pGSAF4 and pGSAF5 carried 50, 107, 173, 283 and 563 bp of the upstream region of *aspS* gene, respectively (Table 1). The structures of the resultant DNA fragments are shown in Fig. 2A. The cultured transformants showed highest β-galactosidase activity in that harboring pGSAF3 (173 bp upstream from ATG) (Fig. 2B).

3.2 Truncated *aspS* gene from *A. pasteurianus* SKU1108 on cell morphology

Figure 3 shows *E. coli* DH5α cells grown at 30, 37 and 42˚C and harboring either pUC595 (synthesizing complete 595 residues), pUC138 or pGEM-T138 (synthesizing 138 residues of C-terminal fragments of AspRS). The pUC595 cells at all temperatures showed normal rod shapes (Fig. 3A). Those cells harboring pUC138 or pGEM-T138 were also normal rods but 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 spGEM-T138 was constructed by deletion of 0.6 kb downstream region from the stop codon of *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.

249

250 3.3 Disruption of *aspS* gene and cell morphology

251

252 The recombinant plasmids pUC595N::Km^r, pUC595C::Km^r, pGEM-T138C::Km^r
253 which coded the AspRS protein with Km^r insertion at a position 44, 508 and 535 downstream
254 from the first amino acid (methionine) were introduced into *E. coli* DH5α. The morphology
255 of *E. coli* DH5α harboring pUC595N::Km^r was changed from short rod to a few long
256 filaments when grown in LB medium at 37 and 42˚C (Fig. 4A). Nevertheless, cells harboring
257 pUC595C::Km^r in which the AspRS protein was disrupted at the C-terminal but carrying the
258 normal motif 3 exhibited the same morphological change (Fig. 4B) as those cells harboring
259 pUC595N::Km^r, but not in *E. coli* harboring pUC595.
260 Moreover, Km^r insertion into pGEM-T138C::Km^r at amino acid position 535 adjacent
261 to the motif 3 ATP binding domain produced short rod morphology (Fig. 4C).

262

263 3.4 Effect of altered amino acid residue at ATP-binding motif

264

265 Site-directed mutagenesis based on the PCR fusion method was used to determine an
266 essential role of ATP-binding motif in the truncated C-terminal AspS protein. A plasmid,
267 designated as pD539V, was prepared encoding mutated ATP-binding motif. At the conserved
268 motif 3(Gly-Val-Asp-Arg), a single amino acid alteration was presented in pD539V, in which
269 539Asp was changed to Val. *E. coli* DH5α harboring the mutagenic plasmid was grown on
270 LB agar at 37°C for 18 h, and the cell morphology was compared (Fig. 5). These cells
271 harboring pGEM-T138 displayed abnormal short and long filamentous shapes at 37˚C (Fig.
272 5A). *E. coli* DH5α harboring a mutated ATP-binding motif, one site amino acid alteration in
273 pD539V plasmid, showed normal shapes at 37˚C (Fig. 5B).

3.5 Overexpression of truncated *aspS* gene on cell morphology

The 1.3 kb DNA fragment carrying the partial *aspS* gene synthesizing 138 amino acids of the C-terminal 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 in *E. coli*. However, the truncated C-terminal fragment of AspRS in pCM138 inhibited division of *E. coli* DH5α cells at high temperature, an effect which was more severe than in *A. pasteurianus* SKU1108 (Fig. 6).

4. 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. Thus all the short 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 does not with Km^r cassette inserts at amino acid position 44 and 508. A Km^r cassette attached directly

298 to motif 3 restored normal cell morphology, presumably by interfering with ATP binding so
299 the amount of cytosolic ATP was adequate to support normal cell morphology.
300 The amino acid sequence of AspRS from *A. pasteurianus* SKU1108 showed 99%
301 sequence similarity with *A. pasteurianus* NBRC 3283. These two strains have led to a
302 physiological understanding of the relationships between thermotolerance and phylogeny via
303 genome-wide phylogenetic analysis (Matsutani *et al*., 2012).
304 Deletion analysis of upstream regions of *aspS* gene suggested that the tentative *aspS*
305 gene promoter might be located at around 173 bp upstream from the start codon. However,
306 the enzyme activity is reduced when it contain more sequences in upstream region it might be
307 due to contain repressor binding site.
308 Excess of intact *aspS* in *E. coli* DH5α harboring pUC595, overexpressing the
309 complete gene sequence, preserved normal cell morphology which may reflect feedback
310 regulation of further gene expression, perhaps thereby limiting the excessive binding of ATP.
311 Such feedback regulation has been observed in yeast by Frugier *et al*. (2005) who cloned the
312 *aspS* gene into a yeast null strain for AspRS.
313 Insertion of the Km^r cassette in pUC595N::Km^r and pUC595C::Km^r created abnormal
314 *E. coli* cells implying excessive functional ATP binding at motif 3. This ATP binding domain
315 plays an important role in amino acid activation in aminoacylation during protein synthesis
316 (Ibba and Söll, 2000; Martin *et al.*, 1997; Metlitskaya *et al.*, 2006). Sequence analysis of
317 yeast *aspS* mutants shows that charged residues at the C-terminus are necessary for ATP
318 binding and stabilizing the reaction transition states (Cavarelli *et al.*, 1994). Eriani *et al*.
319 (1990) showed that all residues in motif 3 (Gly-Leu-Asp-Arg) are required for ATP binding.
320 Thus, a single amino acid change causes a dramatic loss of activity and reduced ATP affinity
321 (Ador *et al.*, 1999). In this study, we have confirmed by site-directed mutagenesis that all
322 residues in the ATP binding domain are necessary for ATP binding. Here, C-terminal

323 fragments of AspRS truncation only affects cell function at higher temperatures (for *A.*
324 *pasteurianus*) as judged by the effect on cell morphology.

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

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

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

345 There are several limitations to our work, as well as further studies that need to be
346 undertaken to determine the hypothesized role of ATP binding in the morphological changes
347 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.

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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., Bignon, C., Lapointe, J., Giegé, R., and Campillau, C. 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.

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. *PNAS.* **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.

447

448

449 **Figure legends**

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

457

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

461

462 Fig. 3. Microscopic appearance of *E. coli* DH5α harboring pUC595 (A), pUC138 or pGEM-
463 T138 (B) and scanning electron microscopic appearance of the cells from B (C) grown on LB
464 medium containing 50 μg/ml ampicillin at 30, 37 and 42˚C. The bacterial cells were observed
465 under light and scanning electron microscopes with magnifying power of 1,000x (scale bars =
466 10 μm) and 15,000x (scale bars = 1μm).

467

468 Fig. 4. Microscopic appearance of *E. coli* DH5α harboring recombinant plasmids carrying
469 disrupted *aspS* gene. Plasmids pUC595N::Km^r (A), pUC595C::Km^r (B) and pGEM-
470 T138C::Km^r (C) carrying Km^r cassette 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 microscopes, Axio Imager. A1, ZEISS, with magnifying power of 1,000x.

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

Table legends

Table 1. Bacterial strains and plasmids used in this study.

*Figure

Fig. 1.

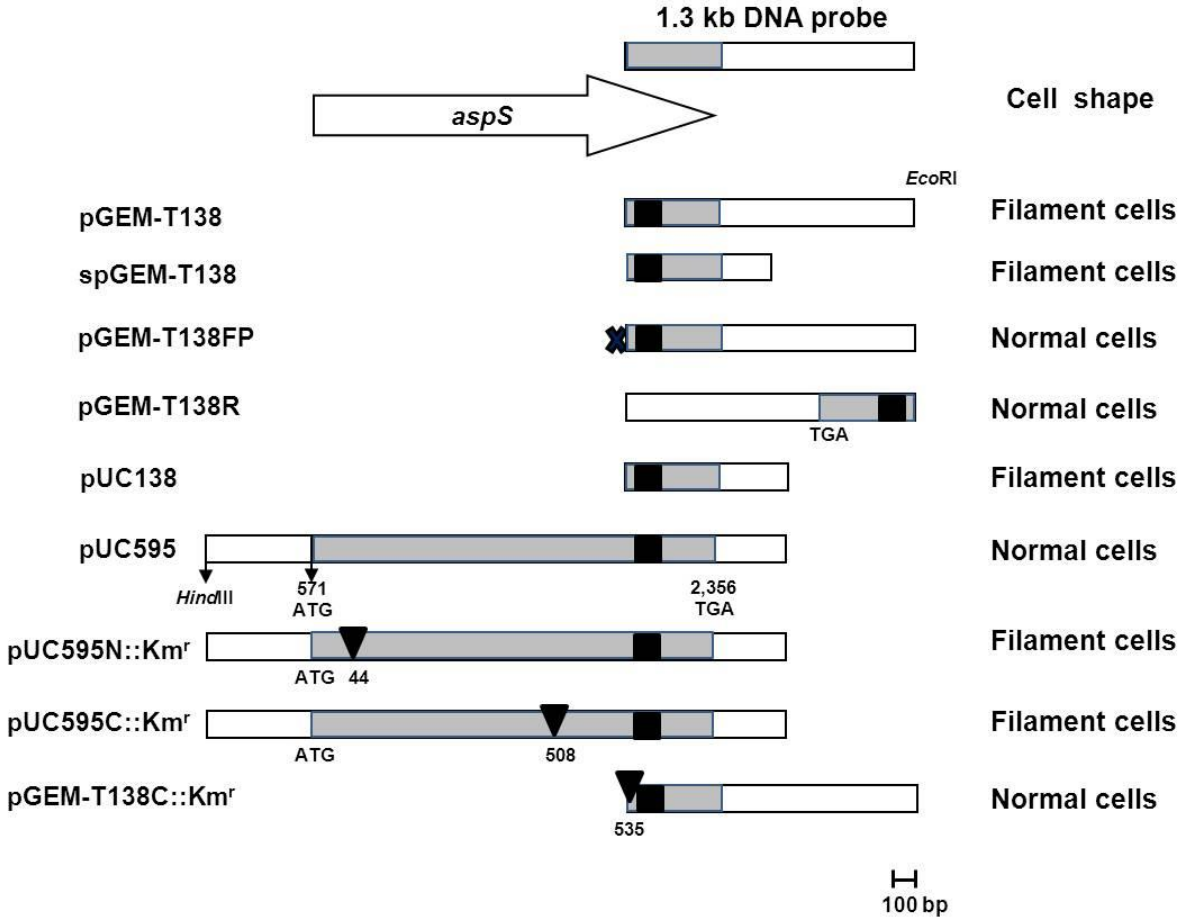

1.3 kb DNA probe
aspS
Cell shape
EcoRI
pGEM-T138
Filament cells
spGEM-T138
Filament cells
pGEM-T138FP
Normal cells
pGEM-T138R
Normal cells
TGA
pUC138
Filament cells
pUC595
Normal cells
HindIII
571
ATG
2,356
TGA
pUC595N::Kmr
Filament cells
ATG 44
pUC595C::Kmr
Filament cells
ATG
508
pGEM-T138C::Kmr
Normal cells
535
100 bp

Fig. 2.

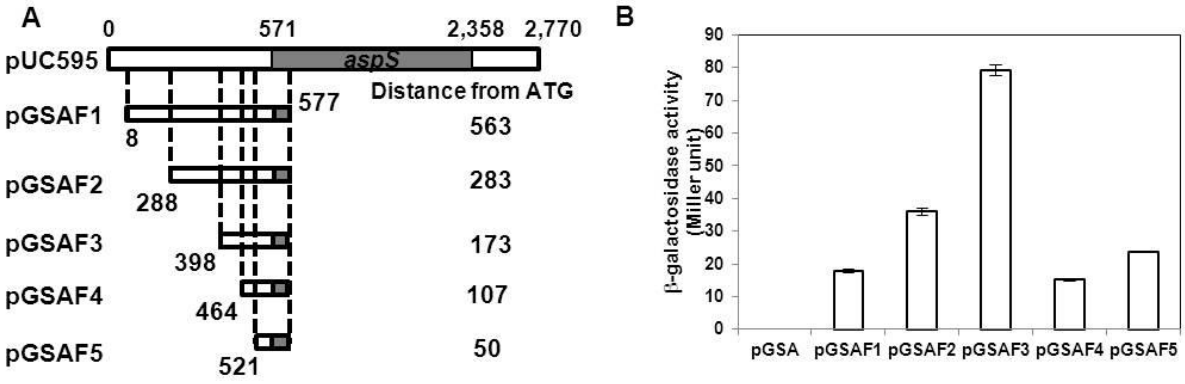

A
0
571
2,358
2,770
pUC595
aspS
Distance from ATG
pGSAF1
577
8
563
pGSAF2
288
283
pGSAF3
398
173
pGSAF4
464
107
pGSAF5
521
50
B
β-galactosidase activity (Miller unit)
90
80
70
60
50
40
30
20
10
0
pGSA
pGSAF1
pGSAF2
pGSAF3
pGSAF4
pGSAF5

Fig. 3.

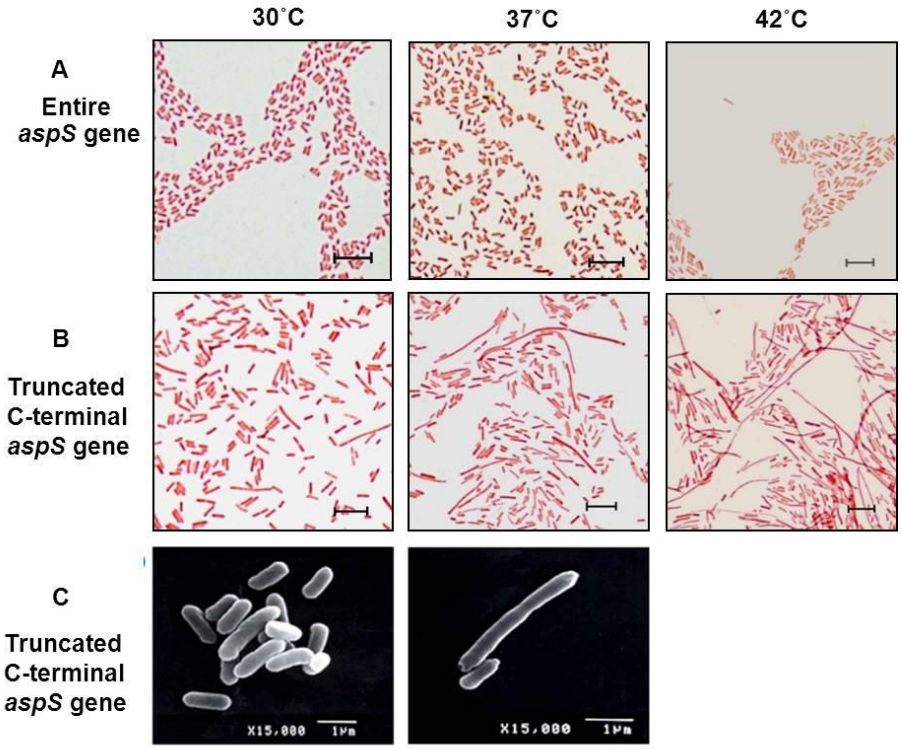

30˚C
37˚C
42˚C
A
Entire
aspS gene
B
Truncated
C-terminal
aspS gene
C
Truncated
C-terminal
aspS gene
X15,000 1μm
X15,000 1μm

Fig. 4.

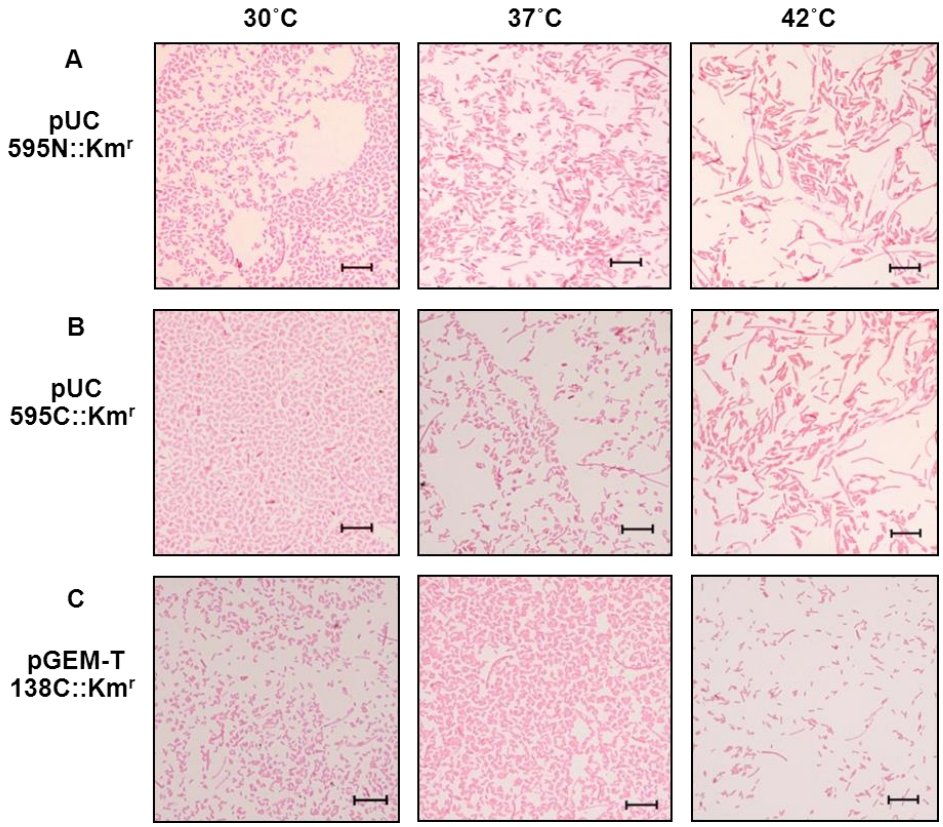


Fig. 5.

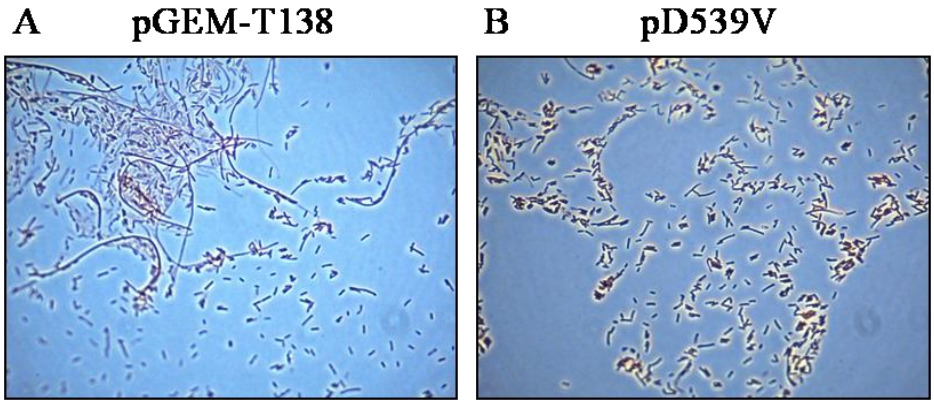
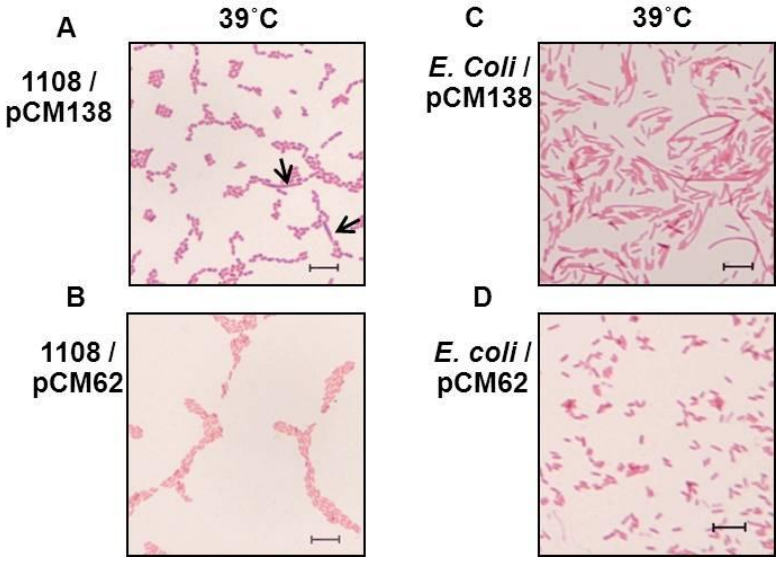


Fig. 6.


A
39˚C
1108 /
pCM138
C
39˚C
E. Coli /
pCM138
B
1108 /
pCM62
D
E. coli /
pCM62

*Table

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)