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# Role of *galE* on biofilm formation by *Thermus* spp.

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ABSTRACT

*Thermus thermophilus* and *Thermus aquaticus* are thermophilic bacteria that are frequently found to attach to solid surfaces in hot springs to form biofilms. Uridine diphosphate (UDP)-galactose-4′-epimerase (GalE) is an enzyme that catalyzes the conversion of UDP-galactose to UDP-glucose, an important biochemical step in exopolysaccharide synthesis. We expressed GalE obtained from *T. thermophilus* HB8 in *Escherichia coli* and found that the enzyme is stable at 80 °C and can epimerize UDP-galactose to UDP-glucose and UDP-*N*-acetylgalactosamine (UDP-GalNAc) to UDP-*N*-acetylglucosamine (UDP-GlcNAc). Enzyme overexpression in *T. thermophilus* HB27 led to an increased capacity of biofilm production. Therefore, the *galE* gene is important to biofilm formation because of its involvement in epimerizing UDP-galactose and UDP-*N*-acetylgalactosamine for exopolysaccharide biosynthesis.



## Background

Bacteria from the genus *Thermus* are often found in hot springs. Several studies have been carried out on *Thermus thermophilus* strains HB8 and HB27, and their genomes have been sequenced [1]. The optimum growth temperature of this species is 50–80 °C. These bacteria often produce yellow pigment and form biofilms in hot springs [2,3]. A biofilm is a community of bacteria surrounded by an extracellular matrix containing bacterial exopolysaccharide (EPS) [4]. Therefore, studying the composition of EPS is important for understanding biofilm formation [5]. Previous studies have shown that the EPS components colanic acid and alginate found in *Escherichia coli* [6] and *Pseudomonas aeruginosa*, respectively, are crucial for biofilm formation [5]. Moreover, uridine diphosphate (UDP)-galactose, UDP-glucose, and dTDP-rhamnose are important intermediates in the EPS biosynthesis pathways [7]. In most microorganisms, galactose is converted to glucose via the Leloir pathway, which involves three enzymes: galactokinase (GalK), galactose-1-P uridyltransferase (GalT), and UDP-galactose 4′-epimerase (GalE) [8]. The initial phosphorylation of galactose to galactose-1-P is catalyzed by GalK. Galactose-1-P is then converted to glucose-1-P by GalT with a cofactor, UDP-glucose. The conversion of UDP-glucose to UDP-galactose is catalyzed by GalE [8,9]. Analysis of the genomes of the *T. thermophilus* strains HB8 and HB27revealed that *galE* and *galK* are not present in the same operon but are located adjacent to each other on the chromosome. Further, *galT* is encoded by a plasmid, pTT27 [1]. The genes responsible for galactose metabolism are clustered in an operon arranged in different orders in *Thermoanaerobacter tengcongensis* [10], *E. coli* [11], *Lactobacillus casei*, *Lactococcus lactis* [12], and several other lactic acid bacteria such as *Streptococcus thermophilus* [13,14]. However, the *galT* gene of *Acidithiobacillus ferrooxidans* does not coexist in the same operon of the *galEK* gene [15], and the *galE* gene of *Campylobacter jejuni* is present in the same operon of *wlaBCDEG* for polysaccharide synthesis but not with the other genes of the Leloir pathway [16,17]. On the other hand, in *Neisseria meningitidis* [18] and *Neisseria gonorrhoeae* [19], which do not use galactose as a carbon source, the *galE* gene of the Leloir pathway is preserved only for EPS and lipopolysaccharide (LPS) production. Consequently, the exact mechanism of regulation of gene expression in *T. thermophilus* remains unknown.

Previous studies have shown that a *galE* mutant of *L. lactis* was unable to grow in galactose-containing minimum medium. Further, this mutant formed long chains of at least 50 cells each, leading to substantially reduced growth rate when cultured on media containing glucose or maltose as the sole carbon source [12]. In another study, a *galE* mutant of *Bradyrhizobium japonicum* inoculated into soybeans exhibited an altered lipopolysaccharide profile and reduced nodule formation ability [20]. In the dental plaque-forming bacterium *Porphyromonas gingivalis*, *galE* mutation decreased

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the length of the O antigen but led to an increase in the biofilm-forming ability [21,22]. Furthermore, a *galE* mutant in *Vibrio cholerae* led to a reduction in biofilm-forming ability by at least five times that of the wild-type strain [23]. In the present study, we show the importance of GalE in biofilm formation and demonstrate that overexpression of GalE increases the biofilm-forming ability of *T. thermophilus* HB27.

## Materials and methods

*Bacteria and growth conditions. Escherichia coli* strains were cultured at 37 °C in Luria broth (LB) or on plates containing antibiotics such as ampicillin (50 μg/ml) for selection. pGEM plasmids (Promega, WI, USA) were used for genetic cloning, and recombinant GalE overexpression was studied using pQE60 (Qiagen, Hilden, Germany). *Thermus* spp. were cultured at 60 °C in modified *Thermus* medium as described previously [24]. *Thermus* plates were supplemented with 2% agar to prolong the incubation period at high temperatures. The pWUR112/77-1 plasmid bearing thermostable bleomycin-resistant marker was a gift from Brouns and colleagues [25]. Zeocin, which was obtained from Invitrogen (CA, USA), was used as a selection antibiotic at a final concentration of 25 μg/ml for both pWUR112/77-1-containing *E. coli* and *Thermus* spp.

*Construction of galE gene expression plasmid.* All genetic manipulation was performed according to standard protocols or according to

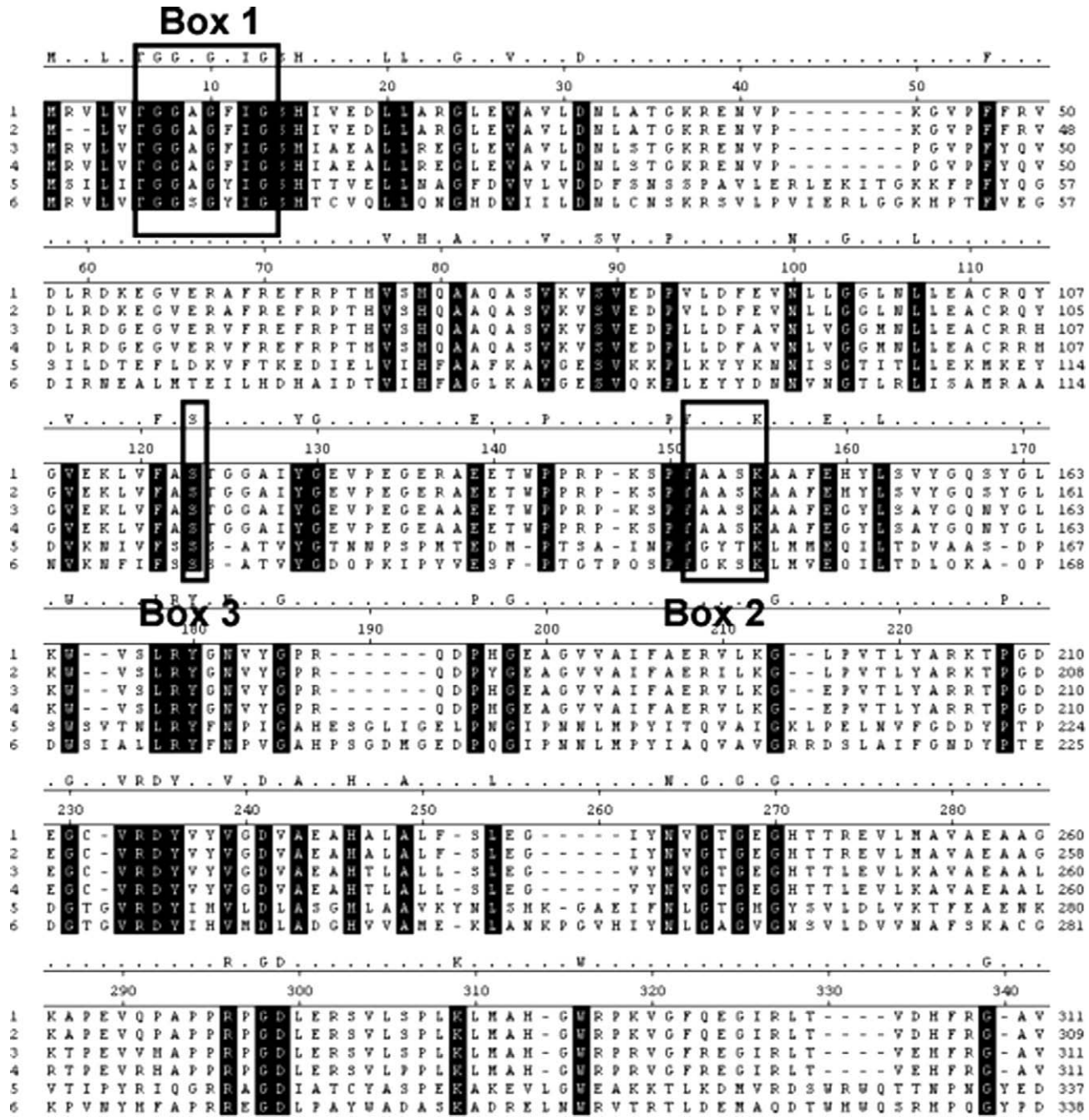


**Fig. 1.** Multiple sequence alignments of UDP-glucose-4′-epimerase. GalE proteins obtained from the following microorganisms: (1) *T. thermophilus* HB8, (2) *T. thermophilus* HB27, (3) *T. aquaticus* YT-1, (4) *T. aquaticus* NTU103, (5) *Streptococcus thermophilus*, and (6) *E. coli*. K-12. Residues conserved in all of the sequence are shaded in black. Box 1, GxxGxxG motif; Box 2, serine residue responsible for proton transfer; Box 3, Y-xxx-K played a role in UDP-glucose or UDP-galactose binding.

the manufacturer's instructions. Plasmid isolation and chromosomal DNA kits were purchased from Qiagen (Hilden, Germany), and restriction enzymes were purchased from New England Biolabs (MA, USA). The total DNAs of *T. thermophilus* HB8, *T. thermophilus* HB27, *Thermus aquaticus* YT-1, and *T. aquaticus* NTU103 were isolated for use as templates for PCR amplification of *galE* genes from the individual strains. Primers for *galE* (*TTHA0591*) construction were designed according to the published sequences of *T. thermophilus* HB8. Primers TTHA0591-F (5′-ATCCATGGGCGTGCTGGTGA CGGGCG-3′) and TTHA0591-R (5′- ATGGATCCTACGGCGCCCCGGAA CTA-3′) were used for gene cloning, where underlines denote the sites for restriction enzymes. Polymerase chain reaction was performed for a total of 30 cycles at 94 °C for 1 min, 50 °C for 30 s, and 72 °C for 90 s. The PCR product of *galE* from *T. thermophilus* HB8 was digested by the restriction enzymes NcoI and BamHI and cloned into the same restriction sites of pQE60 to obtain the pMG0616 plasmid for protein overexpression. The *galE* genes obtained from different *Thermus* spp. were also amplified by the same primer but cloned into pGEM3Zf (−) (Promega, WI, USA) for sequences analysis.

*Recombinant GalE purification.* The *galE*-containing plasmids pQE60 and pMG0616 were transformed into M15 (pRep4) (Qiagen, Hilden, Germany) for protein expression to obtain sufficient amount of recombinant GalE protein. Recombinant GalE was induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). For protein purification, overexpressed cells were suspended in 4 ml of 1× binding buffer containing 5 mM imidazole, 0.5 mM NaCl, and 20 mM Tris–HCl (pH 8.0) and treated with three cycles of freezing and thawing at −80 °C and room temperature, respectively. The thawed cells were sonicated at 0 °C for 60 cycles of 5 s each with 10-s intervals with an output control setting of 3 with a sonicator (Microson ultrasonic cell disruptor, Misonix, NY, USA). The cell extract was then separated from the cell debris by centrifugation at 17,000*g* for 30 min at 4 °C (Avanti J-25 Centrifuge, JA25.5 rotor, Beckman, CA, USA). The GalE-containing cell extract was then purified by Ni-affinity chromatography (Novagen, WI, USA). Purified fractions were analyzed by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and stained with Coomassie blue G250.

*Enzymatic analysis of GalE.* To perform the enzymatic activity of UDP-galactose-4′-epimerase, the coupled method was employed, as described previously [26,27]. Purified protein (1 μg) was added to 600 μl of final reaction volume containing 100 mM glycine–NaOH (pH 8.9), 4 mM UDP-galactose, 1 mM β-$NAD^+$, 8.3 mM $MgCl_2$, and 5.4 U of UDP-glucose dehydrogenase (Sigma, MO, USA). The reaction was initiated by adding UDP-galactose to the reaction mixture and the increase in absorbance was measured at $A_{340}$ using a temperature control spectrophotometer (Genesys 10 UV, Thermo Spectronic, Garfoth, UK). NADH production was cal-

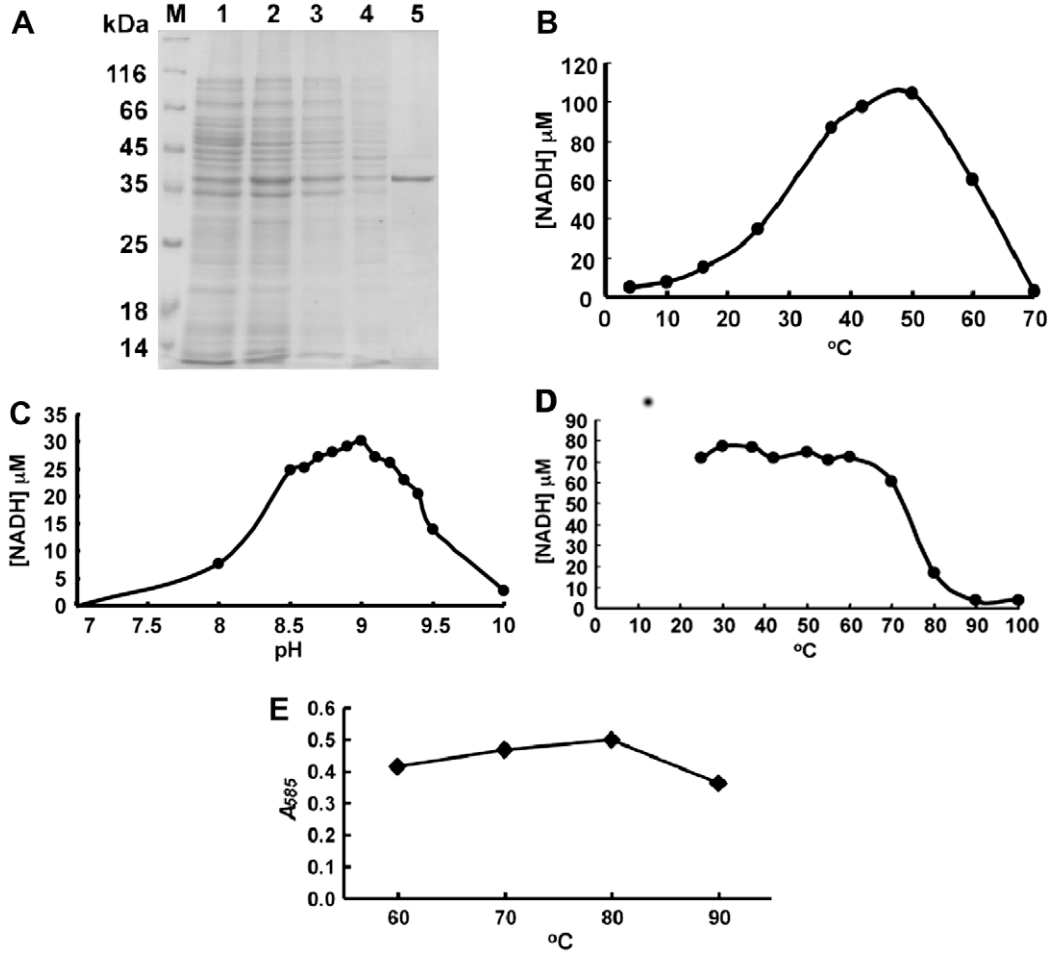


**Fig. 2.** Properties of GalE. (A) Expression and purification of His-tagged recombinant GalE. Cell extracts obtained from cells before (lane 1) and after (lane 2) 1 mM IPTG induction and proteins washed by binding buffer (lane 3), washing buffer (lane 4), and eluted by elution buffer (lane 5) were analyzed in 12% SDS–PAGE and stained using Coomassie blue G250. The sizes of the molecular mass markers (M) are shown to the left of the gel. (B) Optimum reaction temperature, (C) pH, and (D). Stability of GalE at different temperatures for 30 min before enzymatic analysis. (E) Stability of GalE at different temperatures for 10 min before enzymatic analysis by the Morgan-Elson method. A total of 1 μg purified GalE was applied for every reaction.

culated by the molar extinction coefficient ($E_{340}$) of 6220 (molar absorption coefficient of NADH) $M^{-1}$ $cm^{-1}$.

Subsequently, another method modified from the Morgan-Elson reaction, which is a colorimetric assay for the production of GlcNAc, was performed to confirm the enzymatic activity of UDP-*N*-acetylglucosamine-4′-epimerase. Purified GalE (1 μg) was added to 0.5 ml of reaction mixture with 10 mM glycine, 1 mM $MgCl_2$, 0.1 mM ethylenediamine tetraacetic acid (EDTA), and 0.1 mM UDP-GalNAc. The reaction was initiated after UDP-GalNAc was added to the mixture and incubated for 5–10 min. The reaction was then stopped by the addition of 0.8 μl concentrated HCl. This enzymatic reaction was performed by measuring the colorimetric changes at $A_{585}$ using a spectrophotometer [11,28].

*Biofilm formation assays and observation of biofilm architecture.* Biofilm formation was quantitatively analyzed in 96-well microtiter plates as described previously [29]. *Thermus* spp. was cultured in 3 ml *Thermus* medium at 60 °C for 12 h. Subsequently, bacteria were subcultured into 96-well plates (TPP, Denmark) with an initial optical density less than 0.6 and subcultured with serial dilutions of up to 10-fold. After culturing for different periods of time, the bacteria were stained with 0.1% crystal violet for 10 min at room temperature. The cells were then washed three times with distilled water, and the stained cells attached to the microtiter plates were dissolved using 95% alcohol and incubated for another 15 min. The optical density was determined by a spectrophotometer at $A_{595}$. Biofilm architecture was also observed using an atomic force microscope (AFM). Bacteria were cultured on glass slides in a six-well microtiter plate (TPP, Denmark) at 60 °C for 3 h. The glass slides were washed two times with distilled water and dried overnight in a moister buster cabinet prior to atomic force microscopy. A NanoWizardII AFM (JPK Instruments, Germany) controlled in contact mode in air was used to capture the image of biofilm architecture. The nanoprobe cantilevers were made of silicon nitride ($Si_3N_4$) with a spring constant of $k$ = 0.58 N/m (DNP-S20, Veeco Instruments Inc., NY, USA). The topographic images were analyzed by NanoWizardII image processing software (version 3.1) with AFM image resolution of 512 × 512 pixels [30].

## Results and discussion

### *Comparison of amino acid sequences of GalE from four Thermus strains*

The *galE* genes from *T. thermophilus* HB8, *T. thermophilus* HB27, *T. aquaticus* YT-1, and *T. aquaticus* NTU103 were amplified by PCR and cloned in pQE60 and pGEM3Zf(−). Amino acid sequence comparisons showed that the GalE sequences of *T. thermophilus* strains HB8 and HB27 were 99.4% identical and those of *T. aquaticus* strains NTU103 and YT-1, 99% identical. Further, the GalE sequences of *T. thermophilus* HB8 and *T. aquaticus* NTU103 were 88.4% identical and those of *T. aquaticus* NTU103 and *E. coli* K12 were 33.5% identical. The crystal structures of GalE from *E. coli* [31], yeasts [32], and humans [33] indicated that GalE is a homodimer coupled with an $NAD^+$ cofactor. These enzymes contain a GxxGxxG motif (Box 1) for binding to $NAD^+$ [34]. In the GalE enzyme obtained from *E. coli*, Ser-124 (Box 2) is involved in proton transfer and the Tyr149-xxx-Lys153 (Box 3) motif is responsible for UDP-glucose or UDP-galactose binding [35]. These sequences and motifs are located in the *T. thermophilus* HB8 GalE regions Ser-117 and Tyr142-xxx-Lys146, respectively (Fig. 1).

### *Overexpression of GalE from T. thermophilus HB8 in E. coli*

*Escherichia coli* M15 (pMG0616) was cultured in LB medium to the mid-log phase. The expression of histidine-tagged GalE (GalE-His) from pMG0616 was induced by IPTG treatment. Approximately 8 mg of GalE-His was purified from 500 ml of culture using Ni–NTA affinity chromatography (Fig. 2A). MALDI-TOF mass spectrometry verified that the 35-kDa band in the SDS–PAGE gel loaded with the proteins purified by Ni–NTA affinity chromatography was indeed *T. thermophilus* GalE.

### *Dual functions of recombinant GalE*

Enzymatic function of recombinant GalE was carried out by the coupling method as reported previously [26,27]. The enzyme had an optimal temperature of 50 °C (Fig. 2B) and pH of 9.0 (Fig. 2C). The optimal temperature for *T. thermophilus* HB8 GalE is higher than that of *Aeromonas hydrophila* [28]. Under these conditions, enzyme kinetic analysis was performed with 3 nmol of GalE protein and using UDP-galactose (UDP-Gal) as a substrate. The results showed that the enzyme has a $k_m$ of 258 μM, $k_{cat}$ of 3.319 ($min^{-1}$), and $k_{cat}$ ($min^{-1}$)/$k_m$ (mM) of 0.013. Since UDP-glucose dehydrogenase is necessary for converting UDP-glucose (UDP-Glc) to UDP-glucuronate to generate NADH for colorimetric measurement by $OD_{340}$, this was used as the limiting step for enzymatic assay. To test the stability of GalE under different temperatures, we treated this enzyme at different temperatures for at least 30 min. Treatment at 80 °C for 30 min reduced the enzyme activity by 78% (Fig. 2D). In comparison, the activity of *A. hydrophila* GalE was completely destroyed after treatment at 60 °C for 30 min [28], showing that despite the low degree of sequence homology between the enzymes from *A. hydrophila* and *T. thermophilus*, the two enzymes show distinctive heat stability.

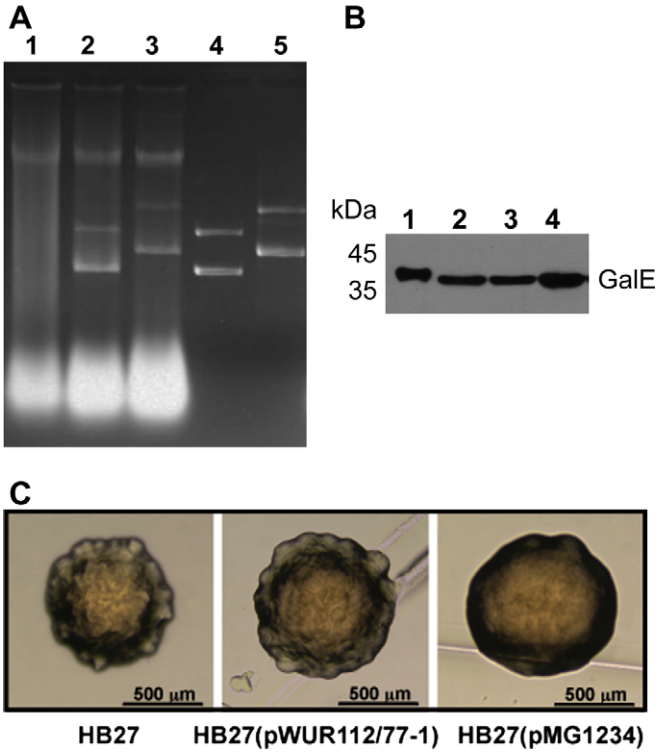


**Fig. 3.** Properties of *T. thermophilus* HB27 containing pMG1234. (A) Total DNA extracted by the methods described by Kado and Liu [39] using 0.8% agarose gel electrophoresis. Lane 1, *T. thermophilus* HB27; lane 2, pWUR112/77-1 bearing *T. thermophilus* HB27; lane 3, *T. thermophilus* HB27 containing pMG1234; and lanes 4 and 5, purified DNA of pWUR112/77-1 and pMG1234 plasmids, respectively, as the positive control. (B) Western bolt analysis of total protein. Lane 1, purified recombinant GalE and lanes 2, 3, and 4, total protein of *T. thermophilus* HB27, pWUR112/77-1-bearing *T. thermophilus* HB27, and pMG1234-bearing *T. thermophilus* HB27, respectively. (C) Colony morphology of different strains. Bar represents 500 μm.

The *galE* gene from *Bacillus subtilis* is known to epimerize UDP-*N*-acetylgalactosamine (UDP-GalNAc) [36]. To determine whether the GalE from *T. thermophilus* has a similar function, in this study, UDP-GalNAc was used as a substrate for *T. thermophilus* GalE for epimerization assay carried out by colorimetrically measuring UDP-*N*-acetylglucosamine (UDP-GlcNAc) produced at $OD_{585}$ [11,28]. The results showed that *T. thermophilus* GalE epimerized UDP-GalNAc in a dose-dependent manner. The enzyme was stable at 90 °C; after incubating at this temperature for 10 min, 90% of the enzymatic activity remained (Fig. 2E). Both enzymatic activities revealed that GalE from *T. thermophilus* HB8 showed dual functions for catalyzing conversion of UDP-Glc to UDP-Gal and between their *N*-acetylated forms. This condition was observed not only in *B. subtilis* GalE [36] but also in the *Yersinia enterocolitica* O:8 Gne [37] and *Pseudomonas aeruginosa* O:6 WbpP proteins [38].

*Increased biofilm-forming ability in galE-overexpressing clones*

To investigate how GalE synthesis influences biofilm formation, we cloned *galE* along with a 500-bp sequence upstream, which probably contained the promoter of the gene, into pWUR112/77-1 [25]to construct pMG1234 (Fig. 3A). Transforming the pMG1234 plasmid into *T. thermophilus* HB27 did not elicit any changes in the growth rate of the cells. Western blotting showed high expression levels of GalE (Fig. 3B). The colony morphology of *T. thermophilus* HB27 (pMG1234) was smoother than *T. thermophilus* HB27 with or without pWUR112/77-1 3 days after inoculation (Fig. 3C). This indicates that GalE overexpression may have contributed to EPS production. Moreover, results also showed that transforming pMG1234 increased biofilm formation by at least 1.8-fold at 24 h after inoculation. *T. thermophilus* HB27 (pMG1234) demonstrated early detachment of the biofilm at 48 h after inoculation (Fig. 4A). To directly assess whether GalE overexpression affects the attachment of *T. thermophilus* HB27 (pMG1234), we observed biofilm architecture by atomic force microscopy 3 h after inoculation. A significantly higher number of *T. thermophilus* HB27 (pMG1234) cells were attached to the solid surface as compared to *T. thermophilus* HB27 and *T. thermophilus* HB27 (pWUR112/77-1) at the same time after inoculation (Fig. 4B). Our findings are consistent with the previous finding that a *galE* mutation in *V. cholerae* led to reduced biofilm formation [23]. GalE is essential for the conversion of UDP-glucose to UDP-galactose that is vital for the production of the major building blocks of LPS in different bacteria; however, LPS of *V. cholerae* do not contain galactose because the *galE* gene in this microorganism is not required for LPS synthesis. Meanwhile, previous studies reported that *galE* is essential for biofilm formation in a spontaneous phage-resistant rugose variant of *V. cholerae*. In contrast, the *galE* mutant of *Porphyromonas gingivalis* increased the biofilm-forming ability by at least sixfold after incubation for 48 h and altered the LPS profile by reducing the O-polysaccharide length and resulting in cell aggregation [21]. Our unpublished data also revealed that galactose and *N*-acetyl galactosamine composed the major proportion of polysaccharide that forms the biofilm in *Thermus* spp. These results suggest that *galE* plays a similar role as *galE* in *V. cholerae* is critical to not only biofilm formation but also EPS synthesis in *Thermus* spp.

In the present study, we attempted to determine the relationship between *galE* expression and biofilm formation. Our results revealed that GalE from *T. thermophilus* HB8 is a thermostable enzyme with specificity for 2 substrates and can convert UDP-galactose to UDP-glucose as well as epimerize its *N*-acetylated forms. Furthermore, our results illustrating the relationship between GalE

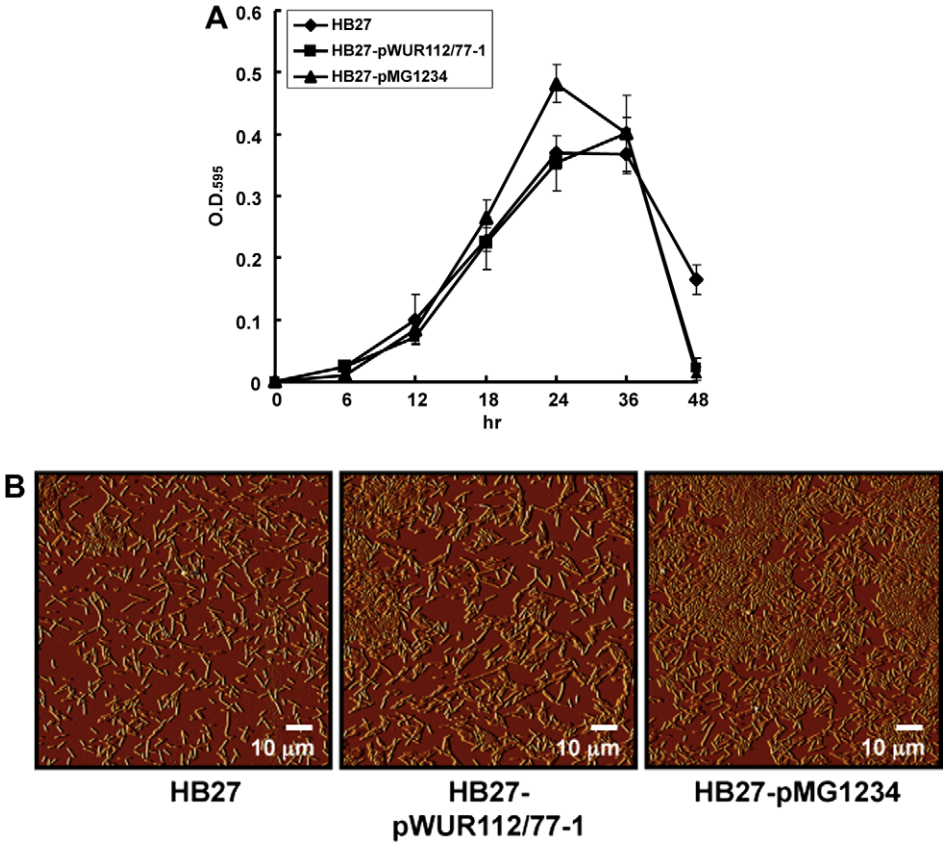


**Fig. 4.** Kinetics and micrographs of biofilm formation of *T. thermophilus* HB27, pWUR112/77-1- and pMG1234-containing strains. (A) Kinetics of biofilm formation was analyzed at different time points of those three strains. Data are the means of three independent experiments with each sample tested in triplicate. (B) Biofilm architecture was captured by AFM. The tested bacteria were cultured on the glass slides in six-well microtiter plates. Images were taken after 3 h of incubation using AFM.

and biofilm formation may provide important clues towards understanding the mechanism involved in biofilm formation by *Thermus* spp.

## Acknowledgments

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