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EXPERIMENTAL ARTICLES

Effect of Site-Directed Mutagenesis of *citB* on the Expression and Activity of *Bacillus subtilis* Aconitase[1]

Weihua Gao, Sen Dai, Quanli Liu, Haijin Xu, Yanlin Bai, and Mingqiang Qiao[2]

The Key Laboratory of Molecular Microbiology and Technology, Ministry of Education of China

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Abstract—*Bacillus subtilis* aconitase, encoded by the *citB* gene, is a bifunctional enzyme, which can not only interconvert citrate and isocitrate, but also has the RNA binding function similar to the eukaryotic protein IRP-1 (iron regulatory protein 1). Homology analysis between eukaryotic aconitase and *B. subtilis* aconitase indicates that the amino acids 741–745 probably have important function for the *B. subtilis* aconitase. To analyse the exact effect of these amino acids for aconitase activity, a site-directed mutagenesis of the *citB* is constructed, in which, the Arg741 and Gln745 are both changed into Glu. The resulting strain exhibits an increased enzymatic activity of aconitase comparing to that of the wild-type strain. Western blotting shows that the aconitase protein expression level is significantly increased in the mutant strain. By β-Galactosidase activity assay, the transcription level of *citB* is also increased. These results indicate that the mutation of *citB* gene has significant effect on *B. subtilis* aconitase transcription, expression and enzymatic activity.

Keywords: *Bacillus subtilis, citB*, aconitase.

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Aconitase is an enzyme that catalyses the intercorvesion of citrate and isocitrate in the citric acid cycle and plays a great role in the cell metabolic and regulation network [1, 2]. *Bacillus subtilis* aconitase, encoded by the *citB* gene, is structurally highly related to eukaryotic IRP-1, an iron-responsive RNA binding protein [3]. Compelling evidence demonstrates that *B. subtilis* aconitase is both an enzyme of the citric acid cycle and an RNA binding protein which is similar to its eukaryotic counterpart [2]. A null mutation in *B. subtilis citB* causes a block in Spo0A-phosphate-dependent gene expression [4, 5] and accumulation of citrate [4]. In addition, *B. subtilis* aconitase can bind some iron relative genes, such as *qoxD* and *feuAB* [2]. It has also been reported that the untranslated region (UTR) of *gerE* mRNA can form a secondary structure similar to the IRE. Thus, aconitase may affect sporulation by stabilizing the mRNA of the GerE transcription factor [6].

In some other prokaryotes, aconitase also shows complex effect on the cell phenotype. *Escherichia coli* aconitases A and B are both proved to be RNA binding proteins and are autoregulatory at the protein production level [7]. They also have opposite regulation effects on SodA synthesis [8]. In *Salmonella enterica serovar Typhimurium*, the RNA binding ability of aconitase is involved with the transcription of a protease, which is important for flagella control [9]. It has also been reported that in some pathogenic bacteria, such as *Staphylococcus aureus, Pseudomonas aeruginosa, Xanthomonas campestris* pv. *campestris*, aconitase is involved in the regulation of pathogenicity factor production [10–12].

Previous study indicates that the Arg728 and Arg732 are critical for interaction of IRP-1 with RNA targets [13]. Homology analysis between eukaryotic aconitase and *B. subtilis* aconitase indicates that the amino acids 741–745 probably have similar function for the *B. subtilis* aconitase [6]. To analyse the exact function of amino acids 741–745 of *B. subtilis* aconitase, a site-directed mutagenesis of *citB* is introduced to the chromosome DNA. Our results uncover that the mutation of *citB* gene has a remarkable impact on *B. subtilis* aconitase transcription, expression and enzymatic activity.

MATERIALS AND METHOD

Bacterial strains, plasmids, medium and growth condition. The *B. subtilis* strains used in this study are described in table and were routinely grown at 37°C in LB medium. The same medium with the addition of agar were used for growth of bacteria on plates. *E. coli* strain DH5α was used for isolation of plasmids and was grown in LB medium. The antibiotics used, when appropriate, erythromycin (Ery), 0.3 mg/ml for *B. subtilis* strains, and ampicillin (Amp), 50 mg/ml for *E. coli* strains.

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2 Corresponding author; e-mail: e-mail: mingqiangqiao@yahoo.com.cn

Strains and plasmids

Strains or plasmids	Characterization	Source
B. subtilis strain		
NK1010	wild-type	lab store
GWH1025	*citBw*-his6:: pMutin4 (Ery^r)	this study
GWH1026	*citBm*-his6 (R740E Q744E) :: pMutin4 (Ery^r)	this study
E. coli strain		
DH5α	supE44 DlacU169 (f80 *lacZ*DM15) hsdR17 RecAl endAl gyrA96 thi-l relAl Host for cloning vector	lab store
Plasmid		
pMUTIN4	integrative vector	BGSC
pMCl	pMUTIN4 plus C-terminal of *citB*, Ery^r	this study
pMC2	pMUTIN4 plus C-terminal of *citB*(R741E Q745E), Ery^r	this study

Construction of *citB* mutant strain. To construct a strain with site-directed mutagenesis of *citB*, the C-terminal 1.2 kb fragment of *citB* was amplified from *B. subtilis* NK1010 chromosomal DNA by PCR with primers of MCP1: 5'-TTATCCCGGGCTCTAAA-GTCGTTAC-3' and MCP2 5'-GCG**GGATCC**-CCTATTGATTCATCAGTGATGATGGTGATGGTG-GGACTGCTTCAT-3', which contains a *Bam*HI site (in bold) and 6-histidine codons (shown by the underline) at the end of the *citB* ORF. The amplified fragment was purified with a PCR purification kit (Solarbio) and restricted with *Bam*HI and *Hin*dIII, which in the amplified fragment. After digestion, the DNA fragment was purified with the same kit and subcloned into the integrative vector pMUTIN4 digested by *Hin*dIII and *Bam*HI. The ligated plasmid was transformed into *E. coli* DH5α to amplify and then resulted in integrative plasmid pMC1. The parent plasmid pMC1 was subjected to inverse PCR using *pfu* ultra polymerase with the invert primers MCP3. 5'-GAGGAACATTT-GCAAACATCGAGATCAAAAACGAAATCGCACC-GG-GTACAG-3' and MCP4: 5'-CTGTACCCGGGT-GCG-ATTTCGTTTTTGATCTCGATGTTTGCA-AATGTTCCTC-3', to construct site-directed mutagenesis, in which the Arg741 and Gln745 were both changed into Glu. The methylated and hemimethylated plasmid pMC1 was digested with *Dpn* I and the PCR resulting plasmid pMC2 was transformed into *E. coli* DH5α to make amplification. The mutant plasmid pMC2 and parental plasmid pMC1 were identified by Sequencing and then transformed into wide-type strain *B. subtilis* NK1010 respectively. Ery resistance mutants with the integration at the *citB* locus by homologous recombination were screened and then we got the two strains GWH1025 and GWH1026.

Aconitase activity assay. Enzymatic activity was determined as described method with little modification [1]. Samples of cell extract were incubated in a 3 ml reaction containing 90 mM Tris-HCL (pH 8.0) and isocitrate (20 mM) as substrate. The accumulation of cis-aconitate was measured as the change in absorbance at 240 nm. One unit of activity was defined as a change in absorbance of 0.0033 per minute. Aconitase-specific activity is expressed in units of activity per minute per milligram of protein.

Western-blot analysis of the aconitase. For western-blot analysis, cells were grown in LB medium, harvested at the indicated times, washed with ice-cold 10 mM Tris-HCL, (pH 7.6), and then ultrasound-assisted extract was obtained. Proteins content was quantified by Coomassie brilliant blue G-250. Proteins were resolved by polyacrylamide gel electrophoresis and transferred onto Nitrocellulose Blotting Membranes (Millipore). The membranes were blocked in phosphate-buffered saline (PBS) which contains 0.1% Tween 20 and 5% fat-free dry milk and incubated with the primary antibody for 2 hours and then incubated with horseradish peroxidase-conjugated secondary antibodies for an hour. Specific proteins were visualized with enhanced chemiluminescence detection reagent (Trangene Biotechnology).

β-galactosidase activity assay. To assay β-galactosidase activity, we diluted overnight cultures in 300 ml LB medium, incubated in 1L flasks with shaking at 37°C, 200 rpm, and samples were taken at regular intervals. At each time point, 1 ml cell suspension was harvested for analysis and the resulting cell pellet was stored at −20°C. The β-galactosidase assays were developed as described previously. Cells were suspended in equal volume of Z buffer (40 mM NaH_2PO_4, 60 mM Na_2HPO_4, 1 mM $MgSO_4$, 10 mM KCl and β-mercaptoethanol, pH 7.0 50 mM). Lysozyme was added to each sample to a final concentration of 0.2 mg/ml and samples were incubated at 30°C for 15 min. Each sample was diluted in Z-buffer to a final volume of 500 μl and the reaction was started with 100 μl of 4.0 mg/ml *o*-Nitrophenyl-β-*D*-galactoside in Z buffer and stopped with 250 μl of 1 M Na_2CO_3. The OD_{420} of the reaction mixture was measured and the β-galactosidase activity was calculated according to the equation: (OD_{420} × dilution factor × 1000)/(time × OD_{600}).

RESULTS

Site-directed mutagenesis of *citB*. As previously reported that the R741 and Q745 are important for the RNA binding ability of *B. subtilis* aconitase [6], a site-directed mutagenesis strategy (Fig. 1a) which converts both of these residues to glutamate was taken. In order to check the expression level of aconitase protein in different genetic background, a His6 tag was incorporated into the mutant and wild-type C-terminal *citB* respectively. Integrative plasmid pMC1 and pMC2

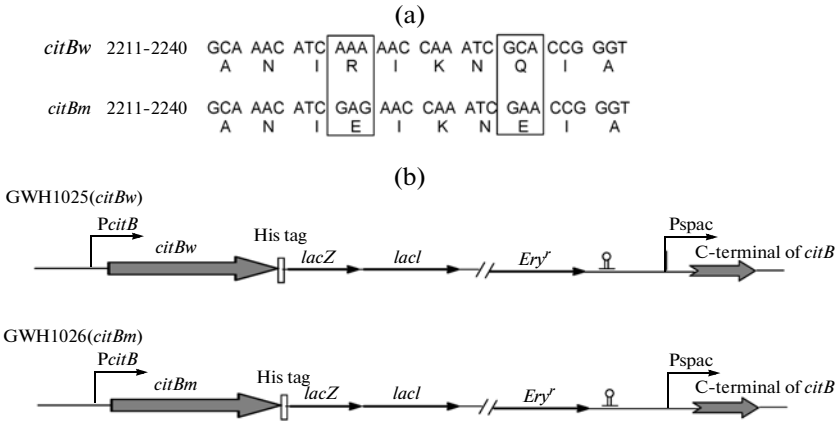

Fig. 1. Schematic presentation of site-directed mutagenesis of *citB*. (a) The site-directed mutagenesis of the *citB* C-terminal fragment. R741 and Q745 were changed into Glu. (b) The mutant and wild-type C-terminal fragments with His6 tag at the end of *citB* ORF were integrated into chromosome DNA through vector pMUTIN4 by a single crossover event. A reporter gene *lacZ* was transcriptionally fused to the *citB* to determine the *citB* expression in GWH1026 and GWH1025 respectively.

were transformed and integrated by homologous recombination at the *citB* locus. In the resulting strains GWH1025 and GWH1026, a *lacZ* reporter gene coming from the integrative vector pMUTIN4 was fused to the *citB* to analysis the expression of *citB* (shown in Fig. 1b) [14].

Effect of *citB* mutation on the enzymatic activity of aconitase. The appearance of aconitase activity in strains GWH1025(*citB*w) and GWH1026(*citB*m) were analyzed in LB medium during exponential growth and stationary phase (Fig. 2). We can see from the result that the enzymatic activity of aconitase mutant strain GWH1026 was apparently higher than that of the wild-type strain NK1020. Two possibilities may probably explain the aconitase activity increase. One was that the site-directed mutation of *citB* increased intrinsic specific activity of aconitase. The other explanation was that there may be a higher level of aconitase protein than the wild type strain, caused by a higher expression level of *citB* in GWH1026.

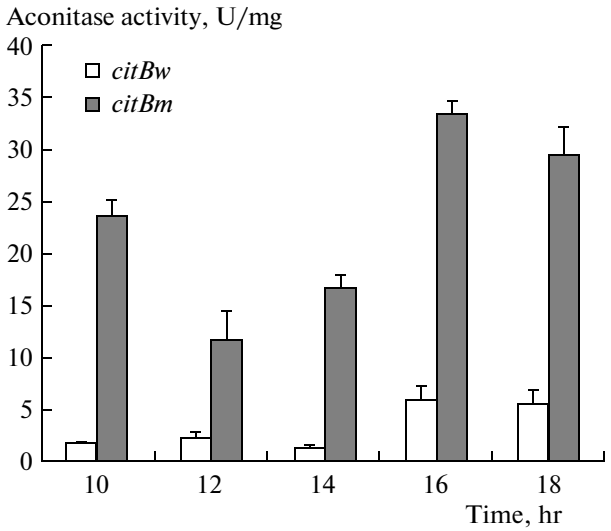

Fig. 2. Activity of aconitase in cell extracts. Strain GWH1026 (*citBm*) and GWH1025 (*citBw*) were grown on LB medium. The cells were harvested from the exponential phase (10 h) to the stationary phase (after 14 h) and ultrasonic disrupted.

Effect of mutation on the protein expression of aconitase. To investigate the protein level of aconitase and make sure the factors accounting for the enhanced enzymatic activity, western-blot was performed with antibody specific to the His6 tag fused to both the wild-type and mutant aconitases. As shown in Fig. 3, the expression level of the mutant aconitase protein was evidently higher than that of the wild-type at the different growth time points respectively. The difference of protein level between the wild-type and mutant strain was approximately consistent with the result of enzymatic assay. The result illustrates that the

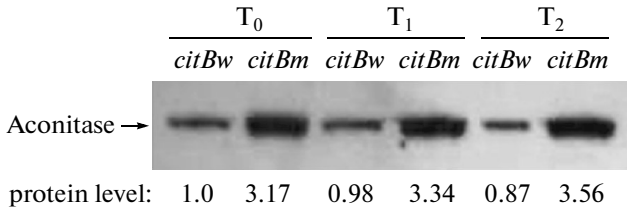

Fig. 3. Western-blot with antibody to His6 fused to aconitase. GWH1026 (*citBm*-His6) and GWH1025(*citBw*-His6) were grown on LB medium at 37°C. Samples of cell extract were isolated at the time indicated (T_0 = 14 h, T_1 = 16 h, T_2 = 18 h) and 10 μg of total protein of the samples were taken to SDS-PAGE and analyzed by Western-blot with antibody specific to His6 tag. The intensity of protein bands was analyzed by the ImageJ software and corrected by subtracting the measured intensity with the background intensity.

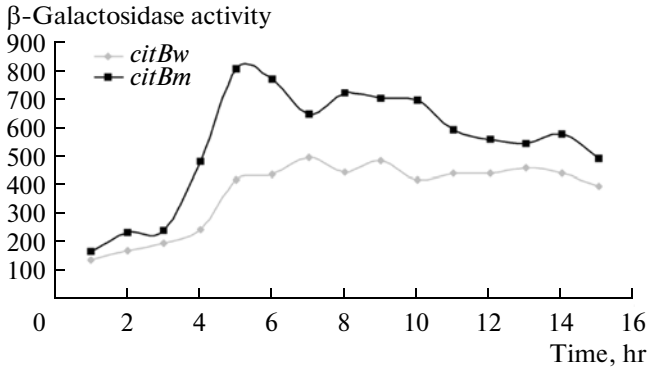

Fig. 4. β-Galactosidase assays of the GWH1026(*citB*m), GWH1025(*citB*w). The *citB-lacZ* fusion schematically shown in Fig. 1b was used to monitor the expression of the *citB* operon in different genetic backgrounds.

site-directed mutant of *citB* increased the protein level of aconitas and the enhanced protein level increased the aconitase enzymatic activity in the mutant strain GWH1026.

Effect of mutation on *citB* transcription. The remarkable effect of mutation on aconitase protein expression suggests that the mutation may affect the *citB* transcription level. To test this hypothesis, the transcription level of *citB*, expressed from *LacZ* transcriptionally fused to the *citB*, was test by β-Galactosidase activity assay.

We found that in the mutant strain GWH1026, the β-galactosidase activity was significantly higher than that of GWH1025 during the entire growth process (Fig. 4). The result indicated that the *citB* promoter of GWH1026 exhibited a higher transcription level than that of the strain GWH1025, Therefore, we may conclude that the increased aconitase protein level in mutant strain may be partly due to the increased transcription level of *citB*. And the transcription level was of *citB* was affected by the site-directed mutation of aconitase.

DISCUSSION

As one of the major enzyme of citric acid cycle, aconitase plays a great role in the metabolic network. In addition, the aconitase protein structure highly resembles to the eukaryotic protein IRP-1 and was postulated to have post-regulation effect on some gene expression. It has been identified that the Arg728 and Arg732 are critical for the regulatory function of IRP-1. In this study, a site-directed mutagenesis in the *citB* is introduced to the *B. subtilis* chromosome DNA. In the result strain, the Arg741 and Gln745 of aconitase, which have similar function with the Arg728 and Arg732 in IRP-1, are both changed into Glu. Enzymatic activity assay indicates that the mutant strain GWH1026 exhibits higher enzymatic activity of aconitase than that of the wild-type strain NK1020. In an earlier report, amino acid substitutions of R741E, Q745E, F662L, I810T, and V865A increased the enzymatic activity of aconitase greatly[6]. This phenomenon was ascribed to the assumed structure change of the mutant aconitase. However, a higher protein level of aconitase is detected in our study by western-blot analysis. This finding indicates that the increased enzymatic activity is at least partly due to a higher protein level of aconitase in the mutant strain. Further investigation reveals that the transcription level of the *citB* promoter is also increased in strain GWH1026. These results suggest that the amino acid residues Arg741 and Gln745 play great role in the *B. subtilis* aconitase function.

There were complex regulation mechanisms of aconitase expression, which involved with several regulators, such as *Fur* [15, 16], *CcpC*, *AbrB*, *CodY* and *GlnA* [17, 18]. These regulators perform their functions varying with growth conditions. Considering the homologous similarity between aconitase and IRP-1, the change of Arg741 and Gln745 may affect the RNA binding ability of aconitase and regulatory network of *B. subtilis*. This effect may affect the transcription, translation and enzymatic activity of aconitase.

Since the aconitase is important for the citric acid cycle and the citric acid cycle is responsible for the production of biosynthetic intermediates and ATP for the cell growth after late exponential phase, the mutation of aconitase would affect the cell post-exponential-phase growth. Study of the aconitase gives us a better understanding of growth properties of *B. subtilis*.

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