The global regulator CodY responds to oxidative stress by the regulation of glutathione biosynthesis in *Streptococcus thermophilus*

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ABSTRACT

CodY_{st} is a global transcriptional regulator that modulates the metabolic network in *Streptococcus thermophilus* ST2017. In this study, experimental data showed that the cell survival of the *codY_{st}* defective mutant obviously declined at the presence of 10 mM H₂O₂, suggesting CodY_{st} was involved in response to the oxidative stress. To investigate this phenomenon, transcriptome analysis and real time-quantitative PCR were performed and the results indicated that the transcriptional level of a bifunctional glutathione synthetase gene (*gshF*) was downregulated by about 3-fold in the *codY_{st}* defective mutant, along with a decrease by 20% of the glutathione yield compared with the wild-type in minimal chemical defined medium, whereas half of the viable cells remained after H₂O₂ challenge. In vitro gel shift assays showed that the purified CodY_{st} could bind to the promoter region of *gshF*, with a conserved CodY_{st} box, confirming the regulation of CodY_{st} on the *gshF* gene. To our knowledge, this is first report of CodY_{st} in response to oxidative stress mediated by the regulation of *gshF* in *S. thermophilus*.

Key words: CodY_{st} regulator, oxidative stress, glutathione biosynthesis, *Streptococcus thermophilus*

INTRODUCTION

Streptococcus thermophilus is a major dairy starter commonly used in combination with *Lactobacillus delbrueckii* ssp. *bulgaricus* or other lactobacilli for the manufacture of yogurt and cheese (Tamime and Deeth, 1980). It is a generally recognized as safe species, and about 10²¹ live cells are used as starter cultures inoculated into pasteurized milk to control the fermentation and obtain high-quality end-products (Hols et al., 2005). The activity of the starter cultures directly affects the texture of the final fermented products. However, during the manufacture, fermentation, and

storage processes, *S. thermophilus* often suffers various environmental stresses, including acidity, oxidization, osmotic pressure, cold and heat shock, leading to the decrease of production capacity (van de Guchte et al., 2002). Among these stresses, oxidization is the most lethal for *S. thermophilus*. In vivo, oxygen is converted into toxic reactive oxygen species, including superoxide anions (O₂^{•−}), hydroxyl radical (•OH), and hydrogen peroxide (H₂O₂), which attack cellular biomolecules, such as proteins, nucleic acids, and lipids, impairing their biological functions and subsequently affecting the cell growth and survival (Condon, 1987).

CodY, a highly conserved regulatory protein present in the lowest GC group of gram-positive bacteria, makes cells adapt to the adverse conditions of poor nutrient availability and also regulates the catabolic pathways, environmental response, and virulence of some strains (Guédon et al., 2005; Hendriksen et al., 2008). Molecular evidence has indicated that the regulatory function of CodY as a repressor was achieved by directly binding to the conserved CodY box in promoter regions of target genes (Shivers and Sonenshein, 2004; Guédon et al., 2005), whereas the detailed mode of action about the mechanism of transcriptional activation of CodY is limited. Recently, we identified a functional ortholog of CodY in *S. thermophilus* ST2017, termed CodY_{st}. Transcriptome data and experimental evidences confirmed that CodY_{st} was involved in globally controlling the interconversion between carbon metabolism and nitrogen metabolism (Lu et al., 2015). In addition, bioinformatics analysis also suggested CodY_{st} had functions in control of resistance to environmental stresses. However, the target genes transcriptionally regulated by CodY_{st} for response to environmental stresses were unknown in *S. thermophilus*.

Glutathione (γ-glutamyl-cystemyl-glycine; **GSH**) is an important nonprotein thiol compound widely distributed in various organisms. Glutathione acts as an active factor to reduce cellular disulfide bonds for keeping the intracellular redox homeostasis and to protecting cells against oxidative damage (Li et al., 2003). Glutathione is also an electron donor for glutathione peroxidase, enabling a reduction of H₂O₂ into H₂O, thus

Received April 11, 2017.

Accepted June 17, 2017.

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preventing cells from damage by H_2O_2 and its derivatives (Pophaly et al., 2012). Recently, we found that GSH synthesis or utilization machinery extensively existed in *S. thermophilus*, and it was an important member of antioxidant compounds for this species to tolerate oxidative stress (Wang et al., 2015). GshF is an ortholog of bifunctional glutathione synthetase in most gram-positive bacteria, which catalyzes the biosynthesis of GSH using glutamate, cysteine and glycine as substrates (Vergauwen et al., 2006; Borgo et al., 2013). Although a novel bifunctional glutathione synthetase, GshF has been discovered to catalyze GSH biosynthesis in *S. thermophilus* (Li et al., 2011), its regulation still remains unclear. The aim of our study was to find the target genes involved in oxidative stress regulated by CodY_{st} in *S. thermophilus* ST2017, and to prove the bifunctional glutathione synthetase gene (*gshF*) was a target gene transcriptionally regulated by CodY_{st} in response to oxidative stress.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Streptococcus thermophilus ST2017 and its *codY_{st}* defective mutant were used in this study (Lu et al., 2015) and routinely propagated in M17 broth (Oxoid, Basingstoke, UK) supplemented with 1% (wt/vol) lactose (LM17 broth) statically at 42°C. To detect the GSH biosynthesis, *S. thermophilus* strains were propagated in the minimal chemically defined medium (mCDM) or mCDM supplemented with glutamate and glycine (Letort and Juillard, 2001). *Escherichia coli* DH5 α and *E. coli* BL21 were cultivated in Luria-Bertani (Oxoid, Basingstoke, UK) broth aerobically at 37°C. When appropriate, antibiotics were added at following final concentrations: erythromycin, 2.5 $\mu\text{g}/\text{mL}$ for *S. thermophilus*; chloramphenicol, 5 $\mu\text{g}/\text{mL}$ for *S. thermophilus* and *E. coli*; and ampicillin, 100 $\mu\text{g}/\text{mL}$ for *E. coli*.

Detection of Cell Survival Against Oxidative Stress

Cell survival under H_2O_2 treatment was detected as described previously (Thibessard et al., 2001). Briefly, after overnight static incubation at 42°C, *S. thermophilus* ST2017 and its *codY_{st}* defective mutant were 2% inoculated into 5 mL of fresh LM17 broth or mCDM with or without additive glycine and glutamate, and incubated statically at 42°C. When the optical density at 600 nm (OD_{600}) reached 0.4, cell cultures were treated with H_2O_2 to final concentrations of 2 and 10 mM for 30 min. When inoculated into mCDM, the cells in LM17 broth were harvested by centrifugation at 6,000

$\times g$ for 5 min at room temperature, washed twice, and resuspended in mCDM to a final OD_{600} of 0.1. The survival of *S. thermophilus* cells against oxidative stress was calculated by pipetting samples onto plates and determining the numbers of colony-forming units according to a previous method (Zhang et al., 2015).

Determination of GSH Content

To detect the GSH content, *S. thermophilus* cells grown in 5 mL of mCDM, with or without additive glutamate and glycine, were harvested at OD_{600} of 1.0 at 6,000 $\times g$ for 5 min at room temperature, washed twice, and resuspended in 1 mL of sodium phosphate buffer (0.2 M Na_2HPO_4 , 0.2 M NaH_2PO_4 , 2 mM EDTA, pH 7.4). Then, 0.1 g of glass beads (Sigma-Aldrich, St. Louis, MO) was added to the suspension and shaken in a bullet blender (Precellys 24, Bertin, French) for 30 s, 3 times. The supernatant was recovered by centrifugation at 13,000 $\times g$ for 10 min at 4°C and subjected to the GSH assay.

The GSH assay was carried out as previously described with minor modifications (Tietze, 1969; Griffith 1980; Li et al., 2003). Briefly, a 50- μL reaction system containing 35 μL of 0.3 mM NADPH, 10 μL of sample, and 5 μL of 6 mM 5,5-dithiobis(2-nitrobenzoic acid) was equilibrated at 25°C. Subsequently, 1 μL of 50 U/mL glutathione reductase from baker's yeast was added to the prewarmed solution. Finally, the resulting 51- μL solution was transferred to a cuvette with a 1-cm light path, and the change of absorbance at 412 nm was continuously monitored (UV-2012, Unico, Dayton, NJ). The NADPH, 5,5-dithiobis(2-nitrobenzoic acid), GSH, and glutathione reductase (Sigma-Aldrich) were prepared in phosphate buffer (0.125 M potassium phosphate, 6.3 mM EDTA, pH 7.5). The protein concentrations of the samples were determined by K5600 micro-spectrophotometer at the wavelength of 280 nm (Biolinker, Shanghai, China).

Analysis of Transcriptional Level of *gshF*

To analyze the transcriptional level of *gshF* in *S. thermophilus* ST2017 and *codY_{st}* defective mutant, transcriptome analysis and real-time quantitative (q) PCR were performed. The Illumina-based (Illumina, San Diego, CA) RNA sequencing and transcriptome data were from Lu et al. (2015). For real-time qPCR, total RNA was extracted using an RNA Simple total RNA kit (Tiangen, Beijing, China) according to the manufacturer's protocols. The appropriate mass of the total RNA was subjected to perform reverse transcription to synthesize cDNA, using a PrimeScript RT

reagent kit (Takara, Tokyo, Japan) according to the manufacturer's instructions. Real-time qPCR was carried out with SYBR Premix Ex TaqII (Takara) in the real-time PCR detection system (Roche LightCycler 480 system, Roche, Basel, Switzerland) according to the manufacturer's protocols. The *gshF* transcription was PCR amplified with the primers RT-*gshF*-F (5'-CAGTGACCACGGCTATGT) and RT-*gshF*-R (5'-TTGTCCACGGAAACGAAC). The 16S rRNA gene, as an internal standard, was amplified with the primers RT-16sF (5'-CTAACTACGTGCCAGCAGC) and RT-16sR (5'-GGTTGAGCCACAGCCTTTA). Relative mRNA level was normalized to the constitutive level of the 16S rRNA and was calculated by the comparative threshold cycle (C_T) method ($2^{-\Delta\Delta C_T}$).

Heterologous Expression and Purification of CodY_{st} Protein

The *codY_{st}* without its stop codon was PCR amplified from genomic DNA of *S. thermophilus* ST2017 with the primers CodYF and CodYR (Lu et al., 2015). The PCR product was restricted with *Bam*HI and *Xho*I, and inserted into the compatible site of pET-PPA, a vector consisting of an N-terminally 6 histidine-tagged and PreScission protease (PPA) cleavage site. The resultant plasmid pET-PPA-CodY_{st} was introduced into chemically competent *E. coli* BL21 (DE3) cells, generating the recombinant strain *E. coli* BL21/pET-PPA-CodY_{st}. To express CodY_{st}, *E. coli* BL21/pET-PPA-CodY_{st} was grown at 37°C aerobically to an OD₆₀₀ of 0.7, and then a final concentration of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce the overexpression of N-terminal His-tagged CodY_{st} at 16°C. The His-tagged CodY_{st} was purified by Ni-NTA affinity chromatograph. The N-terminal His-tag was excised by PreScission protease to obtain purified CodY_{st} protein.

Electrophoretic Mobility Shift Assays

The gel mobility shift assay was performed essentially as described previously (Pagels et al., 2010). Briefly, the upstream regions of *gshF* gene were amplified by PCR with primers P_{*gshF*}-F (5'-CGTGTTCATTCAGT-GATAGA) and P_{*gshF*}-R (5'-AGAATCACCTTTTCATC-TAATG). The DNA products (100 ng) were incubated with purified CodY_{st} protein at concentrations of 0, 100, 250, or 500 ng per reaction in binding buffer [10 mM Tris-HCl (pH 7.5), 5% (vol/vol) glycerol, 100 mM KCl, and 1 mM dithiothreitol] for 30 min at room temperature. To confirm specific protein-DNA interactions, replicates were carried out with 10 μg of sheared salmon testis DNA. After incubation, the reaction mixtures

were analyzed in a 5% nondenaturing polyacrylamide gel. Gel electrophoresis was performed at 100 V for 60 min in 0.5× Tris-acetate-EDTA buffer; DNA fragments and binding complexes were visualized by ethidium bromide staining.

Statistical Analysis

Each experiment was carried out at least in triplicate. Statistical analysis were performed by using the paired Student *t*-test. *P*-values of <0.05 were considered statistically significant.

RESULTS

Effects of CodY_{st} on Cell Survival Under H₂O₂ Stress

To determine whether CodY_{st} was associated with the oxidative stress response, the *codY_{st}*-deficient mutant was constructed (Lu et al., 2015), and cell survival under different H₂O₂ stresses was compared in *S. thermophilus* ST2017 and its *codY_{st}* defective strain. The results showed that, under the mild oxidative stress of 2 mM H₂O₂, cell survival of both wild-type strain and *codY_{st}* defective mutant was similar and just decreased by 1 order of magnitude compared with untreated strains (Figure 1). When challenged with severe oxidative stress of 10 mM H₂O₂, cell survival showed a sharp decline in *codY_{st}* defective mutant of approximately 3 orders of magnitude compared with the wild-type strain

Figure 1. Cell survival of *Streptococcus thermophilus* ST2017 (black bar) and *codY_{st}* defective mutant (gray bar) under oxidative stress induced by 2 and 10 mM H₂O₂ for 30 min was determined in LM17 broth (Oxoid, Basingstoke, UK). Data are shown as the mean ± SD, n = 3.

(Figure 1). These results demonstrated that CodY_{st} contributed to defending severe oxidative stress in *S. thermophilus* ST2017.

Analysis of Transcriptome and Real-Time qPCR of *gshF*

In general, CodY_{st} functions as a regulator by controlling the transcription of various target genes. To identify the target gene of CodY_{st} regulating responses to oxidative stress, the transcriptome data of *S. thermophilus* ST2017 against its *codY_{st}*-deficient mutant was analyzed. Twelve out of 86 genes controlled by the CodY_{st} regulator related to environmental stress responses were identified (Table 1), in which there were heat-shock-related orthologs, such as *hrcA*, *dnaK*, and *groES*, osmotic-stress-related orthologs, including *mscS* and *STND_1785*, some important regulators and other functional proteins and enzymes directly involved in stress responses and invasion. Among the functional genes related to stress responses, only *gshF* associated with oxidative stress response was downregulated by 2.9 fold in the *codY_{st}* defective mutant ($P < 0.05$). Real-time qPCR analysis showed that the transcriptional level of *gshF* in *codY_{st}* defective mutant was lower than that in the wild-type ST2017, with an approximately 3.3 fold reduction. These results indicated that CodY_{st} could activate the transcription of *gshF* in *S. thermophilus* ST2017.

GSH Content in *S. thermophilus* ST2017 and CodY_{st} Defective Mutant

Glutathione is an important antioxidant compound in *S. thermophilus*. As the transcription of *gshF* could be regulated by CodY_{st}, we speculated that CodY_{st} responded to the oxidative stress by the control of GSH biosynthesis. To confirm this hypothesis, the intracel-

Figure 2. The intracellular glutathione (GSH) concentrations of *Streptococcus thermophilus* ST2017 and *codY_{st}* defective mutant ($\Delta codY_{st}$) grown in minimal chemically defined medium (mCDM; white bar) and mCDM containing glutamate and glycine (gray bar) were determined in mCDM with glutamate and glycine. Data are shown as the mean $\pm$ SD, $n = 3$.

lular GSH concentrations for both wild-type strain and *codY_{st}* defective mutant were determined in mCDM. As shown in Figure 2, in mCDM without glutamate and glycine the intracellular GSH was not synthesized efficiently whether in wild-type strain or *codY_{st}* defective mutant, whereas when supplemented with glutamate and glycine the intracellular GSH could be produced and the GSH content of wild-type was 1.2 fold higher than *codY_{st}* defective mutant. These results suggested that the availability of adding glutamate and glycine was critical for the yield of GSH, and demonstrated that CodY_{st} was able to promote GSH biosynthesis in *S. thermophilus* ST2017.

Table 1. Functions and changes in expression of the genes related to stress response and regulation controlled by CodY_{st} in *Streptococcus thermophilus* ST2017

Locus (gene)	Assignment	Ratio [ST2017/ $\Delta codY_{st}$ log ₂ (fold change)]	P-value
<i>hrcA</i>	Heat-inducible transcriptional repressor HrcA	-1.54	1.23E-88
<i>grpE</i>	Putative Hsp-70 cofactor GrpE protein	-1.06	8.21E-92
<i>dnaK</i>	Hsp70-like molecular chaperone DnaK	-1.13	8.79E-182
<i>mscS</i>	Mechanosensitive ion channel (MscS) family	1.53	1.35E-06
<i>groES</i>	10 kDa molecular chaperone GroEL	-1.10	0
<i>covS</i>	Two-component sensor histidine kinase	-1.74	2.63E-105
STND_0658	CRISPR-associated type II RNA-guided endonuclease Cas9	-3.50	1.80E-68
<i>gntR</i>	Transcription regulator GntR family	1.40	5.91E-12
<i>gshF</i>	Bifunctional glutamate-cysteine ligase/glutathione synthetase	1.54	1.12E-40
<i>uspA</i>	Universal stress protein UspA	2.23	2.54E-48
STND_1784	Transcriptional regulator	2.31	2.27E-15
STND_1785	Permease	1.20	1.85E-196

Effects of GSH Against Oxidative Stress by CodY_{st}

To detect whether the change for GSH biosynthetic level regulated by CodY_{st} influenced cell survival under oxidative stress, the numbers of viable cells of wild-type strain and *codY_{st}* defective mutant were determined in mCDM under 10 mM H₂O₂ challenge. The results showed that the 2 strains displayed a similar survival rate when cultured in mCDM, whereas when adding exogenous glutamate and glycine into the medium the higher survival rate was observed in the wild-type strain compared with *codY_{st}* defective mutant (Figure

3). These results suggested that CodY_{st} endowed *S. thermophilus* cells with capability to defend oxidative stress by controlling the biosynthesis of GSH.

Binding of CodY_{st} to the Promoter of *gshF* In Vitro

Electrophoretic mobility shift assays were used to demonstrate the direct binding of CodY_{st} to the putative promoter region of the target genes. According to the website Softberry (<http://linux1.softberry.com>), about a 300-bp DNA fragment from the upstream region of *gshF* was predicted as the putative promoter, P_{*gshF*}. The CodY_{st} protein was expressed in *E. coli* BL21 and purified by His columns. As shown in Figure 4A, incubation of the P_{*gshF*} DNA fragment with increasing amounts of purified CodY_{st} led to retardation in the mobility of the DNA fragment. Two kinds of bands, representing free DNA and DNA-protein complexes, were observed, which was likely due to CodY_{st} anchoring at the binding-site of the promoter P_{*gshF*}. To verify the specificity of the interaction, CodY_{st} binding to an unrelated promoter was analyzed using identical experimental conditions as the control, where an absence of binding was observed (Figure 4B).

Due to the discovery of the interaction between CodY_{st} and P_{*gshF*} by electrophoretic mobility shift as-

Figure 3. Cell survival of *Streptococcus thermophilus* ST2017 and *codY_{st}* defective mutant ($\Delta codY_{st}$) challenged with 10 mM H₂O₂ for 30 min was determined in minimal chemically defined medium (mCDM) with or without additive glutamate (Glu) and glycine (Gly). Data are shown as the mean $\pm$ SD, $n = 3$.

Figure 4. Binding of CodY to DNA promoter region with (A) electrophoretic mobility shift assays analysis of CodY_{st} using about 300 bp of DNA fragments of the putative promoter, P_{*gshF*}, and (B) negative control.

livJ TATGATTGAC**AAATTTCTGATAATT**CTGTAACCTATAAGGTAGACTTTTTTAAGAAAATATTAAGGAGAAATCTTA-ATG
thrC AAATCCGGAT**TTTTTCCGATATTT**AAATGAAATTAACAAGTAAATCCGAATGATTCTCTCACAAGTTACAA
TATGTGTAGAA**AAATTTCTGACAATT**TATGCT-N39-ATG
ilvA ATAGATTGCT**ATTTTTCTGAAAATT**TGTGATACAAT AGAAATAGTTTGAATTTTGTGTCAAAGGAGAGCCA-ATG
thrB AAATGGCAGAA**AATATTCTGTCAATT**TACGATAGAAATATGGTACAATTAATAAATGTATG-N20-ATG
gdhA ACTTGACAAT**AAGATTCTGAAAATT**TATACTATTTCATGTGATTAAATTACTAAGGTAATT-N20-ATG
gshF ACTCATTCAA**AATATTCCGAAATTC**CTCTTAACCGTAGCAGAAATTTGTTTTCTGTTCCCTATTTATAA-N36-ATG

Consensus **AWTWTTCGANAWTT**

Figure 5. The sequence alignment of putative CodY_{st}-boxes of *gshF* and other CodY_{st}-dependent genes were shown in bold and underlined. The consensus was deduced from the alignment of the CodY_{st} boxes; W (A or T), K (T or C), N (A, T, or C).

say, the fragment of promoter P_{*gshF*} was sequenced and analyzed through bioinformatics. Through the sequence alignment with other regulated genes, the existence of a putative CodY_{st} box (AATATTCGGAAATTC) was observed in the promoter region of *gshF* (Figure 5). These results confirmed the regulation effect of CodY_{st} on the *gshF* by binding to its promoter region.

DISCUSSION

Bacteria possess a variety of regulators of gene expression to respond to different environmental signals. In many low G+C species of gram-positive bacteria, CodY, a global transcriptional factor, plays a pivotal regulatory role in cellular metabolism, environmental adaption, and virulence, as well as stress responses (Petranovic et al., 2004; Lemos et al., 2008; Stenz et al., 2011). Transcriptome analysis of CodY_{st} in *S. thermophilus* ST2017 showed that, in addition to mainly regulating the transcriptional expression of genes encoding enzymes involved in AA biosynthesis and transport for nitrogen supply (Lu et al., 2015), CodY_{st} was also involved in environmental stress responses. In this study, we demonstrated the CodY_{st} response to the oxidative stress was mediated by GSH through transcription analysis, determination of GSH content, and cell survival in vivo. Furthermore, the regulatory mechanism of CodY_{st} for *gshF* was proven to directly bind to its promoter region.

Although CodY plays an important role in nitrogen metabolism, the responses to environmental stresses are mostly indirectly controlled by other transcriptional regulators mediated by CodY (Brandenburg et al., 2002; Guédon et al., 2005). Recently, it has been found that CodY was able to directly regulate the urease operon to answer the change of acidic pH in *Strep-*

tococcus salivarius (Huang et al., 2014). Therefore, we speculated that whether CodY_{st} exerted a direct regulatory mechanism for environmental stress responses. Through detecting the responses of CodY_{st} to various stresses, we found that CodY_{st} was sensitive to oxidative stress induced by 10 mM H₂O₂, indicating that CodY_{st} enabled to respond to severe oxidative stress. As a facultative anaerobic bacterium, *S. thermophilus* can tolerate low concentration of H₂O₂ (Thibessard et al., 2001); thus, the 2 mM H₂O₂ appeared to have little effect on cell survival of both wild-type strain and *codY_{st}* defective mutant.

Recently, a novel bifunctional glutathione synthetase (GshF) was discovered to catalyze GSH biosynthesis and respond to oxidative stress in *S. thermophilus* (Wang et al., 2015). That the intracellular GSH concentration in *codY_{st}* defective mutant was lower than wild-type strain further proved that CodY_{st} could positively contribute to GshF activity and GSH formation in vivo. However, we found that GSH synthesis yield was basically the same between the wild-type strain and *codY_{st}* defective mutant in mCDM. Thus, we speculated that the production of GSH existed a certain threshold value, which was the lowest concentration for survival of *S. thermophilus*, and the lowest concentration of GSH could be synthesized by GshF using limited AA produced through catabolism and anabolism in vivo and equilibrated by the glutaredoxin system (Pophaly et al., 2012).

A previous study demonstrated that GSH could protect *S. thermophilus* cells against oxidative stress (Wang et al., 2015). Here, we found that the tolerance of *codY_{st}* defective mutant for oxidative pressure declined in mCDM with additive glutamate and glycine, confirming that CodY_{st} had a positive effect to defend oxidative stress through promoting the GSH activity

in *S. thermophilus* ST2017. Although GSH synthesis yield of *S. thermophilus* was not induced by oxygen (Wang et al., 2015), *codY_{st}* defective mutant decreased oxidative tolerance through the downregulation of GSH biosynthesis under the conditions stimulated by high concentrations of H₂O₂. Corresponding to the urease expression mediated by CodY (Huang et al., 2014), such regulation of CodY_{st} for GSH biosynthesis was a kind of depth control, which played a major role only in severe oxidative stimulation. Due to the lowest concentration of GSH, cell survival of both wild-type and mutant was similar in mCDM.

CodY_{st} exerts the transcriptional regulation by interaction with CodY box located on the promoter region of target genes (Guédon et al., 2005; Huang et al., 2014). *Streptococcus thermophilus* ST2017 also possesses a conserved 15-bp CodY_{st} box (A[AT]A[AT]TTC[TC]GA[ACT]A[AT]TT), which has been shown to serve as a high-affinity binding site for CodY_{st} (Lu et al., 2015). Through analyzing the DNA sequence of the promoter P_{*gshF*}, a conserved CodY_{st} box (AATATTCG-GAAATTC) was also found in P_{*gshF*}. Generally, CodY acted as a transcriptional repressor that controlled the transcription of a wide variety of genes by directly binding to the CodY box (Shivers and Sonenshein, 2004; Barbieri et al., 2015), and the regulatory effect of CodY was enhanced by its interaction with either of 2 ligands, GTP or branched-chain AA (Guédon et al., 2001; Hendriksen et al., 2008). However, the regulatory mechanism of the transcriptional activation of CodY has not yet been elucidated. Here, we speculated that the activated genes by CodY might be under direct control dependent on the CodY box as well.

CONCLUSIONS

We demonstrated the response to oxidative stress by the global transcriptional regulator CodY_{st} and verified the regulated gene, *gshF*, involved in oxidative stress response in *S. thermophilus* ST2017. CodY_{st} could accelerate the GSH biosynthesis by the way of depth control, so as to protect *S. thermophilus* cells against severe oxidative burst. Molecular evidence was provided to further indicate that CodY_{st} was able to positively control the transcription of *gshF* gene by binding to its promoter region.

ACKNOWLEDGMENTS

This work was supported by National Natural Science Foundation of China (31471715). Public Service Sectors (Agriculture) Special and Scientific Research Projects (201503134).

REFERENCES

- Barbieri, G., B. Voigt, D. Albrecht, M. Hecker, A. M. Albertini, A. L. Sonenshein, E. Ferrari, and B. R. Belitsky. 2015. CodY regulates expression of the *Bacillus subtilis* extracellular proteases Vpr and Mpr. *J. Bacteriol.* 197:1423–1432.
- Borgo, F., A. Carpen, C. Ferrario, S. Iametti, and M. G. Fortina. 2013. Genomic analysis reveals the biotechnological ability of *Enterococcus italicus* to produce glutathione. *J. Ind. Microbiol. Biotechnol.* 40:489–494.
- Brandenburg, J. L., L. V. Wray Jr., L. Beier, H. Jarmer, H. H. Saxild, and S. H. Fisher. 2002. Roles of PucR, GlnR, and TnrA in regulating expression of the *Bacillus subtilis* ure P3 promoter. *J. Bacteriol.* 184:6060–6064.
- Condon, S. 1987. Responses of lactic acid bacteria to oxygen. *FEMS Microbiol. Lett.* 46:269–280. <https://doi.org/10.1111/j.1574-6968.1987.tb02465.x>.
- Griffith, O. W. 1980. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal. Biochem.* 106:207–212.
- Guédon, E., P. Serror, S. D. Ehrlich, P. Renault, and C. Delorme. 2001. Pleiotropic transcriptional repressor CodY senses the intracellular pool of branched-chain amino acids in *Lactococcus lactis*. *Mol. Microbiol.* 40:1227–1239. <https://doi.org/10.1046/j.1365-2958.2001.02470.x>.
- Guédon, E., B. Sperandio, N. Pons, S. D. Ehrlich, and P. Renault. 2005. Overall control of nitrogen metabolism in *Lactococcus lactis* by CodY, and possible models for CodY regulation in *Firmicutes*. *Microbiology* 151:3895–3909. <https://doi.org/10.1099/mic.0.28186-0>.
- Hendriksen, W. T., H. J. Bootsma, S. Estevão, T. Hoogenboezem, A. deJong, R. de Groot, O. P. Kuipers, and P. W. Hermans. 2008. CodY of *Streptococcus pneumoniae*: Link between nutritional gene regulation and colonization. *J. Bacteriol.* 190:590–601. <https://doi.org/10.1128/JB.00917-07>.
- Hols, P., F. Hancy, L. Fontaine, B. Grossiord, D. Prozzi, N. Leblond-Bourget, B. Decaris, A. Bolotin, C. Delorme, S. D. Ehrlich, E. Guédon, V. Monnet, P. Renault, and M. Kleerebezem. 2005. New insights in the molecular biology and physiology of *Streptococcus thermophilus* revealed by comparative genomics. *FEMS Microbiol. Rev.* 29:435–463.
- Huang, S.-C., R. A. Burne, and Y.-Y. M. Chen. 2014. The pH-dependent expression of the urease operon in *Streptococcus salivarius* is mediated by CodY. *Appl. Environ. Microbiol.* 80:5386–5393. <https://doi.org/10.1128/AEM.00755-14>.
- Lemos, J. A., M. M. Nascimento, V. K. Lin, J. Abranches, and R. A. Burne. 2008. Global regulation by (p)ppGpp and CodY in *Streptococcus mutans*. *J. Bacteriol.* 190:5291–5299. <https://doi.org/10.1128/JB.00288-08>.
- Letort, C., and V. Juillard. 2001. Development of a minimal chemically defined medium for the exponential growth of *Streptococcus thermophilus*. *J. Appl. Microbiol.* 91:1023–1029. <https://doi.org/10.1046/j.1365-2672.2001.01469.x>.
- Li, W., Z. Li, J. Yang, and Q. Ye. 2011. Production of glutathione using a bifunctional enzyme encoded by *gshF* from *Streptococcus thermophilus* expressed in *Escherichia coli*. *J. Biotechnol.* 154:261–268.
- Li, Y., J. Hugenholtz, T. Abee, and D. Molenaar. 2003. Glutathione protects *Lactococcus lactis* against oxidative stress. *Appl. Environ. Microbiol.* 69:5739–5745.
- Lu, W. W., Y. Wang, T. Wang, and J. Kong. 2015. The global regulator CodY in *Streptococcus thermophilus* controls the metabolic network for escalating growth in the milk environment. *Appl. Environ. Microbiol.* 81:2349–2358.
- Pagels, M., S. Fuchs, J. Pané-Farré, C. Kohler, L. Menschner, M. Hecker, P. J. McNamarra, M. C. Bauer, C. von Wachenfeldt, and M. Liebeke. 2010. Redox sensing by a Rex-family repressor is involved in the regulation of anaerobic gene expression in *Staphylococcus aureus*. *Mol. Microbiol.* 76:1142–1161. <https://doi.org/10.1111/j.1365-2958.2010.07105.x>.

- Petranovic, D., E. Guédon, B. Sperandio, C. Delorme, D. Ehrlich, and P. Renault. 2004. Intracellular effectors regulating the activity of the *Lactococcus lactis* CodY pleiotropic transcription regulator. *Mol. Microbiol.* 53:613–621. <https://doi.org/10.1111/j.1365-2958.2004.04136.x>.
- Pophaly, S. D., R. Singh, S. D. Pophaly, J. K. Kaushik, and S. K. Tomar. 2012. Current status and emerging role of glutathione in food grade lactic acid bacteria. *Microb. Cell Fact.* 11:114.
- Shivers, R. P., and A. L. Sonenshein. 2004. Activation of the *Bacillus subtilis* global regulator CodY by direct interaction with branched-chain amino acids. *Mol. Microbiol.* 53:599–611. <https://doi.org/10.1111/j.1365-2958.2004.04135.x>.
- Stenz, L., P. Francois, K. Whiteson, C. Wolz, P. Linder, and J. Schrenzel. 2011. The CodY pleiotropic repressor controls virulence in gram-positive pathogens. *FEMS Immunol. Med. Microbiol.* 62:123–139. <https://doi.org/10.1111/j.1574-695X.2011.00812.x>.
- Tamime, A., and H. Deeth. 1980. Yogurt technology and biochemistry. *J. Food Prot.* 43:939–977.
- Thibessard, A., A. Fernandez, B. Gintz, N. Leblond-Bourget, and B. Decaris. 2001. Hydrogen peroxide effects on *Streptococcus thermophilus* CNRZ368 cell viability. *Res. Microbiol.* 152:593–596. [https://doi.org/10.1016/S0923-2508\(01\)01234-7](https://doi.org/10.1016/S0923-2508(01)01234-7).
- Tietze, F. 1969. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal. Biochem.* 27:502–522.
- van de Guchte, M., P. Serror, C. Chervaux, T. Smokvina, S. D. Ehrlich, and E. Maguin. 2002. Stress responses in lactic acid bacteria. *Antonie van Leeuwenhoek* 82:187–216. <https://doi.org/10.1023/A:1020631532202>.
- Vergauwen, B., D. De Vos, and J. J. Van Beeumen. 2006. Characterization of the bifunctional γ -Glutamate-cysteine Ligase/Glutathione Synthetase (GshF) of *Pasteurella multocida*. *J. Biol. Chem.* 281:4380–4394.
- Wang, T., W. W. Lu, S. Y. Lu, and J. Kong. 2015. Protective role of glutathione against oxidative stress in *Streptococcus thermophilus*. *Int. Dairy J.* 45:41–47. <https://doi.org/10.1016/j.idairyj.2015.01.015>.
- Zhang, C., Y. P. Xin, Y. Wang, T. T. Guo, S. Y. Lu, and J. Kong. 2015. Identification of a novel dye-decolorizing peroxidase, EfeB, translocated by a twin-arginine translocation system in *Streptococcus thermophilus* CGMCC 7.179. *Appl. Environ. Microbiol.* 81:6108–6119.