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# The Global Regulator CodY in *Streptococcus thermophilus* Controls the Metabolic Network for Escalating Growth in the Milk Environment

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**CodY is a transcriptional regulator conserved in the low-GC group of Gram-positive bacteria. In this work, we demonstrated the presence in *Streptococcus thermophilus* ST2017 of a functional member of the CodY family of global regulatory proteins, *S. thermophilus* CodY (CodY$_{St}$). The CodY$_{St}$ regulon was identified by transcriptome analysis; it consisted predominantly of genes involved in amino acid metabolism but also included genes involved in several other cellular processes, including carbon metabolism, nutrient transport, and stress response. It was revealed that CodY$_{St}$ repressed the transformation of the central metabolic pathway to amino acid metabolism and improved lactose utilization. Furthermore, the glutamate dehydrogenase gene (*gdhA*), repressed by CodY$_{St}$, was suggested to coordinate the interconversion between carbon metabolism and amino acid metabolism and to play an important role on the optimal growth of *S. thermophilus* ST2017 in milk. A conserved CodY$_{St}$ box [AA(T/A)(A/T)TTCTGA(A/C)AATT] was indeed required for *in vitro* binding of CodY$_{St}$ to the target regions of DNA. These results provided evidence for the function of CodY$_{St}$, by which this strain coordinately regulates its various metabolic pathways so as to adapt to the milk environment.**

*Streptococcus thermophilus* is a major dairy starter traditionally used in combination with *Lactobacillus delbrueckii* subsp. *bulgaricus* or *Lactobacillus helveticus* for the manufacture of yogurt and cheeses (1, 2). *S. thermophilus* is a GRAS ("generally recognized as safe") species, and $>10^{21}$ live cells are ingested annually. For long-term growth in milk, *S. thermophilus* forms a unique carbon metabolic system to utilize lactose efficiently and to produce energy for its growth (3, 4). At the same time, *S. thermophilus* is a fastidious microorganism, requiring exogenous sources of amino acids or peptides for optimal growth. Since milk is poor in these low-molecular-weight compounds, the growth of this organism depends largely on a proteolytic system to hydrolyze the casein in milk (5). Accordingly, *S. thermophilus* has evolved a well-developed nitrogen pathway for *de novo* biosynthesis of amino acids and has acquired cell envelope proteinase to enable it to adapt to the milk environment (6–8). Finally, the carbon and amino acid metabolisms and their interconversion facilitate the growth of *S. thermophilus*. Fortunately, all bacteria have evolved additional layers of control to coordinate the use of nutrients by using global regulators (9, 10). CodY, a protein highly conserved in the low-GC group of Gram-positive bacteria, belongs to a unique family of regulatory proteins; it helps bacteria to adapt to conditions of poor nutrient availability and also controls the catabolic pathways, environmental response, and virulence of strains (11–16). The effect of CodY is stimulated by its interaction with either of two ligands, GTP or branched-chain amino acids (BCAA). However, the mechanism of CodY regulation is species dependent (17–20).

Analysis of the annotated genome sequences identified a conserved gene designated *codY*$_{St}$ (*S. thermophilus codY*). Here we focused on the properties and metabolic regulation of the global regulator CodY$_{St}$ in *S. thermophilus* ST2017. The carbon and amino acid metabolism regulatory networks were elucidated by transcriptome analysis, and the regulatory mechanism of CodY$_{St}$ was investigated by electrophoretic mobility shift assays (EMSA) and isothermal titration calorimetry (ITC). Furthermore, we determined that CodY$_{St}$ coordinated the interconversion of the carbon and amino acid metabolisms through glutamate dehydrogenase to enable adaptation to the milk environment.

## MATERIALS AND METHODS

**Bacterial strains, culture, and transformation conditions.** The *S. thermophilus* strains and plasmids used in this study are listed in Table 1. *S. thermophilus* strains were routinely cultured at 37°C in M17 broth (Oxoid) supplemented with 1.0% lactose or sterile milk (Yili, China). Where appropriate, *S. thermophilus* cultures were supplemented with erythromycin at a concentration of 2.5 μg/ml. The complete chemically defined medium (CDM) was adapted from that described by Letort and Juillard (21). *Escherichia coli* strains were grown in Luria-Bertani (LB) broth with shaking at 37°C. Transformants were selected by plating onto LB agar containing erythromycin at 250 μg/ml or ampicillin at 100 μg/ml.

**Construction of the *codY*$_{St}$ mutant in *S. thermophilus* ST2017.** Chromosomal deletion mutants were constructed in *S. thermophilus* by plasmid integration and two-crossover homologous recombination using the method described by Biswas et al. (22). To construct the integration

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**TABLE 1** Strains and plasmids used in this study

| Strain or plasmid | Relevant feature(s) | Source or reference |
|---|---|---|
| Bacterial strains | | |
| *E. coli* DH5α | Host strain for general cloning | 36 |
| *E. coli* BL21 | Expression host | Novagen |
| *S. thermophilus* ST2017 | Wild-type strain isolated from fermented dairy products | Preserved in the laboratory |
| *S. thermophilus* ST2017Δ*codY* | Δ*codY* mutant strain | This study |
| *S. thermophilus* ST2017Δ*codY-codY*exp | $codY_{St}$ overexpression strain | This study |
| *S. thermophilus* ST2017Δ*gdhA* | *gdhA* inactive in *S. thermophilus* ST2017 | This study |
| *S. thermophilus* ST2017Δ*codY*Δ*gdhA* | *gdhA* inactive in *S. thermophilus* ST2017Δ*codY* | This study |
| Plasmids | | |
| $pG^+$host9 | $Erm^r$; pWV01 replicon (temp sensitive) | 22 |
| $pG^+$host-Up-Down | $Erm^r$; $codY_{St}$ knockout vector | This study |
| $pG^+$host-gdhAin | $Erm^r$; vector with inactive *gdhA* | This study |
| pSEC | $Cm^r$; nisin-inducible expression vector | 37 |
| pSEC-*codY* | $codY_{St}$ under the control of the *nox* promoter | This study |

vector, the 1,135-bp Up fragment was PCR amplified from the chromosomal DNA with primers UpF (5′-ATGGTCGACTATCCTCTTTGGAC AGC) and UpR (5′-CTTCCAAGCTTTCTACTGAGCGTTG), and the 1,214-bp Down fragment was PCR amplified with primers DownF (5′-T CGTAAGCTTGGTATGAAAGGCAC) and DownR (5′-GGGAATTCTA GCACCTTCTGGCAAC). The Up fragment and Down fragment were digested with SalI and HindIII and with HindIII and EcoRI, respectively, and were subsequently inserted into plasmid pBluescript II SK(+). Then the SalI/EcoR I fragment was ligated into the temperature-sensitive vector $pG^+$host9, resulting in the knockout plasmid $pG^+$host-Up-Down.

Plasmid $pG^+$host-Up-Down was introduced into *S. thermophilus* ST2017 by electroporation (23). The transformants were first selected at 28°C (permissive temperature) with 2.5 μg/ml of erythromycin. To obtain single-crossover integrants, appropriate diluted cultures were plated onto LM17 medium at 42°C (nonpermissive temperature) with the selective pressure of erythromycin. Cultures were plated onto the same medium at the same temperature without erythromycin in order to count the rate of integration. One clone of the erythromycin-resistant integrant was propagated at 28°C without erythromycin to stimulate the second-crossover integration. The cultures were transferred several times, and culture was extended over 50 generations. Dilutions of the cultures were plated at 42°C without erythromycin to select the second-crossover integrants eliminating the excised vector. As a control, the same diluted cultures were plated with erythromycin to calculate the percentage of plasmid excision. The clones in the plates without erythromycin were plated in the presence or absence of erythromycin to select the $Erm^s$ clones (two single-crossover integrants). Specialized primers were used for PCR amplification to verify the integrants.

**Construction of a $codY_{St}$ overexpression strain in *S. thermophilus* ST2017Δ*codY*.** To construct the $codY_{St}$ overexpression vector, the engineered promoter of the NADH oxidase gene (24) was PCR amplified using primers NoxF (5′-GTTAGATCTAGAAACTATGTGGCAAGC) and NoxR (5′-ACTAATAGGTCTCCTTTAAATG), and the $codY_{St}$ gene was PCR amplified using primers codYF (5′-CATTTAAAGGAGACCTA TTAGTATGGCAAATTTGCTTGATA) and codYR (5′-ATGCTCGAGT TATTCGTATTCTTTCAA). The two fragments were fused by overlapping PCR and were then inserted into plasmid pSEC, yielding the expression vector pSEC-codY. Finally, the vector was introduced into *S. thermophilus* ST2017Δ*codY* to construct the overexpression strain *S. thermophilus* Δ*codY-codY*exp.

**Construction of the *gdhA* mutant in *S. thermophilus* ST2017 and *S. thermophilus* ST2017Δ*codY*.** To construct the integration vector, the 735-bp fragment in the middle of the *gdhA* gene was PCR amplified from the genomic DNA by using primers gdhAin F (5′-AGTGAATTCTCAAA ACAGCCACGAG) and gdhAin R (5′-GAGTCGACACATGTCACAGCT TTAGC). The fragment was digested with SalI and EcoRI and was inserted into $pG^+$host9, resulting in the inactive plasmid $pG^+$host-gdhAin. Plasmid $pG^+$host-gdhAin was introduced into *S. thermophilus* ST2017 and *S. thermophilus* ST2017Δ*codY*. The transformants were first selected at 28°C (permissive temperature) with 2.5 μg/ml of erythromycin. To produce the *gdhA* mutant, appropriate diluted cultures were plated onto LM17 medium at 42°C (nonpermissive temperature) with the selective pressure of erythromycin. Specialized primers were used for PCR amplification to verify the integrants.

**Illumina-based RNA sequencing and transcriptome analysis.** Overnight cultures of the *S. thermophilus* ST2017 and ST2017Δ*codY* strains were diluted in fresh LM17 medium (1:50) and were grown at 42°C. Cells were harvested by centrifugation (10,000 × *g*, 1 min, 4°C) until the optical density at 600 nm ($OD_{600}$) reached 0.4. To isolate the RNA, cell pellets were first resuspended in Tris-EDTA (TE) buffer containing 5 mg/ml lysozyme and then incubated for 10 min at 30°C. Total RNA was isolated according to the manufacturer's instructions (Total RNA Midi kit; Omega, USA). To remove rRNA, a Ribo-Zero rRNA removal kit (Epicentre, Madison, WI, USA) was used according to the manufacturer's instructions. The mRNA was fragmented ultrasonically, and fragmented mRNAs were converted into a transcriptome-sequencing (RNA-seq) library by using the mRNA-seq library construction kit (Illumina Inc., San Diego, CA, USA). RNA sequencing was performed on an Illumina HiSeq 2000 platform (Illumina, San Diego, CA, USA) using a standard paired-end protocol. Clean reads were first obtained from the raw reads by filtering dirty reads, which contained adapters, low-quality bases, or unknown bases, and then imported into the DEGseq package. The MARS model in the DEGseq package was used to calculate the expression levels of each gene in *S. thermophilus* ST2017 and *S. thermophilus* ST2017Δ*codY* (25). The false discovery rate (FDR) was used to determine the threshold of the *P* value. We identified differentially expressed genes as those that were significant at an FDR of $<0.001$, a *P* value of $<0.05$, and a $\geq$2-fold normalized change.

**qPCR.** Transcriptome-sequencing results were verified via quantitative PCR (qPCR) analysis. Total RNA was extracted using the Bacterial RNA kit (Omega, USA). Then 1 μg of the total-RNA sample was used to synthesize cDNA according to the kit instructions (PrimeScript RT-PCR kit; TaKaRa, Japan). qPCR was carried out by using a TaKaRa SYBR Premix *Ex Taq* kit in the Roche LightCycler 480 system according to the manufacturer's instructions. After qPCR, relative mRNA expression was normalized to the constitutive expression of the 16S rRNA housekeeping gene and was calculated by the comparative threshold cycle ($C_T$) method ($2^{-\Delta\Delta CT}$ method). For the 26 selected genes of interest, qPCR was performed at least in duplicate on RNAs purified from 2 independently grown cultures.

**Purification of the $CodY_{St}$ protein and gel mobility shift assay.** The $codY_{St}$ gene was PCR amplified with primers CodYF (5′-TTGGATCCATGGCAAATTTGCTTGATAA) and CodYR (5′-ATGCTCGAGTTATTCGTATTCTTTCAA). The PCR product was cloned into the BamHI/XhoI sites of pETPPA, a vector in which a gene encoding an N-terminally six-histidine-tagged, PreScission protease (PPA) cleavage site-linked version of *S. thermophilus* $CodY_{St}$ is under the control of the T7 promoter. *Escherichia coli* strain BL21(DE3) harboring pETPPA-$CodY_{St}$ was grown in 500 ml of LB broth containing ampicillin until the $OD_{600}$ reached 0.4. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM, and the temperature was lowered to 16°C for the induction of $CodY_{St}$ overexpression. After 24 h of induction, the cells were pelleted by centrifugation at 8,000 × *g* and 4°C. One hundred milliliters of binding buffer (20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole [pH 8.0]) was added to the pellets. Cells were disrupted by sonication, and cellular debris was removed by centrifugation at 10,000 × *g* and 4°C. For the purification of $CodY_{St}$, a 1-ml HisTrap FF column (GE Healthcare) was prepared according to the manufacturer's instructions. Following application of the cleared bacterial lysate and extensive washing, purified $CodY_{St}$ was eluted by using PreScission protease digestion overnight. Protein samples were stored at 4°C until required.

The gel mobility shift assay was performed essentially as described previously (26), with slight modifications. Briefly, the 253-bp upstream region of the *gdhA* gene (ortholog of *STND_0425*) was PCR amplified with primers PgdhAF (5′-TTTCAAACAAATTCAGCTTCAAG) and PgdhAR (5′-TGTTGTCATATCAATGTTTCCTT). One-hundred-nanogram DNA probes were added to 20-μl reaction mixtures containing the binding buffer and purified $CodY_{St}$ protein at a concentration of 0, 25, 50, 75, 100, 150, 200, 300, or 500 ng per reaction. The binding buffer was composed of 10 mM Tris-HCl (pH 7.5), 5% (vol/vol) glycerol, 100 mM KCl, and 1 mM dithiothreitol. To confirm specific protein-DNA interactions, replicates were carried out with 10 μg sheared salmon testis DNA. Immediately after incubation for 30 min at room temperature, samples were loaded onto a 5% nondenaturing polyacrylamide gel. Gel electrophoresis was performed at 100 V for 60 min in 0.5× Tris-acetate-EDTA (TAE) buffer. The binding complex was visualized by ethidium bromide staining.

**Isothermal titration calorimetry.** To determine the $K_d$ (dissociation constant) value and the thermodynamic parameters of the interaction of $CodY_{St}$ with the $CodY_{St}$ box (ATTGACAATTTTCTGATAATTCTGTAACCT [the $CodY_{St}$ box within the *livJ* promoter region is underlined]) in *S. thermophilus* ST2017, the protein was titrated with the $CodY_{St}$ box in a MicroCal iTC200 system (GE Healthcare) equipped with a 300-μl sample cell and a 40-μl syringe. The purified protein was thoroughly dialyzed at 4°C against Tris-HCl buffer (100 mM Tris-HCl, 100 mM KCl [pH 7.5]). The protein solution (50 μM, relating to the CodY dimer) was titrated at 30°C with a double-stranded DNA (dsDNA) solution containing 5 μM $CodY_{St}$ box or $CodY_{St}$ box mutant (ATTGACCATTTTATGCTCATTCTGTAACCT [the $CodY_{St}$ box mutant is underlined]) (13 injections of 2 μl after the first injection of 0.5 μl), using a time interval of 120 s between injections. The heat effects from a blank experiment (injection of $CodY_{St}$ into dialysis buffer) were subtracted before the titration curves were fit to a nonlinear least-squares function. From these curve fits, $K_d$, the change in binding enthalpy ($\Delta H$), and the binding stoichiometry were determined. The change in Gibbs free energy ($\Delta G$) and the change in entropy ($\Delta S$) were calculated using the equations $\Delta G = RT \ln K_d$ and $\Delta G = \Delta H - T\Delta S$, where $R$ is the universal molar gas constant and $T$ is the temperature (in kelvins).

**Data analysis.** The data were analyzed for statistical significance by using the paired Student *t* test. *P* values of $<0.05$ indicated statistical significance.

**RNA-seq data accession number.** All RNA-seq data from both conditions have been deposited in the Sequence Read Archive under accession number SRP051756.

## RESULTS

**Demonstration of a functional $CodY_{St}$ protein in *S. thermophilus* ST2017.** Analysis of the annotated *S. thermophilus* genome sequences identified a conserved gene, designated $codY_{St}$. Comparison of $CodY_{St}$ to the CodY protein sequences of *Streptococcus pneumoniae* ($CodY_{Sp}$) and *Bacillus subtilis* ($CodY_{Bs}$) revealed that at the amino acid level, $CodY_{St}$ has 67% sequence similarity to $CodY_{Sp}$ and 53% sequence similarity to $CodY_{Bs}$. In particular, the C terminus contained a conserved helix-turn-helix motif, which mediated the binding of CodY protein to DNA. To investigate the functions of CodY in *S. thermophilus* ST2017, the $pG^+$host system was used to knock out the $codY_{St}$ gene in this strain. As a result, the putative $codY_{St}$ mutant generated (ST2017Δ*codY*) showed a 115-bp PCR fragment with specific primers for the integrated $codY_{St}$ gene, in contrast to a 786-bp DNA fragment from wild-type ST2017, suggesting that a 671-bp internal region in the $codY_{St}$ gene was deleted in the ST2017Δ*codY* mutant (Fig. 1A). The sequencing results further confirmed the deletion of the $codY_{St}$ gene in the *S. thermophilus* ST2017 genome.

To evaluate the effect of the $codY_{St}$ gene on growth, the growth curves of *S. thermophilus* strains ST2017, ST2017Δ*codY*, and ST2017Δ*codY-codY*exp in LM17 medium were compared. Figure 1B shows that knockout of the $codY_{St}$ gene resulted in a decrease in the growth of *S. thermophilus* ST2017, whereas normal growth was restored by overexpression of the $codY_{St}$ gene in *S. thermophilus* ST2017Δ*codY*. The results revealed that $CodY_{St}$ is a functional protein in *S. thermophilus* ST2017.

**Function of the $CodY_{St}$ regulon.** It has been reported that CodY in low-G+C Gram-positive bacteria regulates genes involved in a wide variety of metabolic pathways and cellular processes. To identify the genes of the $CodY_{St}$ regulon in *S. thermophilus* ST2017, we compared the transcriptional profile of *S. thermophilus* ST2017 with that of its mutant ST2017Δ*codY* by transcriptome analysis. The genes that showed a 2-fold or greater change in the transcript level with a *P* value of $<0.05$ are listed in Table 2. The transcriptome analysis showed that $CodY_{St}$ functions mainly as a transcriptional repressor, since 59 of the 86 differentially expressed genes were found to be upregulated in the $codY_{St}$ mutant, whereas the rest were activated by $CodY_{St}$. These 86 regulated genes were assigned to different groups based on function, as follows. (i) The first group comprises amino acid biosynthesis and transport genes. The genes involved in the biosynthesis of BCAA, tryptophan, cysteine, aspartic acid, serine, and threonine were repressed. The guanosine hydratase gene (*hutU*), which acts during the histidine degradation process, was the sole gene activated by $CodY_{St}$ in amino acid metabolism. (ii) The second group comprises genes involved in the Embden-Meyerhof-Parnas (EMP) pathway and the Leloir pathway, including the glyceraldehyde-3-phosphate dehydrogenase and phosphate mutase genes. (iii) In the third group, the glutamate dehydrogenase gene (*gdhA*) was significantly derepressed in the $codY_{St}$ mutant. (iv) The fourth group consists of a number of genes, including orthologs of *STND_0202* and *-0203*, orthologs of *STND_1235*, *STND_1233*, and *STND_1232*, and an ortholog of *STND_0733*, that encode ABC transporters or ion channels and were derepressed by $CodY_{St}$. (v) The fifth group comprises genes that respond to environmental stresses. The DnaK and GroESL chaperone operons were significantly upregulated in the $codY_{St}$ mutant. The osmotic-stress-related ortholog of *STND_0113*, the ortholog of

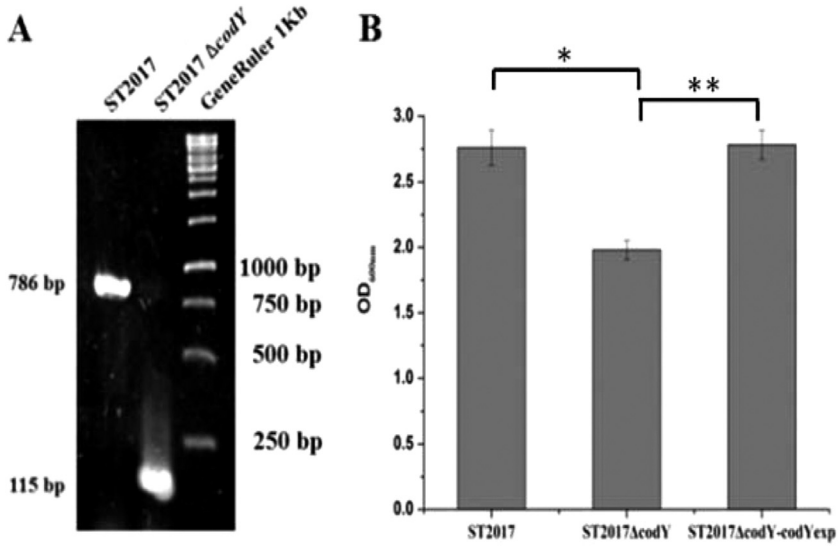


**FIG 1** Functional CodY protein in *S. thermophilus* ST2017. (A) PCR validation of the *codY* mutant. (B) Final biomass levels of *S. thermophilus* ST2017, ST2017Δ*codY*, and ST2017Δ*codY-codY*exp. Asterisks indicate statistically significant differences (*, $P < 0.001$; **, $P < 0.01$) between the values obtained for the Δ*codY* mutant strain and those obtained for wild-type (ST2017) and complemented (Δ*codY-codY*exp) cells.

*STND_0135*, and the oxidative-stress-related ortholog of *STND_1346* were downregulated in the $codY_{St}$ mutant. (vi) The sixth group included the two-component system TCS01 (orthologs of *STND_0306* and *-0307*), involved in environmental stress response. (vii) In the seventh group, the transcription of the hypoxanthine guanine *de novo* IMP synthesis operon was activated by $CodY_{St}$, while the transcription of the uracil synthesis operon was repressed. (viii) The eighth group comprises genes encoding hypothetical proteins. In conclusion, the global regulator $CodY_{St}$ was involved in the regulation of various metabolic processes, especially in amino acid metabolism. At the same time, $CodY_{St}$ also regulated genes responding to environmental stresses. The reliability of the RNA sequence data was also tested by checking the consistency of expression levels within each cluster and performing additional measurements by qPCR (see Fig. S1 in the supplemental material).

**$CodY_{St}$ coordinates the flux of carbon and amino acid metabolisms.** *S. thermophilus* has a complete metabolic pathway from pyruvate to α-oxoglutarate, and the metabolite α-oxoglutarate stands at the crossroads between carbon metabolism and amino acid metabolism. α-Oxoglutarate, one of the substrates of glutamate dehydrogenase, provides the *de novo* carbon skeleton for glutamate. Moreover, as a product of amino acid metabolism, α-oxoglutarate can be the entry point into the central metabolism. The transcriptome data and qPCR analysis showed that the glutamate dehydrogenase gene (*gdhA*; ortholog of *STND_0425*) was significantly repressed by $CodY_{St}$ (see Table S2 in the supplemental material). We also determined the glutamate dehydrogenase activities (27) of *S. thermophilus* ST2017 and the $codY_{St}$ mutant. We found that deletion of $codY_{St}$ led to an increase in enzyme activity from 190 U to 330 U, while overexpression of $codY_{St}$ in *S. thermophilus* ST2017Δ*codY* decreased enzyme activity to 110 U (Fig. 2). These results indicated that $CodY_{St}$ directly inhibited the expression of the *gdhA* gene. EMSA analysis further confirmed that $CodY_{St}$ played a direct role in the binding of the promoter of *gdhA* (ortholog of *STND_0425*) (see Fig. S2 in the supplemental material), suggesting that $CodY_{St}$ may affect *gdhA* transcription.

To determine the role of glutamate dehydrogenase in carbon and amino acid interconversion, the *gdhA* gene was inactivated in *S. thermophilus* ST2017 and ST2017Δ*codY*, yielding the *gdhA* mutant strains ST2017Δ*gdhA* and ST2017Δ*codY*Δ*gdhA*. Furthermore, we compared the growth of *S. thermophilus* ST2017, ST2017Δ*codY*, ST2017Δ*gdhA*, and ST2017Δ*codY*Δ*gdhA* under carbon- or nitrogen-limiting conditions. Here, chemically defined medium (CDM) supplemented with exogenous histidine, cysteine, proline, and methionine was adapted to maintain the growth of *S. thermophilus* ST2017, with nitrogen as its growth constraint. When the lactose concentration in CDM increased from 0.5% to 1.0%, the cell density ($OD_{600}$) of *S. thermophilus* ST2017 increased from 0.768 ± 0.011 to 1.048 ± 0.061, while that of ST2017Δ*codY* increased from 0.822 ± 0.017 to 1.381 ± 0.023. The biomass levels of the *gdhA* mutant strains ST2017Δ*gdhA* and ST2017Δ*codY*Δ*gdhA* in CDM with 0.5% lactose were lower, with $OD_{600}$ values of 0.305 ± 0.034 and 0.531 ± 0.041, respectively. When the lactose concentration increased to 1.0%, the biomass levels of both mutants increased slightly, to 0.328 ± 0.009 and 0.581 ± 0.104, respectively (Fig. 3A). The results suggested that under nitrogen-limiting conditions, *S. thermophilus* ST2017 could convert a carbon source into amino acids for bacterial growth through glutamate dehydrogenase, and the deletion of $codY_{St}$ resulted in an increase in the percentage of the carbon source converted into amino acids. When the link between the carbon and nitrogen metabolic pathways was blocked (by an inactive *gdhA* gene), the growth of mutant strains was severely decreased. On the other hand, under carbon-limiting conditions (CDM containing 0.1% lactose and 0.25% or 0.5% tryptone), the $OD_{600}$ values of *S. thermophilus* ST2017 and ST2017Δ*codY* reached 1.340 ± 0.067 and 1.324 ± 0.017, respectively, when the concentration of tryptone increased from 0.25% to 0.5%, while the growth of strains ST2017Δ*gdhA* and ST2017Δ*codY*Δ*gdhA* increased only to $OD_{600}$ values of 1.188 ± 0.004 and 1.164 ± 0.044, respectively (Fig. 3B). These results confirmed that the capacity for converting nitrogen to carbon was reduced in Δ*gdhA* mutants, consequently limiting bacterial growth.

**TABLE 2** Functions and changes in expression of the genes regulated by $CodY_{St}$ in *S. thermophilus* ST2017

| Locus (gene) | Assignment | ST2017/ST2017Δ*codY* $\log_2$ (normalized fold change) | Presence of $CodY_{St}$ box |
|---|---|---|---|
| *leuA* | 2-Isopropylmalate synthase | −2.44 | |
| *leuB* | 3-Isopropylmalate dehydrogenase | −2.32 | |
| *leuC* | 3-Isopropylmalate dehydratase, large subunit | −3.08 | |
| *leuD* | 3-Isopropylmalate dehydratase, small subunit | −3.14 | |
| *ilvA* | Threonine dehydratase biosynthetic | −1.58 | + |
| *ilvB* | Acetolactate synthase, catalytic subunit | −2.26 | + |
| *ilvH* | Acetolactate synthase, small subunit | −2.65 | |
| *ilvC* | Ketol-acid reductoisomerase | −2.61 | |
| *livJ* | Branched-chain amino acid ABC transporter substrate binding protein | −3.34 | + |
| *livH* | Branched-chain amino acid ABC transporter permease protein | −3.87 | |
| *livM* | ABC-type transport system, permease component | −3.23 | |
| *livG* | ABC-type branched-chain amino acid transport, ATPase component | −2.54 | |
| *livF* | ABC-type branched-chain amino acid transport, ATPase component | −2.51 | |
| *bcaT* | Branched-chain amino acid aminotransferase | −2.06 | + |
| *trpA* | Tryptophan synthase, alpha chain | −2.79 | |
| *trpB* | Tryptophan synthase, beta chain | −1.13 | |
| *trpF* | Anthranilate isomerase | −1.81 | |
| *trpC* | Indole-3-glycerol phosphate synthase | −1.62 | |
| *trpD* | Anthranilate phosphoribosyltransferase | −2.53 | |
| *trpG* | Anthranilate synthase glutamine amidotransferase, component II | −1.38 | |
| *trpE* | Anthranilate synthase, component I | −1.25 | + |
| *argH* | Argininosuccinate lyase | −1.93 | |
| *argG* | Argininosuccinate synthase | −1.58 | |
| *cysM2* | Cysteine synthase | −2.80 | |
| *metB2* | Cystathionine gamma lyase | −2.66 | |
| *cysE2* | Serine acetyltransferase, putative | −3.36 | |
| *lysC* | Aspartokinase | −2.92 | + |
| *thrB* | Homoserine kinase | −1.55 | + |
| *thrC* | Threonine synthase | −1.75 | + |
| *hutU* | Urocanate hydratase | 2.77 | |
| *gdhA* | Glutamate dehydrogenase GdhA | −1.74 | + |
| Ortholog of *STND_0811* | Chorismate mutase | −2.88 | + |
| *purC* | Phosphoribosylaminoimidazole-succinocarboxamide synthase | 1.91 | |
| *purL* | Phosphoribosylformylglycinamidine synthase | 2.00 | |
| *purF* | Amidophosphoribosyltransferase | 1.71 | |
| *purM* | Phosphoribosylformylglycinamidine cyclo-ligase | 1.57 | |
| *purN* | Phosphoribosylglycinamide (GAR) formyltransferase | 2.2 | |
| *purH* | Phosphoribosylaminoimidazolecarboxamide formyltransferase | 1.61 | |
| *purD* | Phosphoribosylamine-glycine ligase | 1.25 | |
| *purE* | Phosphoribosylaminoimidazole carboxylase carboxyltransferase | 1.74 | |
| *purK* | Phosphoribosylaminoimidazole carboxylase II | 1.65 | |
| *pyrK* | Dihydroorotate dehydrogenase, electron transfer subunit | −2.66 | |
| *pyrDb* | Dihydroorotate dehydrogenase B | −1.53 | |
| *pyrF* | Orotidine 5′-phosphate decarboxylase | −2.77 | |
| *pyrE* | Orotate phosphoribosyltransferase PyrE | −1.22 | |
| Ortholog of *STND_0202* | ABC transporter permease protein | −1.94 | |
| Ortholog of *STND_0203* | ABC-type transport system, ATPase component | −2.17 | |
| Ortholog of *STND_0733* | $Mn^{2+}$ and $Fe^{2+}$ transporter of the NRAMP family | −1.07 | + |
| Ortholog of *STND_1235* | Predicted membrane protein | −2.33 | + |
| Ortholog of *STND_1233* | Predicted membrane protein | −2.16 | |
| Ortholog of *STND_1232* | ATPase component of ABC transporter | −1.24 | |
| *hrcA* | Heat-inducible transcription repressor HrcA | −1.54 | |
| *grpE* | Putative Hsp70 cofactor GrpE protein | −1.06 | |
| *dnaK* | Hsp70-like protein | −1.13 | |
| Ortholog of *STND_0135* | Mechanosensitive ion channel (MscS) | 1.53 | |
| *groES* | 10-kDa chaperonin | −1.10 | |
| Ortholog of *STND_0307* | Sensor protein | −1.73 | |
| Ortholog of *STND_0658* | CRISPR-associated endonuclease, Csn1 family | −3.49 | |
| Ortholog of *STND_0862* | Transcription regulator, GntR family | 1.40 | |
| Ortholog of *STND_1346* | Glutamate–cysteine ligase | 1.54 | |
| Ortholog of *STND_1572* | Universal stress protein UspA | 2.22 | |

(Continued on following page)

**TABLE 2** (Continued)

| Locus (gene) | Assignment | ST2017/ST2017Δ*codY* $\log_2$ (normalized fold change) | Presence of $CodY_{St}$ box |
|---|---|---|---|
| Ortholog of *STND_1784* | Transcriptional regulator | 2.30 | |
| Ortholog of *STND_1785* | Permease | 1.20 | |
| Ortholog of *STND_0113* | Enzyme of poly-gamma-glutamate biosynthesis | 2.15 | |
| Ortholog of *STND_0114* | Cytochrome *c* oxidase, monoheme subunit | 2.65 | |
| Ortholog of *STND_0115* | Glycerol-3-phosphate dehydrogenase $NAD(P)^+$ | 1.94 | |
| Ortholog of *STND_0182* | Putative uncharacterized protein | −3.01 | |
| Ortholog of *STND_0394* | Hypothetical protein | 1.50 | |
| Ortholog of *STND_0393* | Hypothetical protein | 2.31 | |
| Ortholog of *STND_0392* | Hypothetical protein | 1.12 | |
| Ortholog of *STND_0611* | Methenyltetrahydrofolate cyclohydrolase | −1.47 | + |
| Ortholog of *STND_0612* | Predicted sugar kinase | −1.80 | |
| Ortholog of *STND_0663* | Hypothetical protein | 1.16 | |
| Ortholog of *STND_0664* | Hypothetical protein | 1.96 | |
| Ortholog of *STND_0782* | Formate–tetrahydrofolate ligase 2 | 2.24 | |
| Ortholog of *STND_1004* | Phosphoglycerate mutase family protein | −1.65 | |
| Ortholog of *STND_1003* | Phosphoglycerate mutase family protein | −1.76 | |
| Ortholog of *STND_1007* | Phosphatase YbjI | −3.22 | |
| Ortholog of *STND_1008* | Hypothetical protein | −4.52 | |
| Ortholog of *STND_1212* | NADP-dependent glyceraldehyde-3-phosphate dehydrogenase GapN | −1.50 | + |
| Ortholog of *STND_1301* | Hypothetical protein | −1.57 | |
| Ortholog of *STND_1308* | Conserved protein | −2.06 | + |
| Ortholog of *STND_1569* | Amidase nicotinamidase-like protein | −4.11 | |
| Ortholog of *STND_1636* | Peptide chain release factor 2 | 1.90 | |
| Ortholog of *STND_1658* | Hydrolase, haloacid dehalogenase-like family | 1.44 | |
| Ortholog of *STND_1689* | Hypothetical protein | −2.42 | |

From the results presented above, we concluded that glutamate dehydrogenase catalyzed the reversible interconversion of α-oxoglutarate and glutamate, and the Michaelis-Menten constant ($K_m$) of glutamate dehydrogenase was determined. Interestingly, glutamate dehydrogenase catalyzed the conversion of α-oxoglutarate to glutamate with a $K_m$ of 0.131 ± 0.030 mM, while it catalyzed the degradation of glutamate to α-oxoglutarate with a $K_m$ of 1.170 ± 0.088 mM. The $K_m$ of the conversion of α-oxoglutarate to glutamate was much lower than that of glutamate to α-oxoglutarate. This metabolic advantage forced the carbon into nitrogen metabolism and effectively utilized the lactose in milk. To confirm this hypothesis, we compared the growth of *S. thermophilus* ST2017, ST2017Δ*codY*, ST2017Δ*gdhA*, and ST2017Δ*codY*Δ*gdhA* in milk. Figure 4 shows that the inactive *gdhA* gene significantly reduced the cell densities of *S. thermophilus* ST2017 and ST2017Δ*codY*, from $6 \times 10^9$ CFU/ml and $2 \times 10^9$ CFU/ml to $9 \times 10^8$ CFU/ml and $8 \times 10^8$ CFU/ml, respectively. The results suggested that *S. thermophilus* ST2017 could convert the rich carbon source into amino acids for bacterial growth through glutamate dehydrogenase in milk, and they further confirmed the important role of glutamate dehydrogenase in the adaptation of *S. thermophilus* to the milk environment.

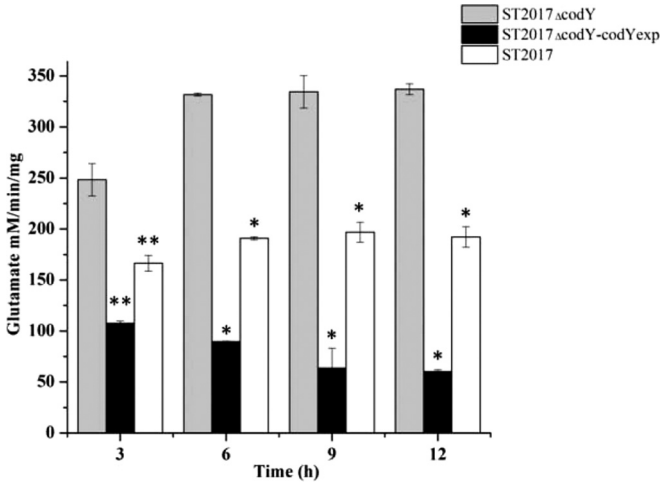


**FIG 2** Assay of glutamate dehydrogenase activity in *S. thermophilus* ST2017, ST2017Δ*codY*, and ST2017Δ*codY-codY*exp. Asterisks indicate statistically significant differences (*, $P < 0.001$; **, $P < 0.01$).

**The $CodY_{St}$ box in *S. thermophilus* ST2017.** EMSA were performed to confirm the direct interaction between $CodY_{St}$ and the promoters of regulated genes. The promoters of both downregulated genes (*purC* [ortholog of *STND_0027*] and the orthologs of *STND_0135*, *STND_1346*, and *STND_1572*) and upregulated genes (*hrcA* [ortholog of *STND_0119*], the ortholog of *STND_0202*, *groES* [ortholog of *STND_0204*], *livJ* [ortholog of *STND_0352*], and the ortholog of *STND_0658*) were retarded in the presence of 200 ng $CodY_{St}$ protein, while no shifted band was observed with the promoter of the control gene (ortholog of *STND_0099*) (Fig.5). This result showed that the global regulator $CodY_{St}$ could regulate the genes directly. In *Lactococcus lactis*, *B. subtilis*, *Streptococcus pneumoniae*, and *Listeria monocytogenes*, a conserved 15-bp palindromic sequence (AATTTTCWGAAAATT) has been shown to serve as a high-affinity binding site for CodY (12). In an attempt to distinguish direct and indirect effects of $CodY_{St}$ on gene expression in the transcriptome data of *S. thermophilus* ST2017, we selected the upstream regions of the 31 repressed gene clusters that were upregulated in the $codY_{St}$ mutant strain. Sixteen

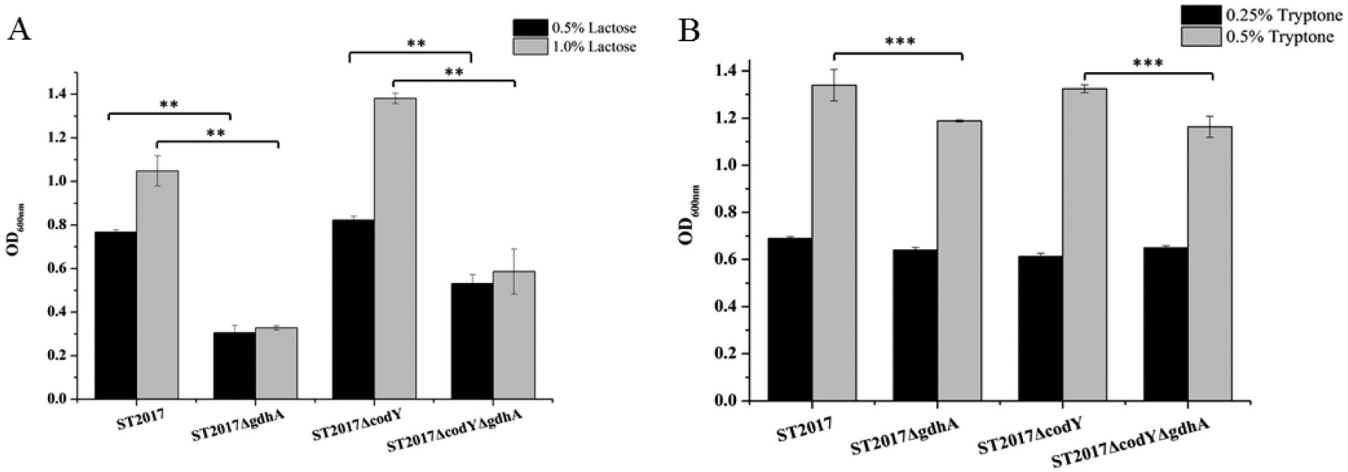


**FIG 3** Final biomass levels of *S. thermophilus* ST2017, ST2017Δ*codY*, ST2017Δ*gdhA*, and ST2017Δ*codY*Δ*gdhA* under nitrogen-limiting conditions (A) or carbon-limiting conditions (B). Asterisks indicate statistically significant differences (**, $P < 0.01$; ***, $P < 0.05$).

of the 31 gene clusters were shown to contain at least a putative $CodY_{St}$ box [AA(T/A)(A/T)TTCTGA(A/C)AATT]. Interestingly, two putative $CodY_{St}$ boxes were also found in the promoter region of the ortholog of *STND_0733* (see Table S1 in the supplemental material). To further confirm the role of the $CodY_{St}$ box in *S. thermophilus* ST2017, the interaction of $CodY_{St}$ with DNA fragments containing the 15-bp conserved motif was monitored by ITC analysis. The results indicated that $CodY_{St}$ and the conserved motif had a significant integration effect. In contrast, the mutated conserved motif lost binding affinity for the $CodY_{St}$ protein, indicating that the conserved motif played a crucial role in the identification of the $CodY_{St}$ protein (Fig. 6). Furthermore, the results revealed that BCAA could effectively enhance the affinity by reducing the dissociation constant ($K_d$) from 780 nM to 8.33 nM and reducing $\Delta H$ from 11.43 ± 1.01 to −5.69 ± 0.33 kcal/mol. Surprisingly, the increase in the stoichiometry of binding to 3 indicated that in the presence of BCAA, $CodY_{St}$ could effectively bind to the conserved motif at a low concentration (Table 3); this result was in agreement with the EMSA results (see Fig. S2 in the supplemental material).

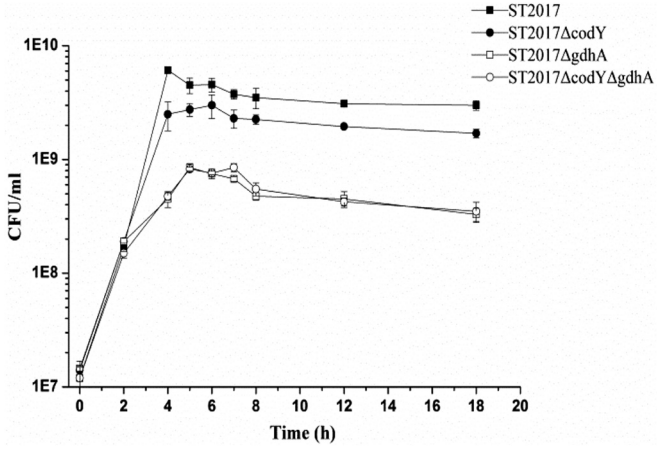


**FIG 4** Growth curves of *S. thermophilus* ST2017, ST2017Δ*codY*, ST2017Δ*gdhA*, and ST2017Δ*codY*Δ*gdhA* in milk.

## DISCUSSION

*S. thermophilus* is the major dairy starter traditionally used for the manufacture of yogurt and cheeses. However, how *S. thermophilus* regulates the metabolic pathways to adapt to the milk environment is still unclear. In low-GC-group Gram-positive bacteria, the CodY regulon has been identified as an important mechanism of environmental adaptation (11, 12, 14, 15). Experimental evidence from the knockout and complementation of the $codY_{St}$ gene demonstrated that *S. thermophilus* ST2017 has a functional $CodY_{St}$ protein. In order to elucidate the role of $CodY_{St}$, transcriptome analysis to identify the extent of the $CodY_{St}$ regulon in *S. thermophilus* ST2017 was carried out. A total of 86 genes organized into 45 apparent transcriptional units were differently expressed in the $codY_{St}$ mutant and thus are thought to be controlled by $CodY_{St}$. The size of the regulon is comparable to those identified in *B. subtilis* (16), *S. pneumoniae* (13), and *L. lactis* (12, 28). Therefore, the global regulator $CodY_{St}$ plays an important role in the regulation of cellular processes in *S. thermophilus*.

**Regulatory network controlled by $CodY_{St}$.** *S. thermophilus* is closely related to *L. lactis*, but it is even more closely related to other streptococcal species (29, 30). The comparative genomics analysis highlights its relatedness to pathogenic species but also reveals that the most important determinants of pathogenicity either are absent or are present as pseudogenes (30–32). Comparative genomics also revealed that the dairy streptococcus species has followed an evolutionary path divergent from that of pathogenic species due to its adaptation to the milk environment (33).

As reported previously, CodY in *L. lactis* controls all pathways for nitrogen supply by modulating *de novo* amino acid biosynthesis and the assimilation of peptides, including their transport and further degradation by peptidases. However, the role of CodY in nitrogen supply is significantly less in *S. thermophilus* than in *L. lactis*, since $CodY_{St}$ controls only the genes involved in amino acid biosynthesis and transport, including BCAA, tryptophan, cysteine, aspartic acid, serine, and threonine, and none of the peptide transporters and peptidases are regulated by $CodY_{St}$. We suppose that *S. thermophilus* is not able to utilize the casein in milk due to the lack of extracellular protease and finally loses control of the proteolytic system. Rather, *S. thermophilus*

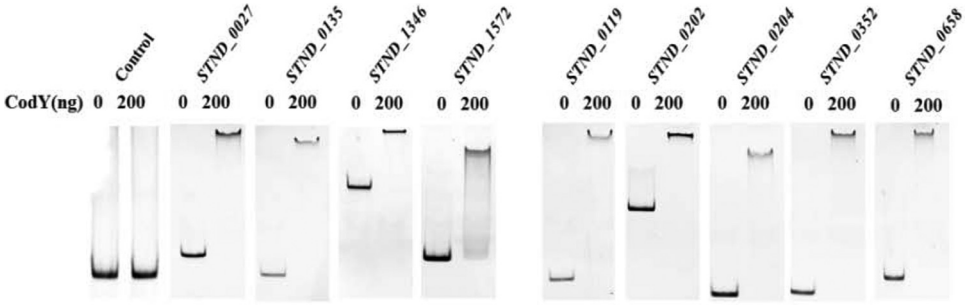


**FIG 5** Gel mobility shift assays for the binding of $CodY_{St}$ to the promoter regions of differentially expressed genes. The promoter regions of $CodY_{St}$-regulated genes (orthologs of *STND_0027*, *STND_0135*, *STND_1346*, *STND_1572*, *STND_0119*, *STND_0202*, *STND_0204*, *STND_0352*, and *STND_0658*) were PCR amplified, and the promoter region of the ortholog of *STND_0099* was used as a control. One hundred nanograms of each promoter region was incubated with $CodY_{St}$ protein, and binding was analyzed by nondenaturing PAGE.

strengthens the regulation of the synthesis and transport of amino acids and controls the intracellular amino acid level effectively. BCAA biosynthesis genes are among those most tightly regulated by $CodY_{St}$. BCAA are the intracellular effectors activating CodY repression in *S. thermophilus*, *L. lactis*, and *B. subtilis*. Thus, CodY exerts feedback control on the pathway involved in the biosynthesis of BCAA (16, 28).

*S. thermophilus* has evolved a well-developed *de novo* pathway of amino acid biosynthesis and a unique carbon metabolic system to efficiently utilize lactose for its adaptation to the milk environment. The transcriptome data showed that the global regulator $CodY_{St}$ controlled not only amino acid metabolism but also lactose utilization processes. Unlike the global regulator CcpA (34), $CodY_{St}$ does not control the expression of the lactose operon directly. Instead, $CodY_{St}$ represses the transcription of phosphomutase genes (orthologs of *STND_1003* and *STND_1004*) and the glyceraldehyde-3-phosphate dehydrogenase gene (*gapN*) to control the carbon flux. Normally, lactose is decomposed into glucose and galactose by β-galactosidase in *S. thermophilus*; glucose is catabolized via the glycolytic pathway, and part of the galactose is used for the synthesis of extracellular polysaccharides, while the rest is exported to the extracellular environment by lactose permease. Our results suggested that derepression of phosphomutase genes (orthologs of *STND_1003* and *STND_1004*) led to an increase in galactose utilization and improved the yield of extracellular polysaccharides and lactic acid as well (data not shown).

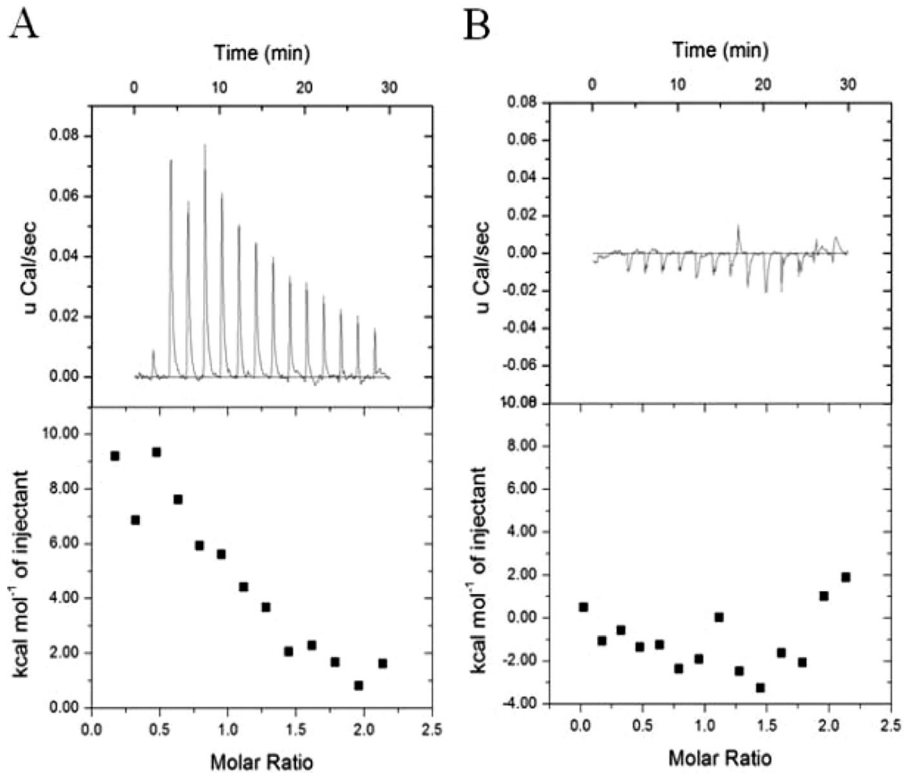


**FIG 6** Affinity of binding of $CodY_{St}$ to the $CodY_{St}$ box. Shown are results of isothermal titration calorimetry experiments titrating *S. thermophilus* $CodY_{St}$ with the $CodY_{St}$ box (A) and a $CodY_{St}$ box mutant (B). (Top) Raw data; (bottom) data fitted using a one-site model.

**TABLE 3** Thermodynamic parameters of the binding of the $CodY_{St}$ box to $CodY_{St}$ at 30°C

| Motif | Thermodynamic parameter of $CodY_{St}$ binding | | | |
|---|---|---|---|---|
| | Stoichiometry | $K_d$ (nM) | $\Delta H$ (kcal/mol) | $\Delta S$ (cal/mol/degree) |
| $CodY_{St}$ box | 1 | 780 | 11.43 ± 1.01 | 61.5 |
| $CodY_{St}$ box containing BCAA | 3 | 8.33 | −5.69 ± 0.33 | 18.2 |

The whole-genome sequence analysis showed that *S. thermophilus* contains a complete metabolic pathway from lactose to glutamate. Glutamate dehydrogenase catalyzes the reversible interconversion of α-oxoglutarate to glutamate and controls the interconversion of carbon metabolism and nitrogen metabolism. Milk is a nutrient-rich and stable environment containing 4.6% lactose and 3.3% casein. Due to the weak activity or absence of extracellular protease in *S. thermophilus*, the nitrogen source became a major limiting factor in the optimal growth of this organism. Our results demonstrate that glutamate dehydrogenase effects the conversion from a carbon source to amino acids in milk, thus conferring optimal growth on *S. thermophilus* in milk.

It has been reported that deletion of the *codY* gene led to low transcription levels of a number of genes in *Streptococcus pyogenes* and *B. subtilis* (15, 20). In *S. thermophilus* ST2017, some genes followed the same pattern, including the purine biosynthetic operon, the poly-gamma-glutamate biosynthetic operon (orthologs of *STND_0113* to *-0115*), a mechanosensitive ion channel gene (ortholog of *STND_0135*), a glutathione synthetase gene (ortholog of *STND_1346*), and a number of hypothetical protein genes (Table 2). Surprisingly, transcription of both the *purCLFMNH* and *purDEK* operons was reduced 4-fold in the $codY_{St}$ mutant. The products of these genes catalyze the *de novo* synthesis of IMP, the precursor of GTP and ATP. This suggests a link between $CodY_{St}$ and the synthesis of GTP and ATP within the cells. Poly-gamma-glutamate and the mechanosensitive ion channel protein are involved in the osmotic stress response. Glutathione synthetase (encoded by the ortholog of *STND_1346*) catalyzes the synthesis of glutathione, which is related to bacterial resistance to reactive oxygen species. Therefore, we suppose that *S. thermophilus* ST2017 responds to environmental stresses via the global regulator $CodY_{St}$.

**Mechanism of regulation by $CodY_{St}$ in *S. thermophilus* ST2017.** To find out whether the genes differentially expressed in the $codY_{St}$ mutant were under the direct or indirect control of $CodY_{St}$, the upstream regions of a number of regulated genes were tested for their abilities to interact with purified $CodY_{St}$ (Fig. 5). $CodY_{St}$ protein could bind to the promoters of both downregulated and upregulated genes. Surprisingly, only those genes that are derepressed in the $codY_{St}$ mutant led to the identification of a conserved inversely repeated motif, AA(T/A)(A/T)TTCTGA(A/C)AATT, which shows a high degree of homology to the canonical CodY box (AATTTTCWGAAAATT) in *L. lactis* and *B. subtilis* (12, 20). ITC analysis proved that this conserved motif, designated the $CodY_{St}$ box, had a crucial role in the identification of $CodY_{St}$ in *S. thermophilus* ST2017. Further analysis shows that multiple copies of the $CodY_{St}$ box are present in the intergenic regions of some genes, and we suppose that the presence of multiple $CodY_{St}$ binding sites strengthens the control of the target genes by $CodY_{St}$. Interestingly, the absence of a putative $CodY_{St}$ box in genes downregulated in the $codY_{St}$ mutant suggests that these genes may be under the direct control of a regulator that is regulated by $CodY_{St}$, or that $CodY_{St}$ may be able to function as a transcriptional activator in another way.

Since it has been shown that BCCA and GTP are the effectors of CodY in *B. subtilis*, while the activity of lactococcal and streptococcal CodY proteins seems to be modulated solely by BCAA and not by GTP (13, 19, 35), we performed gel mobility shift assays to determine the effects of BCCA and GTP on the efficiency of the binding of $CodY_{St}$ to target regions in *S. thermophilus* ST2017. It was possible to demonstrate that only BCAA stimulated $CodY_{St}$ binding; the effect of GTP was insignificant. The mechanism of CodY in *S. thermophilus* ST2017 is similar to those in *L. lactis* and *S. pneumoniae* (12, 15). Sequence similarity analysis of CodY proteins suggests that mutations in *L. lactis*, *S. pneumoniae*, and *S. thermophilus* $Phe^{40}$ and $Ser^{129}$ were related to failure to respond to GTP (18), and our results prove the hypothesis in *S. thermophilus*.

In summary, we have identified the $CodY_{St}$ regulon in *S. thermophilus* ST2017; we show the important roles of $CodY_{St}$ in regulating carbon and nitrogen metabolism; and we further reveal that $CodY_{St}$ could control the interconversion of carbon and amino acids through glutamate dehydrogenase as an important mechanism of adaptation to the milk environment.

## ACKNOWLEDGMENTS

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