Involvement of the arginine repressor in lysine biosynthesis of *Thermus thermophilus*

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Lysine biosynthesis of *Thermus thermophilus* proceeds in a similar way to arginine biosynthesis, and some lysine biosynthetic enzymes from *T. thermophilus* so far investigated have the potential to function in arginine biosynthesis. These observations suggest that arginine might regulate the expression of genes for lysine biosynthesis. To test this hypothesis, the *argR* gene encoding the regulator of arginine biosynthesis was cloned from *T. thermophilus* and its function in lysine biosynthesis was analysed. The addition of arginine to the culture medium inhibited the growth of an arginase gene knockout mutant of *T. thermophilus*, which presumably accumulates arginine inside the cells. Arginine-dependent growth inhibition was not alleviated by the addition of ornithine, which is a biosynthetic intermediate of arginine and serves as a peptidoglycan component of the cell wall in *T. thermophilus*. However, the growth inhibition was cancelled either by the simultaneous addition of lysine and ornithine or by a knockout of the *argR* gene, suggesting the involvement of *argR* in regulation of lysine biosynthesis in *T. thermophilus*. Electrophoretic mobility shift assay and DNase I footprinting revealed that the ArgR protein specifically binds to the promoter region of the major lysine biosynthetic gene cluster. Furthermore, an α -galactosidase reporter assay for this promoter indicated that arginine repressed the promoter in an *argR*-dependent manner. These results indicate that lysine biosynthesis is regulated by arginine in *T. thermophilus*.

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INTRODUCTION

Two pathways are known for lysine biosynthesis: the diaminopimelate pathway found in most bacteria and plants, and the α -aminoadipate (AAA) pathway found in fungi and yeasts. Our recent studies revealed that the extremely thermophilic bacterium *Thermus thermophilus* synthesizes lysine through AAA. The pathway differs from the known AAA pathway found in lower eukaryotes. The *Thermus* pathway starts from the condensation of 2-oxoglutarate with acetyl-CoA to synthesize homocitrate, and proceeds in a manner similar to that in lower eukaryotes until the step of AAA synthesis (Kobashi *et al.*, 1999; Kosuge & Hoshino, 1999). However, the subsequent part of the pathway is totally different from that in lower eukaryotes, but similar to ornithine synthesis from glutamate, a step in arginine biosynthesis (Fig. 1) (Miyazaki *et al.*, 2001, 2002).

Amino acid biosynthesis is usually controlled by an end product in the pathway by two different systems: inhibition of a biosynthetic enzyme and repression of its gene

expression. In most cases, a single end product controls its own biosynthesis. However, in the lysine biosynthesis of *T. thermophilus*, all enzymes so far analysed were found to be able to catalyse the reactions in the tricarboxylic acid (TCA) cycle and arginine biosynthesis (Miyazaki *et al.*, 2001, 2002, 2003, 2004; Wulandari *et al.*, 2002), and 2-oxoglutarate, which is provided through the TCA cycle, is a precursor of both arginine and lysine. This suggests that lysine biosynthesis of *T. thermophilus* is regulated by arginine as well as lysine. Homocitrate synthase (Hcs), which catalyses the first reaction in the pathway, is strictly inhibited by lysine (Wulandari *et al.*, 2002). In addition to the inhibition by lysine, we also found moderate inhibition of Hcs by arginine (Wulandari *et al.*, 2002). As for transcriptional control, we recently demonstrated that the promoter of the homocitrate synthase gene (*hcs* promoter), which is the promoter of the major lysine biosynthetic gene cluster, is regulated by lysine through leader-peptide-mediated transcriptional attenuation (Tsubouchi *et al.*, 2005). A remaining question is whether the *hcs* promoter is also regulated by arginine. In this paper, we report the involvement of ArgR, a global arginine regulator, in the transcriptional regulation of the *hcs* promoter, depending on the availability of arginine.

Abbreviations: AAA, α -aminoadipate; EMSA, electrophoretic mobility shift assay; TCA, tricarboxylic acid.

Fig. 1. Lysine biosynthetic pathway of *T. thermophilus* (right) and related metabolic pathways. Enzymes responsible for the corresponding reactions in the TCA cycle and arginine metabolism are shown as gene products in *E. coli* in most cases. Arginase, which catalyses the conversion of arginine to ornithine, is boxed.

METHODS

Bacterial strains and growth media. *T. thermophilus* TSU130 (a mutant of the *agaT* gene encoding α -galactosidase derived from *T. thermophilus* HB27) was constructed by methods described previously (Fridjonsson & Mattes, 2001; Fridjonsson *et al.*, 2002), and used as the host for the reporter assay. *T. thermophilus* cells were cultivated in TM medium (Koyama *et al.*, 1986) or MM medium (Tsubouchi *et al.*, 2005) supplemented with or without various additions. *Escherichia coli* JM105 [*endA1 thi rpsL sbcB15 hsdR4* Δ (*lac-proAB*)/F' *traD36 proAB lacZ* Δ M15] was used for DNA manipulation, and *E. coli* BL21(DE3) [F[−] *ompT hsdSB* ($r_B^- m_B^-$) *dcm gal* λ (DE3)] was used as the host for gene expression. For the cultivation of *E. coli*, 2 × YT medium, containing Bactotryptone 1.6%, yeast extract 1.0% and NaCl 0.5%, was generally used. All chemicals were purchased from Wako Pure Chemical, Kanto Chemicals or Sigma. Enzymes for DNA manipulation were purchased from Takara Shuzo.

Knockout of the arginase and *argR* genes in *T. thermophilus*. The nucleotide sequences of oligonucleotides used in this study are shown in Table 1. The plasmids for the knockout of arginase were constructed as follows. Two independent PCRs using ANSM1/ANSM2 for amplifying 500 bp of a 5'-portion of the arginase gene TTC1132 of *T. thermophilus* HB27 and ANSU1/ANSU2 for amplifying 500 bp of a 3'-portion of the arginase gene as primers were performed with *T. thermophilus* TSU130 chromosomal DNA as the template. After the amplified 5'- and 3'-fragments had been cloned into pT7Blue (Novagen), the resulting plasmids were digested with *Xba*I/*Eco*RI and *Hind*III/*Hinc*II, respectively. Two fragments were cloned into pBluescript II SK(+) (Stratagene) to yield pRNSMU. An additional PCR for amplifying the heat-stable hygromycin B

Table 1. Oligonucleotides used in this study

Name	Sequence
ANSM1	5'-CGCTCTAGATGGCGCCTCCTTCGGTGCCCATAG-3'
ANSM2	5'-CCAGAATTCATGGGGGGCATGGTAGCAGAAACG-3'
ANSU1	5'-CGGAAGCTTGGACGAGCGCAACCGCACCGCCGAGA-3'
ANSU2	5'-CGGGTCGACTCCCCCTAAGCCTCCTCCTCATG-3'
ANSCN-F	5'-GGAGGTGGCCTGTGGTGATCGCAAGCCCTT-3'
ANSCN-R	5'-ATCATGGCGCCTCCTTCGGTGCCCATAGT-3'
HygR-F	5'-GAATTCATTGCGCCCAAGGTTTACAAAATC-3'
HygR-R	5'-CCAAGCTTCTATTCCCTTGGCCCTCGGACGA-3'
HCSUP	5'-GAGGAGCTACCAGGCCCGGAGAGGCGGTT-3'
HCSOWN	5'-GCATGCACCCCTCCCCAAAAGAAAACCG-3'
RFUP	5'-GAATTCGCGGCCGCTTCATAGACCAAGTGG-3'
RFDWN	5'-GAATTCATATGGCCTTAAGCATACCCCGT-3'
RRKOM1	5'-GTATCGATCTGGAGATGCGAAAGGCCCTCC-3'
RRKOM2	5'-GTAAGCTTGACCACGAAGAGGGTCAGGAA-3'
RRKOU1	5'-CTGAATTCAGTGCTGGAGCTCTTTCTCAAG-3'
RRKOU2	5'-CAGGATCCGTGAAAAGGACCACGCGGAAGC-3'
RRKOCN-F	5'-CTAGACCCGGTGCGCTTCCTCT-3'
RRKOCN-R	5'-TCTTCCAGGTCTAGAGGAGGCC-3'
TtArgRN	5'-GCTCTAGAAAGGAGGTGCATATGGGCAACAAGGCGGAACGGCACCCGC-3'
TtArgRHisC	5'-CCCAAGCTTTCAGTGGTGGTGGTGGTGGTGGTGGGAACCGGAAAGCGCCCGCCGAG-3'
CLWT	5'-CTCGCGGCGTTTCGTATACCCCTACGGGCAAGATG-3'
CLWT-C	5'-CATCTTGCCCGTAAGGGTATACGAAACGCCGCGAG-3'
CLMUT	5'-CTCGCGGCGTTTCGCCTAGGCTTACGGGCAAGATG-3'
CLMUT-C	5'-CATCTTGCCCGTAAGCCTAGGCGAAACGCCGCGAG-3'

phosphotransferase gene was performed using HygR-F/HygR-R as the primers and p8S-T31-hph5 (Nakamura *et al.*, 2005) as the template. An amplified fragment was cloned into pT7Blue to produce pHygRT. pRNSMU was digested with *EcoRI*/*HindIII*, and the larger DNA fragment was blunt-ended and ligated with the *KpnI*-*HindIII* smaller DNA fragment of pHygRT, which was also blunt-ended. The resulting plasmid, pRNMHygU, was used to knock out the arginase gene of *T. thermophilus* TSU130 by the method described previously (Hidaka *et al.*, 1994). An arginase gene knockout mutant of *T. thermophilus* TSU130 verified by PCR with a set of primers ANSCN-F/ANSCN-R was named *T. thermophilus* ADK1.

The plasmid for the knockout of *argR* was constructed as follows. Two independent PCRs using RRKOM1/RRKOM2 for amplifying 446 bp of a 5'-portion of the *argR* gene and RRKOU1/RRKOU2 for amplifying 446 bp of a 3'-portion of the *argR* gene were performed with pBM4K710, carrying the *argR* gene previously cloned from *T. thermophilus* HB27 (unpublished), as the template. The amplified 5'- and 3'-fragments were cloned into pBluescript II SK(+) in the same manner as described above except that the fragments were digested with *Clal*/*EcoRI* and *BamHI*/*HindIII*, respectively. The resulting plasmid, named pWRAB, was digested with *EcoRI*/*HindIII*, blunt-ended, treated with calf intestine alkaline phosphatase, and ligated with the blunt-ended *HindIII* fragment of pUC19-39-442KmR (Maseda & Hoshino, 1998) containing the heat-stable kanamycin nucleotidyltransferase gene (Liao *et al.*, 1986). The resulting plasmid, named pWRAB-KmRF, was used to knock out the *argR* gene of *T. thermophilus* TSU130 in the same way. Disruption of the *argR* gene in the resulting mutant was confirmed by PCR with primers RRKOCN-F and RRKOCN-R. pWRAB was also used to remove the heat-stable kanamycin nucleotidyltransferase gene from the chromosomal DNA of the *argR*-knockout mutant. Three kanamycin-sensitive colonies were isolated from about 1000 colonies. Deletion of the heat-stable kanamycin nucleotidyltransferase gene was confirmed by PCR with the same primers. The resulting *argR*-knockout mutant was named *T. thermophilus* WRK07. *T. thermophilus* ADK2 (Δ *argR*, Δ arginase gene) was generated by the knockout of the arginase gene of *T. thermophilus* WRK07 by using pRNMHygU.

Phenotypic analysis of the arginase gene knockout mutant.

The arginase gene knockout mutant, ADK1, was cultured for 10 h in 5 ml TM medium containing 1.6 mM CaCl_2 and 1.6 mM MgCl_2 . A small portion (1 ml) was taken from the culture, centrifuged at 15 000 g for 30 s, and the precipitate was washed with MM medium three times. Cell suspension equivalent to 10 μl culture was added to 5 ml MP medium (MM medium supplemented with 100 μM proline) and incubated at 70 °C. Proline was added to MM medium to accelerate the growth. Growth was analysed by monitoring the OD_{600} of the culture at appropriate intervals.

Overexpression and preparation of His-tagged ArgR protein.

To express the *argR* gene in a form with a His₈-tag at the COOH terminus, PCR was performed with the primer pairs TtArgRN and TtArgRHIS, using pBM4K710 as the template. The PCR product with the correct nucleotide sequence was chosen and cloned as an *NdeI*-*HindIII* fragment into pET26b(+) (Novagen) to yield pET-ArgRHIS. *E. coli* BL21(DE3) cells harbouring pET-ArgRHIS were pre-cultured in 5 ml 2 $\times$ YT medium with kanamycin at 50 $\mu\text{g ml}^{-1}$ overnight. The culture was transferred to 1 l LB medium containing kanamycin at 50 $\mu\text{g ml}^{-1}$. When the culture had grown to an OD_{600} of 0.6, IPTG was added at a final concentration of 0.4 mM. After cultivation for 14 h at 25 °C, cells were harvested, suspended in 30 ml STU buffer (500 mM NaCl, 20 mM Tris/HCl pH 8.0, 500 mM urea), disrupted by sonication, and heated at 70 °C for 20 min. Debris was removed by centrifugation at 40 000 g for 30 min and phenylmethylsulfonyl fluoride (final 2 mM) was added to the supernatant. The enzyme solution was applied onto a

Ni-NTA resin (Novagen), and washed with 10 vols STU buffer followed by washing with 2 vols STU buffer containing 20 mM imidazole. Elution was done with STR buffer (150 mM NaCl, 20 mM Tris/HCl pH 8.0, 5 mM arginine) supplemented with 0.4 M imidazole. The eluted fractions were dialysed against STR buffer, and used as purified ArgR. The purity of ArgR was verified by SDS-PAGE. Protein concentration was determined by the method of Bradford (1976). Gel filtration was performed to estimate the quaternary structure, with a Superose 12HR 10/30 gel exclusion column (GE Healthcare Bio-Sciences) equilibrated in ST buffer with or without 5 mM arginine.

Electrophoretic mobility shift assays (EMSAs). A 205 bp fragment containing the *hcs* promoter region and a 290 bp fragment containing the *argF* promoter region were amplified from *T. thermophilus* TSU130 chromosomal DNA by PCR with primers HCSUP/HCSOWN and RFUP/RFOWN, and cloned into pT7Blue to yield pHcsUT and pRFUT, respectively. To construct the *hcs* promoter mutant, two independent PCRs were performed using RPHCSR/CLMUT and RPHCSR/CLMUT-C as primers, and cloned into pT7Blue. Portions of the two reaction products were mixed together and subjected to additional PCR using RPHCSR and RPHCSR as primers. An amplified fragment was cloned into pT7Blue to produce pHcsUTmut. The *hcs* promoter region, the mutated *hcs* promoter region and the *argF* promoter region were amplified by PCRs from pHcsUT, pRFUT and pHcsUTmut with primers HCSUP/HCSOWN, HCSUP/HCSOWN and RFUP/RFOWN, respectively. After purification these fragments were endlabelled with [γ -³²P]ATP. The reaction mixture (total 20 μl) containing Tris/HCl pH 7.8, 500 μM arginine, 2 mM MgCl_2 , 1 mM EDTA, 50 mM NaCl, 1 mM dithiothreitol, 250 μg bovine serum albumin ml^{-1} , 10% (v/v) glycerol, 1% sucrose, Nonidet P-40 and 0.05 μg poly(dI-dC) ml^{-1} was mixed with the labelled probe (1 nM) and 0–400 nM ArgR. Binding of ArgR to the labelled DNA fragment was started by adding the labelled fragment to the reaction mixture preincubated for 10 min at 70 °C and the mixture was further incubated at 70 °C at least 30 min. The reaction mixture was used for 5% PAGE in TAE (40 mM Tris/acetate pH 8.0, 2 mM EDTA) buffer. Arginine was added at 5 mM to the electrophoresis buffer only in the experiment for Fig. 3(a). Band shift on the gel was analysed by an FLA-3000G fluoro/image analyser (Fuji Film). In the competition assay, 2.5 μM unlabelled probe was added. Each competition probe was prepared by annealing each complement pair of 35 bp nucleotides, CLWT/CLWT-C and CLMUT/CLMUT-C, by incubation at 95 °C for 5 min, 65 °C for 5 min and 37 °C for 5 min.

Construction of reporter plasmid and α -galactosidase assays.

After pRFUT was digested with *NdeI*/*NotI*, the resulting smaller fragment was ligated with pET26-5714/*Nde*-*Not* (Tsubouchi *et al.*, 2005) previously digested with *NdeI*/*NotI*. An *EcoRI*-*BglII* fragment from the resulting plasmid was cloned into the appropriate portion of pOF5714 (Fridjonsson & Mattes, 2001) to yield pTtRF. *T. thermophilus* cells harbouring reporter plasmids, pTthcs-plp (Tsubouchi *et al.*, 2005) or pTtRF were grown at 60 °C in TM medium supplemented with 1.6 mM CaCl_2 , 1.6 mM MgCl_2 and 50 μg kanamycin ml^{-1} until the mid-exponential phase. Cells were harvested by centrifugation at 15 000 g and washed with MM medium three times. Cells were suspended in MM medium or MP medium containing 50 μg kanamycin ml^{-1} and various additives, and the culture was continued for an additional 24 h. Cells were harvested by centrifugation at 15 000 g for 30 s, washed and suspended in 0.1 M HEPES buffer (pH 7.5). Crude extract was prepared by sonication, and debris was removed by centrifugation at 15 000 g for 10 min. Proteins were determined by the method described above. α -Galactosidase activity was determined by the methods previously described (Tsubouchi *et al.*, 2005), except that 5 mM 4-nitrophenyl α -D-galactopyranoside was used as the

substrate. One unit of activity was defined as the amount of enzyme required to release 1 μmol *p*-nitrophenol min^{-1} , and the specific activity of crude extract was expressed as units $(\text{mg protein})^{-1}$.

DNase I footprinting. DNase I footprinting was performed using non-radioactive probes containing the IRD800 label at the 5'-end. DNA probes were prepared as follows. pHcsUT was used as a template for two PCRs with primers IRD800-labelled T7 promoter primer (MWG-Biotech)/M4p and IRD800-labelled M13-29 primer (Li-Cor)/T7p. These PCRs produced DNA fragments that contain an 188 bp *hcs* promoter region from -73 to +115 with respect to the transcriptional start point. The DNA fragment also contains an approximately 80 bp or 60 bp extension originating from pT7Blue at the upstream and the downstream ends, respectively, at which the IRD800 label was attached. After purification by Microspin S-400 HR columns (GE Healthcare Bioscience), 50 ng of the resulting DNA was used for DNase I footprinting. Binding of ArgR (0.06–7 μM) to the DNA fragment was performed as in the EMSA experiments (see above) with minor modification: the total volume was changed to 50 μl , poly(dI-dC) was omitted, and the arginine concentration was set to 10 mM. DNase I cleavage was done by adding 3 μl of a solution containing 160 mM MgCl_2 and 0.25 units DNase I. After 1 min incubation at room temperature, the DNase I reaction was stopped by addition of 50 μl 5 M ammonium acetate and 30 mM EDTA. The DNA was extracted with 100 μl phenol and 100 μl chloroform, precipitated with ethanol, and washed with 70 % ethanol. The pellet, dissolved in 1 μl of formamide loading buffer, was heated at 95 °C for 10 min, rapidly chilled on ice, and applied to a Li-Cor automated DNA sequencer (model 4000L) using a 6 % KBPlus 41 cm denaturing sequence gel (Li-Cor) at 50 °C.

RESULTS

Identification of TTC1194 as the *argR* gene of *T. thermophilus*

The transcriptional regulation of arginine biosynthesis is highly conserved in bacteria: a repressor called ArgR interacts with a specific operator sequence (called the ARG box) in the promoter region (Charlier, 2004; Czaplewski *et al.*, 1992; Dimova *et al.*, 2000; Dion *et al.*, 1997; Larsen *et al.*, 2004, 2005; Lim *et al.*, 1987; Lu *et al.*, 1992; Morin *et al.*, 2003; Tian *et al.*, 1994; Xu *et al.*, 2003). For *T. thermophilus*, Sanchez *et al.* (2000) previously suggested that the *argF* promoter is regulated by ArgR. For the initial investigation, we aligned the sequences between the *argF* promoter region containing the ARG box and the *hcs* promoter region (Fig. 2a). The result of the alignment suggested the existence of an ARG box in the *hcs* promoter region, suggesting that the *hcs* promoter is regulated by ArgR. The whole genome sequence determined for *T. thermophilus* HB27 (Henne *et al.*, 2004) indicates that *T. thermophilus* possesses only a single homologue of *argR* as TTC1194. We have already cloned a DNA fragment containing the *argR* homologue of *T. thermophilus* HB27 as a different research project, and therefore used the gene for the following expression experiment.

We constructed a plasmid for the expression of the *argR* homologue as a COOH-terminally His₈-tagged protein. Ten milligrams of the recombinant ArgR homologue was prepared through purification using Ni-NTA resin from

Fig. 2. ArgR binds to the *argF* promoter DNA. (a) Alignment of the *hcs* -10 promoter region and a putative ARG box in the *argF* -35 promoter region. The *hcs* promoter -10 region and *argF* promoter -35 regions are indicated. Identical nucleotides are boxed. (b) SDS-PAGE of purified ArgR. Lane 1, purified ArgR; lane 2, molecular mass markers: phosphorylase *b* (97.0 kDa), bovine serum albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), soybean trypsin inhibitor (20.1 kDa) and cytochrome *c* (14.4 kDa). (c) EMSA of ArgR for *argF* promoter of 290 bp from *T. thermophilus*. The concentrations of purified ArgR protein added are: lane 1, 0 nM; lane 2, 12 nM; lane 3, 25 nM; lane 4, 50 nM; lane 5, 100 nM; lane 6, 200 nM; lane 7, 400 nM. The positions of free DNA (F) and bound DNA (B) are indicated. The assay was performed without arginine in the electrophoresis buffer and the acrylamide gel.

1 l of culture with over 95 % purity on SDS-PAGE (Fig. 2b). We carried out gel filtration to estimate the quaternary structure of the ArgR homologue using a column equilibrated with ST buffer with or without 5 mM arginine. The *argR* homologue encodes an 18 kDa protein with 164 amino acid residues. According to the elution volume, the ArgR homologue has a molecular mass of 60 kDa without arginine, suggesting the configuration of a trimer; however, in the presence of 5 mM arginine the ArgR homologue was eluted in the fraction with a molecular mass of 100 kDa. This result shows that arginine affects the quaternary structure of the ArgR homologue. ArgR proteins, such as AhrC from *Bacillus subtilis* (Holtham *et al.*, 1999) and ArgRs from *Bacillus stearothermophilus* (Dion *et al.*, 1997) and *Thermotoga neapolitana* (Dimova *et al.*, 2000) are isolated as trimers and are in rapid equilibrium with hexamers. The oligomeric state of some

Fig. 3. Specific binding of ArgR protein to the *hcs* promoter -10 region. (a) EMSA in the presence or absence of arginine in the electrophoresis buffer. Lanes 1–10, 0 mM arginine; lanes 11–19, 5 mM arginine. Concentrations of purified ArgR protein added are: lanes 1 and 11, 0 nM; lanes 2 and 12, 0.8 nM; lanes 3 and 13, 1.5 nM; lanes 4 and 14, 3 nM; lanes 5 and 15, 6 nM; lanes 6 and 16, 12 nM; lanes 7 and 17, 24 nM; lanes 8 and 18, 48 nM; lanes 9 and 19, 96 nM; lane 10, 196 nM. (b) Schematic representation of the mutated positions (dotted boxes). Sequences of DNAs added as competition probes are underlined. A putative ARG box is shown by a solid box. (c) EMSA with wild-type DNA region -72 to +101 (+1 represents the *hcs* transcriptional start point) (lanes 1–4) or the 4 bp-mutated form of this region (lanes 5–8) as the probe. ArgR concentrations are 0 nM for lanes 1 and 5, 40 nM for lanes 2 and 6, 100 nM for lanes 3 and 7, and 400 nM for lanes 4 and 8. The positions of free DNA (F) and bound DNA (B) are indicated. (d) EMSA in the presence of competitor oligonucleotides. Additions are: lane 1, none; lane 2, ArgR; lane 3, 2500-fold excess of unlabelled 35 bp wild-type fragment; lane 4, 2500-fold excess of the unlabelled 35 bp mutated fragment. ArgR concentrations were 0 nM for lane 1 and 100 nM for lanes 2–4. Only the positions of free DNA (F) and fast-migrating complexes of DNA (B) are indicated, because slow-migrating complexes are not observed at 100 nM ArgR as shown in (c) (lane 3).

ArgR proteins might be concentration dependent and affected by the presence of arginine and/or target DNA. Binding of six arginine molecules per hexamer has been shown for the *E. coli* and *B. stearothermophilus* ArgR proteins by X-ray crystallographic analysis (Ni *et al.*, 1999; Van Duyne *et al.*, 1996). Therefore, we assume that the ArgR homologue from *T. thermophilus* is as the hexamer in the arginine-bound form.

To examine binding of the ArgR homologue to the *argF* promoter *in vitro*, EMSA was performed with DNA fragments covering the *argF* promoter as the probe. The ArgR homologue was able to shift the target probe DNA in a dose-dependent manner with a K_d of approximately 30–50 nM (in monomer equivalent) (Fig. 2c). Then, to confirm that the *argR* homologue is involved in arginine regulation *in vivo*, we constructed an *argR* homologue knockout mutant (named WRK07) of TSU130, and used it as the host for the reporter assay. In TSU130 harbouring pTtRF, which carries the α -galactosidase gene under the control of the *argF* promoter, α -galactosidase activity was

decreased by the addition of 0.5 mM arginine. In the *argR* knockout mutant WRK07, however, 50-fold increased α -galactosidase activity that was not affected by the addition of 0.5 mM arginine was detected (Table 2). Similar deregulated overexpression was observed for arginine regulons in *argR* mutants of *E. coli* and *Lactococcus lactis* (Larsen *et al.*, 2004; Tian *et al.*, 1994). Thus, the *argR* homologue is shown to be an *argR* orthologue. We hereafter call the gene *argR*. The ArgR protein from *T. thermophilus* shows identity in amino acid sequence to the orthologues of *E. coli*, *B. stearothermophilus*, *B. subtilis* and *Thermotoga neapolitana* of 30.2, 43.3, 41.5, and 38.8 %, respectively.

ArgR binds to the *hcs* promoter region

ArgR identified above was used for EMSA for the *hcs* promoter region. EMSA clearly showed that ArgR binds to the *hcs* promoter region (Fig. 3a). The addition of higher concentrations of ArgR caused the formation of a more slowly migrating complex that might correspond to the hexamer–operator complex, whereas the faster-migrating

Table 2. Reporter assay for the *argF* promoter

Strain	Genotype	Supplement in MM medium	α -Galactosidase specific activity* [mU (mg protein) ⁻¹]
TSU130	Δ <i>agaT</i>	None	2.7 $\pm$ 0.2
		0.5 mM arginine	1.6 $\pm$ 0.2
WRK07	Δ <i>agaT</i> Δ <i>argR</i>	None	132.4 $\pm$ 10.3
		0.5 mM arginine	123.0 $\pm$ 22.1

*Means $\pm$ SD. Three independent experiments were performed.

band might correspond to the trimer-operator complex, as suggested previously by others (Dimova *et al.*, 2000; Morin *et al.*, 2003). When electrophoresis for EMSA was done in electrophoresis buffer containing 5 mM arginine, the slower-migrating band appeared at lower concentrations of ArgR (Fig. 3a). Apparent equilibrium dissociation constants (K_d) were around 30 nM and 100 nM in the presence and absence of 5 mM arginine in the buffer, respectively. Thus, arginine enhances the affinity of ArgR to the target DNA to some extent. In addition, in the presence of arginine, the complex forms a sharper band compared to the broad and diffuse band observed in its absence, suggesting that the complex is more stable in the presence of arginine. To elucidate the specificity of the binding, we prepared DNA fragments with 4 bp substitutions in the putative ARG box around the *hcs* promoter -10 region (Fig. 3b), and used them for EMSA. In contrast with the fragments with a wild-type sequence, the 4 bp-substituted DNA fragment showed lower affinity to ArgR (Fig. 3c). The observation that no fast-migrating band was formed for the mutant operator suggests that ArgR protein may bind to the mutant ARG box stably only in a hexameric form. In any case, it should be noted that ArgR from *T. thermophilus* binds to both *hcs* and *argF* promoters with very similar affinity.

To determine the region of ArgR binding more precisely, a 35 bp DNA fragment around the *hcs* promoter -10 region was used as a competitive probe. The band shifts observed without the competitive probe were diminished by the addition of the 35 bp DNA fragment with the wild-type sequence, whereas the 35 bp DNA fragment with 4 bp mutations had little effect on the shift band of ArgR (Fig. 3d). Furthermore, DNase I footprinting indicated that ArgR protected regions from -23 to -5 for the sense strand and from -19 to -1 for the antisense strand with respect to the transcriptional start point (Fig. 4a, b). These results show that ArgR specifically binds to the *hcs* promoter -10 region. ArgR also protected the upstream region (site B). This will be discussed below.

Arginine regulates the *hcs* promoter *in vivo*

To elucidate the role of ArgR in the *hcs* promoter function, we further investigated the effect of arginine on the *hcs* promoter by reporter gene assay in *T. thermophilus* TSU130.

T. thermophilus TSU130 cells carrying pTthcs-plp exhibited α -galactosidase specific activity of 40.9 $\pm$ 7.9 mU (mg protein)⁻¹. When a similar assay was performed with the

Fig. 4. DNase I footprinting for the *hcs* promoter region. (a) DNase I footprinting. The concentrations of ArgR protein added are: lanes 1 and 6, 0 μ M; lanes 2 and 7, 7.0 μ M; lanes 3 and 8, 1.4 μ M; lanes 4 and 9, 0.28 μ M; lanes 5 and 10, 0.06 μ M. Bars indicate regions protected by ArgR. Dotted lines indicate the sequences derived from pT7Blue. (b) Schematic representation of the regions protected by ArgR (site A). Lower and upper bars indicate regions in the sense strand or antisense strand, respectively, protected by ArgR from DNase I digestion.

argR mutant WRK07, the level of α -galactosidase activity was increased to 95.2 ± 15.2 mU (mg protein) $^{-1}$, and no enhanced expression [activity 113.4 ± 13.5 mU (mg protein) $^{-1}$] was observed by addition of arginine. From these results, it can be concluded that ArgR is involved in the regulation (repression) of *hcs* promoter activity.

When 0.1 mM arginine was added to the culture of *T. thermophilus* TSU130 carrying pTthcs-plp, the response to arginine varied, with large deviation (data not shown). This observation suggests that a subtle change in culture conditions affects the *hcs* promoter in *T. thermophilus* TSU130. Considering that arginine degradation in cells might be the possible main reason for the large deviation of the response, we constructed an arginase gene knockout mutant, *T. thermophilus* ADK1, of *T. thermophilus* TSU130 and used it as the host for the same reporter assay. As expected, in the arginase-minus genetic background, a reproducible response to arginine was observed. In ADK1, with the knockout of the arginase gene, the α -galactosidase activity [36.6 ± 1.2 mU (mg protein) $^{-1}$] obtained in the absence of arginine was decreased threefold [to 11.9 ± 3.4 mU (mg protein) $^{-1}$] by addition of arginine. On the other hand, when a different mutant, ADK2, defective in both *argR* and arginase genes was constructed and used as the host for the reporter assay, α -galactosidase activity of 113.2 ± 16.5 mU (mg protein) $^{-1}$ was obtained in the absence of arginine. Addition of arginine did not decrease α -galactosidase activity but increased the activity twofold, to 234.2 ± 10.4 mU (mg protein) $^{-1}$.

Growth inhibition of the arginase knockout mutant by arginine

The above results indicate the involvement of ArgR in *hcs* promoter activity. These results also suggest that *T. thermophilus* may show a lysine-auxotrophic phenotype when arginine is added to the culture, due to repression of *hcs* promoter activity. To examine this possibility, growth of *T. thermophilus* TSU130, ADK1 and ADK2 was monitored.

Fig. 5. Growth inhibition of the arginase-knockout mutant by arginine. Growth curves are shown for the wild-type TSU130 (○) and the arginase-deficient mutant ADK1 (●) in MP medium. Growth of ADK1 in MP medium supplemented with 0.1 mM arginine (□), both 0.1 mM lysine and 0.1 mM arginine (△), both 0.1 mM ornithine and 0.1 mM arginine (■), and all three amino acids (lysine, arginine and ornithine, 0.1 mM each) (▲) is also shown.

As expected, the growth of the arginase mutant *T. thermophilus* ADK1 was inhibited by the addition of arginine to the minimal medium (Fig. 5), which contrasted with the cases of the wild-type strain *T. thermophilus* TSU130 and the mutant ADK2, defective in both arginase and *argR* genes, where no inhibition by arginine was observed (Table 3). Ornithine is an intermediate of arginine biosynthesis, and its synthesis is therefore controlled by arginine. Furthermore, ornithine is a peptidoglycan component in *T. thermophilus* (Quintela *et al.*, 1995). We anticipated that an increase in the intracellular arginine pool could activate the ArgR function to repress the arginine biosynthetic gene expression and, as a result, lead to the short supply of ornithine as a cell wall component. We therefore added 0.1 mM ornithine to MP medium, and examined its effect on the growth of the arginase mutant ADK1; however, it had only a slight effect on growth

Table 3. Generation time of ADK1 in MP medium supplemented with arginine, ornithine, and/or lysine

Strain	Genotype	Addition (0.1 mM)	Generation time (min)
TSU130	Δ <i>agaT</i>	None	139
		Arginine	106
ADK1	Δ <i>agaT</i> Δ <i>arginase</i>	None	160
		Arginine	> 300*
		Arginine, ornithine	> 300*
		Arginine, lysine	191
		Arginine, lysine, ornithine	146
ADK2	Δ <i>agaT</i> Δ <i>arginase</i> Δ <i>argR</i>	None	160
		Arginine	158

*Generation times of ADK1 in the presence of arginine, or arginine plus ornithine, could not be determined due to the extremely slow growth.

(Fig. 5). We next examined the growth of ADK1 in MP medium supplemented with 0.1 mM lysine or both 0.1 mM lysine and 0.1 mM ornithine. The slow growth of ADK1 was partially restored by the addition of 0.1 mM lysine, and fully restored by the addition of both 0.1 mM lysine and 0.1 mM ornithine. When generation times were compared, addition of arginine prolonged the generation time of ADK1 significantly (Table 3). The prolonged generation time was partially shortened by addition of lysine and completely normalized by simultaneous addition of lysine and ornithine. On the other hand, the generation time of ADK2, with the knockout of both *argR* and arginase genes, was not affected by arginine. These results also support the idea that ArgR regulates lysine biosynthesis *in vivo*.

DISCUSSION

In this study, we characterized ArgR as a transcriptional regulator for lysine biosynthesis in *T. thermophilus*. ArgR from *T. thermophilus* was purified as a trimeric protein, and shown to become a hexamer in the presence of arginine. As shown in Fig. 3(a), the bands in the EMSA were somewhat broad and smeary in the absence of arginine, and addition of arginine in the electrophoresis buffer gave clear band profiles. Thus, arginine may assure the formation of a stable ArgR–DNA complex. However, apparent *K*_d was not so significantly affected by arginine (30 nM and 100 nM in the presence or in the absence of arginine, respectively). Recently Charlier and colleagues classified arginine regulators into three major types (Charlier, 2004; Morin *et al.*, 2003). Among them, ArgR from *Thermotoga*, which is the only ArgR classified into class III, is defined as serving as a general regulator exhibiting broad target specificity and low arginine-dependent activation of DNA-binding capacity. In *T. thermophilus* ArgR, a Gln residue, which is highly conserved in class I ArgR and involved in the highly arginine-dependent activation of DNA-binding ability, is replaced by Ser (Fig. 6). Substitution of Gln for the Ser of ArgR from *T. neapolitana* resulted in a mutant protein exhibiting a marked need for arginine as co-factor and an enhanced target specificity (Morin *et al.*, 2003). It is also shown that the *T. thermophilus* ArgR is different from other class ArgR proteins in that it can interact with a single ARG box sequence. Arg from *T. thermophilus* also has a serine residue at the corresponding position and shows low arginine-dependent activation of DNA-binding capacity. The observation that the band shift in EMSA was diminished by addition of a short competitor of 35 bp having only a single ArgR-binding site around the *hcs*

promoter –10 region may also suggest that ArgR interacts with a single ARG box sequence. DNase I footprinting indicates that ArgR binds to the *hcs* promoter –10 region (site A) as expected. The analysis revealed that another region (site B) was also protected by ArgR (Fig. 4a, b). However, the protection was found at a region connecting the *hcs* promoter upstream sequence with the cloning site of the vector pT7Blue. Actually, only a small portion, from –73 to –68 in the sense strand and from –73 to –70 in the antisense strand, was protected for the *hcs* promoter upstream region, and the other protection was found in the vector region. This observation indicates that the putative ArgR-binding site in the *hcs* promoter upstream region is artificially generated in our DNase I footprinting experiment. Although the DNase I footprinting could not rule out the possibility that ArgR may bind to other regions which were not tested in this study, our results are consistent with there being a single ARG box covering the *hcs* promoter –10 region, which regulates *hcs* promoter activity. From these observations, we may conclude that ArgR of *T. thermophilus* is a class III regulator.

The addition of arginine inhibited the growth of the arginase gene knockout mutant ADK1 (Fig. 5). Addition of lysine partially overcomes the growth inhibition by arginine, and the simultaneous addition of both lysine and ornithine restores the growth of ADK1 completely, even in the presence of arginine (Fig. 5). It could be that addition of arginine to ADK1 causes diversion of the excess arginine to a pathway that produces a toxic compound and that this pathway is repressed by lysine. However, in both the wild-type and the double mutant, ADK2, defective in arginase and *argR*, arginine inhibition was not observed (Table 3). The most likely explanation of these phenomena is that the growth inhibition by arginine in the arginase mutant is attributable to the function of ArgR, therefore suggesting that lysine biosynthesis as well as ornithine synthesis is regulated by ArgR in *T. thermophilus*. Lysine biosynthesis through AAA is also found in fungi, and AAA synthesis from 2-oxoglutarate proceeds in the same way in fungi and *T. thermophilus*; however, subsequent synthesis, from AAA to lysine, is totally different between these organisms. The *T. thermophilus* pathway is similar to a step in arginine biosynthesis, while the pathway in fungi starts from adenylation of the α-amino group of AAA and contains saccharopine as an intermediate. It should be noted that similar growth inhibition by arginine has been reported in an arginase mutant of the fungus *Neurospora crassa*, but inhibition is restored by only ornithine (Davis *et al.*, 1970).

			Class
<i>Escherichia coli</i>	94	AVVVIHTSPGAAQLIARLLDSL	115
<i>Moritella profunda</i>	92	CLIVIQTSPGSAQLIARLLDSL	113
<i>Bacillus subtilis</i>	92	HMIVLKTMPGNAQAIGALMDNL	113
<i>Streptomyces coelicolor</i>	118	NLVVLRTFPGAACFLASAIQDA	139
<i>Thermotoga maritima</i>	95	NLIVIKTIPGTASGIARVIDRL	116
<i>Thermus thermophilus</i>	94	NLLVVKTAEGHASGIAYLLDRL	115

Fig. 6. Partial alignment of ArgR amino acid sequences in three classes. Glutamine residues conserved in class I and II ArgR proteins and serine residues at the corresponding position in class III ArgR proteins are boxed.

This suggests the presence of a different regulatory system in *Neurospora*, although it should be taken into account that the fungus has compartmentalization and a specific transport system, and therefore the situation may be totally different.

By EMSA and DNase I footprinting, we demonstrated that ArgR specifically bound to the DNA fragment containing the *hcs* promoter –10 region (Fig. 3). For further elucidation of the role of ArgR in *hcs* promoter regulation *in vivo*, reporter gene assays were carried out. In ADK1, with the knockout of the arginase gene, the addition of arginine repressed the *hcs* promoter. In ADK2, with the additional mutation of *argR*, no repression was observed, whereas the addition of arginine activated the *hcs* promoter. In WRK07, with an *argR* mutation, no response to arginine was observed. These results may indicate not only the involvement of ArgR in the regulation of lysine biosynthesis in *T. thermophilus*, but also the presence of some other regulatory systems that sense arginine or its metabolites to activate lysine biosynthesis. As described in Results, the involvement of ArgR in the regulation of the *hcs* promoter was not verified in the parent strain of *T. thermophilus*; in some cultures, arginine seemed to enhance α -galactosidase expression, while it decreased the expression in others. Since such a variable effect was not observed with the addition of other amino acids in the reporter assay, we assume that there are multiple systems to sense arginine and/or its metabolites, and therefore arginine might play a central role in the metabolic flux of related amino acids in *T. thermophilus*. We have very recently found a transcriptional factor, which also regulates lysine biosynthesis, and are analysing its regulatory mechanism (unpublished results). Through detailed research of at least two transcriptional factors, regulatory mechanisms for lysine biosynthesis should be elucidated in the near future.

The lysine biosynthesis of *T. thermophilus* has an evolutionary relationship with the biosynthesis of arginine and leucine, and the TCA cycle (Nishida *et al.*, 1999). Furthermore, the fact that a similar lysine biosynthesis pathway is also present in archaea (Brinkman *et al.*, 2002; Lombo *et al.*, 2004; Nishida *et al.*, 1999), which are phylogenetically positioned close to the root of the universal tree of evolution (Woese *et al.*, 1990), suggests that lysine biosynthesis was present in the earliest organisms. We may assume that the ancestral enzymes involved in the processes related to current *Thermus* lysine biosynthesis functioned in lysine and arginine biosynthesis as well as the TCA cycle. In previous studies, we have shown that *T. thermophilus* lysine biosynthetic enzymes have the ability to recognize intermediates of the TCA cycle and arginine biosynthesis as substrates, suggesting that the enzymes have additional roles in those metabolisms even now. It is of interest that an enzyme involved in lysine biosynthesis may play a role in biosynthesis of another basic amino acid, arginine; however, there are other reported cases of such functional overlap. Ledwidge & Blanchard (1999) showed that the *argD* gene

encoding *N*-acetylornithine aminotransferase in the arginine biosynthesis pathway is the most probable candidate for *dapC* gene encoding *N*-succinyl-L,L-diaminopimelate:2-oxoglutarate aminotransferase in a major root (DAP pathway) of the lysine biosynthesis pathway in *E. coli*. The *argD* gene is regulated by ArgR (Charlier *et al.*, 1992). However, it is unclear whether this gene is regulated by lysine. In this study, we showed that ArgR, a repressor of arginine biosynthesis, has the ability to regulate lysine biosynthesis in *T. thermophilus*, although the regulation is apparent in an arginase gene knockout background. Cumulative data on the expression profile of lysine biosynthetic and related genes of *T. thermophilus* will elucidate the complicated regulatory mechanisms managed by multiple transcriptional machinery.

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