



Role of ClpB From *Corynebacterium crenatum* in Thermal Stress and Arginine Fermentation

Mingzhu Huang^{1,2}, Yue Zhao¹, Lin Feng¹, Lingfeng Zhu¹, Li Zhan¹ and Xuelan Chen^{1,2*}

¹ Department of Life Science, Jiangxi Normal University, Nanchang, China, ² Key Laboratory of Functional Small Organic Molecule of Ministry of Education, Jiangxi Normal University, Nanchang, China

OPEN ACCESS

Edited by:

Kesen Ma,
University of Waterloo, Canada

Reviewed by:

Nicolai Kallscheuer,
Radboud University Nijmegen,
Netherlands
Sung Sun Yim,
Columbia University, United States
Fernando Pérez-García,
Norwegian University of Science
and Technology, Norway

*Correspondence:

Xuelan Chen
xuelanchen162@163.com

Specialty section:

This article was submitted to
Microbial Physiology and Metabolism,
a section of the journal
Frontiers in Microbiology

Received: 19 December 2019

Accepted: 25 June 2020

Published: 17 July 2020

Citation:

Huang M, Zhao Y, Feng L, Zhu L,
Zhan L and Chen X (2020) Role
of ClpB From *Corynebacterium*
crenatum in Thermal Stress
and Arginine Fermentation.
Front. Microbiol. 11:1660.
doi: 10.3389/fmicb.2020.01660

ClpB, an ATP-dependent molecular chaperone, is involved in metabolic pathways and plays important roles in microorganisms under stress conditions. Metabolic pathways and stress resistance are important characteristics of industrially -relevant bacteria during fermentation. Nevertheless, ClpB-related observations have been rarely reported in industrially -relevant microorganisms. Herein, we found a homolog of ClpB from *Corynebacterium crenatum*. The amino acid sequence of ClpB was analyzed, and the recombinant ClpB protein was purified and characterized. The full function of ClpB requires DnaK as chaperone protein. For this reason, *dnaK/clpB* deletion mutants and the complemented strains were constructed to investigate the role of ClpB. The results showed that DnaK/ClpB is not essential for the survival of *C. crenatum* MT under pH and alcohol stresses. The ClpB-deficient or DnaK-deficient *C. crenatum* mutants showed weakened growth during thermal stress. In addition, the results demonstrated that deletion of the *clpB* gene affected glucose consumption and L-arginine, L-glutamate, and lactate production during fermentation.

Keywords: ClpB, DnaK, *Corynebacterium crenatum*, thermal stress, arginine

INTRODUCTION

Molecular chaperones are essential for homeostasis in living cells. Their fundamental role is to aid proteins in achieving their functional and final conformations (Hartl and Hayer-Hartl, 2002). ClpB is an ATP-dependent molecular chaperone that reactivates and disaggregates aggregated proteins with the DnaK chaperone system (Mogk et al., 2018). Similar to other ATP-dependent molecular chaperones, ClpB forms a hexameric ring structure to mediate protein disaggregation. The mechanism of the ClpB-catalyzed protein disaggregation is the coupling of ATP hydrolysis with the translocation of polypeptide substrates through the central channel of barrel-shaped hexamers (Li et al., 2015).

Proteolysis is essential under conditions where many misfolded and damaged proteins are likely to accumulate, particularly oxidizing environments, non-physiological pH, or elevated temperatures (Raivio, 2018). The survival rate of bacteria decreases tremendously without ClpB when cells are under various stressful conditions, and ClpB is involved in supporting the virulence of some bacterial pathogens (Squires et al., 1991; Athar et al., 2018; Sangpuui et al., 2018). Multiple enzymes of the central carbon metabolism are potential substrates of ClpB. Hence, this protein has regulatory activity in terms of the metabolism. For example, the majority of ClpB-interacting proteins in *Leptospira interrogans* is associated with metabolic pathways, such as the tricarboxylic

acid (TCA) cycle, amino acid metabolism, and glycolysis–gluconeogenesis (Joanna et al., 2018). ClpB orthologs have been identified in plants, fungi, bacteria, and protozoa (Ranaweera et al., 2018). Nevertheless, in microbes employed on the industrial scale, the role of ClpB has not been sufficiently investigated. Industrially relevant bacteria also experience various stresses during fermentation (Oide et al., 2015), and studies on metabolic pathways are extremely important for the production of compounds using industrially relevant bacteria. Thus, the relevance of ClpB in industrially relevant bacteria is worth to be investigated in detail.

Corynebacterium glutamicum, an industrial bacterium, has a long history in the industrial production of various amino acids (Lee and Wendisch, 2017). *C. glutamicum* mutant strains have been constructed for a number of useful metabolites, such as polyphenols, organic acids, and alcohols (Litsanov et al., 2012; Kallscheuer et al., 2016; Vogt et al., 2016). The *C. glutamicum* genome contains structural genes coding for chaperone systems including *clpC*, *clpB*, *clpX*, *dnaK*, *clpP1*, and *clpP2* (Engels et al., 2004). Some chaperone systems have been studied in *C. glutamicum*. For example, a *clpC*-deficient *C. glutamicum* mutant exhibited higher levels of gene expression compared with the wild type (Engels et al., 2004). The expression of *clpC* was clearly upregulated at pH 6.0 (Peng et al., 2019). However, most chaperone systems have not been studied in *C. glutamicum*. *Corynebacterium crenatum* is a close relative of *C. glutamicum*, and its mutant strains can also produce various industrial compounds (Su et al., 2014). In this study, we found a ClpB ortholog (an ATP-dependent molecular chaperone) from *C. crenatum*. The *clpB* gene was cloned and heterologously expressed in *Escherichia coli*, and the C-terminal His-tagged fusion protein was purified and characterized. We constructed *dnaK/clpB* deletion mutants and the complemented strains to investigate the role of ClpB and DnaK in *C. crenatum* MT (mutant strain that is auxotrophic for biotin and produces L-arginine, laboratory stock). Our investigation demonstrated that ClpB and DnaK are not essential for the survival of *C. crenatum* MT under pH and alcohol stresses. However, *dnaK/clpB* deletion mutants showed weakened growth during thermal stress. In addition, we showed that the deletion of the *clpB* gene affected glucose consumption and L-arginine, L-glutamate, and lactate production during fermentation.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

All strains and plasmids used in this study are listed in **Table 1**. *C. crenatum* strains were grown in Luria–Bertani (LB) medium (containing per liter of distilled water: 10 g of tryptone, 10 g of NaCl, 5 g of yeast extract, pH 7.0) at 30°C. *E. coli* DH5 α /BL21 (DE3) strains were cultured in LB medium at 37°C. When required, kanamycin and chloramphenicol (Solarbio, China) were used at a concentration of 12.5 μ g/ml for *C. crenatum* and 25 μ g/ml for *E. coli*. BHI medium (Land bridge, China) was used for the transformation of *C. crenatum* via electroporation.

Sucrose medium was used to remove pK18*mobsacB* containing *sacB*, which is a sucrose lethal gene (Chen et al., 2015). The fermentation medium (per liter) was composed of 120 g of glucose, 40 g of corn steep liquor, 1.5 g of KH₂PO₄, 20 g of (NH₄)₂SO₄, 0.5 g of MgSO₄·7H₂O, 0.02 g of FeSO₄·7H₂O, 0.05 g of MnSO₄·2H₂O, 30 g of CaCO₃, 5 \times 10⁻⁴ g of thiamine, 8 \times 10⁻⁵ g of biotin, and pH 7.0. The seed medium (per liter) of *C. crenatum* consisted of 40 g of (NH₄)₂SO₄, 20 g of corn steep liquor, 1.5 g of urea, 30 g of glucose, 1 g of KH₂PO₄, 0.5 g of MgSO₄·7H₂O, and pH 7.0 (Chen et al., 2015). Growth analyses were performed using the defined medium CGXII (containing per liter of distilled water: 20 g of (NH₄)₂SO₄, 40 g of glucose, 5 g of urea, 1 g of K₂HPO₄, 1 g of KH₂PO₄, 0.25 g of MgSO₄·7H₂O, 10 mg of MnSO₄·H₂O, 10 mg of FeSO₄·7H₂O, 0.25 mg of CaCl₂·2H₂O, 0.2 mg of CuSO₄·5H₂O, 1 mg of ZnSO₄·7H₂O, 0.02 mg of NiCl₂·6H₂O, 0.2 mg of biotin, 40 g of 3-(*N*-morpholino) propanesulfonic acid, pH 7.0).

Construction of Plasmids and Strains

The primers applied in this study are shown in **Table 2**. The *clpB* and *dnaK* genes in *C. crenatum* MT were deleted using pK18*mobsacB* (Schafer et al., 1994). In brief, we amplified upstream and downstream sequences of *clpB* and *dnaK* by specific primers. The upstream and downstream sequences were fused using overlapping PCR. The fused fragments were ligated into pK18*mobsacB* by NovoRec[®]Plus PRC kit (Novoprotein, China). The recombinant plasmids were used for the transformation of *C. crenatum* MT using electroporation as previously reported (Zhang et al., 2017). The single exchange clones were selected using kanamycin, whereas the double crossover clones were checked using PCR. The coding sequences of *clpB* and *dnaK* were amplified by the primers with His-tag at the C-terminus. The PCR-amplified *clpB* and *dnaK* were ligated into the *E. coli*–*C. glutamicum* shuttle vector pXMJ19. The recombinant vectors were used for the transformation of *C. crenatum* Δ *clpB* and *C. crenatum* Δ *dnaK* as complemented strains, respectively. The plasmid was used for the transformation of *E. coli* BL21 (DE3) via heat shock. The *clpB* gene was expressed in *E. coli* BL21 (DE3), and the ClpB protein was purified as a hexamer as previously reported (Lupoli et al., 2016; Rizo et al., 2019). In brief, *E. coli* BL21 (DE3)-*clpB* was incubated in LB medium for heterologous gene expression at 37°C, supplemented with 0.5 mM isopropyl- β -D-thiogalactoside, and cultivated for 12 h. The proteins were harvested and analyzed by SDS-PAGE (Wang et al., 2020). Whole cells were disrupted using sonication (Wang et al., 2020). Subsequently, the recombinant protein was purified using Ni-NTA affinity chromatography (Sangon Biotech, China). Total protein content was determined using a BCA protein assay kit (Nanjing Jiancheng, China).

Cell Growth Analyses

For liquid growth experiments, the strains were inoculated in LB medium for approximately 18 h and were subsequently transferred into 100 ml of fresh CGXII medium to an initial OD at 562 nm of 0.06. Approximately 3 ml of each culture was collected every hour for OD measurement. For solid growth experiments, the strains were inoculated in LB medium for approximately 18 h.

TABLE 1 | Strains and plasmids in this study.

Strains/plasmids	Characteristics	Resources
Plasmids		
pK18 <i>mobsacB</i>	Mobilizable vector, allows for selection of double crossover in <i>C. crenatum</i> , Km ^R , <i>sacB</i>	Biovector
pXMJ19	Shuttle vector for overexpression, Chl ^R	Biovector
pK18 <i>mobsacB-clpB</i>	A derivative of pK18 <i>mobsacB</i> , harboring <i>clpB</i> fragments	This work
pK18 <i>mobsacB-dnaK</i>	A derivative of pK18 <i>mobsacB</i> , harboring <i>dnaK</i> fragments	This work
pXMJ19- <i>clpB</i>	A derivative of pXMJ19, harboring <i>clpB</i> gene fragments	This work
pXMJ19- <i>dnaK</i>	A derivative of pXMJ19, harboring <i>dnaK</i> gene fragments	This work
Strains		
<i>E. coli</i> DH5 α	Clone host strain	Invitrogen
<i>E. coli</i> BL21(DE3)	Recombinant expression	Invitrogen
<i>E. coli</i> BL21 (DE3)- <i>clpB</i>	Harboring pXMJ19- <i>clpB</i>	This work
<i>C. crenatum</i> MT	mutation strain with auxotrophy for biotin, and producing L-Arg	Lab Stock (Chen et al., 2015)
<i>C. crenatum</i> Δ <i>clpB</i>	<i>C. crenatum</i> MT with a deletion of the <i>clpB</i> gene	This work
<i>C. crenatum</i> Δ <i>dnaK</i>	<i>C. crenatum</i> MT with a deletion of the <i>dnaK</i> gene	This work
<i>C. crenatum</i> Δ <i>dnaK</i> Δ <i>clpB</i>	<i>C. crenatum</i> MT with deletion of the <i>dnaK</i> and <i>clpB</i> genes	This work
<i>C. crenatum</i> Δ <i>clpB</i> (pXMJ19- <i>clpB</i>)	Complemented strain of <i>C. crenatum</i> Δ <i>clpB</i>	This work
<i>C. crenatum</i> Δ <i>dnaK</i> (pXMJ19- <i>dnaK</i>)	Complemented strain of <i>C. crenatum</i> Δ <i>dnaK</i>	This work
<i>C. crenatum</i> (pXMJ19)	<i>C. crenatum</i> MT harboring pXMJ19	This work

TABLE 2 | Primers and sequences in this study.

Primers	Primer sequences (5'-3')	Target
<i>clpB</i> -up-F	AACGACGGCCAGTGCCAAAGCTCAATGAGTATGGCGTGGCGTA	Upstream fragment of <i>clpB</i>
<i>clpB</i> -up-R	CAATGACAGGATCGAAACGTACTCATCGCCTAACTC	Upstream fragment of <i>clpB</i>
<i>clpB</i> -down-F	GATGAGTACGTTTCGATCCTGTCTTGGCCGTGAC	Downstream fragment of <i>clpB</i>
<i>clpB</i> -down-R	CGGTACCCGGGGATCCTCTAGCGTTCTTGGAAAGCTGCATCG	Downstream fragment of <i>clpB</i>
<i>dnaK</i> -up-F	AACGACGGCCAGTGCCAAAGCTGACGTGACGTAGGAATTGACCTTGG	Upstream fragment of <i>dnaK</i>
<i>dnaK</i> -up-R	ATGCGCTGGAAGTCTGCACGGGTCAAACGATACGCTGATCCCAGTCGT	Upstream fragment of <i>dnaK</i>
<i>dnaK</i> -down-F	ATCAGCGTATCGTTGACCCGTGCAGAGTTCAGCGCATCAC	Downstream fragment of <i>dnaK</i>
<i>dnaK</i> -down-R	CGGTACCCGGGGATCCTCTAGGACAGACCCGGAGCCGTCCTGAATG	Downstream fragment of <i>dnaK</i>
<i>clpB</i> -F	AAACAGAATTAATTAAGCTTATGAGTTCATTCAATCCAACCTACCA	ORF of <i>clpB</i>
<i>clpB</i> -R	GTACCCGGGGATCCTCTAGATTAATGATGATGATGATGATGATGGACCG CCTTGGAAACGTCGAGCTTC	ORF of <i>clpB</i>
<i>dnaK</i> -F	AACAGAATTAATTAAGCTTATGGGACGTGACGTAGGAATT	ORF of <i>dnaK</i>
<i>dnaK</i> -R	TAAATGATGATGATGATGATGATGCTTCTTATCCTCACCATTGTC	ORF of <i>dnaK</i>
pXMJ19 check-F	CGGCTCGTATAATGTGTGGA	Recombinant pXMJ19 vector detecting
pXMJ19 check-R	ATCTTCTCTCATCCGCCAAA	Recombinant pXMJ19 vector detecting
M13-F	CGCCAGGGTTTTCCAGTCACGAC	Recombinant pK18 <i>mobsacB</i> vector detecting
M13-R	GAGGGGATAACAATTTACACAGG	Recombinant pK18 <i>mobsacB</i> vector detecting

Subsequently, 1 ml of the medium was transferred into 50 ml of fresh LB medium. When an OD of 1 was reached, the cultures were diluted using phosphate-buffered saline containing 10 mM Na₂HPO₄, 137 mM NaCl, 2.7 mM KCl, and 2 mM KH₂PO₄, pH 7.4 (10⁻²–10⁻⁶). Approximately 5 μ l of the dilutions was spot-plated on CGXII solid media (Mashruwala et al., 2019). Cultures of complemented strains were grown in medium with additional chloromycetin and IPTG.

ATPase Test

The fusion ClpB protein was incubated with buffer A (10 mM MgCl₂, 1 mM dithiothreitol, BSA 0.1 mg/ml, 25 mM Tris, 0.05–2.5 mM ATP; pH 7.6) for 10 min at 30°C in accordance with

Kajfasz and Santagata's method (Santagata et al., 1999; Kajfasz et al., 2009) with slight modifications. EDTA (25 mM) was added to stop the catalytic reaction, and the reaction buffer was frozen at –80°C. The release of inorganic phosphate from ATP was measured using a Micro Tissue Inorganic Phosphorus Content assay kit (Solarbio, China) following the manufacturer's protocol.

Fermentation in Shake Flasks

The strains were activated for 24 h on LB solid media. The activated strains were cultured in a seed medium for 18 h at 30°C with agitation at 200 rpm. Approximately 2 ml of each seed culture was transferred into 25 ml of fermentation medium. The fermentations were incubated at 30°C and 200 rpm, and 200 μ l

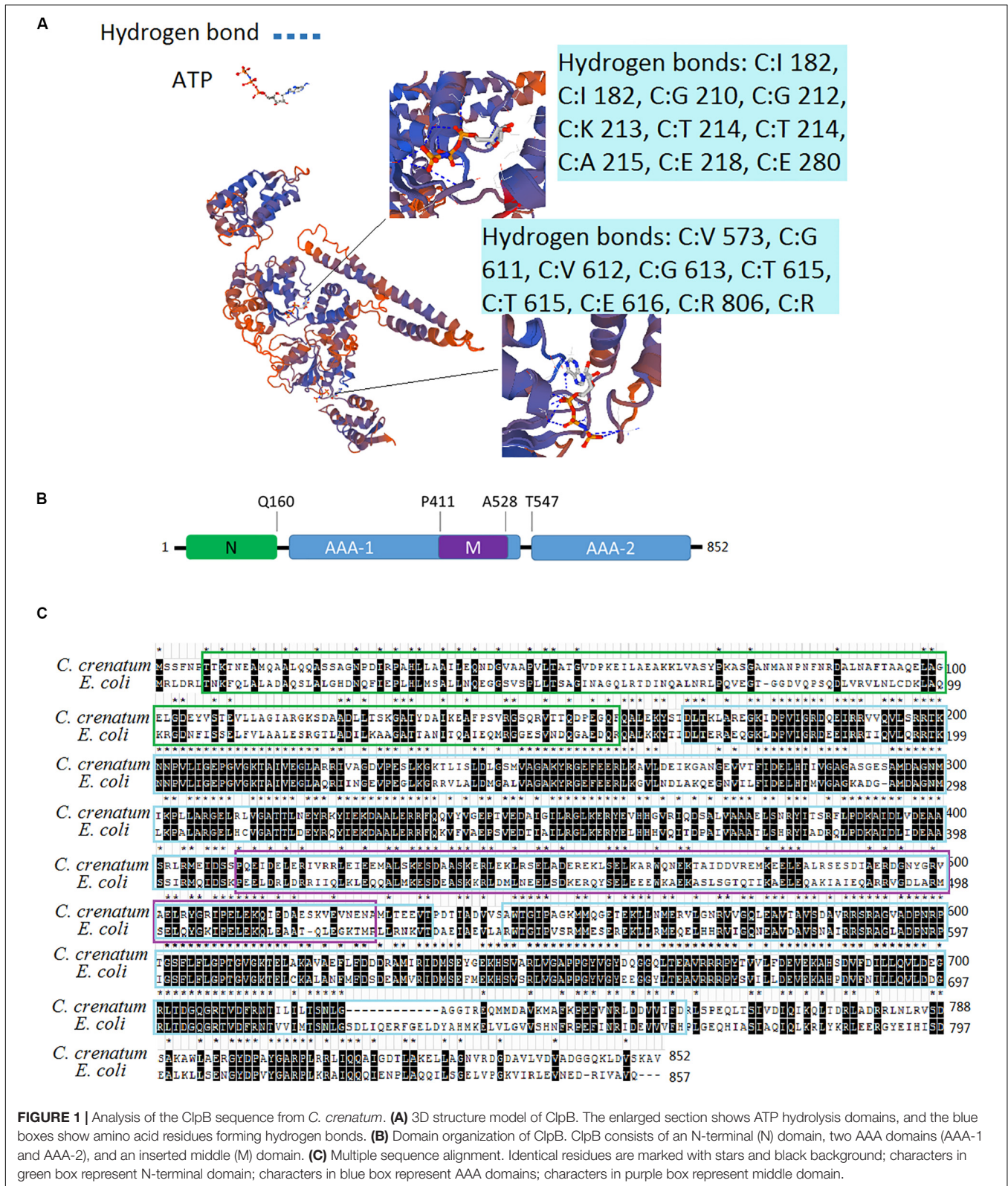
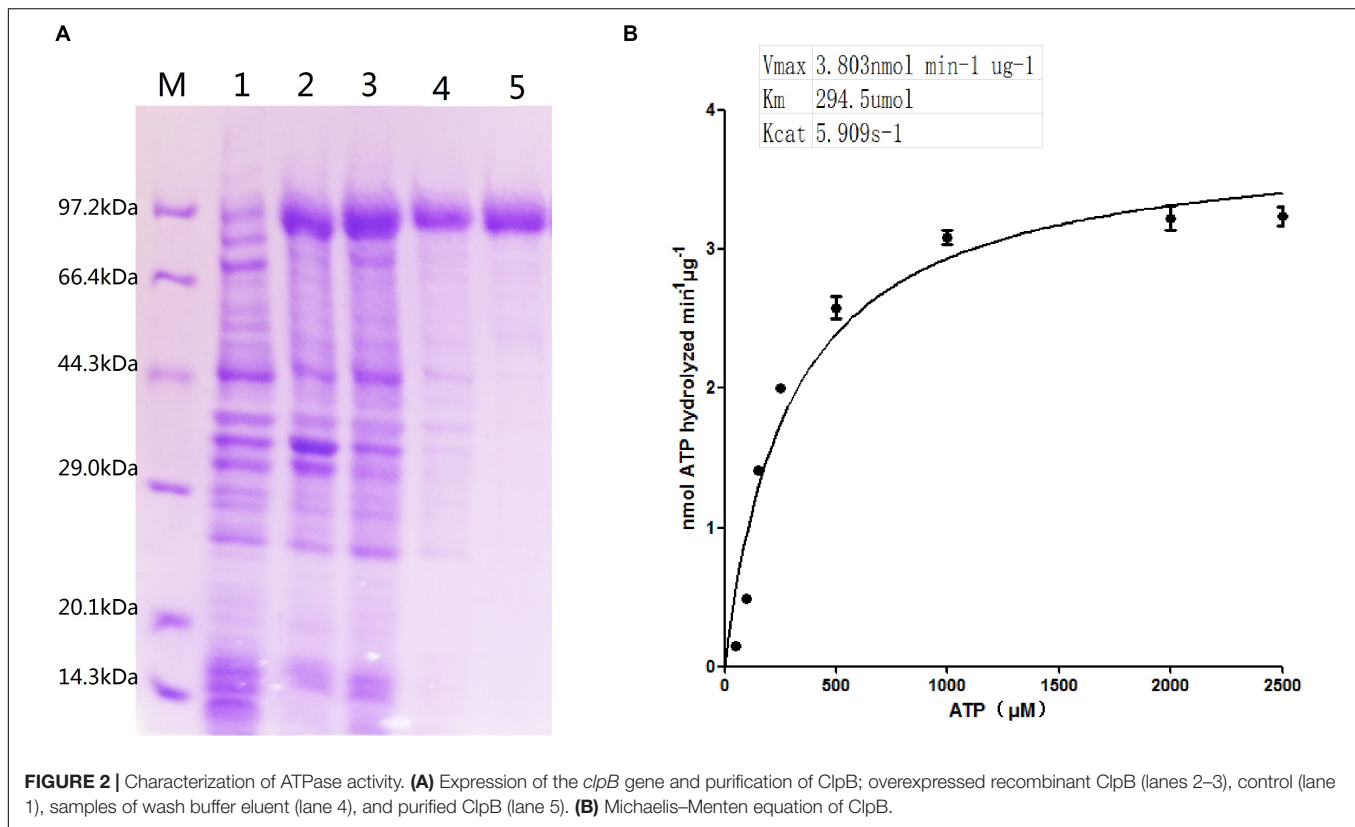


FIGURE 1 | Analysis of the ClpB sequence from *C. crenatum*. **(A)** 3D structure model of ClpB. The enlarged section shows ATP hydrolysis domains, and the blue boxes show amino acid residues forming hydrogen bonds. **(B)** Domain organization of ClpB. ClpB consists of an N-terminal (N) domain, two AAA domains (AAA-1 and AAA-2), and an inserted middle (M) domain. **(C)** Multiple sequence alignment. Identical residues are marked with stars and black background; characters in green box represent N-terminal domain; characters in blue box represent AAA domains; characters in purple box represent middle domain.

of cultures was collected every 12 h. Glucose concentration was determined by 3,5-dinitrosalicylic acid colorimetry (Chen et al., 2015). The concentrations of L-arginine were determined with a

Sykam S-433D amino acid analyzer (Sykam Co., Ltd., Germany) (Chen et al., 2015). L-glutamate and lactate were measured enzymatically using a bioanalyzer (SBA-40E, Shandong, China).



Cell growth was monitored by measuring the OD₅₆₂ (1 OD₅₆₂ = 0.375 g/L dry cell weight) with a spectrophotometer (BG-XM496, Beijing, China) after dissolving CaCO₃ with HCl.

Bioinformatics and Statistical Datum Analysis

The 3D structure model of ClpB was constructed through protein alignment with SWISS-MODEL (Bienert et al., 2017; Bertoni et al., 2017). The differences among groups were determined by ANOVA. All statistical data were analyzed using GraphPad Prism 5. All experiments were conducted in triplicate, and all data are presented as mean ± standard deviation.

RESULTS AND DISCUSSION

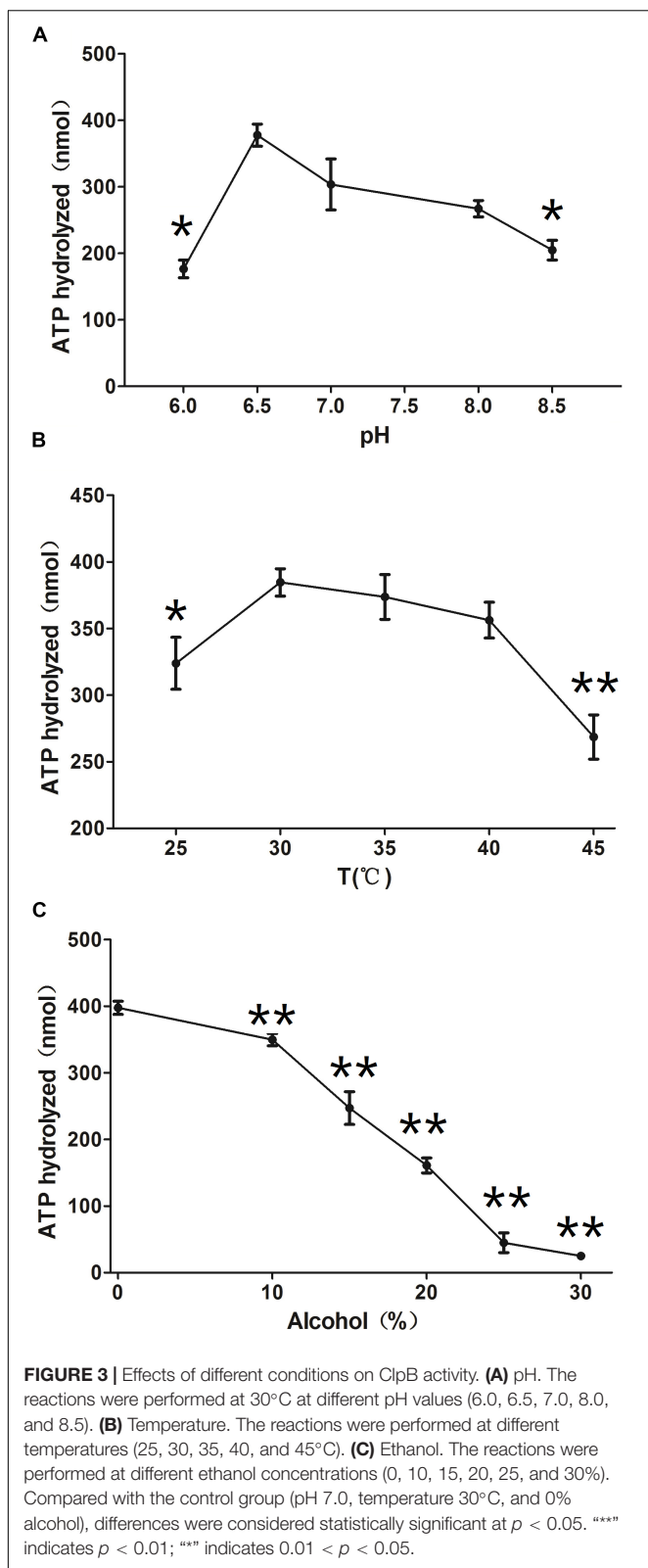
Analysis of the ClpB Sequence

We obtained the DNA sequence from the whole genome shotgun sequence of *C. crenatum* (National Center of Biotechnology Information, NCBI Reference Sequence: GCA_000380545.1). The 3D structure model of ClpB was constructed on the basis of the sequences of ClpB proteins and the crystal structure of ClpB from *Mycobacterium tuberculosis* (Figure 1A). ClpB belongs to Hsp100 or the Clp family (caseinolytic protease). A distinct characteristic of Hsp100 is the presence of ATP hydrolysis domains (AAA domain) (Baker and Sauer, 2006). ClpB of *C. crenatum* MT contains two conserved ATP hydrolysis domains, AAA1 and AAA2, forming 12 and 10 hydrogen bonds,

respectively. AAA1 and AAA2 provide ClpB with the ability of ATP hydrolysis required for restructuring substrates (Baker and Sauer, 2006). Conserved substrate-binding “pore loops” in the ATP hydrolysis domains contain essential tyrosine residues that mechanically couple ATP hydrolysis to translocation (Tessarz et al., 2008). Besides the two ATP hydrolysis domains, the domains of ClpB (Figure 1B) include an N-terminal domain (N) and a coiled-coil middle domain (M). The N-terminal domain provides ClpB with the ability to bind substrates (Gates et al., 2017). The middle region within the first AAA cassette contains a bundle of four helices approximately 120 amino acids long, which is required for ClpB interactions with DnaK and mediates allosteric functions during disaggregation and hydrolysis (Haslberger et al., 2007). The two sequence alignments indicated that the protein sequences of ClpB between *C. crenatum* MT and *E. coli* are highly conserved in the two ATP hydrolysis domains (Figure 1C), in which all 15 amino acid residues forming hydrogen bonds are conserved. The N-terminal region showed low similarity, which is loosely associated with the core domain and is not necessary for ClpB activity (Nagy et al., 2006).

ATPase Activity

The recombinant ClpB protein with His-tag at the C-terminus was purified from *E. coli* BL21 (DE3) -ClpB by using Ni-NTA affinity resin (Figure 2A). Compared with the control (lane 1), an apparent ~93 kDa protein band in lanes 2–3 was visualized, demonstrating that the *clpB* gene was successfully expressed. A single protein band (lane 5) showed successfully



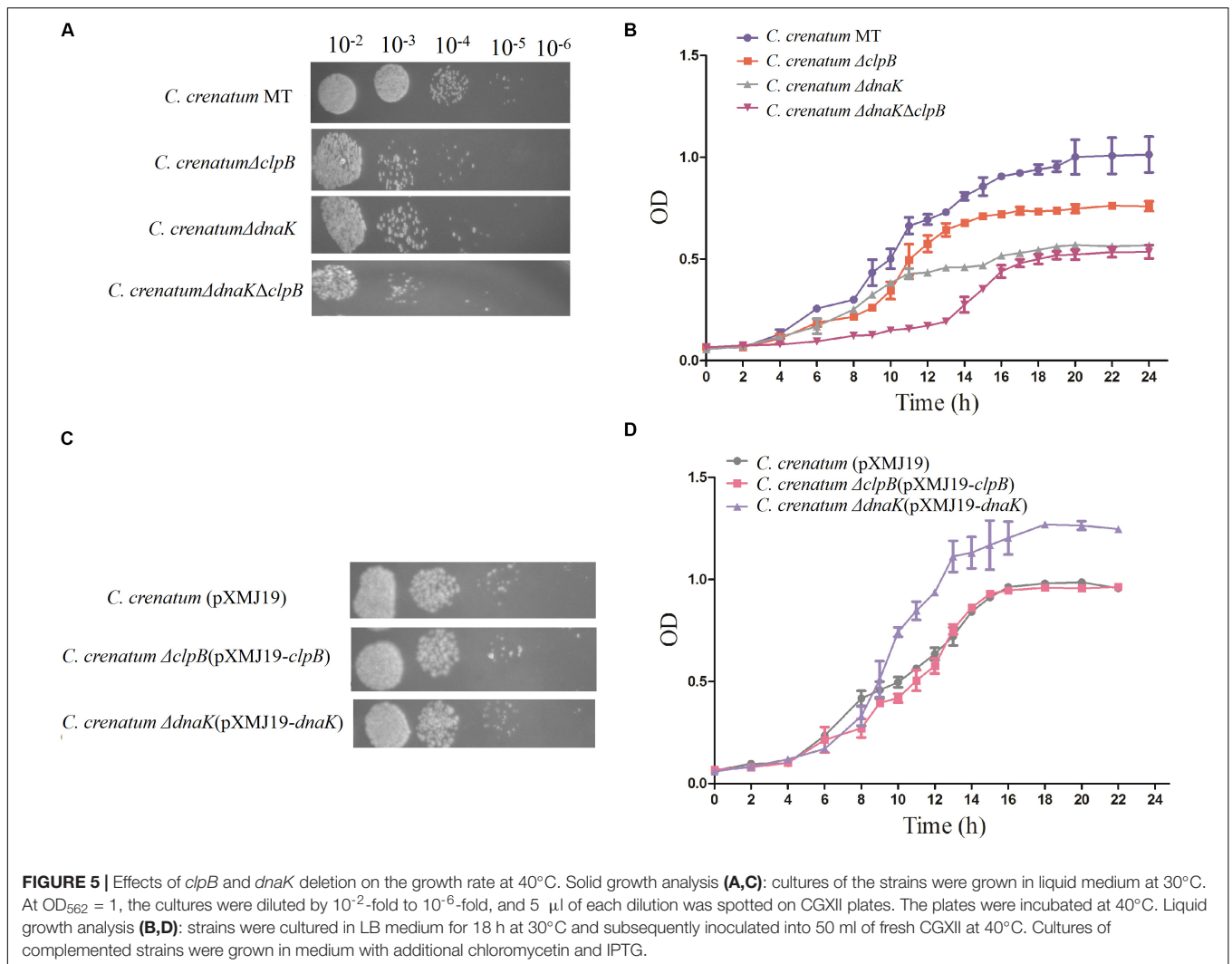
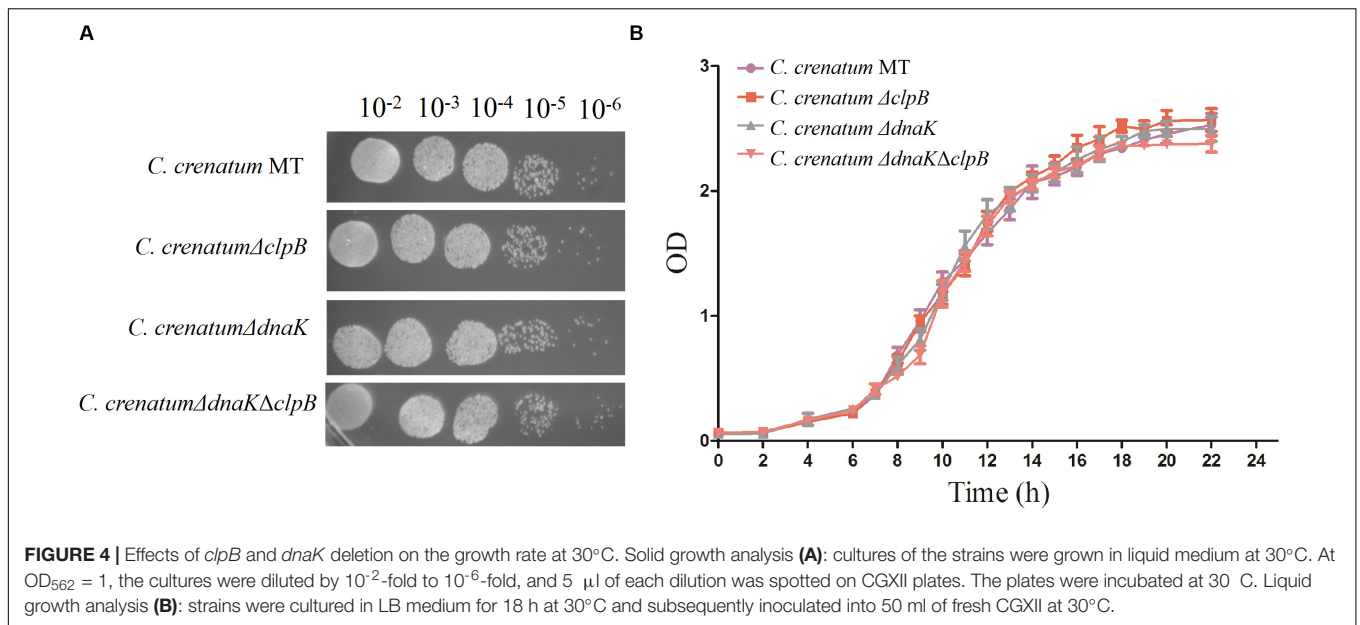
purified recombinant ClpB. For the measurement of ClpB activity, 20 μg of recombinant ClpB protein was incubated with 1 ml of buffer A at 30°C for 10 min. The Michaelis–Menten

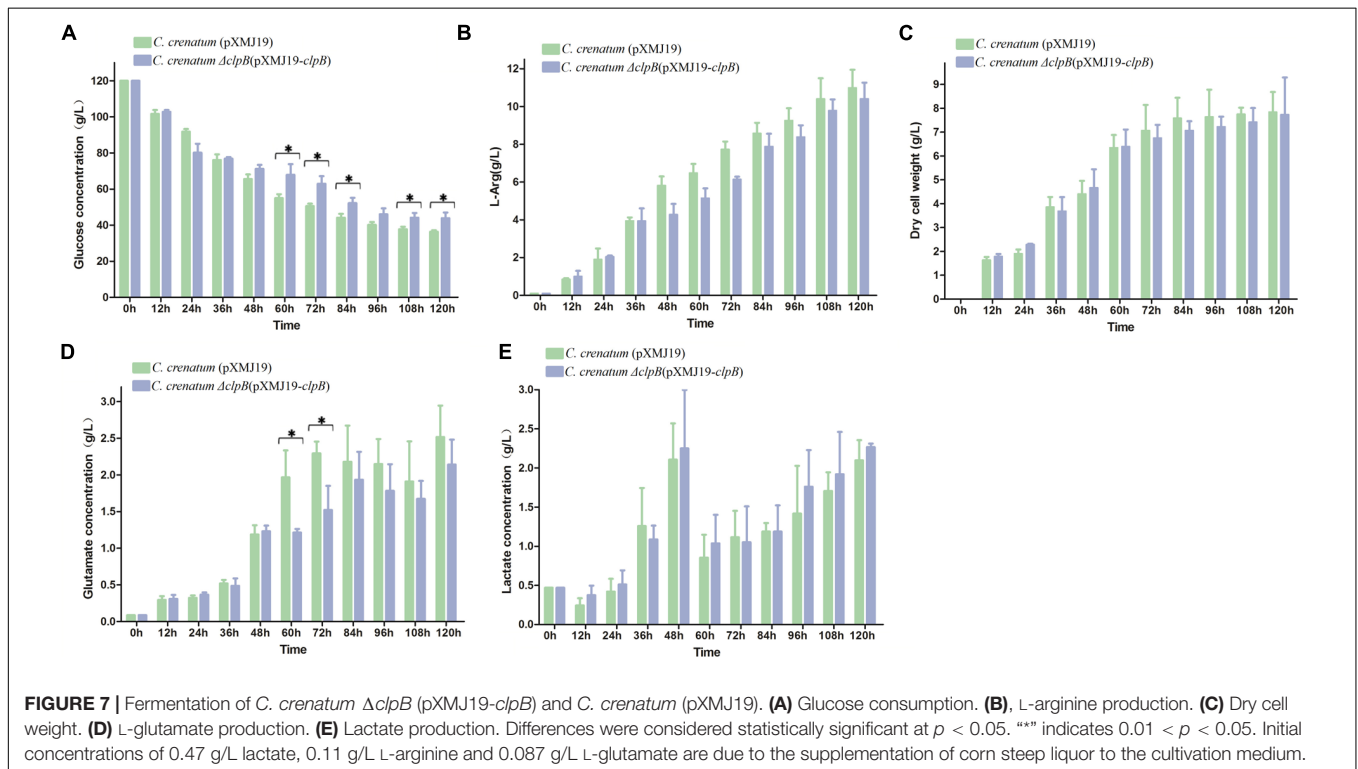
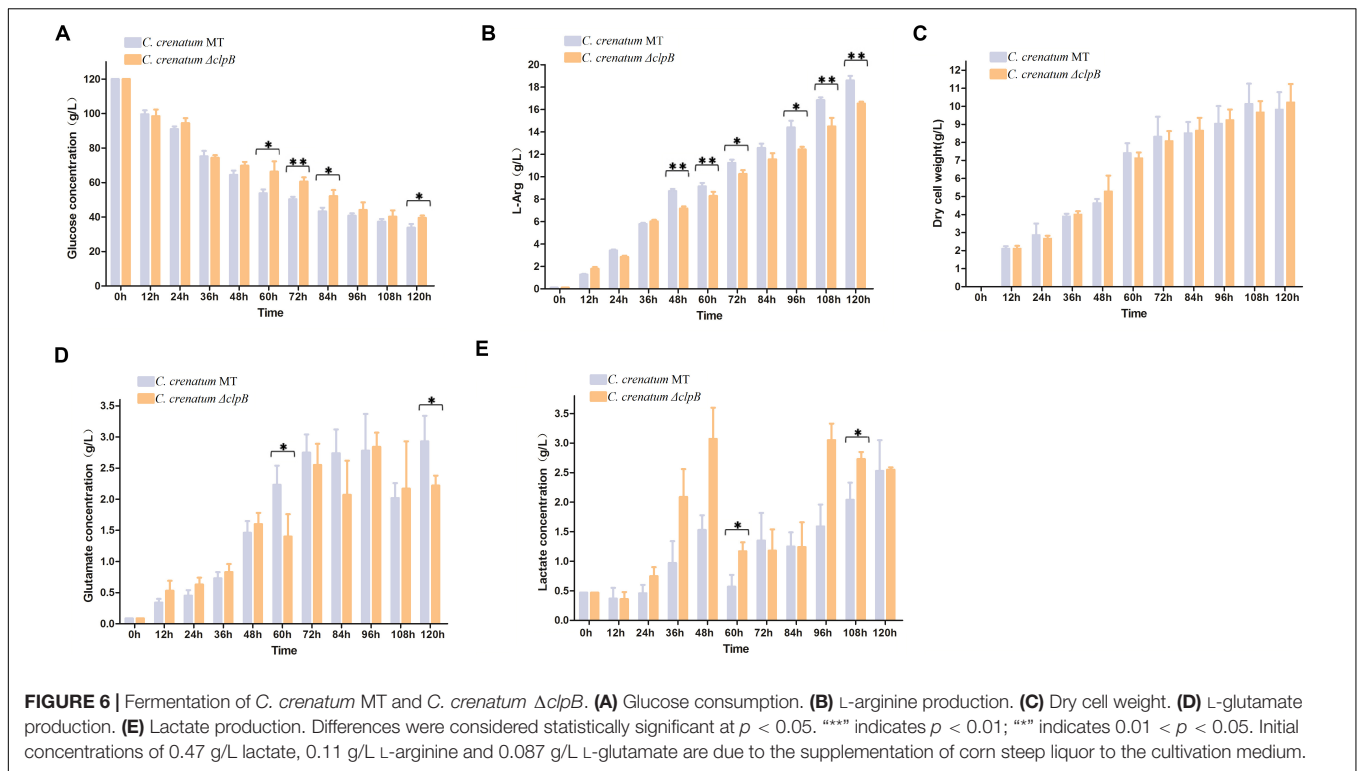
equation of ClpB shown in **Figure 2B** revealed that ClpB hydrolyzed ATP with $V_{\text{max}} = 3.803 \text{ nmol min}^{-1} \mu\text{g}^{-1}$ and $K_m = 294.5 \mu\text{mol}$, corresponding to 1 molecule of ClpB hydrolyzing 5.909 molecules of ATP in 1 s.

ClpB is involved in the response to thermal, oxidative, osmotic, pH, ethanol, and starvation stresses of microorganisms and protects vital cellular proteins encountering stress conditions (Meibom et al., 2008; Pan et al., 2012; Krajewska et al., 2017; Tripathi et al., 2020). Therefore, we measured the ATPase activity of ClpB under different conditions to reflect different types of stresses. Different pH affects the stability of all cellular proteins and their function. *C. glutamicum*, a close relative of the here investigated *C. crenatum*, is an acid-sensitive strain (Liu et al., 2016). However, **Figure 3A** shows that the groups of pH = 9.0 and 8.5 were significantly reduced compared with the group of pH = 7.0, and the optimal pH of ClpB was 6.5. This finding indicates that ClpB is still able to maintain high activity for the homeostasis of cellular proteins in acidic conditions. Athar et al. (2018) also certified that ClpB performs important functions during low-pH stress response. Many previous observations showed that ClpB contributes to the survival of bacteria during heat stress (Squires et al., 1991; Athar et al., 2018; Sangpui et al., 2018). Our results showed (**Figure 3B**) that the activity of ClpB was relatively unaltered at 30–40°C and decreased evidently at 25 and 45°C. ATPase activity decreased by only 7% at 40°C relative to the highest value (at 30°C), which revealed that ClpB efficiently functioned during heat stress, but not during cold stress. Improving resistance to alcohols is an important aspect in engineering strains for producing alcohols (Borden and Papoutsakis, 2007). Hence, the ATPase activity of ClpB was determined in presence of different ethanol concentrations (**Figure 3C**). **Figure 3C** indicates that the activity of ClpB was unstable under alcohol conditions, and ClpB is probably useless in *C. crenatum* MT under high alcohol concentrations.

Inactivation of ClpB Confers a Growth Defect Under Thermal Stress

ClpB plays a vital role in the survival of microorganisms during stressful conditions (Squires et al., 1991; Athar et al., 2018; Sangpui et al., 2018). In general, bacterial ClpB cooperates with DnaK cochaperone to refold and rescue aggregated proteins, thereby helping cells survive under stressful conditions (Lupoli et al., 2016). Therefore, we constructed *dnaK/clpB* deletion mutants and complemented strains to investigate the role of ClpB and DnaK in stress. However, our study demonstrated that ClpB and DnaK from *C. crenatum* MT are not essential for the survival of *C. crenatum* MT under alcohol and pH stress (**Supplementary Figures S1, S2**). The cooperation of ClpB and chaperones in substrate disaggregation is species-specific (Schlee et al., 2004; DeSantis and Shorter, 2012; Ngansop et al., 2013). The M domain of ClpB corresponds to the species-specific cooperation with chaperones (Miot et al., 2011). The two-sequence alignment showed that the sequence identity between *C. crenatum* MT and *E. coli* within the middle domain of ClpB was only ~42% (**Figure 1C**), which indicated species-specific function. In general, industrial fermentation is carried out below 35°C.





These processes demand large quantities of water to cool the heat generated by the metabolism of microbes, particularly during hot seasons and in tropical regions (Oide et al., 2015). Thus, studying

the thermal stress tolerance of microorganisms reduces the costs of maintaining optimal fermentation temperature (Oide et al., 2015). We first determined whether the growth of *C. crenatum*

$\Delta clpB$, *C. crenatum* $\Delta dnaK$, and *C. crenatum* $\Delta dnaK \Delta clpB$ differ from that of *C. crenatum* MT at an optimal temperature. Thus, the strains were cultured for solid and liquid growth analyses at 30°C (Figures 4A,B). The growth of *C. crenatum* $\Delta clpB$, *C. crenatum* $\Delta dnaK$, and *C. crenatum* $\Delta dnaK \Delta clpB$ showed no significant difference compared with that of *C. crenatum* MT. The number of colonies of *C. crenatum* $\Delta clpB$, *C. crenatum* $\Delta dnaK$, and *C. crenatum* $\Delta dnaK \Delta clpB$ was smaller than that of *C. crenatum* at 40°C (Figure 5A). The differences of growth were obvious through liquid growth analyses (Figure 5B), and *C. crenatum* $\Delta dnaK$ and *C. crenatum* $\Delta dnaK \Delta clpB$ showed the weakest growth rate, indicating that ClpB and DnaK contribute to thermal stress tolerance. Compared with the wild type of *Salmonella typhimurium*, $\Delta clpB$ strains did not show any sensitivity to a temperature of 37°C but were hypersusceptible ($p < 0.001$) to a temperature of 42°C (Sangpuii et al., 2018). When subjected to high temperature (50°C), the survival of the $\Delta clpB$ mutant of *Francisella tularensis* was compromised, whereas the CFU of the wild-type strains did not drop significantly (Athar et al., 2018). Thermal stress results in the massive misfolding of proteins and the aggregation of proteins, which is toxic for bacteria. Cell survival during heat stress requires the reactivation of aggregated proteins by ClpB and DnaK, which indicates the essential role of ClpB and DnaK in thermotolerance (Weibezahn et al., 2004; Acebrón et al., 2008). Furthermore, the growth rate under thermal stress was observed through the complemented strains (Figures 5C,D). The deficient growth rate was restored in *C. crenatum* $\Delta clpB$ (pXMJ19-*clpB*). Unexpectedly, the growth rate of *C. crenatum* $\Delta dnaK$ (pXMJ19-*dnaK*) was significantly increased compared with that of *C. crenatum* (pXMJ19). These results suggested that the DnaK/ClpB chaperone system plays an important role in thermotolerance, but DnaK possesses a broader function with other cochaperones. DnaK also collaborates with GrpE and DnaJ to prevent protein aggregation and solubilize protein aggregates, which is critical in the resistance to thermal stress (Acebrón et al., 2008).

Fermentation in Shake Flasks

Various valuable chemical compounds have been produced using glucose through fermentation. Therefore, the understanding of glucose metabolism is essential for industrially relevant bacteria (Sekine et al., 2001). *C. crenatum* $\Delta clpB$ displayed relatively lower glucose consumption and L-arginine production than *C. crenatum* MT during the late period of fermentation (Figures 6A,B). L-arginine is biosynthesized from L-glutamate through citrulline and ornithine in cellular metabolic pathways (Chen et al., 2015). Figure 6C shows that *clpB* deletion affected the L-glutamate production of *C. crenatum* MT, especially at 60 and 120 h. Lactate is one of the major organic acids excreted to the broth during fermentation (Xu et al., 2009). At the early phase of fermentation, the concentration of lactate showed net consumption, which may be caused by low-density cells and pure medium containing a certain amount of lactic acid (0.47 g/L). The accumulation of lactate was significantly increased in *C. crenatum* $\Delta clpB$ strain at 60 and 108 h (Figure 6D). Reduction of glutamate and increase of lactic acid may be the reasons for the decrease of arginine production. As a complement, the fermentation of

C. crenatum $\Delta clpB$ (pXMJ19-*clpB*) and *C. crenatum* (pXMJ19) was performed, and the cultures were collected and tested. Deletion of *clpB* did not affect the dry cell weight of *C. crenatum* during fermentation (Figures 6C, 7C). Figure 7 shows that the deficient L-arginine production was restored in *C. crenatum* $\Delta clpB$ (pXMJ19-*clpB*). The glucose consumption of *C. crenatum* $\Delta clpB$ (pXMJ19-*clpB*) was significantly increased compared with that of *C. crenatum* (pXMJ19); however, L-glutamate production decreased due to the overexpression of the *clpB* gene. The strains might encounter a disadvantageous circumstance with the accumulation of toxic metabolites (Wu et al., 2016) and consumption of nutrition at the late period of fermentation (108–120 h), resulting in massive protein misfolding. The interactional proteins of ClpB include enzymes for major metabolic pathways, such as glycolysis–gluconeogenesis, TCA cycle, and amino acid metabolism (Joanna et al., 2018). The metabolism of *C. crenatum* $\Delta clpB$ may be subjected to protein misfolding.

CONCLUSION

We found a homolog of ClpB from *C. crenatum* MT. The amino acid sequence of ClpB was analyzed, and the recombinant ClpB protein was purified and characterized. The full function of ClpB requires DnaK as chaperone protein. For this reason, *dnaK/clpB* deletion mutants and complemented strains were constructed to investigate the role of ClpB. Our investigation demonstrated that ClpB is not essential for the survival of *C. crenatum* MT under pH and alcohol stresses. The growth of *C. crenatum* $\Delta clpB$, *C. crenatum* $\Delta dnaK$, and *C. crenatum* $\Delta dnaK \Delta clpB$ showed no significant difference compared with that of *C. crenatum* MT at optimal temperature. However, growth test under thermal stress indicated that ClpB and DnaK contribute to thermal stress tolerance. Deletion of *clpB* affected glucose consumption and L-glutamate, lactate, and L-arginine production during fermentation. The strains encountered a disadvantageous circumstance with the accumulation of toxic metabolites and consumption of nutrition at the late period of fermentation (108–120 h), which might lead to protein aggregation, and the metabolism of *C. crenatum* $\Delta clpB$ may be subjected to protein misfolding. The underlying mechanism behind the ClpB protein function remains unknown, and the ClpB of industrially relevant bacteria must be further investigated.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the National Center of Biotechnology Information/NZ_AQPS01000020.1/https://www.ncbi.nlm.nih.gov/nuccore/NZ_AQPS01000020.1.

AUTHOR CONTRIBUTIONS

MH and XC contributed to research work and manuscript writing. MH contributed to construction of plasmids and strains and bioinformatics analysis. YZ and LF contributed to

fermentation. LZhu and LZha contributed to the testing of ATPase and cell growth. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Natural Science Foundation of China (31660019 and 31960014) and “5511”

REFERENCES

- Acebrón, S. P., Fernández-Sáiz, V., Taneva, S. G., Moro, F., and Muga, A. (2008). DnaJ recruits DnaK to protein aggregates. *J. Biol. Chem.* 283, 1381–1390. doi: 10.1074/jbc.M706189200
- Athar, A., Igor, G., Eram, J., and Anders, S. (2018). ClpB mutants of *Francisella tularensis* subspecies *holarctica* and *tularensis* are defective for type VI secretion and intracellular replication. *Sci. Rep.* 8:11324. doi: 10.1038/s41598-018-29745-4
- Baker, T. A., and Sauer, R. T. (2006). ATP-dependent proteases of bacteria: recognition logic and operating principles. *Trends Biochem. Sci.* 31, 647–653. doi: 10.1016/j.tibs.2006.10.006
- Bertoni, M., Kiefer, F., Biasini, M., Bordoli, L., and Schwede, T. (2017). Modeling protein quaternary structure of homo- and hetero-oligomers beyond binary interactions by homology. *Sci. Rep.* 7:10480. doi: 10.1038/s41598-017-09654-8
- Bienert, S., Waterhouse, A., de Beer, T. A. P., Tauriello, G., Studer, G., Bordoli, L., et al. (2017). The SWISS-MODEL Repository – new features and functionality. *Nucleic Acids Res.* 45, D313–D319. doi: 10.1093/nar/gkw1132
- Borden, J. R., and Papoutsakis, E. T. (2007). Dynamics of genomic-library enrichment and identification of solvent tolerance genes for *Clostridium acetobutylicum*. *Appl. Environ. Microb.* 73, 3061–3068. doi: 10.1128/AEM.02296-06
- Chen, M., Chen, X., Wan, F., Zhang, B., Chen, J., and Xiong, Y. (2015). Effect of Tween 40 and DtsR1 on l-arginine overproduction in *Corynebacterium crenatum*. *Microb. Cell. Fact.* 14:119. doi: 10.1186/s12934-015-0310-9
- DeSantis, M. E., and Shorter, J. (2012). The elusive middle domain of Hsp104 and ClpB: location and function. *BBA Mol. Cell Res.* 1823, 29–39. doi: 10.1016/j.bbamcr.2011.07.014
- Engels, S., Schweitzer, J. E., Ludwig, C., Bott, M., and Schaffer, S. (2004). *clpC* and *clpP2* gene expression in *Corynebacterium glutamicum* is controlled by a regulatory network involving the transcriptional regulators *clgR* and *hspR* as well as the ECF sigma factor σ^H . *Mol. Microbiol.* 52, 285–302. doi: 10.1111/j.1365-2958.2003.03979.x
- Gates, S. N., Yokom, A. L., Lin, J. B., Jackrel, M. E., Rizo, A. N., Kendersky, N. M., et al. (2017). Ratchet-like polypeptide translocation mechanism of the AAA+ disaggregase Hsp104. *Science* 357, 273–279. doi: 10.1126/science.aan1052
- Hartl, F. U., and Hayer-Hartl, M. (2002). Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* 295, 1852–1858. doi: 10.1126/science.1068408
- Haslberger, T., Weibezahn, J., Zahn, R., Lee, S., and Mogk, A. (2007). M domains couple the ClpB threading motor with the DnaK chaperone activity. *Mol. Cell* 25, 247–260. doi: 10.1016/j.molcel.2006.11.008
- Joanna, K., Zbigniew, A., Michal, Z., and Sabina, K. M. (2018). Isolation and identification of putative protein substrates of the AAA+ molecular chaperone ClpB from the pathogenic spirochaete *Leptospira interrogans*. *Int. J. Mol. Sci.* 19:1234. doi: 10.3390/ijms19041234
- Kajfasz, J. K., Martinez, A. R., Rivera-Ramos, I., Abranches, J., Koo, H., Quivey, R. G., et al. (2009). Role of clp proteins in expression of virulence properties of *Streptococcus mutans*. *J. Bacteriol.* 191, 2060–2068. doi: 10.1128/JB.01609-08
- Kallscheuer, N., Vogt, M., Stenzel, A., Gätgens, J., Bott, M., and Marienhagen, J. (2016). Construction of a *Corynebacterium glutamicum* platform strain for the production of stilbenes and (2S)-flavanones. *Metab. Eng.* 38, 47–55.
- Krajewska, J., Modrak-Wojcik, A., Arent, Z. J., Więckowski, D., Zolkiewski, M., Bzowska, A., et al. (2017). Characterization of the molecular chaperone ClpB

Superior Technology Innovation Team Project of Jiangxi (20165BCB19004).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2020.01660/full#supplementary-material>

- from the pathogenic spirochaete *Leptospira interrogans*. *PLoS One* 12:e0181118. doi: 10.1371/journal.pone.0181118
- Lee, J. H., and Wendisch, V. F. (2017). Production of amino acids—genetic and metabolic engineering approaches. *Bioresour. Technol.* 245, 1575–1587. doi: 10.1016/j.biortech.2017.05.065
- Li, T., Lin, J., and Lucius, A. L. (2015). Examination of polypeptide substrate specificity for *Escherichia coli* ClpB. *Proteins Struct. Funct. Bioinform.* 83, 117–134. doi: 10.1002/prot.24710
- Litsanov, B., Kabus, A., Brocker, M., and Bott, M. (2012). Efficient aerobic succinate production from glucose in minimal medium with *Corynebacterium glutamicum*. *Microb. Biotechnol.* 5, 116–128.
- Liu, Y., Yang, X., Yin, Y., Lin, J., Chen, C., Pan, J., et al. (2016). Mycothiol protects *Corynebacterium glutamicum* against acid stress via maintaining intracellular pH homeostasis, scavenging ROS, and S-mycothiolating MetE. *J. Gen. Appl. Microbiol.* 62, 144–153. doi: 10.2323/jgam.2016.02.001
- Lupoli, T. J., Fay, A., Adura, C., Glickman, M. S., and Nathan, C. F. (2016). Reconstitution of a *Mycobacterium tuberculosis* proteostasis network highlights essential cofactor interactions with chaperone DnaK. *Proc. Natl. Acad. Sci. U.S.A.* 113, E7947–E7956. doi: 10.1073/pnas.1617644113
- Mashruwala, A. A., Eilers, B. J., Fuchs, A. L., Norambuena, J., Earle, C. A., Guchte, A., et al. (2019). The ClpCP complex modulates respiratory metabolism in *Staphylococcus aureus* and is regulated in a SrrAB-dependent manner. *J. Bacteriol.* 201:e00188-19. doi: 10.1128/JB.00188-19
- Meibom, K. L., Dubail, I., Dupuis, M., Barel, M., Lenco, J., Stulik, J., et al. (2008). The heat-shock protein ClpB of *Francisella tularensis* is involved in stress tolerance and is required for multiplication in target organs of infected mice. *Mol. Microbiol.* 67, 1384–1401. doi: 10.1111/j.1365-2958.2008.06139.x
- Miot, M., Reidy, M., Doyle, S. M., Hoskins, J. R., Johnston, D. M., Genest, D., et al. (2011). Species-specific collaboration of heat shock proteins (Hsp) 70 and 100 in the rmtolerance and protein disaggregation. *Proc. Natl. Acad. Sci. U.S.A.* 108, 6915–6920. doi: 10.1073/pnas.1102828108
- Mogk, A., Bukau, B., and Kampinga, H. H. (2018). Cellular handling of protein aggregates by disaggregation machines. *Mol. Cell* 69, 214–226. doi: 10.1016/j.molcel.2018.01.004
- Nagy, M., Akoev, V., and Zolkiewski, M. (2006). Domain stability in the AAA+ ATPase ClpB from *Escherichia coli*. *Arch. Biochem. Biophys.* 453, 63–69. doi: 10.1016/j.abb.2006.03.004
- Ngansop, F., Li, H., Zolkiewska, A., and Zolkiewski, M. (2013). Biochemical characterization of the apicoplast-targeted AAA+ ATPase ClpB from *Plasmodium falciparum*. *Biochem. Biophys. Res. Commun.* 439, 191–195. doi: 10.1016/j.bbrc.2013.08.064
- Oide, S., Gunji, W., Moteki, Y., Yamamoto, S., Suda, M., Jojima, T., et al. (2015). Thermal and solvent stress cross-tolerance conferred to *Corynebacterium glutamicum* by adaptive laboratory evolution. *Appl. Environ. Microbiol.* 81, 2284–2298. doi: 10.1128/AEM.03973-14
- Pan, H., Luan, J., He, X., Lux, R., and Shi, W. (2012). The *clpB* gene is involved in the stress response of *Myxococcus xanthus* during vegetative growth and development. *Microbiol. SGM* 158, 2336–2343. doi: 10.1099/mic.0.060103-0
- Peng, F., Liu, X., Wang, X., Chen, J., Liu, M., Yang, Y., et al. (2019). Triple deletion of *clpC*, *porB*, and *mepA* enhances production of small ubiquitin-like modifier-N-terminal pro-brain natriuretic peptide in *Corynebacterium glutamicum*. *J. Ind. Microbiol. Biot.* 46, 67–79. doi: 10.1007/s10295-018-2091-8
- Raivio, T. L. (2018). Regulation of proteolysis in the Gram-negative bacterial envelope. *J. Bacteriol.* 200:e00639-17. doi: 10.1128/JB.00639-17.4

- Ranaweera, C. B., Przemyslaw, G., Taihao, Y., and Michal, Z. (2018). Interaction of substrate-mimicking peptides with the AAA+ Atase ClpB from *Escherichia coli*. *Arch. Biochem. Biophys.* 655, 12–17. doi: 10.1016/j.abb.2018.08.002
- Rizo, A. N., Lin, J., Gates, S. N., Tse, E., Bart, S. M., Castellano, L. M., et al. (2019). Structural basis for substrate gripping and translocation by the ClpB AAA+ disaggregase. *Nat. Commun.* 10:2393. doi: 10.1038/s41467-019-10150-y
- Sanguini, L., Dixit, S. K., Kumawat, M., Apoorva, S., Kumar, M., et al. (2018). Comparative roles of clpA and clpB in the survival of *S. Typhimurium* under stress and virulence in poultry. *Sci. Rep.* 8:4481. doi: 10.1038/s41598-018-22670-6
- Santagata, S., Bhattacharyya, D., Wang, F. H., Singha, N., Hodtsev, A., and Spanopoulou, E. (1999). Molecular cloning and characterization of a mouse homolog of bacterial ClpX, a novel mammalian class II member of the Hsp100/Clp chaperone family. *J. Biol. Chem.* 274, 16311–16319. doi: 10.1074/jbc.274.23.16311
- Schafer, A., Tauch, A., Jager, W., Kalinowski, J., Thierbach, G., and Pühler, A. (1994). Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pk18 and pk19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* 145, 69–73. doi: 10.1016/0378-1119(94)90324-7
- Schlee, S., Beinker, P., Akhrymuk, A., and Reinstein, J. (2004). A chaperone network for the resolubilization of protein aggregates: direct interaction of ClpB and DnaK. *J. Mol. Biol.* 336, 275–285. doi: 10.1016/j.jmb.2003.12.013
- Sekine, H., Shimada, T., Hayashi, C., Ishiguro, A., Tomita, F., and Yokota, A. (2001). H⁺-ATPase defect in *Corynebacterium glutamicum* abolishes glutamic acid production with enhancement of glucose consumption rate. *Appl. Microbiol. Biot.* 57, 534–540. doi: 10.1007/s002530100778
- Squires, C. L., Pedersen, S., Ross, B. M., and Squires, C. (1991). ClpB is the *Escherichia coli* heat shock protein F84.1. *J. Bacteriol.* 173, 4254–4262. doi: 10.1128/jb.173.14.4254-4262.1991
- Su, H., Lu, Q., Zhao, Y., Jiang, J., Zhao, Z., and Wang, M. (2014). Preliminary investigation of metabolic engineering in a novel host bacterium *Corynebacterium crenatum* for alcohol biofuel production. *RSC Adv.* 4, 65021–65030. doi: 10.1039/c4ra08668f
- Tessarz, P., Mogk, A., and Bukau, B. (2008). Substrate threading through the central pore of the Hsp104 chaperone as a common mechanism for protein disaggregation and prion propagation. *Mol. Microbiol.* 68, 87–97. doi: 10.1111/j.1365-2958.2008.06135.x
- Tripathi, P., Singh, L. K., Kumari, S., Hakiem, O. R., and Batra, J. K. (2020). ClpB is an essential stress regulator of *Mycobacterium tuberculosis* and endows survival advantage to dormant bacilli. *Int. J. Med. Microbiol.* 310:151402. doi: 10.1016/j.ijmm.2020.151402
- Vogt, M., Brüsseler, C., van Ooyen, J., Bott, M., and Marienhagen, J. (2016). Production of 2-methyl-1-butanol and 3-methyl-1-butanol in engineered *Corynebacterium glutamicum*. *Metab. Eng.* 38, 436–445.
- Wang, F., Zhang, G., Peng, J., Ji, X., Hai, J., Deng, X., et al. (2020). High cell-density fermentation, expression and purification of bacteriophage lysin TSPphg, a thermostable antimicrobial protein from extremophilic *Thermus* bacteriophage TSP4. *Protein Express. Purif.* 174:105676. doi: 10.1016/j.pep.2020.105676
- Weibezahn, J., Tessarz, P., Schlieker, C., Zahn, R., Maglica, Z., Lee, S., et al. (2004). Thermotolerance requires refolding of aggregated proteins by substrate translocation through the central pore of ClpB. *Cell* 119, 653–665.
- Wu, Y., Xue, C., Chen, L., Yuan, W., and Bai, F. (2016). Improvements of metabolites tolerance in *Clostridium acetobutylicum* by micronutrient zinc supplementation. *Biotechnol. Bioproc. E.* 21, 60–67. doi: 10.1007/s12257-015-0583-1
- Xu, H., Dou, W., Xua, H., Zhang, X., Rao, Z., Shi, Z., et al. (2009). A two-stage oxygen supply strategy for enhanced l-arginine production by *Corynebacterium crenatum* based on metabolic fluxes analysis. *Biochem. Eng. J.* 43, 41–51. doi: 10.1016/j.bej.2008.08.007
- Zhang, B., Ren, L., Yu, M., Zhou, Y., and Ye, B. (2017). Enhanced l-ornithine production by systematic manipulation of l-ornithine metabolism in engineered *Corynebacterium glutamicum* S9114. *Bioresour. Technol.* 250, 60–68. doi: 10.1016/j.biortech.2017.11.017

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Huang, Zhao, Feng, Zhu, Zhan and Chen. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.