A Global Transcriptional Regulator in Thermococcus kodakaraensis Controls the Expression Levels of Both Glycolytic and Gluconeogenic Enzyme-encoding Genes*

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We identified a novel regulator, Thermococcales glycolytic regulator (Tgr), functioning as both an activator and a repressor of transcription in the hyperthermophilic archaeon Thermococcus kodakaraensis KOD1. Tgr (TK1769) displays similarity (28% identical) to Pyrococcus furiosus TrmB (PF1743), a transcriptional repressor regulating the trehalose/maltose ATP-binding cassette transporter genes, but is more closely related (67%) to a TrmB paralog in P. furiosus (PF0124). Growth of a tgr disruption strain (Δtgr) displayed a significant decrease in growth rate under gluconeogenic conditions compared with the wild-type strain, whereas comparable growth rates were observed under glycolytic conditions. A whole genome microarray analysis revealed that transcript levels of almost all genes related to glycolysis and maltodextrin metabolism were at relatively high levels in the Δtgr mutant even under gluconeogenic conditions. The Δtgr mutant also displayed defects in the transcriptional activation of gluconeogenic genes under these conditions, indicating that Tgr functions both as an activator and a repressor. Genes regulated by Tgr contain a previously identified sequence motif, the Thermococcales glycolytic motif (TGM). The TGM was positioned upstream of the transcription factor B-responsive element (BRE)/TATA sequence in glucogenen promoter elements and downstream of it in glycolytic promoters. Electrophoretic mobility shift assay indicated that recombinant Tgr protein specifically binds to promoter regions containing a TGM. Tgr was released from the DNA when maltotriose was added, suggesting that this sugar is most likely the physiological effector. Our results strongly suggest that Tgr is a global transcriptional regulator that simultaneously controls, in response to sugar availability, both glycolytic and gluconeogenic metabolism in T. kodakaraensis via its direct binding to the TGM.

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The abbreviations used are: PFK, phosphofructokinase; FBPane, fructose-1,6-bisphosphate; EM, Embden-Meyerhoff; GAP, glyceraldehyde 3-phosphate; TGM, Thermococcales glycolytic motif; Tgr, Thermococcales glycolytic regulator; ASW, artificial seawater; EMGA, electrophoresis mobility shift assay; TFB, transcription factor B; TBP, TATA-binding protein; Cra, catabolite repressor/activator; ABC, ATP-binding cassette; DTT, dithiothreitol; Mdx, maltodextrin; Pyr, pyruvate; BRE, Transcription factor B-responsive element.
Transcriptional Regulator of Archaeal Sugar Metabolism

FBPase and phosphoenolpyruvate carboxykinase via protein phosphorylation and specific proteolysis.

Although studies on the metabolic regulation in Archaea are still in the initial phase, valuable insight has been obtained on the control of the glycolytic pathway in a number of archaeal strains including those of the Thermococcales and Thermoproteales (for reviews, see Refs. 8 and 9). Thermoproteus tenax, a member of the Thermoproteales that exhibits both autotrophic and heterotrophic modes of growth, utilizes a variant of the Embden-Meyerhof (EM) pathway as well as the semi- and non-phosphorylating Entner-Doudoroff pathways for glycolysis (10). The variant EM pathway is characterized by the absence of allosteric control in the reactions of the PP\textsubscript{i}-dependent PFK and pyruvate kinase. Instead transcriptional and allosteric regulation is observed for the enzymes involved in the conversion between glyceraldehyde 3-phosphate (GAP) and 3-phosphoglycerate (11–13) as well as for phosphoenolpyruvate synthetase and pyruvate, phosphate dikinase (14).

The Thermococcales order is composed of two major genera, Pyrococcus and Thermococcus, and its members are hyperthermophilic, anaerobic sulfur reducers that display growth on complex proteinaceous substrates (15). Some members of this order can also grow on carbohydrates using a modified EM pathway (16), which differs from the classical EM pathway by the presence of several unique enzymes, such as ADP-dependent glucokinase (17), ADP-dependent PFK (18), and GAP:ferredoxin oxidoreductase (19, 20). Pyrococcus furiosus is one of the most studied species of the Thermococcales and is capable of growth on a variety of sugars including maltose, starch, cellulose, and laminarin. The level of activity of enzymes present in the modified EM pathway is generally higher in P. furiosus cells grown on sugars (glycolytic conditions) compared with cells grown on peptides or pyruvate (gluconeogenic conditions) (20–23), and the activity of gluconeogenic enzymes significantly increases under gluconeogenic conditions (23), both indicating tight control. Allosteric regulation has not been observed in the enzymes characterized so far in the modified EM and gluconeogenic pathways of the Thermococcales, and therefore, the regulation of enzyme activity is presumed to be primarily at the transcriptional level (24). We have recently identified a potential cis-regulatory element (TATCAC\textsubscript{n}GTGATA) in the glycolytic promoters of P. furiosus (25). This putative element was not found in Pyrococcus species that have a significantly lower capacity to metabolize sugars (Pyrococcus abyssi and Pyrococcus horikoshii); however, nearly identical motifs are present in the glycolytic and starch-utilizing promoters of the sugar-metabolizing Thermococcales. The sequence motif was thus designated Thermococcales glycolytic motif (TGM).

*Thermococcus kodakaraensis* is a member of the Thermococcales and grows between 60 and 100 °C with an optimum at 85 °C (26, 27). Both glycolytic (starch or maltodextrin) and gluconeogenic (peptides or pyruvate) modes of growth are observed for this archaeon, and the TGM sequences are found on most of the glycolytic and starch-utilizing gene promoters (25). The development of a gene disruption system for *T. kodakaraensis* (28, 29) as well as the availability of gene information (30) makes this archaeon an attractive model organism for the elucidation of the physiological role of unknown gene function in Thermococcales (31–33).

In this study, we characterized a gene (TK1769) disruption mutant of a putative transcriptional regulator in *T. kodakaraensis*. Whole genome microarray analysis and electrophoretic mobility shift assay strongly suggest that TK1769 encodes a transcriptional regulation factor controlling both the glycolytic (modified EM) and gluconeogenic pathways via binding to the TGM motif present in the glycolytic and gluconeogenic genes. The protein is therefore designated Tgr for Thermococcales glycolytic regulator.

**MATERIALS AND METHODS**

**Microorganisms and Culture Conditions**—For general DNA manipulation and sequencing, *E. coli* DH5\textalpha (Invitrogen) was used. For recombinant production of Tgr, *E. coli* strain BL21(DE3) (Novagen, Madison, WI) containing the tRNA accessory plasmid pRIL (Stratagene, La Jolla, CA) was used. *E. coli* cells were cultivated at 37 °C in LB medium (10 g liter\(^{-1}\) tryptophane, 5 g liter\(^{-1}\) yeast extract, and 10 g liter\(^{-1}\) NaCl) supplemented with either ampicillin (100 \mu/ml) or kanamycin (100 \mu/ml) and chloramphenicol (34 \mu/ml) when necessary.

*T. kodakaraensis* strains were routinely cultivated under anaerobic conditions at 85 °C using a nutrient rich medium (MA-YT) or a synthetic medium (ASW-AA). The MA-YT-based medium contained synthetic sea salts (Marine Art SF; Tomita Pharmaceutical, Tokushima, Japan), yeast extract, and tryptophane as described previously (34). The ASW-AA-based medium contained artificial seawater (ASW), vitamin mixture, modified Wolfe’s trace minerals, and 20 amino acids as described previously (26, 35). As members in the Thermococcales are reported to have several tungstoenzymes (36), NaWO\(_4\cdot2\)H\(_2\)O was also added to ASW-AA medium at a final concentration of 10 \mu.

**Construction of the T. kodakaraensis Δtgr Strain**—Disruption of the Tgr gene (TK1769, tgr) by double crossover homologous recombination was performed using the gene disruption system developed for *T. kodakaraensis* (28, 29). The plasmid used for disruption of tgr was constructed as follows. A DNA fragment containing the tgr-coding region together with its flanking regions (about 1,000 bp) was amplified with the primer set TGR-L1 (5’-CGGTTATCACCTTACGTTTCC-3’) and TGR-R2 (5’-GGTGAAAGGCGCTGAGT-3’) using genomic DNA of *T. kodakaraensis* KOD1 as a template and inserted into the HincII site of pUC118. Using the constructed plasmid DNA as a template, the flanking regions of tgr along with the plasmid backbone were amplified using the primer set TGR-L2 (5’-CCCATCATTTTITAATTCTA-3’) and TGR-R1 (5’-CCAAAGACATTTAAGTTC-3’). The amplified fragment was ligated with a PvuII-PvuII restriction fragment (763 bp) containing the pyrF marker gene excised from pU22 (29), resulting in the plasmid for tgr disruption (pUTGR). A *T. kodakaraensis* uracil auxotroph strain, KU216 (29), was used as the host strain for transformation. After transformation, a pyrF\(^+\) strain exhibiting uracil prototrophy was selected. The genotype of the tgr locus was confirmed by PCR amplification using the primer set tgr-US1 (5’-TACCCTGTGAAGACGTGGG-3’) and tgr-DS2 (5’-GCCGTCAGGCAGCTGAAT-).
GGTGC-3'). Genotypes were also confirmed by Southern blot analyses. Two micrograms of genomic DNA from KU216 and ∆tgr were digested with PstI, separated by 1% agarose gel electrophoresis, and transferred to a nylon membrane Hybond™-N+ (GE Healthcare). The preparation of specific probes, hybridization, and signal detection were performed with the DIG DNA Labeling and Detection kit (Roche Diagnostics) according to the instructions from the manufacturer. The constructed ∆tgr strain was designated KGR1.

**Growth Measurements**—Growth characteristics of wild-type cells (strain KOD1) and ∆tgr mutant cells (strain KGR1) were determined as follows. Each strain was precultured in MA-YT medium supplemented with elemental sulfur (S0) (0.2%, w/v) at 85 °C for 10 h. S0 is required by *T. kodakaraensis* during growth on peptides or amino acids (26). After the preculture, cells were inoculated into MA-YT medium supplemented either with S0 (0.5%, w/v) (MA-YT-S0), sodium pyruvate (0.5%, w/v) (MA-YT-Pyr), or maltodextrin (0.5%, w/v) (MA-YT-Mdx). As a source of maltodextrin, Amycol number 3-L (Nippon Starch Chemical, Osaka, Japan), which consists of maltooligosaccharides of 1–12 glucose units, was used. Growth characteristics of KOD1 and KGR1 cells were also determined in synthetic ASW-AA medium containing S0 (0.2%, w/v) (ASW-AA-S0), S0 (0.2%, w/v) and sodium pyruvate (0.5%, w/v) (ASW-AA-S0-Pyr), or S0 (0.2%, w/v) and maltodextrin (0.5%, w/v) (ASW-AA-S0-Mdx). Cell densities (A600) were recorded at appropriate intervals with a UV spectrometer Ultraspec 3300 pro (GE Healthcare).

**Microarray Analysis**—The microarray plate used in this study (Array Tk01) was manufactured at Takara Bio (Otsu, Japan) and covers 2,226 genes among the total predicted 2,506 genes of *T. kodakaraensis* KOD1 (96.5% coverage). DNA fragments of about 300 bp, corresponding to the 3’-terminal regions of each coding region, were spotted on the glass plate. Two identical sets (left and right) were loaded on each plate. Therefore, two sets of data are obtained from each microarray plate. In the data files, individual signal intensity ratios obtained from each set as well as the average ratio value and the SD are shown.

*T. kodakaraensis* KOD1 and KGR1 were cultivated at 85 °C in MA-YT-S0, MA-YT-Pyr, or MA-YT-Mdx medium. Cells were harvested in the early log phase (A600 ≈ 0.2), and total RNA was extracted using the RNasy Midi kit (Qiagen, Hilden, Germany). Fluorescently labeled cDNA used for hybridization was prepared using the RNA Fluorescence Labeling Core kit version 2.0 (Takara Bio). Total RNA (10 μg) was annealed with random hexamers, and reverse transcription was performed in solutions containing CyDye-labeled dUTP (Cy3-dUTP or Cy5-dUTP) (GE Healthcare). RNA was subsequently degraded with RNase H, and the labeled cDNA was purified using a column supplied in the kit according to the manufacturer’s instructions. The labeled cDNA was dissolved in hybridization buffer (30 μl) containing 6X SSC (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.2% SDS, 5X Denhardt’s solution (Sigma-Aldrich), and 0.1 mg/ml denatured salmon sperm DNA. Hybridization was performed under a coverslip (Spaced Cover Glass XL, Takara Bio) in a humidity chamber at 65 °C for 12–15 h. After hybridization, the microarray plates were washed four times with 2X SSC and 0.2% SDS at 55 °C for 5 min, rinsed in 0.05X SSC, and dried by centrifugation. The intensities of the Cy3 and Cy5 dyes were measured by using an Affymetrix 428 Array Scanner (Affymetrix, Santa Clara, CA). The microarray images were analyzed using Imagenes version 5.5 software (BioDiscovery, Marina Del Ray, CA).

**Recombinant Production of Tgr in E. coli**—The tgr gene of *T. kodakaraensis* KOD1 was amplified by PCR from genomic DNA using *Pfu* TURBO polymerase (Stratagene) by standard methods. Primers used were BG2072 (5’-GGGCCGGCTGAGTTTCACGGAGATCTTACACTGAGGATGAACTT-3’) and BG2073 (5’-GCCGCCGGATCTGC TCACTCAAGGAGGATGAACTT-3’) (Ndel and BamHI sites are underlined). BG2073 contained a stop codon to ensure overexpression of Tgr without a histidine tag to prevent interference during DNA binding assays. The PCR-amplified DNA fragment was digested with Ndel and BamHI and ligated in pET26b (Novagen), and the resulting plasmid was named pWUR278. The QIAprep Spin Miniprep kit (Qiagen) was used for plasmid purification. The correct sequence of the construct was verified (Baseclear, Leiden, The Netherlands).

Overexpression of Tgr was achieved by induction of *E. coli* BL21(DE3)/pRIL cells harboring pWUR278. A 1.5-liter culture was grown until A600 ≈ 0.5, and protein expression was induced by addition of 0.5 mm isopropyl β-D-thiogalactoside. After 15 h of incubation at 37 °C, cells were harvested and centrifuged (20 min at 5000 × g at 4 °C). Cells were resuspended in lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 1 mM dithiothreitol (DTT), 100 mM NaCl, pH 8.0) and disrupted by sonication at 0 °C. Insoluble material was removed by centrifugation (30 min at 26,000 × g at 2 °C). Ten millimolar MgCl2 and 0.1 mg/ml DNase I (Ambion, Austin, TX) were added, and the cell-free extract was incubated for 30 min at room temperature. DNase I and contaminating *E. coli* proteins were denatured by a subsequent heat treatment (20 min at 80 °C) and removed by centrifugation (30 min at 26,000 × g at 2 °C). Resulting heat-stable cell-free extract was slowly mixed with 5 ml of cross-linked agarose-heparin resin (Sigma-Aldrich) at 4 °C for 60 min. After mixing, the resin was allowed to settle in a 10-ml syringe. Contaminant proteins were eluted by washing the resin with 10 ml of wash buffer (20 mM Tris-HCl, 2 mM EDTA, 1 mM DTT, 200 mM NaCl, pH 8.0). Tgr was eluted in elution buffer (20 mM Tris-HCl, 2 mM EDTA, 1 mM DTT, 500 mM NaCl, pH 8.0). Partial desalting of the sample was accomplished by dialysis to lysis buffer at 4 °C for 16 h after which the sample was loaded on a Mono Q Column (pre-equilibrated with lysis buffer) (GE Healthcare). Tgr was eluted in a linear gradient to 1 M NaCl in lysis buffer. Samples containing Tgr were collected, pooled, and dialyzed to lysis buffer at 4 °C for 16 h.

**Electrophoresis Mobility Shift Assay**—Promoter sequences of 100 bp of the ADP-dependent PFK (pfp, TK0376), FBPase (fbp, TK2164), and archaenal histone A (hpkA, TK1413) were PCR-amplified using primers BG2113 (5’-GGGCCGGCTGAGTTTCACGGAGATCTTACACTGAGGATGAACTT-3’) and BG2114 (5’-GCCGCCGGATCTGC TCACTCAAGGAGGATGAACTT-3’). BG2115 (5’-GCCGCCGGCTGAGTTTCACGGAGATCTTACACTGAGGATGAACTT-3’) and BG2118 (5’-CCCCGGCATGAGTTTCACGGAGATCTTACACTGAGGATGAACTT-3’) and BG2115 (5’-GGGCCGGCTGAGTTTCACGGAGATCTTACACTGAGGATGAACTT-3’).
The PCR-amplified pfk and fbp promoter fragments contained the TGM (25). DNA was purified with the QIAquick PCR Puri-
fication kit (Qiagen) and radioactively end-labeled with $^{32}$P
with phosphonucleotide kinase (Invitrogen) according to the manu-
facturer’s instructions. Unincor-
porated label was removed by the QIAquick Nucleotide Removal
kit (Qiagen). For electrophoretic
mobility shift assays (EMSAs), 43
nM Tgr was incubated with 0.2 pmol
of labeled DNA in 15 µl of binding
buffer (25 mM HEPES, 150 mM
potassium glutamate, 10% gly-
cerol, 1 mM DTT, 1 µg of poly(dI-
dC)-poly(dI-dC), pH 7.5) at 70 °C
for 30 min. Different concentra-
tions of possible carbohydrate
ligands (analytical grade) were
added as indicated in the text.
After incubation, samples were
allowed to cool to room tempera-
ture for 3 min and loaded onto a prerun 4% native PAGE gel.
Gels were run in 1× TBE (89 mM Tris borate, 2 mM EDTA,
pH 8.3) at 15 mA, 200 V at room temperature until satisfac-
tory migration.

RESULTS

TK1769 Encodes a Protein Similar to TrmB—TrmB is an
archaeal transcriptional regulator involved in sugar metabo-
lism originally identified in Thermococcus litoralis (37). In vitro
studies have indicated that in the absence of trehalose or malt-
ose TrmB blocks transcription of the trehalose/maltose ATP-
binding cassette (ABC) transporter operon, in which the TrmB
gene itself is included, through the direct binding to its pro-
moter region. In the presence of trehalose or maltose, TrmB is
released from the promoter region, resulting in subsequent
transcriptional initiation. A nearly identical trehalose/maltose
ABC transporter operon (PF1739–PF1744, including a TrmB
ortholog) is also present on the genome of
P. furiosus that was
proposed to be the result of lateral gene transfer (38). A recent
in vitro analysis of
P. furiosus
TrmB (encoded by PF1743) indi-
cated a dual function of this protein; it regulates not only the
trehalose/maltose ABC transporter operon but also the malto-
dextrin ABC transporter operon (PF1938–PF1933) (39). The
latter transporter functions in the uptake of maltooligosaccha-
rides with three or more glucose units (40).

T. kodakaraensis contains neither an ortholog of TrmB nor
that of a trehalose/maltose ABC transporter but contains an
ortholog corresponding to the maltodextrin ABC transporter
(TK1771–TK1775). This is consistent with the fact that malto-
triose and longer oligomers, including starch, can support the
growth of T. kodakaraensis, whereas maltose cannot. Despite
the absence of a TrmB ortholog, two TrmB-like genes (paralogs
TK0471 and TK1769) are present on the
T. kodakaraensis
genome. Orthologous genes corresponding to TK0471 are
present on all of the four sequenced genomes of the Thermo-
coccales, whereas TK1769 orthologs are found only in
P. furio-
sus (PF0124) and
T. kodakaraensis. We speculated previously
(25) that the latter TrmB-like gene might encode the transcrip-
tional regulator responsible for controlling the sugar metabo-
lism in *T. kodakaraensis*. This was because (i) a TrmB ortholog is absent in *T. kodakaraensis*, (ii) the TK1769 orthologs are present only in the sugar-metabolizing Thermococcales species, and (iii) the TK1769 gene is located adjacent to the malto-dextrin ABC transporter operon on the *T. kodakaraensis* genome.

TK1769 encodes a protein (Tgr) of 341 amino acids with a calculated molecular mass of 39,386 Da (Fig. 1). Tgr is 67% identical to the PF0124 protein, but it is only 28% identical to TrmB of *P. furiosus*. It is annotated as a “predicted transcription regulator, DUF118 helix-turn-helix family” (30), and its helix-turn-helix motif is positioned at the N terminus (1–100 amino acids) as in the case of TrmB. TrmB-like regulators including TK1769 are assigned to the COG1378 group, whose members are mostly distributed in Archaea.

**Construction of tgr-disrupted Mutant Strain**—To clarify the physiological function of Tgr in *T. kodakaraensis*, its deletion mutant was constructed with a gene disruption system previously developed in this organism (28, 29). A tgr disruption vector that harbors the *pyrF* (pUTGR) was constructed and used to transform the host strain (KU216, S0) by double crossover homologous recombination (Fig. 2A). A transformant exhibiting uracil prototrophy was confirmed to have the expected genotype; PCR amplification resulted in a DNA fragment with a length corresponding to that of the Δtgr locus (Fig. 2B). The genetic recombination at the Δtgr locus and the absence of the tgr gene on the chromosome was confirmed by Southern blot analysis (Fig. 2C), and the mutant was designated KGR1.

**Growth Properties of KGR1**—Growth characteristics of the KGR1 strain under several growth conditions were examined and compared with those of the wild-type strain, KOD1 (Fig. 3). First a nutrient rich MA-YT medium was used to examine the growth rate under gluconeogenic (MA-YT-S0 and MA-YT-Pyr) and glycolytic (MA-YT-Mdx) conditions. However, under all medium conditions tested, no significant difference in growth phenotype was observed between the two strains (Fig. 3, A–C). As yeast extract, which is a component of the MA-YT medium, is presumed to include (poly)saccharides that may affect the growth of the mutant (32), we next examined growth characteristics in a synthetic medium containing 20 amino acids (ASW-AA) under both gluconeogenic (ASW-AA-S0 and ASW-AA-S0-Pyr) and glycolytic (ASW-AA-S0-Mdx) conditions. It should be noted that stable growth of *T. kodakaraensis* cells in ASW-AA medium requires S0 even when sodium pyruvate or maltodextrin is present. In the ASW-AA medium, the growth rate under gluconeogenic conditions was severely affected by the disruption of tgr (Fig. 3, D and E). In contrast, although a prolonged lag time was observed, the KGR1 strain exhibited a growth rate comparable to that of the wild-type strain under glycolytic conditions (Fig. 3F). These results imply a significant function of the Tgr protein for growth under gluconeogenic conditions.

**Transcriptome Analysis of the KOD1 and KGR1 Strains**—To determine the entire set of genes that are controlled by Tgr, a whole genome DNA microarray analysis was performed on the wild-type strain KOD1 and the tgr disruptive strain KGR1. The
media applied for gluconeogenic conditions were MA-YT-S₀ and MA-YT-Pyr, and the medium representing glycolytic conditions was MA-YT-Mdx.

When the wild-type KOD1 strain was grown under glycolytic and gluconeogenic conditions, we observed a significant increase in the transcript levels of the genes encoding enzymes of the modified EM pathway (Fig. 4).

As the effects on growth resulting from the tgr gene disruption were greater under gluconeogenic conditions, we compared the transcript levels of KOD1 and KGR1 cells grown in MA-YT-S₀ or MA-YT-Pyr (see supplemental Tables S1–S3 for specific microarray data).

The results of a biological replicate performed with the strains grown in MA-YT-S₀ are shown in supplemental Tables S4–S6. In both media we observed a large number of genes whose transcript levels increased in the KGR1 strain compared with the KOD1 strain. Among the genes whose signal intensities increased over 4-fold in the KGR1 strain, 12 of 15 genes observed in MA-YT-S₀ and 14 of 16 in MA-YT-Pyr were related to glycolysis or maltodextrin metabolism. When genes constituting these pathways were examined (Fig. 5, A and B; Table 2; supplemental Figs. S5–S8; and supplemental Tables S3 and S6), all genes displayed higher signal intensities in the KGR1 strain with the exception of the pyruvate kinase gene (see below). Among the glycolytic members, genes responsible for the unidirectional reaction steps, such as ADP-dependent glucokinase (TK1110), ADP-dependent PFK (TK0376), and non-phosphorylating GAP dehydrogenase (TK0705), showed high intensity ratios (Atgr/wild type). This may reflect a strong repression of these genes by Tgr under gluconeogenic conditions. Similarly high intensity ratios were observed for genes encoding the maltodextrin ABC transporter system and amylolpullulanase, both confirmed to play major roles in maltopolsaccharide assimilation (41), increased over 4- to 19-fold. In contrast, transcript levels of FBPase, which is essential for gluconeogenesis in *T. kodakaraensis* (32), were much higher under gluconeogenic conditions. The results obtained with the wild-type KOD1 strain were as expected and confirm that the three media although containing low levels of sugars deriving from yeast extract are applicable for transcriptome analysis between glycolytic and gluconeogenic modes of growth.
these genes in the processing and uptake of maltodextrins, as has been reported in *P. furiosus* (42). Moreover the signal intensity ratios of the glycolytic and maltodextrin metabolism genes observed in the absence/presence of the *tgr* gene (Fig. 5, A and B) displayed the same tendencies as those observed in KOD1 grown under glycolytic/glucogenic conditions (Fig. 4, A and B). The results suggest that under glucogenic conditions *Tgr* functions as a global transcriptional regulator that represses the entire gene network involved in glycolysis and maltodextrin utilization.

Cell-free extracts of KOD1 and KGR1 cells were tested for different enzyme activities: ADP-dependent glucokinase, ADP-dependent PFK, non-phosphorylating GAP dehydrogenase, phosphoglucomutase, and maltodextrin phosphorylase. The activity of ADP-dependent glucokinase and non-phosphorylating GAP dehydrogenase in KGR1 cells grown under glucogenic conditions was comparable to the activity observed in KOD1 cells grown under glycolytic conditions (supplemental Fig. S9), indicating that the regulation of these enzymes is predominantly brought about by Tgr. For the phosphoglucomutase and maltodextrin phosphorylase, the activity level in KGR1 cell extracts was 2–7-fold higher than the activity level measured in KOD1 cell extracts (both under glucogenic conditions; supplemental Fig. S9), indicating that Tgr plays a major role in regulating these enzyme activities. However, the activity levels were still 25–55% of the levels observed in KOD1 cells grown under glycolytic conditions, indicating possible additional post-transcriptional regulation. The strongest effect was observed with the ADP-dependent PFK; derepression after disruption of *tgr* in KGR1 cells was initially observed to only a low extent (supplemental Fig. S9). However, we found that activity levels increased severalfold when glucose 1-phosphate (0.4 mM) was added to the assay mixtures in KGR1 cell extracts, suggesting allosteric regulation (supplemental Fig. S10) in addition to the above mentioned Tgr-mediated transcriptional control (Fig. 5A). However, as appears to be the case of phosphoglucomutase and maltodextrin phosphorylase, activity levels were still 20–30% of those observed in KOD1 cells grown under glycolytic conditions, again suggesting additional post-transcriptional regulation in the case of PFK.

As described above, a common sequence motif (TGM) has been identified on almost all genes in *P. furiosus* and *T. kodakaraensis* that is predicted to be involved in glycolysis and maltodextrin metabolism. The pyruvate kinase gene (TK0511) was an exception, and a TGM sequence could not be found on its promoter. The gene also exhibited exceptional characteristics in our transcriptome analyses; no significant increase in transcript levels was observed when KOD1 cells were grown under glycolytic/gluconeogenic conditions (Fig. 4, A and B).

Table 1

| Gene product | Gene ID | Mean intensity ratio (log₂ ± S.D.)* |
|--------------|---------|-------------------------------------|
| Glycolysis (the modified EM pathway) | | |
| ADP-dependent glucokinase | TK1110 | 2.36 ± 0.22 | 2.37 ± 0.13 |
| Glucose-6-phosphate isomerase | TK1111 | 1.67 ± 0.18 | 1.78 ± 0.11 |
| ADP-dependent phosphofructokinase | TK0576 | 3.37 ± 0.03 | 3.05 ± 0.04 |
| Fructose-1,6-bisphosphate aldolase | TK0989 | 4.36 ± 0.27 | 2.97 ± 0.08 |
| Triose-phosphate isomerase | TK2129 | 2.24 ± 0.02 | 1.88 ± 0.09 |
| GAP ferredoxin oxidoreductase | TK2163 | 1.95 ± 0.04 | 2.45 ± 0.04 |
| GAP dehydrogenase (non-phosphorylating) | TK0705 | 2.99 ± 0.14 | 1.74 ± 0.01 |
| Phosphoglycerate mutase | TK0866 | 2.03 ± 0.05 | 1.31 ± 0.10 |
| Endolase | TK2106 | 1.63 ± 0.00 | 1.03 ± 0.08 |
| Phosphoenolpyruvate synthase | TK1292 | 1.21 ± 0.01 | 0.76 ± 0.04 |
| Pyruvate kinase | TK0511 | 0.07 ± 0.02 | 0.52 ± 0.11 |
| Maltodextrin metabolism | | |
| α-Amylase | TK1884 | 0.05 ± 0.09 | 0.38 ± 0.05 |
| Pululactase type II, GH13 family | TK0977 | 1.63 ± 0.30 | 1.27 ± 0.02 |
| Cyclomaltoextrin glucanotransferase | TK2172 | 1.80 ± 0.04 | 1.84 ± 0.12 |
| Maltodextrin-binding protein precursor | TK1771 | 4.26 ± 0.11 | 2.86 ± 0.04 |
| Maltodextrin transport system, permease component | TK1772 | 3.82 ± 0.02 | 2.56 ± 0.05 |
| Maltodextrin transport system, permease component | TK1773 | 3.51 ± 0.16 | 2.47 ± 0.03 |
| Amylopululactase | TK1774 | 4.02 ± 0.00 | 1.97 ± 0.06 |
| Maltodextrin transport system, ATPase component | TK1775 | 3.35 ± 0.15 | 2.62 ± 0.01 |
| Cyclomaltoextrinase | TK1770 | 0.17 ± 0.02 | 0.53 ± 0.02 |
| 4-α-Glucanotransferase | TK1809 | 3.09 ± 0.07 | 2.87 ± 0.04 |
| Maltodextrin phosphorylase | TK1406 | 3.06 ± 0.04 | 2.82 ± 0.03 |
| α-Glucosidase | TK2148 | 0.99 ± 0.03 | 0.53 ± 0.02 |
| Phosphohexomutase | TK1108 | 2.29 ± 0.01 | 2.06 ± 0.13 |
| Glucogenesis | | |
| Fructose-1,6-bisphosphatase | TK2164 | −4.25 ± 0.04 | −3.91 ± 0.06 |
| GAP dehydrogenase (phosphorylating) | TK0765 | −2.57 ± 0.09 | −2.34 ± 0.02 |
| 3-Phosphoglycerate kinase | TK1146 | −0.94 ± 0.00 | −0.41 ± 0.04 |

* The mean intensity ratio is expressed as a log₂ value with S.D.
Although few in number, we observed the presence of genes whose transcript levels decreased in the \( \Delta tgr \) strain under gluconeogenic conditions. It was of particular interest that genes encoding gluconeogenic enzymes (TK2164 encoding FBPase, TK0765 encoding phosphorylating GAP dehydrogenase, and TK1146 encoding phosphoglycerate kinase), which were upregulated under gluconeogenic conditions in the KOD1 strain (Fig. 4 and Table 1), were included in this group (Fig. 5 and Table 2). The microarray data presented here suggest another aspect of the Tgr protein functioning as a transcriptional activator for genes involved in gluconeogenesis. Consistent with this role, we found that the increase in FBPase activity observed in KOD1 cells under gluconeogenic conditions could not be detected in KGR1 cells (supplemental Fig. S9).

The presence of a TGM in the FBPase gene promoter is described elsewhere (25). We have found additional TGM-like sequences in the promoter regions of the other gluconeogenic genes (TK0765: \((-65)^{\text{TTTCACN}}_{N+5} \text{GTGAAA} \) TK1146: \((-65)^{\text{AAC-}}_{N+5} \text{CANCN} \text{GTGTTC} \) (bases matching the consensus sequence are underlined; numbers in parentheses indicate the positions relative to the initiation codon of each gene)). These TGM sequences, including the TGM present in the FBPase promoter, are located upstream of the predicted Transcription factor B-responsive element (BRE/TATA sequence). The TGM present in the promoters of glycolytic and maltodextrin-metabolizing genes is positioned downstream of the predicted BRE/TATA sequence in the near vicinity of the transcriptional initiation site. The relative position of TGM with respect to the BRE/TATA sequence might determine the role of the Tgr protein, functioning as a repressor for the glycolytic genes and as an activator for the gluconeogenic genes (see “Discussion”).

Besides the genes indicated in Table 2, there are a large number of genes whose transcription levels are affected by \( tgr \) gene disruption. Genes that had been reported previously to harbor TGM sequences in their promoters along with genes that exhibited particularly high increases in transcript levels are shown in Table 3. Among the former group, genes with increased transcript levels in KGR1 were TK1404 (phosphosugar mutase with unknown substrate specificity) (43), TK1436 (branching enzyme, glycoside hydrolase family 57) (44), and TK1743 (glycoside hydrolase family 57), all likely to be involved in \(-\text{glucan metabolism. The branching enzyme and the phosphohexomutase} (\text{TK1108; shown in Table 2}) \text{are key enzymes for glycogen biosynthesis from glucose 6-phosphate, suggesting that glycogen synthesis is repressed by Tgr under gluconeogenic conditions. Genes that displayed a decrease in transcript levels were those encoding a putative thiol protease (TGM downstream of the predicted BRE/TATA sequence, whereas the TGMs of activated genes were positioned upstream of the BRE/TATA sequence. Although TGM sequences had been identified in their promoter regions, a few genes apparently do not respond to the presence or absence of Tgr.}

A number of genes that had not been identified as harboring a TGM were also found to be affected by \( tgr \) gene disruption. Five genes with the highest intensity ratios (\( \Delta tgr/wild \) type) are shown in Table 3. Genes encoding putative sugar-phosphate nucleotidytransferase (TK0955), bifunctional mannose-6-phosphate isomerase/mannose-1-phosphate guanylyltrans-

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Gene} & \textbf{Function} & \textbf{Expression}
\hline
TK1404 & Phosphosugar mutase & Upregulated
\hline
TK1436 & Branching enzyme & Upregulated
\hline
TK1743 & Glycoside hydrolase & Upregulated
\hline
TK0955 & Sugar-phosphate nucleotidytransferase & Downregulated
\hline
TK0955 & Bifunctional mannose-6-phosphate isomerase/mannose-1-phosphate guanylyltrans-
\hline
\end{tabular}
\caption{Genes with the highest intensity ratios (\( \Delta tgr/wild \) type) affected by Tgr gene disruption.}
\end{table}
ferase (TK1109), and myo-inositol-1-phosphate synthase (TK2278) fit well to the presumed role of Tgr as they are expected to be involved in biosynthetic pathways activated in the presence of abundant sugar. The relationship of the ABC-type manganese/zinc transport system, ATPase component (TK0803) and conserved protein, radical S-adenosylmethione-
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A

Tgr-DNA complex

Free DNA

1 2 3 4 5 6

FIGURE 6. A, EMSAs of Tgr with DNA fragments corresponding to the 5′-flanking regions of the pfk (lanes 1 and 2), fbp (lanes 3 and 4), or hpkA (lane 5 and 6) genes. The concentration of DNA was 13 nM, and Tgr was added (lanes 2, 4, and 6) at a concentration of 43 nM. B, left panel, titration experiment. Tgr at concentrations of 0, 11, 32, or 43 nM (lanes 1, 2, 3, and 4) was incubated with the 5′-flanking region of the pfk gene (13 nM). Right panel, determination of the binding affinity of Tgr with the 5′-flanking region of the pfk gene. Phosphorimage software was used to quantify the amount of DNA bound to Tgr. C, Tgr (43 nM) was incubated with the 5′-flanking region of the pfk gene (13 nM) in the presence of maltotriose (left panel) and maltose (right panel). Lanes 1 and 7, DNA only; lanes 2 and 8, DNA with Tgr; lanes 3–6, DNA with Tgr and maltotriose added at concentrations of 100, 250, 500, and 1000 μM, respectively; lanes 9–12, DNA with Tgr and maltose added at concentrations of 100, 250, 500, and 1000 μM, respectively.

online superfamly (TK1135) with sugar metabolism cannot be understood at present. A detailed search in the upstream regions of these genes led to the identification of putative TGM sequences in four of the genes.

The Tgr gene (TK1769) itself contains the TGM sequence. When we compared the microarray signals of TK1769 using KODI grown on pyruvate (MA-YT-Pyr) and maltodextrin (MA-YT-Mdx), a higher signal was detected for cells grown on maltodextrin (log₂(Mdx/Pyr) = 0.57 ± 0.03), suggesting that transcription of Tgr gene is autoregulated. It was also reported in P. furiosus that the Tgr gene ortholog (PF0124) is more actively transcribed in maltose-grown cells than in peptide-grown cells (log₂(maltose/peptide) = 0.4) (24).

Electrophoretic Mobility Shift Assay—To test whether Tgr has the ability to bind to the TGM sequence, EMSAs were performed. T. kodakaraensis Tgr was overexpressed and purified to homogeneity as confirmed by SDS-PAGE analysis. Tgr had a tendency to partially break down in two parts of ~27 and 12 kDa in the absence of EDTA and DTT and when being concentrated (data not shown).

Using the purified Tgr, EMSAs were performed with three different DNA fragments, i.e., promoters of the ADP-dependent PFK (pfk, TK0376), FBPase (fbp, TK2164), and archaeal histone A (hpkA, TK1413). A clear band exhibiting the formation of Tgr-DNA complex was detected when TGM-containing promoters (pfk and fbp) were used (Fig. 6A). The hpkA promoter does not contain a TGM, and indeed no interaction could be detected. The motifs in the pfk promoter (AGTCACTGTGATA) and in the fbp promoter (TATCATGTGATA) both slightly deviate from the TGM consensus in T. kodakaraensis. The EMSAs revealed that, at least under the conditions tested, Tgr has a higher affinity for the pfk promoter fragment (Fig. 6A). When the pfk promoter was titrated with increasing amounts of Tgr, the amount of retarded DNA clearly increased. Tgr protein bound to pfk promoter with an apparent dissociation constant (Kₐ) of 31 nM (Fig. 6B).

Several maltooligosaccharides (maltose, isomaltose, maltotriose, maltotetraose, and maltopentaose) were included in the Tgr-mediated EMSA, and their effects on the binding affinity of Tgr were analyzed. When maltose was added at various concentrations, no change in the amount of Tgr-DNA complex was observed (Fig. 6C). Similarly additions of isomaltose, maltotetraose, and maltopentaose gave no change in the complex formation (supplemental Fig. S11). However, when maltotriose was added, the complex formation between Tgr and pfk promoter was clearly inhibited in a concentration-dependent manner (Fig. 6C), suggesting that maltotriose functions as a physiological effector to regulate Tgr-mediated transcriptional activation/repression. Maltotriose inhibited the binding of Tgr with a Kₐ value of ~520 μM. The addition of glucose in the above described EMSA experiment did not affect the observed retardation patterns (supplemental Fig. S12), indicating that glucose is not a co-repressor in the case of Tgr, unlike the effect that was recently reported for P. furiosus TrmB (45).

DISCUSSION

In the modified EM and gluconeogenic pathways of the Thermococcales, allosteric control has not been observed in the key enzymes characterized so far, including PFK (18, 46), FBPase (47), and pyruvate kinase.4 As the transcript levels of these genes have been found to respond to glycolytic/gluconeogenic conditions in P. furiosus, it has been suggested that regulation occurs primarily at the transcriptional level (24). Although our results have raised the possibilities of post-transcriptional regulation for several specific enzymes, the transcriptome analyses clearly indicated that all glycolytic/glu-

4 J. E. Tuininga and S. W. Kengen, personal communication.
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A Gluconeogenic conditions

- RNAP
- Tgr blocks RNAP recruitment
- Tgr
- OFF

- RNAP
- Tgr supports recruitment of RNAP, TFB and/or TBP
- OFF

 Glycolytic genes

B Glycolytic conditions (with maltodextrin)

- RNAP
- Tgr-Maltotriose complex
- ON

- RNAP
- Low affinity
- OFF

 Glycolytic genes

FIGURE 7. A model of the mechanisms by which Tgr regulates gene transcription in *T. kodakaraensis* under gluconeogenic (A) and glycolytic (B) growth conditions. RNAP, RNA polymerase.

coneogenic genes in *T. kodakaraensis* are controlled at the transcription level via Tgr function. The presence of TGM sequences and a Tgr ortholog on the *P. furiosus* genome suggests that a similar regulon is also present in *P. furiosus* and possibly in other sugar-metabolizing Thermococcales species (25). The Thermococcales glycolytic regulon, or the Tgr regulon, of *T. kodakaraensis* comprises more than 30 genes and is the largest regulon that has presently been identified in the Archaea.

A model illustrating the regulation mechanism of Tgr-mediated transcriptional repression/activation in *T. kodakaraensis* is shown in Fig. 7. Under gluconeogenic conditions, Tgr binds to the TGM of target genes. When TGM is downstream of the BRE/TATA sequence, the Tgr most likely blocks RNA polymerase recruitment, leading to transcriptional inhibition. There is also the possibility that Tgr affects the binding of transcription factor B (TFB) and/or TATA-binding protein (TBP) to their respective binding sites. In *T. kodakaraensis*, two TFB paralogs (TK1280 and TK2287) whose functions cannot be distinguished (48) and one TBP ortholog (TK0132) are present. When TGM is upstream of the BRE/TATA sequence (in the case of the gluconeogenic genes; Fig. 7A, right), transcription is activated. Tgr may exhibit affinity toward RNA polymerase, TFB, and/or TBP, facilitating recruitment of these components to the promoter regions. When maltodextrins are available in the medium, the intermediate maltotriose binds to the Tgr protein, leading to displacement of Tgr from TGM. Dissociation relieves the inhibiting effects of Tgr on the glycolytic and maltodextrin-metabolizing genes (Fig. 7B, left) while diminishing the activating effect of Tgr as in the case of the gluconeogenic genes (Fig. 7B, right).

Tgr is the first archaeal transcription regulator that displays both activation and repression activities in a single protein. A transcription factor with similar dual activities has been identified in *E. coli* that also controls a series of genes involved in gluconeogenesis (49). The control mechanism of this protein, Cra (catabolite repressor activator; alternative name FruR), is very similar to that of Tgr. Cra binds to the operator regions upstream of glycolytic genes, and derepression takes place when Cra is released from its binding site by binding to specific inducers such as fructose 1-phosphate and fructose 1,6-bisphosphate. Furthermore transcriptional activation by Cra occurs in cases when the Cra-binding operator sequence precedes the RNA polymerase-binding site, whereas transcriptional repression occurs when it overlaps or follows the RNA polymerase-binding site (49). Despite the functional similarity between Tgr and Cra, comparison of their amino acid sequences indicates that these proteins are classified into distinct families of transcriptional factors; Cra is a member of the LacI-GalR family and assigned to the COG1609 group, whereas Tgr is assigned to the COG1378 group. Therefore, it can be presumed that Tgr and Cra originally evolved independently of one another but have come to adopt similar mechanisms of function, an example of convergent evolution.

Dissociation of TrmB from its operator sequence occurs upon binding of sugar ligands to TrmB (37, 39). The present results suggest that a similar binding/release mechanism also occurs in the case of Tgr. The sugar interaction domain of TrmB (TrmB<sub>12–109</sub>) binds strongly with maltose and to a lesser degree with sucrose and maltotriose (50). Determination of the crystal structure of a maltose-TrmB<sub>12–109</sub> complex revealed that the C-terminal sugar-binding site of TrmB involved seven amino acid residues, six of them recognizing the non-reducing glucosyl residue (50). Two amino acid residues (Gly<sup>320</sup> and Glu<sup>326</sup>) in TrmB that were necessary for the complex formation are also conserved in Tgr (corresponding to Gly<sup>324</sup> and Glu<sup>330</sup>) (Fig. 1). As for the other residues involved in sugar binding, similar amino acids are conserved in Tgr, suggesting a possibility that the basic mechanisms for sugar recognition are shared by Tgr and TrmB. On the other hand, the other TrmB-like protein in *T. kodakaraensis*, TK0471, lacks a greater part of the corresponding sugar-binding site, indicating that this regulator may be triggered by a distinct effector in *T. kodakaraensis*.

Most archaeal transcriptional regulators characterized thus far act in a negative manner, and little is known about positive regulators. Ptr2 of *Methanocaldococcus jannaschii*, an Lrp family regulator, is one of the best studied positive regulators in the Archaea (51–53). Two genes activated by Ptr2 (rb2 and rbr) have a binding site just upstream of the BRE/TATA sequence, whereas binding site exists further upstream of the rb2 promoter. The presence of a binding site immediately upstream of the BRE/TATA sequence is reported in the *lysWXJK* operon of *Sulfolobus solfataricus* that is controlled by a proposed positive effect.
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regulator, LysM (54), and in the haloarchaeal hop gene promoter, which is activated by Bat (55). The position of these binding sites, including that of TGM in the gluconeogenic genes, suggests a direct interaction of regulator proteins with TFB and/or TBP. Actually an in vitro reconstitution experiment indicated that binding of Ptr2 to the operator sequence enhances the recruitment of TBP to a weak TATA box (53). At present, the molecular mechanisms of the anticipated interaction between archaeal activators and TFB and/or TBP are still unclear and will be an important subject of research for the elucidation of the mechanisms governing archaeal transcriptional activation.

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Addendum—During the reviewing process of this manuscript, Lee et al. (56) reported an in vitro study on the Tgr ortholog (TrmBL1) in P. furiosus. The results of the study are consistent with those obtained in the in vitro analysis of Tgr reported in this study and also display through footprint analysis that TrmBL1 recognizes TGM.

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