Systematic characterization of a novel *gal* operon in *Thermoanaerobacter tengcongensis*

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On the basis of the *Thermoanaerobacter tengcongensis* genome, a novel type of *gal* operon was deduced. The gene expression and biochemical properties of this operon were further characterized. RT-PCR analysis of the intergenic regions suggested that the transcription of the *gal* operon was continuous. With gene cloning and enzyme activity assays, TTE1929, TTE1928 and TTE1927 were identified to be GalT, GalK and GalE, respectively. Results elicited from polarimetry assays revealed that TTE1925, a hypothetical protein, was a novel mutarotase, termed MR-Tt. TTE1926 was identified as a regulator that could bind to two operators in the operon promoter. The transcriptional start sites were mapped, and this suggested that there are two promoters in this operon. Expression of the *gal* genes was significantly induced by galactose, whereas only MR-Tt expression was detected in glucose-cultured *T. tengcongensis* at both the mRNA and the protein level. In addition, the abundance of *gal* proteins was examined at different temperatures. At temperatures ranging from 60 to 80 °C, the level of MR-Tt protein was relatively stable, but that of the other *gal* proteins was dramatically decreased. The operator-binding complexes were isolated and identified by electrophoretic mobility shift assay-liquid chromatography (EMSA-LC) MS-MS, which suggested that several regulatory proteins, such as GalR and a sensory histidine kinase, participate in the regulation of the *gal* operon.

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INTRODUCTION

Many prokaryotes can utilize galactose as a carbon source. Several different uptake systems transport galactose into the cytoplasm, while a unique pathway, the Leloir pathway (Frey, 1996), subsequently converts the galactose to glucose 1-phosphate. The Leloir pathway is known to be composed of gene products from either the *lac-gal* regulon (Vaillancourt *et al.*, 2002; Vaughan *et al.*, 2001) or the *gal* operon (Ajdic & Ferretti, 1998; Bettenbrock & Alpert, 1998; Mackie & Wilson, 1972; Rohde *et al.*, 2000; Weickert & Adhya, 1993). The *lac-gal* regulon consists of the genes involved in lactose and galactose metabolism, such as *galRKTEM-lacSZ* in *Streptococcus thermophilus* (Vaughan *et al.*, 2001), while the *gal* operon contains the genes that participate in galactose metabolism, such as *galETKM* in

Escherichia coli. Up to now, the *lac-gal* regulon and the *gal* operon have been reported in Proteobacteria and Firmicutes. The molecular regulation of the *gal* operon in Proteobacteria such as *E. coli* is well known (Roy *et al.*, 2004; Semsey *et al.*, 2002, 2004, 2006), but the study of Firmicute *gal* operons is considered to be uncharted territory. Firmicutes are broadly divided into two classes, bacilli and clostridia. Five operons related to galactose metabolism have been characterized in lactic acid bacteria, such as *Lactobacillus casei* (Bettenbrock & Alpert, 1998), *Lactococcus lactis* (Luesink *et al.*, 1998), *Streptococcus mutans* (Ajdic & Ferretti, 1998), *Streptococcus salivarius* (Vaillancourt *et al.*, 2002) and *S. thermophilus* (Vaillancourt *et al.*, 2002), including three *gal-lac* regulons and two *gal* operons. These operon structures are different from those of *E. coli* because of the proximity of the GalR of bacilli to other structural genes. In contrast, neither the *gal* operon nor the *gal-lac* regulon has ever been reported in clostridia.

Thermoanaerobacter tengcongensis belongs to the clostridia, and can survive temperatures from 50 to 80 °C (Xue *et al.*, 2001). Its genomic sequence was determined in 2002 (Bao

Abbreviations: EMSA, electrophoretic mobility shift assay; HTH, helix-turn-helix; LC, liquid chromatography; 5' RACE, 5' rapid amplification of cDNA ends.

Two supplementary figures, showing gene expression of the *gal* operon at different culture temperatures and phylogenetic analysis of *gal* genes in *T. tengcongensis* alongside *gal* genes in other thermophiles, are available with the online version of this paper.

et al., 2002), and the proteome at the optimal temperature has also been determined (Wang *et al.*, 2004). *T. tengcongensis* can utilize several carbon sources, such as glucose, galactose and lactose. Investigations of galactose metabolism in *T. tengcongensis* have revealed the lack of a *lac-gal* regulon, but genomic data suggest the existence of a putative *gal* operon. The putative *gal* operon is composed of five genes, *TTE1929*, *TTE1928*, *TTE1927*, *TTE1926* and *TTE1925*. In addition, the functional domains of these genes have been analysed. *TTE1929*, *TTE1928* and *TTE1927* are annotated as *GalT*, *GalK* and *GalE*, respectively, *TTE1926* as *GalR* of the ROK (repressor, open reading frame and kinase) family (Titgemeyer *et al.*, 1994), and *TTE1925* as a hypothetical protein. On the other hand, the regulator of this putative operon, *GalR*, is predicted to be not, as usual, divergently transcribed (Ajdic & Ferretti, 1998; Mackie & Wilson, 1972; Rohde *et al.*, 2000), but in the same direction as the other four genes. Since this was the first investigation of the *gal* operon in clostridia, it was desirable that the theoretical model could be supported by experimental data. Hence, the regulation of the *T. tengcongensis gal* operon and the functions of its genes were investigated.

METHODS

Culture of *T. tengcongensis*. *T. tengcongensis* strain MB^{4T} (^T, type strain) was cultured in modified complex MB medium (Xue *et al.*, 2001), using mannitol, galactose or glucose as carbon source, and tryptone as nitrogen source. The bacteria were routinely cultured at 75 °C if not otherwise mentioned. The cells were harvested, washed twice with PBS and centrifuged at 3000 g for 5 min at 4 °C, and the pellets were collected for preparation of RNA and protein.

Total RNA extraction from *T. tengcongensis*. Total RNA from *T. tengcongensis* harvested at exponential phase was extracted by TRIzol reagent (Invitrogen). The concentration of RNA was photometrically estimated at A₂₆₀ by an SMG3000 spectrophotometer (BGI). The quality of RNA was examined by conventional agarose electrophoresis.

RT-PCR analysis of intergenic regions of the *gal* operon. RT-PCR was done with M-MLV reverse transcriptase (Promega) according to the manufacturer's instructions. Total RNA templates for RT-PCR were treated with DNase (Takara) multiple times until no DNA product was detected by *Taq* DNA polymerase in a PCR with any of the RT-PCR primers listed in Supplementary Table S1.

Real-time PCR analysis of *gal* genes in *T. tengcongensis*. cDNAs were generated through reverse transcription catalysed by M-MLV reverse transcriptase (Promega) with total RNA as the template. The gene amplification was carried out in a PCR machine (Eppendorf) with designed primers (Supplementary Table S1). To quantitatively estimate *gal* gene expression, real-time PCR was carried out in a PRISM 7300 real-time PCR system (ABI). cDNA libraries of *T. tengcongensis* generated from differently cultured bacteria were used as the templates for real-time PCR, and the lengths of PCR products were ~200 bp. The Ct values for each PCR were normalized against the Ct of 16S rRNA. Data were calculated according to relative gene expression by the $2^{-\Delta\Delta C_t}$ formula (Li *et al.*, 2006). In addition, melting curves for each PCR were analysed carefully to ensure the purity of the amplification products.

5' Rapid amplification of cDNA ends (5' RACE) analysis of *gal* operon promoters. Total RNA of *T. tengcongensis* cultured in galactose medium was extracted as described above. The transcription starting sites of *P_{gal1}* and *P_{gal2}* were determined with a SMART RACE cDNA Amplification kit (Clontech) according to the user manual. SubPCR (second PCR reaction) was carried out with specific primers and with the RACE products as templates. The subPCR products were purified with an AxyPrep DNA Gel Extraction Kit (Axygen) and sequenced (BGI).

Expression of recombinant *gal* proteins and generation of corresponding antibodies. The *gal* genes were overexpressed in *E. coli* BL21 (DE3). Recombinant proteins were purified through an Ni²⁺ affinity column (Qiagen) and used to generate antibodies. The rabbit sera were collected and purified by protein A affinity chromatography (Bio-Rad). Titres and specificities of the antibodies were routinely examined by ELISA and Western blotting.

Protein identification by Western blot analysis. Equal amounts of protein lysate (15 µg) of each sample, quantified by the Bradford method, were separated on 12% SDS-PAGE and transferred onto PVDF membranes (Millipore) for Western blot analysis (Wang *et al.*, 2007). Each Western blot was run in parallel at least three times.

To monitor protein degradation *in vitro*, each recombinant *gal* protein at the same quantity was incubated for 1 h with the lysate of *T. tengcongensis* cultured in galactose medium at 75 or 80 °C, and was detected by Western blotting using anti-His-tag antibody (Cell Signalling).

Electrophoresis mobility shift assay (EMSA) and EMSA-liquid chromatography (LC) MS-MS. Based on bioinformatic analysis, probes were designed for the regions upstream of the operon and potentially interacting with regulatory elements, as follows: 5'-TTTCTTTTGAAGACTTAATTAAAAAATATAATAAAGTCTGTGAGGAAAAGAAAATGACTCAAAAAG, termed *O₁*; and 5'-TAAACC-TTTCACATAAAAACAAAATTTGTTGAGGGTATATTGTAGTGCAT-TAGAAGT, termed *O₂* (potential protein binding sites shown in bold type). Also, another two probes, overlapping *O₁* and *O₂*, were designed: *O₁'*, 5'-TATTGTAAGTCTATTGTTGACATACCAGGC-GCTTTTCTTTTGAAGACTTAATTAAAA; and *O₂'*, 5'-CGAATAT-AATAAAAAAGATGTTAATTAAAAATAATAAACCCTTTCACATAAAAACAAA (overlapping regions underlined). These single-strand DNA probes were synthesized, denatured, labelled with [γ -³²P]ATP by T4 polynucleotide kinase (NEB), and mixed with different concentrations of lysate of galactose-cultured *T. tengcongensis*, or recombinant TTE1926 or TTE1925 protein (as the negative control) for EMSA (Wu *et al.*, 2007).

The DNA-protein complexes in *T. tengcongensis* were analysed by EMSA-LC MS-MS according to Stenger's method, with modifications (Stenger *et al.*, 2004). DNA-protein complexes 1A and 2A, which refer, respectively, to probe *O₁* and *O₂* incubated with lysate of galactose-cultured *T. tengcongensis*, were isolated by EMSA. The shifted bands were then excised and digested with trypsin (Promega). Finally, the peptides were separated by HPLC (UltiMate 3000, Dionex) and identified by MS (HCT Ultra, Bruker). For analysis of LC MS-MS data, stringent criteria were adopted: (1) all the identified proteins should be acquired from duplicate preparations and injections; (2) all the identified proteins should be elicited from more than three matched peptides; (3) all the identified proteins should co-appear under both sets of experimental conditions – *O₁* or *O₂* alone would be considered non-specific.

Enzyme assays. Mutarotase assays were carried out according to the modified method of Majumdar *et al.* (2004) using a P-1030 polarimeter (Jasco). The rate of nonenzymic turnover was subtracted from the initial rate of the enzymic reaction. The first-order rate

constant was obtained from plots of the values of angular rotation against reaction time:

$$K = \ln[(\alpha_0 - \alpha_c)/(\alpha_t - \alpha_c)] \times 1/t$$

where K is the rate constant, α_0 , α_t and α_c the observed angular rotation, and t the reaction time (Brahma & Bhattacharyya, 2004).

Galactokinase (GalK) activity was measured by the NADH-ADP coupling assay (Mustapha *et al.*, 1995; Qian *et al.*, 2006). The kinetic parameters were calculated with SigmaPlot 8.0 (SPSS). The activities of GalT and GalE were assayed as described by Lai *et al.* (1999) and Maxwell *et al.* (1962), and one unit was defined as the change in A_{340} in 1 min in the presence of 1 μ g protein.

RESULTS

Bioinformatic analysis of the putative *gal* operon in *T. tengcongensis*

Based upon the genomic data, a putative *gal* operon in the region from 1539 to 7763 bp in section 170 of 244 sections of the complete genome was deduced. As shown in Fig. 1(a), *TTE1929* was annotated as galactose-1-phosphate uridylyl-transferase (*galT*), catalysing the formation of glucose 1-phosphate and UDP-galactose from UDP-glucose and galactose 1-phosphate. *TTE1928* was annotated as galactokinase (*galK*), catalysing the formation of galactose 1-phosphate from galactose. *TTE1927* was annotated as UDP-glucose 4-epimerase (*galE*), catalysing the interconversion of UDP-glucose and UDP-galactose. Analysis of the *TTE1929* upstream sequence by Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html) revealed one putative promoter and two putative operators. At the -10 region of the predicted promoter, the sequence TATAAT was a typical Pribnow box. The sequence TTGACG was predicted to be the -35 region (Fig. 1b). Downstream of *TTE1925*, a stem-loop structure with a calculated free energy (ΔG^0) of -10.01 kcal mol $^{-1}$ (-41.9 kJ mol $^{-1}$) (data not shown) was located as a potential transcriptional terminator. *TTE1926* was noted to be a member of the ROK family, with a helix-turn-helix (HTH) structure in the N terminus, the typical DNA-binding motif in regulators such as GalR. Moreover, transcription of *GalR* in most reported *gal* operons is regulated by its own promoter and the direction of transcription is divergent to that of the other *gal* genes (Vaillancourt *et al.*, 2002; Weickert & Adhya, 1993), but *TTE1926* is located within the *gal* operon, and is co-transcribed with the other *gal* genes in the same direction and without its own promoter. Such a novel structure of the *gal* operon has, to our knowledge, never been reported in thermophilic bacteria. Through sequence analysis, no conserved domain was detected for *TTE1925*. Hence, exploring the function of *TTE1925* required biochemical experiments. Taking all the bioinformatic data together, it is proposed that *T. tengcongensis* possesses a novel type of *gal* operon.

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Gene expression of the *T. tengcongensis gal* operon induced by galactose

If the genes were located within an operon, they would be co-transcribed. Thus, four pairs of PCR primers (2928,

Fig. 1. Components of the *T. tengcongensis gal* operon, as confirmed by intergenic analysis with RT-PCR. (a) Gene organization of the *gal* operon in *T. tengcongensis*. Arrows indicate the direction of gene transcription. The lengths (in bp) of genes (upper numbers) and intergenic regions (lower numbers) are given. (b) Nucleotide sequences of *P_{gal1}* and *P_{gal2}*. TSS, transcriptional start site. (c) Agarose gel electrophoresis of RT-PCR amplification products of the intergenic regions of the *gal* operon. Lanes: M, DNA ladder; NC, negative control reaction performed without reverse transcriptase to test for RNA purity; 1, 2, 3 and 4, amplified intergenic regions of TTE1929–TTE1928, TTE1928–TTE1927, TTE1927–TTE1926 and TTE1926–TTE1925, respectively.

2827, 2726 and 2625; Supplementary Table S1) were designed to amplify the intergenic regions between neighbouring *gal* genes from *T. tengcongensis* cDNA. Also, a negative control was designed to eliminate the possibility of genomic DNA contamination (lane NC in Fig. 1c). As shown in Fig. 1(c), four predicted fragments spanning the intergenic regions were detected, suggesting that all the given pairs were linked in a transcript.

As a *gal* operon, its transcription as well as translation should respond to galactose induction. The expression of the *gal* operon genes in *T. tengcongensis* cultured in glucose or galactose medium was measured at the mRNA and the protein level for comparison of the inducing response. Analysed by real-time PCR, the relative mRNA abundance of *gal* genes acquired from galactose-cultured *T. tengcongensis* was significantly higher than that from glucose-cultured cells (Fig. 2a). Specifically, the fold-increase in mRNA due to galactose induction was 5.6 for TTE1925, 4.5 for TTE1926, 4.5 for TTE1927, 9.2 for TTE1928 and 2.3 for TTE1929. Using Western blotting, the relative protein abundance of the *gal* proteins from galactose-cultured *T. tengcongensis* was also significantly higher than that from glucose-cultured cells (Fig. 2b). Specifically, the fold increases of the protein were 2.9 for TTE1925, infinite for TTE1926, 14.3 for TTE1927, 10.3 for TTE1928 and 13.7 for TTE1929. To confirm that the gene expression of the

gal operon specifically responded to galactose induction, *T. tengcongensis* also was cultured in MB medium containing mannitol, a neutral carbon source. No *gal* gene expression except that of TTE1925 was found. The expression of *gal* proteins was specifically activated by the presence of galactose in the medium (Fig. 3a). These results thus reinforced the argument that *gal* gene expression is only induced by galactose and not by other carbon sources. Interestingly, there was a relatively high background of TTE1925 in the glucose-cultured *T. tengcongensis*. This prompted the question of whether there was a promoter located at upstream of TTE1925.

The dynamic profile of protein expression was then monitored. When *T. tengcongensis* prior-cultured in glucose was transferred to galactose medium, the expression of *gal* proteins responded to the stimulus within a short period. After 2 h galactose induction, the expression of *gal* proteins was greatly increased from an undetectable to a detectable level. After 8 h, the protein content reached a maximum steady state (Fig. 3b, c). As mentioned above, although TTE1925 expression was significantly induced by galactose, it remained at a detectable level in *T. tengcongensis* cultured in glucose medium (Fig. 2b). Moreover, if *T. tengcongensis* prior-cultured in galactose was transferred to glucose medium, the relative protein content of *gal* proteins was significantly decreased after 4 h,

Fig. 2. Gene expression at the mRNA and protein levels of the *gal* operon of *T. tengcongensis* cultured in glucose (Glu) or galactose (Gal) medium. (a) Relative quantitative comparison of the *gal* genes by real-time PCR. The y axis represents the fold differences of transcription between the two media normalized to transcription in glucose medium. Mean changes for each gene were obtained from nine runs. (b) Relative quantitative comparison of the *gal* proteins by Western blotting. The quantitative estimation was based on calibration curves using 10, 20, 30 and 40 ng recombinant protein of each gene in the Western blot. A total of 15 µg protein lysate was loaded into each lane. Mean changes for each protein were obtained from three runs. *Significant differences using Student's *t* test were defined as $P < 0.01$.

Fig. 3. Western blot analysis of the response of *gal* proteins to galactose induction. (a) Western blot analysis of *gal* proteins in neutral medium with or without galactose or glucose. (b) Dynamic Western blot analysis of *gal* proteins expressed at the indicated times after galactose induction. (c) Semiquantitative analysis of the intensities of each band recognized by the corresponding antibody. An asterisk indicates a significant difference ($P < 0.01$) between neighbouring times. Mean changes for each protein were obtained from three runs.

whereas that of TTE1925 remained relatively stable (data not shown).

Determination of transcriptional start sites in the *T. tengcongensis gal* operon

As mentioned above, two promoters have been proposed for the *gal* operon, P_{gal1} for the whole operon and P_{gal2} for TTE1925 alone. To confirm the prediction, a 5' RACE assay was employed to determine the transcription start sites. The amplified DNA fragments from 5' RACE were examined by 2% agarose gel and sequenced (data not shown). The sequencing results revealed that right after the specific 5' RACE primer, an adenine, 11 nt upstream of the initiation codon of TTE1929, and a guanine, 20 nt upstream of that of TTE1925, were the transcription starting sites for P_{gal1} and P_{gal2} respectively (Fig. 1b).

Functional annotation of the *gal* proteins in *T. tengcongensis*

The GalK activity of recombinant TTE1928 protein was determined over a wide range of temperatures from 30 to 95 °C. As shown in Fig. 4(a), GalK displayed activity from 30 to 95 °C, with a maximum at 80 °C. In addition, the activation energy of GalK was estimated using Arrhenius

plots in the temperature range 30–80 °C, and it was found that 44 kJ mol⁻¹ was required for the catalytic reaction (Fig. 4a, insert). The kinetic parameters of GalK were estimated. The K_m for galactose was 2.5 mM and for ATP was 5 mM. The enzyme activities of GalT and GalE were determined, and were calculated to be 0.087 and 0.130 units (data not shown).

TTE1926 was predicted to be the regulator for the *gal* operon. EMSA experiments were designed to test whether this protein could interact with the two predicted operators (O_1 and O_2) and the two control probes (O_1' and O_2') *in vitro*. In lanes 2 and 3 of Fig. 5, obvious gel shifts were observed when the two labelled probes were incubated with native protein lysate of *T. tengcongensis* cultured in galactose medium, implying that a protein was bound to these DNA fragments. No gel shift was observed for the O_1' and O_2' controls (data not shown). In addition, the competition experiment was adapted by adding unlabelled probes to the binding mixture. The shifted DNA bands were attenuated due to the addition of unlabelled probe. This result strengthened the argument that the O_1 and O_2 fragments indeed have affinity sites for some *T. tengcongensis* proteins. As determined above, TTE1926 has a DNA-binding motif, an HTH. To prove that the gel shift was caused by an interaction between TTE1926 and the

Fig. 4. Enzymic activity assays of recombinant GalK and MR-Tt. (a) Temperature-dependent enzyme activity of recombinant GalK. The insert indicates that the theoretical relationship between enzyme activity and temperature was estimated by Arrhenius plot for GalK. (b) Polarimetric assay of the mutarotase activity of recombinant TTE1925. The insert shows the conversion of α - to β -glucose with (□) and without (■) 50 μ g recombinant TTE1925. Cal. Rate. Con., calculated rate constant.

Fig. 5. Interactions between TTE1926 and potential operators, determined by EMSA. Two potential operators were examined, O₁ (left) and O₂ (right). Upper panels indicate additions of EMSA reaction mixtures. Lane 1 was a blank with probes only. Lanes 2 and 3 were loaded with 50 and 25 μ g, respectively, of protein lysate from galactose medium, while lanes 5, 6 and 7 were loaded with 3.75, 37.5 and 375 ng, respectively, of the recombinant TTE1926 protein. Lanes 4 and 8 were competition assays to the mixtures in lanes 2 and 7, respectively, with 10 pmol unlabelled DNA. Lane 9 was loaded with 375 ng recombinant TTE1925 protein as a negative control. Lanes 10, 11 and 12 were supershift assays obtained by adding polyclonal anti-TTE1926 antibody at three dilutions from stock (1 : 12.5, 1 : 25 and 1 : 50, respectively) to the mixture of lane 2. Upper panels, gel shifts monitored by autoradiography; lower panels, free probes.

operators, recombinant TTE1926 and TTE1925 proteins were incubated with the labelled probes. As shown in Fig. 5 (lanes 5–9), two bands were shifted in response to the addition of TTE1926, whereas no shifted band was detected for TTE1925 as the negative control. When the anti-TTE1926 antibody was added to the binding buffer, the supershifted bands appeared on the gels (Fig. 5, lanes 10–12). Taking all the EMSA data together, it was concluded that TTE1926 was likely to interact with the O_1 and O_2 fragments to play a regulatory role in the *gal* operon.

It is well known that α -D-galactose is the only substrate for GalK in bacteria. To efficiently utilize galactose in nature, bacteria usually convert β -D-galactose to α -D-galactose. Mutarotase (GalM; EC 5.1.3.3) is a functional enzyme in many bacteria (Brahma & Bhattacharyya, 2004; Majumdar *et al.*, 2004; Poolman *et al.*, 1990; Thoden & Holden, 2002), and catalyses the interconversion of the α - and β -anomers of galactose and glucose. Also, mutarotase is one of the key elements in many *gal* operons (Bettenbrock & Alpert, 1998; Thoden & Holden, 2002; Vaillancourt *et al.*, 2002; Vaughan *et al.*, 2001). Nevertheless, on the basis of the current genomic data for *T. tengcongensis*, there is no mutarotase gene. Is it possible that TTE1925 functions as the mutarotase in *T. tengcongensis*? A polarimetric assay was carried out. In Fig. 4(b), insert, although spontaneous epimerization of glucose was found without TTE1925, the rotation rates were dramatically stimulated by the addition of TTE1925. The mutarotation rates were concentration-dependent (Fig. 4b). The mutarotation data thus demonstrated that TTE1925 was a novel mutarotase (MR-Tt), even though its protein structure and catalytic mechanism have not yet been fully elucidated.

Protein expression of the *gal* operon in response to temperature changes

T. tengcongensis can be cultured over a wide range of temperatures, from 50 to 80 °C, with an optimal temperature at 75 °C (Xue *et al.*, 2001). A previous investigation that employed a proteomic approach suggested a global change of protein expression in response to temperature elevation (Wang *et al.*, 2007). Expression of the *gal* proteins in *T. tengcongensis* cultured in galactose medium at three temperatures, 60, 75 and 80 °C, was detected by Western blotting as well as real-time PCR. Except for MR-Tt, the relative mRNA abundance of the *gal* genes was positively correlated with elevated temperature (Supplementary Fig. S1a). In contrast to the profile at the mRNA level, the relative protein content of these four *gal* proteins decreased as the temperature rose, as shown in Supplementary Fig. S1(b), and was undetectable at 80 °C. MR-Tt, however, showed a completely different mode, with a relatively higher abundance at 80 °C. These results provoked the question of why the temperature-dependent transcription of the *gal* genes was so different from the trend in translation. The abundance of a protein results from the balance between its synthesis and degradation. An

in vitro protein degradation assay revealed that the recombinant MR-Tt was relatively stable at both 75 and 80 °C, but the other four recombinant *gal* proteins were quickly degraded at 80 °C (Supplementary Fig. S1c). This accounts for the low abundance of the four *gal* proteins at 80 °C.

Proteomic analysis of the DNA–protein complexes of the *gal* operon

To identify the operator-binding protein(s), EMSA-LC MS-MS was employed. As shown in Supplementary Table S2, five proteins were identified in complex 1A and three in complex 2A. As expected, GalR was identified in both complex 1A and complex 2A, and this provided additional evidence that GalR was indeed an operator-binding protein. In complex 2A, a sensory histidine kinase was identified, suggesting that the regulation mechanism for the *gal* operon was a two-component regulatory system. Some of the other proteins identified from the complexes function in RNA or DNA metabolism, such as the ATPase involved in chromosome partitioning, RNA polymerase subunit alpha, DNA polymerase III subunit alpha and DNA gyrase β subunit. These proteins might interact with the probes non-specifically. The ATP:corrinoid adenosyl-transferase in complex 1A has not been reported to be related to DNA binding as well as transcription regulation.

DISCUSSION

In the NCBI database, approximately 50 genomes of thermophilic bacteria are currently available. With a systematic search for genes involved in the Leloir pathway, only seven species containing a relatively complete *gal* operon or *gal-lac* regulon were found. To date, only the *gal-lac* regulon in *S. thermophilus* has been experimentally examined and the related regulation mechanism extensively investigated (Vaillancourt *et al.*, 2002; Vaughan *et al.*, 2001). No report of a *gal* operon in thermophilic bacteria is believed to have been published to date. Furthermore, all the *gal* genes in thermophilic bacteria were analysed by a phylogenetic approach. The phylogenetic trees of GalT, GalK and GalE overlap well, but among the thermophiles GalR is very divergent, indicating that the genes participating in the Leloir pathway are consistent in both mesophiles and thermophiles, although the regulation genes vary in thermophiles (Supplementary Fig. S2). The determination of the unique *gal* operon structure in *T. tengcongensis* could, therefore, enhance our understanding of the molecular mechanism of galactose metabolism in thermophiles.

The mesophilic GalRs, identified either in *gal* operons or in *gal-lac* regulons, belong to the LacI/GalR regulator family. The N terminus of LacI-GalR proteins commonly contains the HTH domain, which can specifically bind to certain DNA sequences. A consensus pattern in the N terminus has been proposed as follows: [LIVM]-x-[DE]-[LIVM]-A-x₍₂₎-[STAGV]-x-V-[GSTP]-x₍₂₎-[STAG]-[LIVMA]-x₍₂₎-

[LIVMFYAN]-[LIVMC]. The C-terminal has several regions that could serve for sugar binding as well as for oligomerization. Through sequence alignment and analysis of conserved domains between GalRs of mesophiles and those of *T. tengcongensis*, a sequence comparable to HTH-LacI, ADIAKETN TPPTVTNIV, was found in the N terminus of GalR, with 90% homology to the consensus sequence. Furthermore, an EMSA experiment was designed to detect the binding of GalR to the putative operators in the *gal* operon, revealing the interactions of this recombinant protein with the O_1 and O_2 fragments. These data are believed to be the first to demonstrate that *T. tengcongensis* possesses a novel GalR.

GalM could catalyse the interconversion of α - and β -anomers of galactose and glucose, and has been identified in various species from bacteria to mammals. The X-ray structure of galactose mutarotase from *L. lactis* at high resolution reveals that the residues Arg⁷¹, His⁹⁶, His¹⁷⁰, Asp²⁴³ and Glu³⁰⁴ are responsible for anchoring the sugar to GalM. In 34 GalM sequences in SWISS-PROT, a histidine residue is generally located at the centre region and another histidine residue is ~60–80 aa distant from the centre one at the N terminus (Thoden & Holden, 2002). For instance, in *E. coli*, the mutation of the centre histidine, His¹⁷⁵, results in complete loss of enzyme activity, and in *L. lactis*, mutation of either the His⁹⁶ or the His¹⁷⁰ residue can lead to a significant decrease in enzyme activity (Beebe & Frey, 1998). Although MR-Tt does not possess any homology to the known GalMs in amino acid sequence, this protein contains a similar pattern of histidine distribution, in which His¹²⁹ is located at the centre and His⁶⁹ is 60 aa away from His¹²⁹. Moreover, the sequence of the recombinant MR-Tt protein exactly followed the predicted ORF (MSVKIEKINFSGWENSIRLS) and then was experimentally proven to have mutarotase activity for α -D-glucose. Hence, MR-Tt is a novel mutarotase and a key component of sugar metabolism in *T. tengcongensis*.

MR-Tt is the last gene in the *gal* operon and is located upstream of the operon terminator. Expression of this gene was strongly induced by addition of galactose, but was still found at a low level, as either mRNA or protein, in *T. tengcongensis* cultured in glucose medium. A question that arises from this phenomenon is whether MR-Tt is a true member of the *gal* operon. Experimentally, MR-Tt was co-transcribed with the other operon components, and its gene expression responded sensitively to galactose induction. Therefore, MR-Tt should be a member of the operon. Furthermore, the possibility of an independent promoter for MR-Tt was confirmed by 5' RACE. Two promoters were deduced in the *gal* operon, the major one located upstream of the entire operon, P_{gal1} , and the minor one upstream of MR-Tt, P_{gal2} . There is no evidence that dual promoters exist widely in *gal* operons. For what is believed to be the first time, therefore, it is reported here that dual promoters are indeed located in the *T. tengcongensis gal* operon.

T. tengcongensis can be cultured from 50 to 80 °C (Xue *et al.*, 2001), and exhibits temperature-responsive changes in its proteome (Wang *et al.*, 2007). Similar to the proteomic observations, the expression of most *gal* proteins was decreased significantly with rising temperature. There are manifold impacts upon protein abundance that result from high temperature, such as a short half-life of mRNA (Love *et al.*, 1988), fast degradation of proteins, and inhibition of transcription or translation. By real-time PCR analysis, the mRNA levels of *gal* genes were found to be upregulated in response to the elevated culture temperatures. Furthermore, an *in vitro* protein degradation assay showed that *gal* proteins were significantly more degraded at 80 °C than at 75 °C, except for MR-Tt. From the above data, it is proposed that the low abundance of the *gal* proteins except for MR-Tt is likely to be one of the reasons for the slow growth of *T. tengcongensis* at 80 °C.

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REFERENCES

- Ajdic, D. & Ferretti, J. J. (1998). Transcriptional regulation of the *Streptococcus mutans gal* operon by the GalR repressor. *J Bacteriol* **180**, 5727–5732.
- Bao, Q., Tian, Y., Li, W., Xu, Z., Xuan, Z., Hu, S., Dong, W., Yang, J., Chen, Y. & other authors (2002). A complete sequence of the *T. tengcongensis* genome. *Genome Res* **12**, 689–700.
- Beebe, J. A. & Frey, P. A. (1998). Galactose mutarotase: purification, characterization, and investigations of two important histidine residues. *Biochemistry* **37**, 14989–14997.
- Bettenbrock, K. & Alpert, C. A. (1998). The *gal* genes for the Leloir pathway of *Lactobacillus casei* 64H. *Appl Environ Microbiol* **64**, 2013–2019.
- Brahma, A. & Bhattacharyya, D. (2004). UDP-galactose 4-epimerase from *Kluyveromyces fragilis*. Evidence for independent mutarotation site. *Eur J Biochem* **271**, 58–68.
- Frey, P. A. (1996). The Leloir pathway: a mechanistic imperative for three enzymes to change the stereochemical configuration of a single carbon in galactose. *FASEB J* **10**, 461–470.
- Lai, K., Willis, A. C. & Elsas, L. J. (1999). The biochemical role of glutamine 188 in human galactose-1-phosphate uridylyltransferase. *J Biol Chem* **274**, 6559–6566.
- Li, N., Guo, R., Li, W., Shao, J., Li, S., Zhao, K., Chen, X., Xu, N., Liu, S. & Lu, Y. (2006). A proteomic investigation into a human gastric cancer cell line BGC823 treated with diallyl trisulfide. *Carcinogenesis* **27**, 1222–1231.
- Love, H. D., Jr, Allen-Nash, A., Zhao, Q. A. & Bannon, G. A. (1988). mRNA stability plays a major role in regulating the temperature-

- specific expression of a *Tetrahymena thermophila* surface protein. *Mol Cell Biol* **8**, 427–432.
- Luesink, E. J., van Herpen, R. E., Grossiord, B. P., Kuipers, O. P. & de Vos, W. M. (1998). Transcriptional activation of the glycolytic *las* operon and catabolite repression of the *gal* operon in *Lactococcus lactis* are mediated by the catabolite control protein CcpA. *Mol Microbiol* **30**, 789–798.
- Mackie, G. & Wilson, D. B. (1972). Regulation of the *gal* operon of *Escherichia coli* by the *capR* gene. *J Biol Chem* **247**, 2973–2978.
- Majumdar, S., Ghatak, J., Mukherji, S., Bhattacharjee, H. & Bhaduri, A. (2004). UDPgalactose 4-epimerase from *Saccharomyces cerevisiae*. A bifunctional enzyme with aldose 1-epimerase activity. *Eur J Biochem* **271**, 753–759.
- Maxwell, E. S., Kurahashi, K. & Kalckar, H. M. (1962). Enzymes of the Leloir pathway. *Methods Enzymol* **5**, 16.
- Mustapha, A., Hutkins, R. W. & Zirnstein, G. W. (1995). Cloning and characterization of the galactokinase gene from *Streptococcus thermophilus*. *J Dairy Sci* **78**, 989–997.
- Poolman, B., Royer, T. J., Mainzer, S. E. & Schmidt, B. F. (1990). Carbohydrate utilization in *Streptococcus thermophilus*: characterization of the genes for aldose 1-epimerase (mutarotase) and UDPglucose 4-epimerase. *J Bacteriol* **172**, 4037–4047.
- Qian, Z., Wang, J. Q., Zhou, C. Q., Ma, Y. H. & Liu, S. Q. (2006). Expression and catalysis of glucokinase of *Thermoanaerobacter tengcongensis* at different temperatures. *Wei Sheng Wu Xue Bao* **46**, 243–248 (in Chinese).
- Rohde, J. R., Trinh, J. & Sadowski, I. (2000). Multiple signals regulate GAL transcription in yeast. *Mol Cell Biol* **20**, 3880–3886.
- Roy, S., Semsey, S., Liu, M., Gussin, G. N. & Adhya, S. (2004). GalR represses *galP1* by inhibiting the rate-determining open complex formation through RNA polymerase contact: a GalR negative control mutant. *J Mol Biol* **344**, 609–618.
- Semsey, S., Geanacopoulos, M., Lewis, D. E. & Adhya, S. (2002). Operator-bound GalR dimers close DNA loops by direct interaction: tetramerization and inducer binding. *EMBO J* **21**, 4349–4356.
- Semsey, S., Tolstorukov, M. Y., Virnik, K., Zhurkin, V. B. & Adhya, S. (2004). DNA trajectory in the Gal repressosome. *Genes Dev* **18**, 1898–1907.
- Semsey, S., Virnik, K. & Adhya, S. (2006). Three-stage regulation of the amphibolic *gal* operon: from repressosome to GalR-free DNA. *J Mol Biol* **358**, 355–363.
- Stenger, D., Gruissem, W. & Baginsky, S. (2004). Mass spectrometric identification of RNA binding proteins from dried EMSA gels. *J Proteome Res* **3**, 662–664.
- Thoden, J. B. & Holden, H. M. (2002). High resolution X-ray structure of galactose mutarotase from *Lactococcus lactis*. *J Biol Chem* **277**, 20854–20861.
- Titgemeyer, F., Reizer, J., Reizer, A. & Saier, M. H., Jr (1994). Evolutionary relationships between sugar kinases and transcriptional repressors in bacteria. *Microbiology* **140**, 2349–2354.
- Vaillancourt, K., Moineau, S., Frenette, M., Lessard, C. & Vadeboncoeur, C. (2002). Galactose and lactose genes from the galactose-positive bacterium *Streptococcus salivarius* and the phylogenetically related galactose-negative bacterium *Streptococcus thermophilus*: organization, sequence, transcription, and activity of the *gal* gene products. *J Bacteriol* **184**, 785–793.
- Vaughan, E. E., van den Bogaard, P. T., Catzeddu, P., Kuipers, O. P. & de Vos, W. M. (2001). Activation of silent *gal* genes in the *lac-gal* regulon of *Streptococcus thermophilus*. *J Bacteriol* **183**, 1184–1194.
- Wang, J., Xue, Y., Feng, X., Li, X., Wang, H., Li, W., Zhao, C., Cheng, X., Ma, Y. & other authors (2004). An analysis of the proteomic profile for *Thermoanaerobacter tengcongensis* under optimal culture conditions. *Proteomics* **4**, 136–150.
- Wang, J., Zhao, C., Meng, B., Xie, J., Zhou, C., Chen, X., Zhao, K., Shao, J., Xue, Y. & other authors (2007). The proteomic alterations of *Thermoanaerobacter tengcongensis* cultured at different temperatures. *Proteomics* **7**, 1409–1419.
- Weickert, M. J. & Adhya, S. (1993). The galactose regulon of *Escherichia coli*. *Mol Microbiol* **10**, 245–251.
- Wu, K., Lai, X., Guo, X., Hu, J., Xiang, X. & Huang, L. (2007). Interplay between primase and replication factor C in the hyperthermophilic archaeon *Sulfolobus solfataricus*. *Mol Microbiol* **63**, 826–837.
- Xue, Y., Xu, Y., Liu, Y., Ma, Y. & Zhou, P. (2001). *Thermoanaerobacter tengcongensis* sp. nov., a novel anaerobic, saccharolytic, thermophilic bacterium isolated from a hot spring in Tengcong, China. *Int J Syst Evol Microbiol* **51**, 1335–1341.

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