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

Y. Wang, H. Y. He, H. H. Li, W. W. Lu, T. T. Guo, and J. Kong[1]
State Key Laboratory of Microbial Technology, Shandong University, Jinan, 250100, P. R. China

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 m*M* H_2O_2, 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 H_2O_2 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 10^{21} 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 ($O_2^{\bullet-}$), hydroxyl radical (•OH), and hydrogen peroxide (H_2O_2), 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 H_2O_2 into H_2O, thus

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[1]Corresponding author: kongjian@sdu.edu.cn

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 *cod*Y_{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* DH5α 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 μg/mL for *S. thermophilus*; chloramphenicol, 5 μg/mL for *S. thermophilus* and *E. coli*; and ampicillin, 100 μg/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 *cod*Y_{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 m*M* for 30 min. When inoculated into mCDM, the cells in LM17 broth were harvested by centrifugation at 6,000 × *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 × *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 m*M* 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 × *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 m*M* NADPH, 10 μL of sample, and 5 μL of 6 m*M* 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 m*M* 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 *cod*Y_{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