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Improving cellular robustness and butanol titers of *Clostridium acetobutylicum* ATCC824 by introducing heat shock proteins from an extremophilic bacterium

Zhengping Liao[a], Yanan Zhang[a], Sheng Luo[a], Yukai Suo[a], Shaozhi Zhang[a], Jufang Wang[a,b,*]

[a] School of Bioscience & Bioengineering, South China University of Technology, Guangzhou 510006, China
[b] Guangdong Provincial Key Laboratory of Fermentation and Enzyme Engineering, South China University of Technology, Guangzhou 510006, China

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ABSTRACT

In recent years, increasing concerns over environment, energy and climate have renewed interest in biotechnological production of butanol. However, growth inhibition by fermentation products and inhibitory components from raw biomass has hindered the development of acetone-butanol-ethanol (ABE) fermentation. Improving the cellular robustness of *Clostridium acetobutylicum* is of great importance for efficient ABE production. In this study, we attempted to improve the robustness and butanol titers of *C. acetobutylicum* ATCC824 by overexpressing GroESL and DnaK from the extremely radioresistant bacterium *Deinococcus wulumuqiensis* R12 and from *C. acetobutylicum* ATCC824 itself. Three recombinant strains were obtained and designated 824(dnaK R12), 824(groESL R12) and 824(groESL824). These three recombinants were found to have significantly improved tolerances to stresses including butanol, furfural, oxidation and acid. Meanwhile, the butanol titers increased to 13.0 g/L, 11.2 g/L and 10.7 g/L, which were 49.4%, 28.7% and 23.0% higher than that from the wild-type strain (8.7 g/L), respectively. For 824(dnaK R12), the production of acetic and butyric acids decreased by 97.1% (1.4 g/L vs. 0.04 g/L) and 100% (0.3 g/L vs. 0 g/L), respectively, compared with the wild-type strain. Overexpressing GroESL and DnaK from *D. wulumuqiensis* R12 also resulted in better growth and ABE production than the wild-type strain on fermentation in the presence of 2.5 g/L furfural. Strain 824(groESL R12) was superior to 824(groESL 824) in diverse types of stress-tolerance and butanol titer, indicating that GroESL from the extremophilic bacterium could perform its function more efficiently in the heterologous host than native GroESL. Our study provides evidence that extremophilic bacteria can be excellent resources for engineering *C. acetobutylicum* to improve its robustness and butanol titer.

1. Introduction

Decreasing petroleum resources and increasing environmental concerns have led to increased interest in biofuel development. Butanol has excellent inherent properties compared with other biofuels: it has a higher octane rating than ethanol, it is soluble with gasoline in any proportion, and it can be directly used in existing supply infrastructure. Thus, it has vast potential for future development. Biobutanol is mainly produced by acetone-butanol-ethanol (ABE) fermentation using *Clostridium acetobutylicum* or *C. beijerinckii*. This process is influenced by many factors, such as the metabolic products including butanol and organic acids, and the inhibitory components generated from pretreatment and hydrolysis of lignocellulose biomass including furfural; these toxic materials result in lower *n*-butanol titers and hinder the industrialization of ABE fermentation. Thus, improving the cellular robustness of *C. acetobutylicum* is of great importance for efficient ABE production (Lee et al., 2008; Xue et al., 2013).

Heat shock proteins (HSPs) play important roles in bacterial stress response. They can be induced by various environmental stresses such as butanol, butyrate and furfural (Janssen et al., 2012; Schwarz et al., 2012; Wang et al., 2013; Zhang and Ezeji, 2013). Recent studies have reported the role of HSPs in resistance to many stresses in various bacteria (Luan et al., 2014; Mann et al., 2012; Singh et al., 2007; Tomas et al., 2003; Zingaro and Terry Papoutsakis, 2013). For instance, homologous overexpression of the *groESL* genes in *C. acetobutylicum* led to 85% increase in butanol tolerance when compared with the

Abbreviations: ABE, acetone-butanol-ethanol; HSPs, heat shock proteins; LB, Luria-Bertani; RCM, Reinforced Clostridial Medium; HPLC, high performance liquid chromatography; GC, gas chromatograph; EMP, Embden-Meyerhof-Parnas pathway

* Corresponding author at: School of Bioscience & Bioengineering, South China University of Technology, Guangzhou 510006, China.
E-mail address: jufwang@scut.edu.cn (J. Wang).

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plasmid control strain, and the final butanol titer was increased by 33% (Tomas et al., 2003). Overexpressing native *C. acetobutylicum grpE* and *htpG* significantly improved tolerance to butanol: after 2 h of 2% (v/v) butanol exposure, the wild-type and plasmid control strains did not survive, while the recombinant strains pT:*grpE* and pT:*htpG* showed 25% and 56% survival, respectively (Mann et al., 2012). Overexpressing the autologous *groESL* of *Escherichia coli* with its natural promoter resulted in cell growth increases of 12-fold under 4% (v/v) ethanol, 2.8-fold under 0.75% (v/v) *n*-butanol, 3-fold under 1.25% (v/v) 2-butanol, and 4-fold under 20% (v/v) 1,2,4-butanetriol (Zingaro and Terry Papoutsakis, 2013). Introducing *groESL* from the solvent-tolerant *Pseudomonas putida* into *E. coli* significantly improved its tolerance of heat and ethanol. In addition, expressing *groESL* from the thermophilic *Thermoanaerobacter tengcongensis* in *C. acetobutylicum* improved its corn cob hydrolysate-tolerance and butanol titer (Luan et al., 2014). Deletion of *dnaK* from *Staphylococcus aureus* resulted in significant growth inhibition at 37 °C and above, more sensitivity to the stresses of oxidation and cell-wall-active antibiotics, and reduced carotenoid production (Singh et al., 2007).

Extremophilic bacteria, which can adapt and survive in extreme environments as a result of long-term natural evolution, show superior robustness under harsh conditions, possibly due to their well-adapted stress-response genes (Egorova and Antranikian, 2005; Podar and Reysenbach, 2006; Stetter, 1999). In recent years, many researchers have identified and used such genes from extremophiles, including efflux pumps, HSPs and transcriptional regulators, to engineer cellular robustness of various microbes (Dong et al., 2015; Dunlop et al., 2011; Luan et al., 2014; Ma et al., 2011; Pan et al., 2009; Wang et al., 2012). In this study, we introduced the HSPs GroESL and DnaK from *Deinococcus wulumuqiensis* R12, an extremely radioresistant bacterium, into *C. acetobutylicum* ATCC824, and successfully improved the cellular robustness and the butanol titer. In addition, for comparative study, we also overexpressed the autologous GroESL and DnaK of *C. acetobutylicum* ATCC824 and tested the resulting butanol production and the cellular robustness. The stress-response proteins from the extremophilic bacterium were superior to the native proteins.

2. Materials and methods

2.1. Bacterial strains and plasmids

Table 1 lists the bacterial strains and plasmids used in this study. *C. acetobutylicum* ATCC 824 and plasmid pAN2 (Heap et al., 2007) were kindly provided by Prof. Shang-Tian Yang (The Ohio State University, Columbus, OH). The genomic DNA of *D. wulumuqiensis*R12 was kindly provided by Ling Jiang (Nanjing University of Technology, Nanjing, China). *E. coli* DH5α was purchased from Tiangen Biotech Company (Beijing, China) and used for vector construction and amplification. *E. coli* TOP10 bearing pAN2 was used for methylation of pMTL82151 (Heap et al., 2009) and recombinant plasmids. All strains were stored at −80 °C in 15% glycerol.

Table 1
Bacterial strains and plasmids.

Strain or plasmid	Relevant characteristics	Source or reference
C. acetobutylicum ATCC 824	Wild type strain	ATCC
Escherichia coli		
DH5α	DeoR, recA1, endA1, hsdR17 (rk^-, mk+)	TIANGEN
Top10	hsd R, mcr A, rec A1, end A1	TIANGEN
Plasmids		
pAN2	Φ3t I, p15a ori, Tet^R	Heap et al. (2007)
pMTL82151	*Clostridium* modular plasmid used for complementation and control; CmR	Heap et al. (2009)
pMTL82151:groESL12	*groESL12* complementation plasmid	This work
pMTL82151:groESL824	*groESL824* complementation plasmid	This work
pMTL82151:dnaK12	*dnaK12* complementation plasmid	This work
pMTL82151: dnaK824	*dnaK824*complementation plasmid	This work

2.2. Cell growth and culture media

E. coli strains were cultured in Luria-Bertani (LB) medium supplemented with chloramphenicol (25 μg/mL) and/or tetracycline (20 μg/mL) when needed, at 37 °C. All *C. acetobutylicum* strains were cultured in liquid Reinforced Clostridial Medium (RCM) (containing 0.5% glucose) or on RCM agar (Ventura et al., 2013) at 37 °C anaerobically; 30 μg/mL thiamphenicol was added to the medium if needed. To maintain anaerobiosis, liquid RCM in serum bottles was purged with nitrogen, and RCM agar plates were incubated in an anaerobic chamber overnight. P2 medium was used for main batch fermentation; the composition of P2 medium in distilled water was: 80 g/L glucose, 1 g/L yeast extract, buffer (2.2 g/L ammonium acetate, 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4), mineral salts solution (0.2 g/L $MgSO_4{\cdot}7H_2O$, 0.01 g/L $FeSO_4{\cdot}7H_2O$, 0.01 g/L $MnSO_4{\cdot}H_2O$ and 0.01 g/L NaCl), and vitamins solution (1 mg/L *p*-Aminobenzoic acid, 1 mg/L thiamine and 0.01 mg/L biotin).

2.3. DNA and plasmid isolation, construction and transformation

The genomic DNA of *C. acetobutylicum* ATCC 824 and *D. wulumuqiensis* R12 were isolated using a genome extraction kit (Sangon, Shanghai, China). Plasmids were isolated using the TIANprep Mini Plasmid Kit II (Tiangen). Isolated chromosomal DNA and plasmids were dissolved in ddH_2O and stored at −20 °C. PCR primers used in this study are listed in Table 2, and were synthesized by Sangon Biotech Company. All restriction enzymes were purchased from Fermentas (Pittsburgh, PA, USA) and used per the manufacturer's instructions.

The PCR amplification procedure for the genes *groESL* and *dnaK* from *C. acetobutylicum* ATCC 824 was: 95 °C predenaturation for 240 s; 30 cycles of 95 °C denaturation for 30 s, 55 °C annealing for 30 s and 72 °C elongation for 150 s; and a final extension at 72 °C for 10 min. The gene fragments of *groESL* and *dnaK* from *C. acetobutylicum* ATCC 824 were amplified with *Bam*HI and *Nco*I sites at the respective ends. The PCR amplification procedure for the genes *groESL* and *dnaK* from *D. wulumuqiensis* R12 was: 95 °C predenaturation for 240 s; 30 cycles of 95 °C denaturation for 30 s, 67 °C annealing for 30 s and 72 °C elongation for 150 s; and a final extension at 72 °C for 10 min. The gene fragments of *groESL* and *dnaK* from *D. wulumuqiensis* R12 were amplified with *Bam*HI and *Hin*dIII sites at the respective ends.

The plasmid pMTL82151 isolated from *E. coli* DH5α was double digested with *Bam*HI and *Nco*I or *Bam*HI and *Hin*dIII. The digested PCR products and pMTL82151 were purified using a TIANquick Midi Purification Kit (Tiangen) and dissolved in 30 μL ddH_2O. Digested pMTL82151 and gene fragments were ligated using T4 DNA ligase

Table 2
PCR primers used for the amplification of *dnaK* and *groESL* genes from *Clostridium acetobutylicum* ATCC 824 and *Deinococcus wulumuqiensis* R12.

Genes	Primers
groESL824	CGCGGATCCATGAAAATTAGACCACTTGGTGAC
	CATGCCATGGTTAGTACATTCCGCCCATTCCCAT
groESL12	GGATCCATGCTGAAACCACTTGGTGACCGC
	AAGCTTTTAGAAGTCCATGCCGCCCATGCC
dnaK824	CGCGGATCCATGTCAAAAGTAATTGGAATTGATTTAG
	CATGCCATGGCTATTTATCATTATCTACTTTAAAGTCAGC
dnaK12	GGATCCATGGCCAAAGCTGTTGGAATCGAC
	AAGCTTTTACTCCGCAGGCTTGAAGTCG
pMTL-F	TGAAGTACATCACCGACGAGCAAG
pMTL-R	TGCTGCAAGGCGATTAAGTTGGGT