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Enhancing *n*-Butanol Tolerance of *Escherichia coli* by Overexpressing of Stress-Responsive Molecular Chaperones

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Abstract
Microbial tolerance to organic solvents is critical for efficient production of biofuels. In this study, *n*-butanol tolerance of *Escherichia coli* JM109 was improved by overexpressing of genes encoding stress-responsive small RNA-regulator, RNA chaperone, and molecular chaperone. Gene *rpoS*, coding for sigma S subunit of RNA polymerase, was the most efficient in improving *n*-butanol tolerance of *E. coli*. The highest OD_{600} and the specific growth rate of JM109/pQE80L-rpoS reached 1.692 and 0.144 h^{-1} respectively at 1.0% (*v*/*v*) *n*-butanol. Double and triple expression of molecular chaperones *rpoS*, *secB*, and *groS* were conducted and optimized. Recombinant strains JM109/pQE80L-*secB-rpoS* and JM109/pQE80L-*groS-secB-rpoS* exhibited the highest *n*-butanol tolerance, with specific growth rates of 0.164 and 0.165 h^{-1}, respectively. Membrane integrity, potentials, and cell morphology analyses demonstrated the high viability of JM109/pQE80L-*groS-secB-rpoS*. This study provides guidance on employing various molecular chaperones for enhancing the tolerance of *E. coli* against *n*-butanol.

Keywords Molecular chaperone · *n*-Butanol tolerance · *Escherichia coli* · RpoS · Coexpression

Introduction

Biofuels are promising alternatives for traditional fossil fuels [1]. Especially, biobutanol (*n*-butanol), a clear and neutral C4 primary alcohol with low viscous, has many attractive features as a biofuel. In addition, biobutanol could be more compatible with the traditional fossil fuels than ethanol, etc. [2, 3]. *Escherichia coli*

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is generally accepted as a platform microorganism for producing valuable biofuels [4, 5]. Enormous efforts have been committed to design and develop novel pathways, circuits, modules, and synthetic genes for enhanced productivity [6–8]. Nevertheless, severe toxicity of the biofuel products (such as ethanol, *n*-butanol) is one serious limitation for the application of *E. coli* in biofuels production. It was reported that the growth of *E. coli* could be significantly influenced under merely 1.0% (*v*/*v*) *n*-butanol [9]. To achieve the production of biobutanol at high titers, process engineering and host engineering are two alternative and effective strategies. Process engineering, such as membrane separation, adsorption gas, and extraction of newly produced *n*-butanol is passive method to alleviate *n*-butanol toxicity [10, 11]. However, host engineering is an active strategy to improve *n*-butanol tolerance [12]. Thus, it is of great importance to develop solvent-tolerant host strains for the production of titer-dependent biofuels.

To resist the toxicity of organic solvents, microorganisms have evolved various patterns [12, 13]. Some bacteria could respond to organic solvents by altering the membrane architecture [14, 15], such as the ratio of saturated to unsaturated fatty acids [16, 17], phospholipid head groups [12, 14, 17–19], and isomerization of *cis* to *trans* unsaturated fatty acids [15, 16]. Also, intruded organic solvents could be discharged from the cells by microbial efflux pumps of resistance-nodulation-cell division (RND) family to avoid the above-mentioned damages [20], which is known as the most efficient mechanism in Gram-negative bacteria [12, 17]. RND transporters are proton-driven efflux systems, mainly consisted of three proteins: intimal transporter, membrane fusion protein, and outer membrane channel protein [21, 22]. RND transporters permit cells to expulse organic solvents via two possible pathways: from cytoplasm to external medium or from periplasm to external medium [23]. Additionally, molecular chaperones, such as GroES, GroL, GrpE, and DnaK, have also been proved to be crucial for organic solvent tolerance (OST) of microorganisms through refolding the misfolded proteins denatured by organic solvents (*e.g.*, ethanol, *n*-butanol, xylene, or toluene) in the cytoplasm and periplasm [13, 24].

In our previous study, a random mutagenesis library of *ropD* was constructed, and *E. coli* strains harboring variants capable of tolerating 2% (*v*/*v*) *n*-butanol and 69% (*v*/*v*) cyclohexane were obtained. Besides, a series of functional genes (such as *yibT*, *yghW*) relating to butanol tolerance in *E. coli* were identified by DNA microarray analyses [21, 25]. Furthermore, molecular chaperones SecB and its variant $SecB_{T10A}$ were proved to significantly enhance the tolerance of *E. coli* against *n*-butanol, in which hydrophobic interaction between SecB and its cargo proteins is critical [26].

Small regulatory RNAs (sRNAs) play crucial roles in microbes [27, 28] through repressing or activating the expression of a great part of genes under environmental stresses [29–31]. Similarly, the small heat shock proteins (sHSPs) are ubiquitous stress-responsive proteins that are capable of protecting cells against high temperature, starvation, organic solvents, etc. [32–37]. The sRNA regulator gene *rpoS* has been reported to manipulate at least 500 genes related to various stress responses in *E. coli* [33]. However, roles of sRNA (such as RpoS) in microbial OST have been rarely investigated. Here, molecular chaperones involved in manipulating folding of sRNAs and proteins were explored for their potentials in enhancing tolerance of *E. coli* against organic solvents.

Material and Methods

Strains and Medium

Strains and plasmids used in this study are listed in Table 1. Primers used in this study are shown in Table S1. All *E. coli* strains were cultivated in Luria-Bertani (LB) medium at 37 °C and 120 rpm in flasks with threaded caps. *n*-Butanol was of analytical grade and purchased from Sinopharm Ltd.

Construction of Recombinant Plasmids for Expression of Single Gene

sRNA genes or molecular chaperone genes including *secB* (*EcsecB*), *rpoS*, *asr*, *cspC*, *cspD*, *grpE*, *proQ*, *hfq*, *fur*, and *nusB* were amplified from *E. coli* JM109 with KOD polymerase (Toyobo co. Ltd., Japan) and primers in Table S1. PCR procedures were set as follows: initial denaturation at 94 °C for 2 min, 30 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s, and extension at 68 °C with time depending on the length of each gene at 0.5 kb min^{-1}, and final extension at 68 °C for 10 min. The resultant PCR product was purified and ligated into pQE80L double digested with *Bam*H I and *Sal* I by CloneExpress II (Vazyme Biotech., Nanjing).

Table 1 Strains and plasmids used in this study.

Strains and plasmids	Description	Source
E. coli JM109(DE3)	*E. coli* JM109 with a DE3 prophage carrying T7 polymerase expression cassette	QIAGEN
pQE80L	empty expression vector	QIAGEN
pQE80L-*secB*	*pQE80L carrying secB* gene from *E. coli* JM109	Xu et al. [26]
pQE80L-*EtsecB*	*secB* gene from *Erwinia tasmaniensis*	This study
pQE80L-*EssecB*	*secB* gene from *Enterobacter* sp. 638	This study
pQE80L-*PasecB*	*secB* gene from *Pseudomonas atlantica*	This study
pQE80L-*asr*	*asr* gene from *E. coli* JM109	This study
pQE80L-*cspD*	*cspD* gene from *E. coli* JM109	This study
pQE80L-*hfq*	*hfq* gene from *E. coli* JM109	This study
pQE80L-*fur*	*fur* gene from *E. coli* JM109	This study
pQE80L-*nusB*	*nusB* gene from *E. coli* JM109	This study
pQE80L-*proQ*	*proQ* gene from *E. coli* JM109	This study
pQE80L-*rpoS*	*rpoS* gene from *E. coli* JM109	This study
pQE80L-*cspC*	*cspC* gene from *E. coli* JM109	This study
pQE80L-*grpE*	*grpE* gene from *E. coli* JM109	This study
pQE80L-*secB-rpoS*	*pQE80L* carrying *secB* and *rpoS* genes	This study
pQE80L-*rpoS-secB*	coexpression of *rpoS* and *secB*	This study
pQE80L-*groS-secB*	coexpression of *groS* and *secB*	This study
pQE80L-*secB-groS*	coexpression of *secB* and *groS*	This study
pQE80L-*groS-secB-rpoS*	coexpression of *groS*, *secB*, and *rpoS*	This study
pQE80L-*groS-rpoS-secB*	coexpression of *groS*, *rpoS*, and *secB*	This study
pQE80L-*rpoS-secB-groS*	coexpression of *rpoS*, *secB*, and *groS*	This study
pQE80L-*rpoS-groS-secB*	coexpression of *rpoS*, *groS*, and *secB*	This study
pQE80L-*secB-rpoS-groS*	coexpression of *secB*, *rpoS*, and *groS*	This study
pQE80L-*secB-groS-rpoS*	coexpression of *secB*, *groS*, and *rpoS*	This study

Construction of pQE80L-secB Expression Vectors

The *secB* genes were amplified from *Erwinia tasmaniensis* Et1/99 (*EtsecB*), *Enterobacter* sp. 638 (*EssecB*), and *Pseudomonas atlantica* T6C (*PasecB*) and double digested with *Bam*H I and *Sal* I. Afterwards, *secB* genes were ligated into expressing plasmid pQE80L double-digested with the same restricted endonucleases.

Construction of Recombinant Plasmids for Coexpression

The *groS* and *rpoS* genes together with the promoter sequence were excised from recombinant plasmids pQE80L-*groS* and pQE80L-*rpoS* using *Xho* I and *Hin*d III. Moreover, plasmid pQE80L-*secB* was linearized with *Sal* I and *Hin*d III. Since *Xho* I and *Sal* I are a pair of isocaudamers, fragments of *groS* and *rpoS* could be ligated into linearized pQE80L-*secB* by T_4 DNA ligase.

SecB, *groS*, and *rpoS* together with promoter element and homologous arm sequences were amplified from the corresponding pQE80L-*secB*, pQE80L-*groS*, and pQE80L-*rpoS*. Furthermore, all the three fragments were ligated into pQE80L digested with *Bam*H I and *Hin*d III by ClonExpress Multis (Vazyme Ltd., Nanjing).

Expression and Analysis of Recombinant Proteins

Recombinant plasmids were transformed into *E. coli* JM109(DE3) and verified by sequencing. Positive strains were picked up and inoculated into LB medium and cultivated at 37 °C and 120 rpm until OD_{600} reached 0.6. Then, 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was supplemented and further cultivated at 30 °C and 120 rpm for inducing the expression of recombinant proteins. Cell pellets were harvested by centrifugation at 8000 rpm and 4 °C for 10 min and resuspended in 10 mL PBS (pH 7.0, 100 mM), subjected to sonication disruption at 150 W for 10 min. Afterwards, 1 mL of sample was withdrawn, and supernatant and precipitant were obtained by centrifugation at 12,000 rpm for 5 min. Appropriate amounts of supernatant and precipitant were mixed with 5 μL 5 × SDS-PAGE loading buffer and heated at 100 °C for 10 min. Expression result of each chaperone was verified by SDS-PAGE.

Determination Tolerance Toward *n*-Butanol

Recombinant strains were cultivated in 30 mL LB medium supplemented with 50 μg mL^{-1} kanamycin overnight at 37 °C and 120 rpm. About 1.0 mL overnight culture was transferred into 100 mL fresh LB medium (50 μg mL^{-1} kan) and incubated at 37 °C and 120 rpm until OD_{600} reached 0.5, followed by 0.2 mM IPTG was added and induced at 30 °C and 120 rpm. Furthermore, when OD_{600} reached 0.75~0.80, 1.0% (*v*/*v*) *n*-butanol was supplemented for all strains and cultivated at 30 °C and 120 rpm. The OD_{600} of each strain was monitored over a period of 10 h. To measure the growth kinetic of different strains, different concentrations of *n*-butanol were added when OD_{600} reached 0.75~0.80. In this study, OD_{600} of each strain reached 0.75~0.80 within 3 h, and the OD_{600} value was defined as the starting value. Specific growth rate (μ) of *E. coli* strain in the initial 4 h was calculated according to Eq. (1) [26]. All the experiments were performed for at least three times. Data represents mean ± standard deviation of three independent biological experiments. Significant difference analysis was evaluated by *t* test with P value < 0.05 as significant difference.

$$\mu\left(\mathrm{h}^{-1}\right)=\frac{\mathrm{LnOD600(4\ h)-LnOD600(0\ h)}}{(4-0)\ \mathrm{h}} \quad (1)$$

Measure of Membrane Potential and Membrane Permeability

The excitation and emission wavelengths of rhodamine 123 (Rh123) are 507 and 529 nm, respectively. Rh123 was prepared with ultrapure water (2.5 mg mL^{-1}) and stored in the dark. Three-milliliter cultures were withdrawn every 1 h, followed by addition of 18 μL of prepared Rh123 solution, shaking at 180 rpm and 37 °C for 30 min in a water bath shaker. Samples were collected by centrifugation at 8000 rpm and 4 °C for 10 min and washed twice with 10 mM PBS. Fluorescence intensity was measured by fluorescence spectrophotometer F-7000.

The excitation and emission wavelength of 3,6-diacetoxyfluoran (FDA) is 488 and 530 nm, respectively. FDA was prepared into a 2-mg mL^{-1} solution with acetone. Three-mililiter sample was taken every 1 h, followed by addition of 450 μL of FDA solution. Samples were thoroughly mixed at room temperature for 5 min in the dark. Then the fluorescence intensity was immediately measured with fluorescence spectrophotometer F-7000.

Cell Morphology Observation Assay

Recombinant *E. coli* JM109(DE3) harboring pQE80L and pQE80L-*groS-secB-rpoS* was cultured in 30 mL LB medium overnight at 37 °C and 180 rpm with 50 μg mL^{-1} kanamycin. About 1.0% (*v*/*v*) of the overnight culture was further inoculated in 100 mL LB fresh medium and cultured at 37 °C and 200 rpm with 50 μg mL^{-1} kanamycin until the OD_{600} reached 0.5; 0.2 mM IPTG was added to all of the recombinant strains to initiate induction at 30 °C. After OD_{600} reached 0.75~0.80, 1.0% (*v*/*v*) *n*-butanol was supplemented and control strain was prepared without addition of *n*-butanol. All the strains were cultivated for 10 h with shaking at 200 rpm. Cells were collected and washed with physiologic saline for four times. And the samples were diluted for cell morphology observation using Hitachi-H7650 transmission electron microscopy (Japan) at × 12,000 magnification. The average cellular sizes of *E. coli* strains were measured on the electron microscope from about 100 cells.

Results and Discussion

Mining for Homologous Proteins of SecB

Previously, *secB* gene from *E. coli* JM109, encoding translocator protein, was identified to be capable of enhancing *n*-butanol tolerance of *E. coli* [26]. Herein, several SecB homologous proteins with > 70% identities, including *Et*SecB from *Erwinia tasmaniensis* Et1/99, *Es*SecB from *Enterobacter sp*. 638, and *Pa*SecB from *Pseudomonas atlantica* T6C, were identified and heterogeneously overexpressed in *E. coli* JM109(DE3) as indicated by SDS-PAGE analysis (Fig. S1). The growth profiles of recombinant and control strains (harboring empty plasmid pQE80L) were monitored in LB medium supplemented with 1.0% (*v*/*v*) *n*-butanol. As shown in Fig. 1, the growth of all

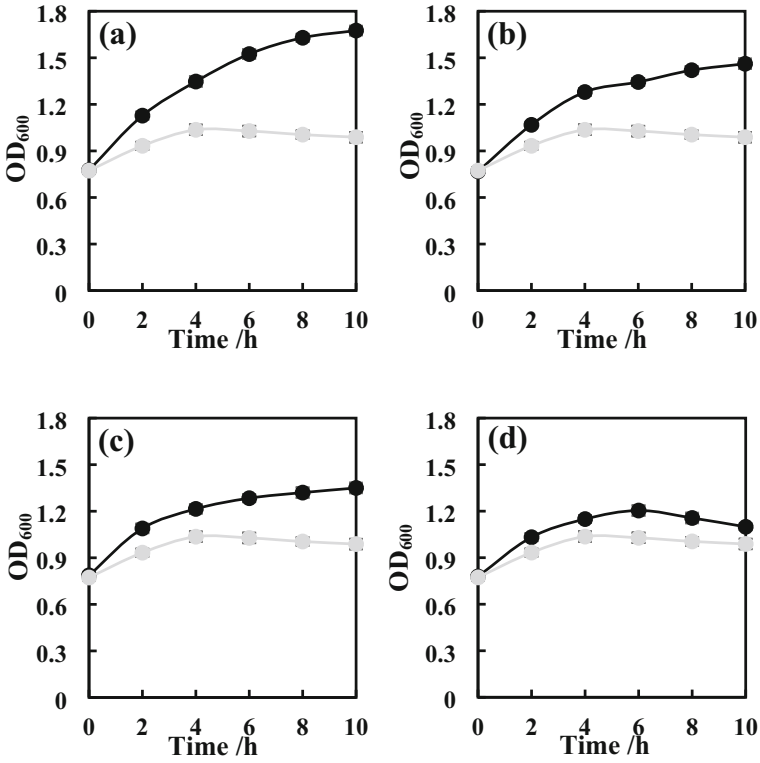

Fig. 1 Growth profiles of *E. coli* JM109(DE3) strains overexpressing *secB* homologous genes from different microbes. *EcsecB* (**a**), *EtsecB* (**b**), *PasecB* (**c**), and *EssecB* (**d**) in the presence of 1% (*v*/*v*) *n*-butanol. Control was performed with *E. coli* harboring empty pQE80L (grey cycle). All strains were cultured in LB medium and induced with 0.2 mM IPTG at 30 °C. Error bars indicate standard deviations of at least three independent replicates

recombinant strains carrying *secB* was better than that of the control. The highest OD_{600} and specific growth rate (μ) of SecB, *Et*SecB, *Es*SecB, and *Pa*SecB strains were 1.676 and 0.138 h^{-1}, 1.463 and 0.097 h^{-1}, 1.204 and 0.109 h^{-1}, and 1.350 and 0.108 h^{-1}, respectively. Under the same conditions, the highest OD_{600} and μ of control strain were only 1.004 and 0.073 h^{-1}. Recombinant strain with *secB* from *E. coli* displayed the best performance considering the highest OD_{600} and μ, which were 1.67- and 1.89-fold of those of control strain. As a result, *secB* from *E. coli* was selected for coexpression with other potential molecular chaperones.

Mining for Molecular Chaperones Modulating Small RNA and Proteins

To explore potential molecular chaperones involved in environmental stress response, nine small RNA-regulated genes, RNA chaperone genes and molecular chaperone genes (*rpoS*, *hfq*, *fur*, *asr*, *nusB*, *cspC*, *cspD*, *grpE*, and *proQ*) were overexpressed in *E. coli* JM109(DE3) and evaluated by SDS-PAGE (Fig. S2). All strains were cultivated in LB medium supplemented with 1.0% (*v*/*v*) *n*-butanol in sealed flasks at 180 rpm and 30 °C. Growth profiles were determined as shown in Fig. 2. The highest OD_{600} and μ of *rpoS*, *asr*, *cspC*, *cspD*, *grpE*, *proQ*, *hfq*, *fur*, and *nusB* were 1.692 and 0.144 h^{-1}, 1.138 and 0.077 h^{-1}, 1.00 and 0.064 h^{-1},

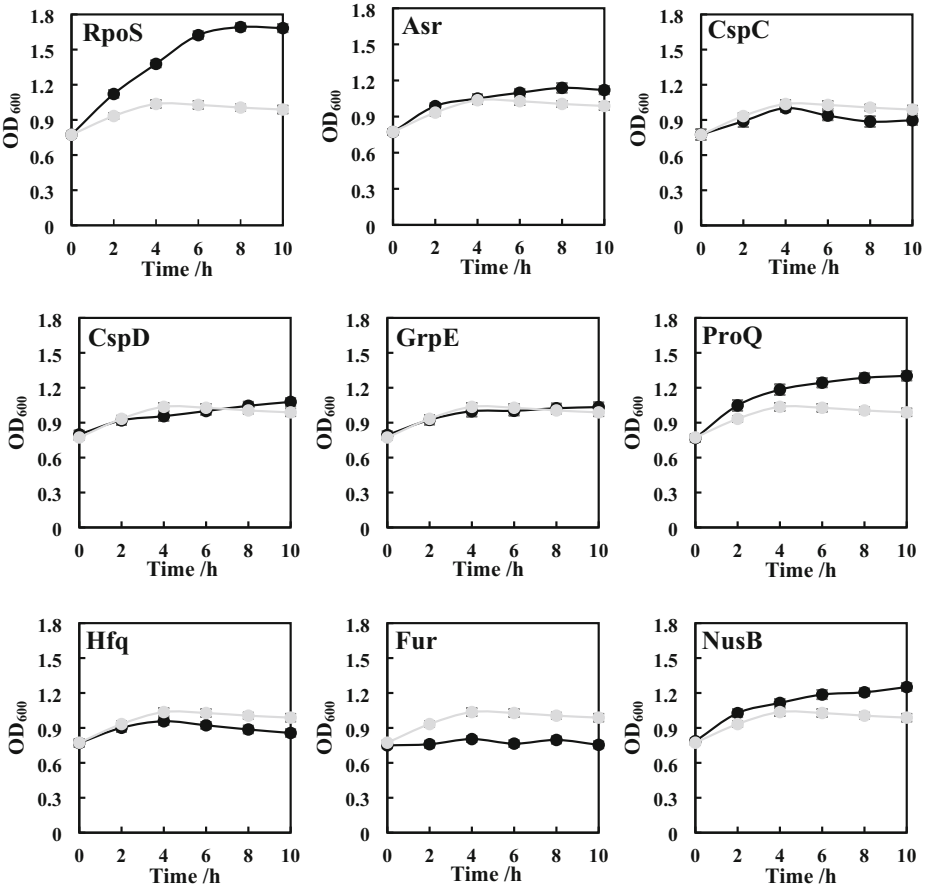

Fig. 2 Growth profiles of *E. coli* strains harboring various molecular chaperones in the presence of 1% (*v*/*v*) *n*-butanol. Control was performed with *E. coli* harboring blank pQE80L (grey cycle). All experiments were performed for at least three times

1.078 and 0.045 h^{-1}, 1.034 and 0.057 h^{-1}, 1.302 and 0.108 h^{-1}, 0.957 and 0.054 h^{-1}, 0.804 and 0.017 h^{-1}, and 1.252 and 0.087 h^{-1}, respectively. In comparison with the control, overexpression of *asr*, *cspC*, *cspD*, and *grpE* did not influence the growth of *E. coli*, *hfq* and *fur* were deleterious to growth, whereas *rpoS*, *proQ*, and *nusB* were favorable for the growth. Especially *rpoS*, coding for RNA polymerase sigma S subunit, displayed the highest μ, 1.97-fold of control strain. Genes *proQ* and *nusB* codes for RNA molecular chaperone and transcription antitermination factor. To the best of our knowledge, this was the first report of *rpoS*, *proQ*, and *nusB* in improving the tolerance of *E. coli* toward *n*-butanol. It was reported that some specific sRNAs could stimulate the translation of *rpoS* and assist bacteria to adapt to various surrounding changes, such as nutrient starvation, variations in temperature, osmolarity, or pH [33]. Additionally, GroS is a small subunit of GroESL and was reported to be effective in enhancing *n*-butanol tolerance of *E. coli* [34, 35]. Therefore, *groS* gene is also a good choice for improving the tolerance to *n*-butanol in *E. coli*. Consequently, RpoS and GroS were chosen to further coexpress with SecB to investigate the synergistic effects.

Effect of Double Expression of *groS*, *rpoS*, and *secB* on *n*-Butanol Tolerance of *E. coli*

Coexpression of *groS* and *rpoS* with *secB* was conducted by construction of recombinant strains harboring pQE80L-*groS*-*secB*, pQE80L-*rpoS*-*secB*, pQE80L-*secB*-*rpoS*, and pQE80L-*secB*-*groS* in different orders. SDS-PAGE analysis revealed *secB*, *groS*, and *rpoS* were all successfully expressed. Then, growth curves of four strains under *n*-butanol stress were monitored and shown in Fig. 3. Coexpression of two genes could lead to a better growth than strains expressing single gene. Under 1.0% (*v*/*v*) *n*-butanol, a longer and faster exponential growth phase was observed for JM109/*secB*-*groS* and JM109/*secB*-*rpoS*. The μ values of JM109/*secB*-*groS* and JM109/*secB*-*rpoS* were 0.148 and 0.164 h^{-1}, which were 2.03- and 2.23-fold of the control strain with blank pQE80L (Fig. 3a, c) and also slightly higher than those of single expression strains. Strains JM109/*groS*-*secB* and JM109/*rpoS*-*secB* showed no obvious improvement on *n*-butanol tolerance when compared with JM109 expressing *secB* alone (Fig. 3b, d). The μ values of JM109/*groS*-*secB* and JM109/*rpoS*-*secB* were 0.142 and 0.143 h^{-1}, lower than those of JM109/*secB*-*groS*, and JM109/*secB*-*rpoS*. It can also be concluded that alteration in the orders of RpoS, GroS, and SecB would affect *n*-butanol tolerance. The above results suggest that double expression of *secB*, *rpoS*, and *groS* could synergistically enhance *n*-butanol tolerance and prolong the exponential growth phase of

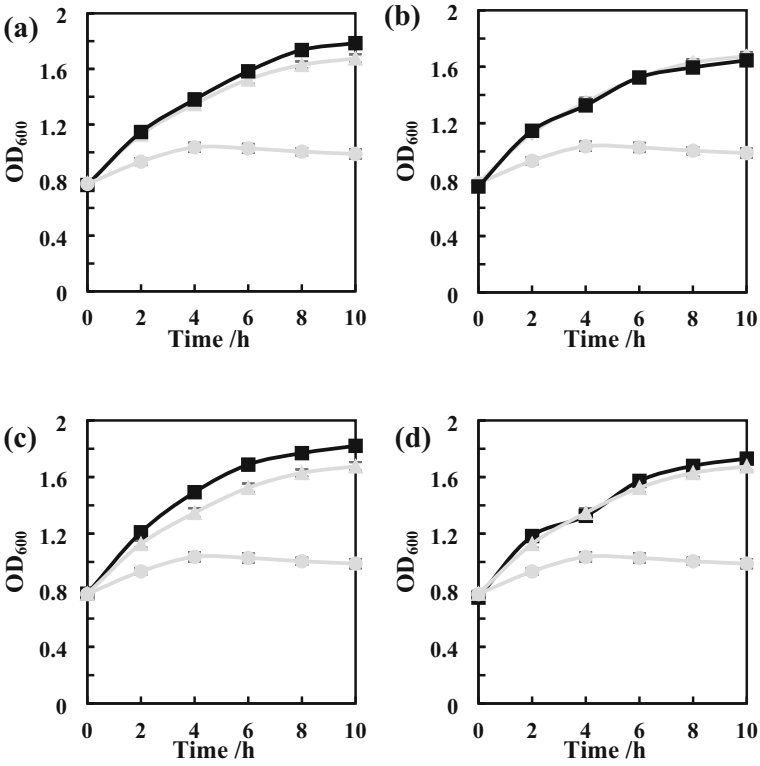

Fig. 3 Growth profiles of *E. coli* strains with double expression of molecular chaperones in the presence of 1% (*v*/*v*) *n*-butanol. **a** JM109/*secB*-*groS*, **b** JM109/*groS*-*secB*, **c** JM109/*secB*-*rpoS*, and **d** JM109/*rpoS*-*secB*. Negative and positive controls were performed with *E. coli* harboring empty pQE80L (grey cycle) and pQE80L-*secB* (grey triangle). All experiments were performed for at least three times

recombinant *E. coli*. In fact, the *n*-butanol tolerance of *E. coli* strain overexpressing *proQ* or *nusB* with *secB* was also investigated; however, all of them showed lower tolerance than expression of *secB* alone.

Considering its better performance, the growth profiles of JM109/*secB-rpoS* at different butanol concentrations were explored. The μ values of JM109/*secB-rpoS* at 0.8% (*v*/*v*) and 1.2% (*v*/*v*) *n*-butanol were 0.200 and 0.083 h^{-1} respectively, whereas the μ values of control strain were 0.128 and − 0.035 h^{-1} under the same conditions. It can be seen that JM109/*secB-rpoS* could tolerate 1.2% *n*-butanol, at which the control strain could not grow. The specific growth rate of JM109/*secB-rpoS* at 0.8% *n*-butanol was 1.56-fold of control strain.

Effect of Triple Expression of *groS*, *rpoS*, and *secB* on *n*-Butanol Tolerance of *E. coli*

To explore the effect of triple expression of molecular chaperones on *n*-butanol tolerance of *E. coli*, *groS*, *rpoS*, and *secB* were inserted into one plasmid in different orders, including JM109/*groS-secB-rpoS*, JM109/*secB-groS-rpoS*, JM109/*groS-rpoS-secB*, JM109/*rpoS-secB-groS*, JM109/*secB-rpoS-groS*, and JM109/*rpoS-groS-secB*. The growth profiles of triple expression strains are shown in Fig. 4. All the recombinant strains expressing three molecular chaperones were higher than SecB recombinant strain, except for JM109/*rpoS-secB-groS* with similar growth profiles. Strain JM109/*groS-secB-rpoS* displayed the highest specific growth rate of 0.165 h^{-1}, followed by JM109/*secB-groS-rpoS* with specific growth rate of 0.160 h^{-1} and JM109/*groS-rpoS-secB* of 0.157 h^{-1}. Furthermore, JM109/*groS-secB-rpoS* and JM109/*secB-groS-rpoS* were subjected to tolerance analysis at different *n*-butanol concentrations (Table 2). Under 0.8% *n*-butanol, the specific growth rates of JM109/*groS-secB-rpoS* and

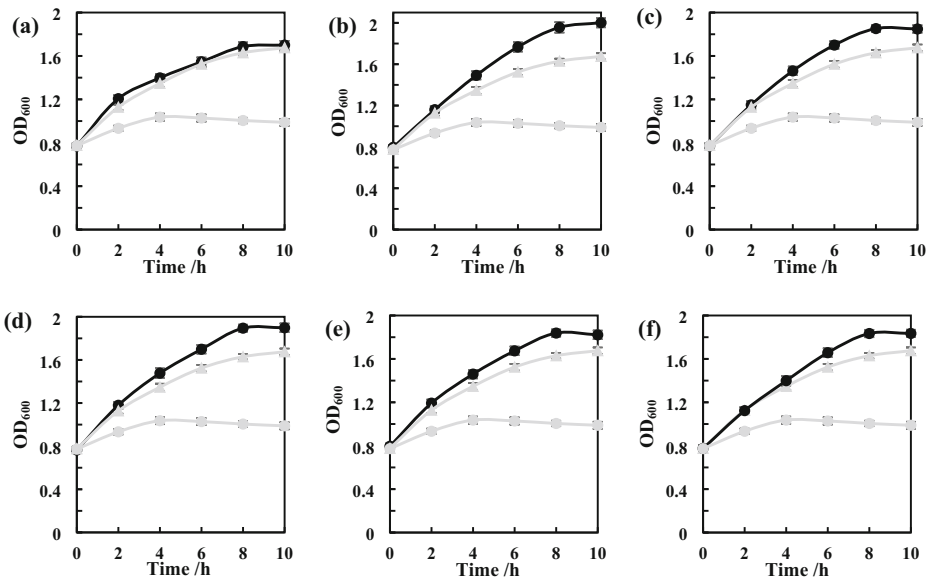

Fig. 4 Growth profiles of *E. coli* strains with triple expression of molecular chaperones in the presence of 1% (*v*/*v*) *n*-butanol. **a** JM109/*rpoS-secB-groS*, **b** JM109/*groS-secB-rpoS*, **c** JM109/*groS-rpoS-secB*, **d** JM109/*secB-groS-rpoS*, **e** JM109/*secB-rpoS-groS*, and **f** JM109/*rpoS-groS-secB*. Negative and positive controls were performed with *E. coli* harboring empty pQE80L (grey cycle) and pQE80L-*secB* (grey triangle). All experiments were performed for at least three times

Table 2 Specific growth rates of *E. coli* JM109 strains coexpressed with molecular chaperones under different *n*-butanol concentration

	Specific growth rate (h^{-1})		
	0.8% *n*-butanol	1.0% *n*-butanol	1.2% *n*-butanol
Control	0.128 ± 0.003	0.073 ± 0.002	− 0.035 ± 0.003
SecB-RpoS	0.200 ± 0.004	0.164 ± 0.003	0.083 ± 0.002
SecB-GroS-RpoS	0.188 ± 0.003	0.160 ± 0.003	0.057 ± 0.003
GroS-SecB-RpoS	0.215 ± 0.005	0.165 ± 0.003	0.078 ± 0.002

JM109/*secB-groS-rpoS* were 0.215 and 0.188 h^{-1}, respectively. Strain JM109/*groS-secB-rpoS* was highly *n*-butanol tolerant, with specific growth rate of 1.68- and 1.07-fold of WT and double expressing strain JM109/pQE80L-*secB-rpoS*, respectively. At 1.2% *n*-butanol, the specific growth rates of JM109/*groS-secB-rpoS* and JM109/*secB-groS-rpoS* were 0.078 and 0.057 h^{-1}, slightly lower than that of JM109/*secB-rpoS*. Considering that the specific growth rates of JM109/*secB-rpoS* and JM109/*groS-secB-rpoS* were similar and higher than those of JM109/*rpoS* and JM109/*secB*, there is a good synergistic effect between *secB* and *rpoS*.

SDS-PAGE analysis revealed that alteration the order of *rpoS*, *groS*, and *secB* in plasmid resulted in different expression levels of them (Fig. S3). Firstly, the expression levels of all three genes decreased to some extent compared with single expression. Secondly, in JM109/pQE80L-*groS-secB-rpoS* and JM109/pQE80L-*secB-groS-rpoS*, the expression levels of *secB* and *rpoS* were relative higher than other triple coexpression strains, consistent with their higher *n*-butanol tolerance. With regard to other triple coexpression strains, the expression levels of *rpoS*, *groS*, and *secB* were similar. SDS-PAGE analysis result could provide evidence for the varied *n*-butanol tolerances of triple coexpression strains. However, this correlation cannot be overestimated considering only the static state at the end of cultivation were collected for SDS-PAGE analysis, while the *n*-butanol stress is dynamic.

As generally accepted, *secB* and *groS* are genes coding for molecular chaperones, which play important roles in protein transmembrane transportation by recognizing and binding with the transporter to facilitate the entire protein transporting process [36, 37]. In addition, SecB can assist in refolding proteins denatured by heating or environmental stress. RpoS is a general stress sigma factor which interacts with core RNA polymerase (RNAP) and controls the expression of specific but large sets of genes in *E. coli*. Various stresses including acid stress, starvation and high osmotic pressure could lead to the induction of specific sRNAs, which might stimulate RpoS translation or induction of small-protein anti-adaptors that stabilize the proteins [35–39]. Actually, RpoS is strictly regulated at all levels, but primarily at the translational and post-translational levels [33]. Herein, coexpression of *groS*, *rpoS*, and *secB* takes full advantage of the important roles of molecular chaperones and sRNAs in responding to environmental stress and enhancing the tolerance of *E. coli* against *n*-butanol.

Changes in Membrane Potential and Permeability

Rh123, a cationic lipophilic fluorescent dye, can pass through cell membrane. Due to the different potentials inside and outside of the cell membrane, Rh123 is capable of specifically adsorbing to the inner membrane of the cell. A decrease in fluorescence intensity of Rh123 indicates a decrease in membrane potential [40, 41]. As a result, the degree of damage to the

cell membrane could be reflected by measuring the changes of fluorescence intensity of Rh123. As shown in Fig. 5a, under 1.0% (*v*/*v*) *n*-butanol, the ratio of fluorescence intensity of JM109/pQE80L-*groS-secB-rpoS* has increased by 18.4% after 5 h of induced cultivation, which was lower than that of control strain (26.7%). From the view of overall profiles, strain harboring three molecular chaperones exhibited higher ratio of fluorescence intensity and lower fluctuation of fluorescence intensity than control strain. Less fluctuation in cell membrane potential indicates that the cell membrane of coexpressed strain is less damaged. Consequently, the integrity and viability of strain JM109/pQE80L-*groS-secB-rpoS* is higher.

Compound 3,6-diacetoxyfluoran (FDA) cannot emit fluorescence. However, it can be degraded by intracellular esterases to produce fluorescence. FDA could be maintained in living cells due to its special polarity. If the cell membrane is damaged by organic solvents, the permeability is increased and the fluorescence is leaked from cytoplasm, resulting in the reducing of FDA fluorescence intensity [40, 41]. Therefore, the fluorescence intensity of FDA can reflect cell membrane integrity and permeability. As shown in Fig. 5b, the fluorescence intensity of both strains decreased upon addition of 1.0% (*v*/*v*) *n*-butanol. After incubation for 6 h, the fluorescence intensity of the coexpressed strain decreased to 86.9%, indicating that the cell membrane permeability was increased by 13.1. Under the same condition, the fluorescence intensity of control strain decreased to 72.2%. Thus, cell membrane permeability of the control was higher than that of the coexpressed strain, suggesting the cell membrane fluidity of coexpressed strain is relatively lower. In summary, the above results showed that coexpression of *groS*, *rpoS*, and *secB* could help maintain the integrity of the cell membrane and improve *n*-butanol tolerance of microorganisms.

Effect of *n*-Butanol on Cell Morphology

Our previous study showed that the presence of organic solvents could change the morphology of cells, and the decrease of specific surface area would lead to improved organic solvent tolerance [21]. As shown in Fig. 6a, b, in the absence of *n*-butanol, JM109/*groS-secB-rpoS* cells exhibited similar morphology with the control, and the cell length and width were 3.43 ± 0.45 μm × 2.26 ± 0.21 μm and 3.22 ± 0.32 μm × 2.13 ± 0.17 μm, respectively. In the presence of 1.0% (*v*/*v*) *n*-butanol, both JM109/pQE80L and JM109/

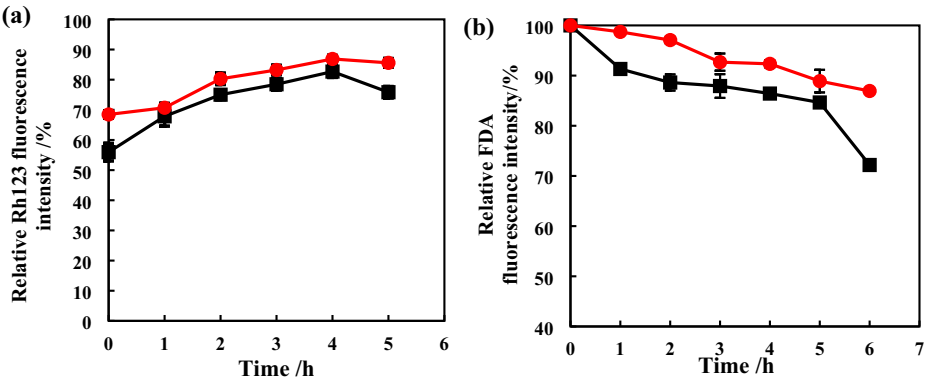

Fig. 5 Effect of *n*-butanol on the cell membrane potential (**a**) and permeability (**b**) of JM109/*groS-secB-rpoS* (red cycle) and JM109/pQE80L. All experiments were performed in triplicate, and the error bars indicate standard deviations

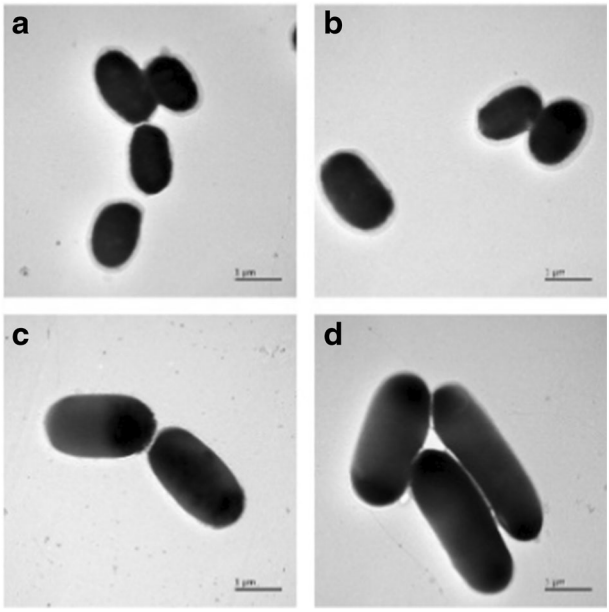

Fig. 6 Effect of *n*-butanol on cell morphology of *E. coli* JM109 at × 12,000 magnification. **a** JM109/pQE80L without *n*-butanol. **b** JM109/*groS-secB-rpoS* without *n*-butanol. **c** JM109/pQE80L with n-butanol. **d** JM109/*groS-secB-rpoS* with *n*-butanol

groS-secB-rpoS cells became longer than that of without *n*-butanol. As shown in Fig. 6c, d, JM109/*groS-secB-rpoS* cells were 2-fold longer than those the control strain, while their width was similar. It has been reported that elongated cells were more compatible with organic solvents [21]. As a result, coexpression of molecular chaperones was favorable for improving the tolerance of *E. coli* toward *n*-butanol.

Conclusions

In summary, molecular chaperone RpoS encoded by *rpoS* was identified with high efficiency in enhancing butanol tolerance of *E. coli*. Coexpression of *secB*, *groS*, and *rpoS* was investigated to further improve the *n*-butanol resistance of *E. coli*. Recombinant strains JM109/*secB-rpoS* and JM109/*groS-secB-rpoS* exhibited the highest tolerance. The specific growth rates of two strains were 0.164 and 0.165 h^{-1} in 1.0% *n*-butanol, much higher than 0.073 h^{-1} of control strain. All the double and triple expression strains could grow at 1.2% *n*-butanol, which could significantly inhibit the growth of *E. coli*. Compared with the control, JM109/*groS-secB-rpoS* also displayed higher integrity and viability, and became longer in cellular morphology. There is good synergistic effect between SecB and RpoS. This study provides evidence for the role of RpoS and offers guidance for employing molecular chaperones to improve *n*-butanol tolerance of *E. coli*.

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Compliance with ethical standards

Conflict of Interest The authors declare that they have no competing interests.

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