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Enhancement of butanol production by sequential introduction of mutations conferring butanol tolerance and streptomycin resistance

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Ribosome engineering, originally applied to *Streptomyces lividans*, has been widely utilized for strain improvement, especially for the activation of bacterial secondary metabolism. This study assessed ribosome engineering technology to modulate primary metabolism, taking butanol production as a representative example. The introduction into *Clostridium saccharoperbutylacetonicum* of mutations conferring resistance to butanol (But^R) and of the *str* mutation (Sm^R; a mutation in the *rpsL* gene encoding ribosomal protein S12), conferring high-level resistance to streptomycin, increased butanol production 1.6-fold, to 16.5 g butanol/L. Real-time qPCR analysis demonstrated that the genes involved in butanol metabolism by *C. saccharoperbutylacetonicum* were activated at the transcriptional level in the drug-resistant mutants, providing a mechanism for the higher yields of butanol by the mutants. Moreover, the activity of enzymes butyraldehyde dehydrogenase (AdhE) and butanol dehydrogenases (BdhAB), the key enzymes involved in butanol synthesis, was both markedly increased in the But^R Sm^R mutant, reflecting the significant up-regulation of *adhE* and *bdhA* at transcriptional level in this mutant strain. These results demonstrate the efficacy of ribosome engineering for the production of not only secondary metabolites but of industrially important primary metabolites. The possible ways to overcome the reduced growth rate and/or fitness cost caused by the mutation were also discussed.

[**Keywords:** Butanol; Ribosome engineering; *rpsL* mutation; *rpoB* mutation; Tolerance; *Clostridium saccharoperbutylacetonicum*]

Drug resistance mutation technology, often called ribosome engineering (1,2), has been widely utilized for microbial strain improvement, especially in the overproduction of antibiotics and the activation of silent genes involved in bacterial secondary metabolite production (3–5). Ribosome engineering is characterized by simplicity, consisting of the isolation of spontaneously developed drug-resistant mutants. Therefore, this method does not require the induction of mutagenesis or any genomic information and provides a rational approach of enhancing bacterial capabilities for industrial applications. To date, however, few reports have focused on utilization of ribosome engineering in the production of primary metabolites (3).

The notion of ribosome engineering was derived from results obtained with *Streptomyces lividans*. Although *S. lividans* normally does not produce antibiotics, it possesses dormant antibiotic biosynthesis genes (6). The introduction of ribosome engineering into *Streptomyces* strains isolated from soil samples was found to activate the dormant abilities of these bacteria to produce antibiotics (7). Furthermore, the bacterial alarmone ppGpp (guanosine 5′-diphosphate 3′-diphosphate), produced on ribosomes in response to nutrient starvation, was found to bind to RNA polymerase, eventually initiating the production of antibiotics (8–10). These observations led to the development of a ribosome engineering technology targeting S12, RNA polymerase, and other ribosomal proteins and translation factors, thus activating or enhancing the production of secondary metabolites. Ribosome engineering technology was found applicable to strain improvement and silent gene activation, resulting in the identification of novel secondary metabolites (7,11–13), as well as to the enhancement of enzyme production and tolerance to toxic chemicals (14,15).

Streptomycin-resistant (Sm^R) *rpsL*-mutant ribosomes, which carry an amino acid substitution in the ribosomal S12 protein that confers high level resistance to streptomycin, are more stable than wild-type ribosomes, indicating that increased stability may enhance protein synthesis during the late growth phase of bacteria (15–17). Increased expression of the translation factor ribosome recycling factor also contributed to the enhanced synthesis of the *rpsL* K88E mutant protein during the transition and stationary growth phase (18). That is, both the greater stability of the 70S ribosomes and the elevated levels of ribosome recycling factor resulting from the *rpsL* K88E mutation were responsible for enhanced protein synthesis during the late growth phase, with the latter being responsible for antibiotic overproduction and silent gene activation. In contrast, the activation of silent genes by rifampicin resistance (Rif^R) *rpoB* mutations in *Streptomyces* has been attributed, at least in part, to the increased affinity of mutant RNA polymerase for silent gene promoters (7). A recent study showed the broad applicability of the Rif^R *rpoB* mutation method to the expression of cryptic secondary metabolite-biosynthetic gene clusters (19).

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Butanol can be efficiently produced by the genus *Clostridium* through acetone-butanol-ethanol fermentation. Commercial butanol fermentation utilizes the classical solventogenic strains of clostridial strains, including *Clostridium acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* (20). Several challenges to the industrial production of butanol must still be overcome, such as overall cost competitiveness and development of higher performance strains with greater butanol tolerance (21,22). Butanol is highly toxic to all microorganisms, including clostridia, and at high concentrations can lead to reduced substrate consumption and decreased overall cellular metabolism. Overcoming butanol toxicity has become a major challenge in the economic production of butanol (23–26). Details of metabolic pathways and biochemistry of clostridial species are well documented in review papers (24,27,28). Employing random chemical mutagenesis and butanol exposure, butanol-tolerant strains of *C. acetobutylicum* were selected and exhibited enhanced butanol production. Genome shuffling and selection in the presence of high butanol concentrations was also effective (29,30). Several studies have demonstrated that butanol production can be improved by manipulating genes other than those directly involved in acetone-butanol-ethanol fermentation pathways (reviewed by Moon et al. (20) and Zheng et al. (31)). However, clostridial strains are, in general, rather difficult to genetically engineer. Strain development through metabolic engineering has also been explored for enhanced production of butanol (32–34). After 100 years of investigations, level of butanol production reached 25.7 g/L by batch fermentation (35–39) (reviewed by Moon et al. (20)).

Although several studies have assessed the ability of ribosome engineering technology to enhance ethanol and butanol production, this study assessed the applicability of ribosome engineering technology (and sequential introduction of mutations conferring butanol tolerance) to improve butanol production as a representative example because of its industrial importance.

MATERIALS AND METHODS

Bacterial strains and culture conditions The wild-type strain *C. saccharoperbutylacetonicum* ATCC 27021 (N1-4) was used for acetone-butanol fermentation. *C. saccharoperbutylacetonicum* was grown in tryptone-yeast extract-acetate (TYA) medium (40) at 30 °C for 1 day under anaerobic conditions, using an anaeropack system (Mitsubishi Gas Chemical Co., Tokyo, Japan). TYA medium was composed of (per liter); 20 g glucose, 2 g yeast extract, 6 g tryptone, 3 g ammonium acetate, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.5 g KH_2PO_4, and 10 mg $FeSO_4 \cdot 7H_2O$. Subsequently, 10% (v/v) volume of culture was inoculated into fresh TYA medium, followed by incubation for 4 days under anaerobic conditions. After 1 day cultivation, glucose was added to a final concentration of 6% (w/v) to enhance acetone-butanol fermentation. Butanol, acetone and ethanol produced in the medium were measured as described below.

Determination of minimum inhibitory concentrations To determine minimum inhibitory concentrations (MICs) of various drugs, 10 μl of full grown culture of *C. saccharoperbutylacetonicum* was spotted onto TYA and inoculation plates, respectively, containing various concentrations of drug. Plates containing *C. saccharoperbutylacetonicum* were incubated at 30 °C for 3 days. The minimum drug concentration able to fully inhibit growth was defined as the MIC. Resistance to butanol and acetone was determined by inoculating 1% (v/v) of full grown culture into TYA liquid medium containing various concentrations of butanol or acetone. After incubation for 3 days at 30 °C, the presence or absence of growth was determined.

Mutagenesis and screening procedure The disparity mutagenesis cocktail (Chitose Laboratory Corp., Kawasaki, Japan), which generates point mutations at higher efficiencies on dispersive chromosome areas than general mutagens (41–43), was used to introduce mutations into *C. saccharoperbutylacetonicum*. The disparity mutagenesis method allows accumulation of the replication errors during the overnight growth in liquid culture. Strains were cultivated to full growth in 5 ml of culture medium containing the mutagen cocktail. *C. saccharoperbutylacetonicum* mutants resistant to butanol, streptomycin, rifampicin or fusidic acid were selected using TYA plates containing each chemical, which were incubated at 30 °C for 3–7 days. The antibiotic concentrations used for mutant isolation are described in Table 1. Mutations in the *rpoB* and *rpsL* genes were determined by DNA sequencing using the primers listed in Table S1.

Measurement of butanol, ethanol, and acetone Culture broth of *C. saccharoperbutylacetonicum* was centrifuged at 15,000 ×*g* for 1 min, and the supernatant was analyzed directly by gas chromatography–mass spectrometry (GC/MS). The analytical conditions were: device, Shimadzu GC-2010; column, GL Science Inert Cap WAX (15 m, inner diameter 0.25 mm); oven temperature, increased from 40 °C to 240 °C at a rate of 10 °C/min; injector temperature, 220 °C carrier gas, helium; flow rate, 2.29 ml/min; detection, *m/z* between 29 and 1090 using a Shimadzu GCMS-QP2010; ion source temperature, 200 °C; interface temperature, 220 °C.

Transcriptional analysis by real-time qPCR Total RNAs were extracted and purified from cells grown for the indicated times, using Isogen reagent (Nippon Gene) according to the manufacturer's protocol. Real-time qPCR was performed as described (44). Following the removal of contaminating DNA by incubation of 2 μg total RNA with 2 U of DNase I (Invitrogen, Carlsbad, CA, USA) for 15 min at 25 °C, RNAs were reverse transcribed using a High Capacity RNA-to-cDNA Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. After terminating the reaction by incubation for 5 min at 95 °C, samples were analyzed using a CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA) and

TABLE 1. Characteristics of mutants of *Clostridium saccharoperbutylacetonicum*.

Strain	Butanol or antibiotic concentration used for selection	Resistance to: (μg/ml)[a]	Resistance to butanol[b] (g/L)	Position of mutation in *rpsL* or *rpoB*	Amino acid substitution
27021 (wild-type)	–[c]	Streptomycin (50) Rifampicin (1) Fusidic acid (0.5)	17.8	–	–
KO-1255 (But^R) (selected by 8 steps of disparity mutagenesis from 27021)	Butanol in plate (23.5–35.6 g/L)		21.1	ND[d]	ND
KO-1256 (But^R Sm^R) (selected for streptomycin resistance from KO-1255)	Streptomycin (100 μg/ml)	Streptomycin (>1000)	21.9	129G→A (*rpsL*)	Lys43→Asn
KO-1257 (But^R Rif^R) (selected for rifampicin resistance from KO-1255)	Rifampicin (5 μg/ml)	Rifampicin (200)	20.3	1514G→A (*rpoB*)	Arg505→Lys
KO-1258 (But^R Fus^R) (selected for fusidic acid resistance from KO-1255)	Fusidic acid (5 μg/ml)	Fusidic acid (20)	20.3	ND	ND

[a] After incubation for 3 days on TYA solid medium.
[b] After incubation for 3 days in TYA liquid medium.
[c] Not applicable.
[d] Not detected.

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