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Native efflux pumps of *Escherichia coli* responsible for short and medium chain alcohol

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ABSTRACT

Efflux pumps (EPs) are an important mechanism responsible for extrusion of toxic compounds from the cell. Engineering of microbial cell EPs is a promising strategy in the production of biofuels and chemicals. In this study, we evaluate the ability of 44 native *Escherichia coli* EPs to enhance tolerance to short and medium carbon chain alcohols (SMCAs). Among EPs studied, MdtJ, Bcr, and MdtH and YdeA transported the majority of tested monohydroxy, dihydroxy, and trihydroxy alcohols, respectively. Overexpression of MdtJ, Bcr, MdtH and YdeA increased the tolerance of strains to SMCAs by 35%–50%. The efficiency of EPs for transporting SMCAs was verified by use of the 1, 2, 4-butanetriol (BT) synthesis system: where overexpression of MdtH and YdeA effectively increased the titers of BT by 31% and 18.5%, respectively. This work demonstrates that the innate EP system of *E. coli* is a resource which might be exploited to improve the synthesis of biofuels and chemicals.

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1. Introduction

With the rising concern of climate change, finite fossil fuel availability and energy security, interest in alternative renewable fuels and chemicals has grown [1]. Advanced biofuels, such as short and medium carbon chain alcohols (SMCA), have higher energy densities, lower hygroscopicity, and vapor pressure, compared to more traditional biofuels [2]. In addition, SMCA biofuels can be manufactured using lignocellulose and are compatible with current fuel compositions used for transportation [3,4]. SMCAs are also used as precursors in chemical synthesis or industrial products. For example, 1, 3-propanediol is the monomer used to manufacture many biodegradable polymers and consequently, demand is gradually rising [5]. 1, 2, 4-butanetriol (BT) is an important precursor in the synthesis of various drugs and chemicals such as 1, 2, 4-butanetriol trinitrate [6,7]. However, considerable challenges exist using engineered microbes to manufacture SMCAs. For example, during the fermentation process, high concentrations of alcohols accumulate and reduce the rate of growth of strains, limiting the yield of target products. Accumulation of alcohols within the fermentation system can result in disorganizing structure of cell membranes, denaturing of proteins, and degradation of RNA [8–10]. Therefore development of new approaches is required to improve the efficacy and efficiency of alcohol bio-production processes. An effective strategy to improve cell tolerance is actively exporting alcohols from the cytoplasm by use of efflux pumps (EPs) [1].

EPs are a class of membrane bound transporters that recognize and export the noxious agents from the cell [11]. Most organisms express multiple EPs [12]. For example, it has been reported that *Escherichia coli* express at least 44 multidrug EPs [13,14] belonging to five categories: ATP-binding cassette family (ABC), major facilitator superfamily (MFS), multidrug and toxic compound extrusion family (MATE), small multidrug resistance family (SMR), and resistance modulation cell division family (RND) [14]. In addition to the transportation of toxic and harmful substances that have penetrated the cell wall of the organism, EPs alleviate the toxic effects of intracellular metabolites [15]. Expression of EPs is tightly regulated by microorganisms, however inducers can be used to increase the expression EPs [15].

Native or heterologous EPs have been overexpressed in microbial strains to impart tolerance phenotypes and/or improve the production of biofuels or chemicals [16]. In *E. coli*, overexpression of MdlB, an ABC-type EP, can increase the yield of isopentenol by 12% [17]. Directed evolution strategies have also been used to improve the export capacity of EPs for specific substrates [18]. For example, variants of the *E. coli* EP AcrB have been engineered to enhance cell growth rates in the presence of *n*-butanol by up to 25% [19]. Further studies have shown that heterologous overexpression of many EPs

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in *E. coli* is less effective for chemical efflux when compared to their native transporters [20,21].

Although multiple EPs have been used in biofuel production [17,19,22–24], few studies have systematically investigated the effects of endogenous *E. coli* EPs on export of SMCAs [16], thus limiting utilization of tolerance engineering in biofuel production by engineered *E. coli*. Therefore in this study, native EPs of *E. coli* were analyzed, and putative SMCA exporters were overexpressed to investigate their effect on *E. coli* tolerance. The BT biosynthesis system was used as a model system to verify the efficiency of selected EPs on improving BT production. This study demonstrates that endogenous EPs of *E. coli* can play important roles in the production of fuels and chemicals.

2. Materials and methods

2.1. Bacterial strains and plasmid construction

Constructed *E. coli* strains and BW25113 derivatives obtained from the Coli Genetic Stock Center (CGSC) (Yale University, New Haven, USA) with disrupted EP genes are listed in Tables 1 and 2, respectively. *E. coli* DH5α was the host strain used to construct recombinant plasmids.

Plasmids used in this study are listed in Table 1. For construction of plasmid harboring EP genes, pBBR1MCS, a low-copy-number and broad host vector, was used. Briefly, pBBR1MCS was digested by use of the restriction enzymes *SalI* and *HindIII*. The genes *mdtJ*, *bcr*, *mdtH, and ydeA*, were amplified from the *E. coli* BW25113 genome using primers listed in Table S1. Primers contained 18–29 bp priming to the target gene and a 20 bp "linker" homologous to pBBR1MCS. The linearized pBBR1MCS and amplified genes were assembled as described by Gibson et al. [25]. Primers were obtained from Sangon Biotech (Shanghai, China). Endonucleases were purchased from Thermo Fisher Scientific (Massachusetts, USA). Gel DNA purification kits and plasmid purification kits were ordered from TianGen (Beijing, China).

For construction of *acrB* null *E. coli* strain BTΔacrB, we selectively deleted *acrB* in the *E. coli* strain MJ133K-1 using λ Red homologous recombination method [26]. Briefly, the DNA fragment containing 500 bp upstream of *acrB*, the FRT-flanked kanamycin resistance (Km^r) cassette, and the 500 bp downstream of *acrB* was amplified using genomic DNA of *E. coli* JW0451-2 and primers PR9 and PR10, and transferred into *E. coli* MJ133 containing plasmid pKD46 for genomic recombination to generate the *acrB* deletion mutation strain, to which the plasmid pTMX was transferred resulting in the *E. coli* BTΔacrB. The plasmids pKD46 and pCP20 were used as helper plasmid for the deletion of the gene *acrB* in *E. coli* genome.

2.2. Growth inhibition assay

E. coli BW25113 and its derivatives with EP genes deleted, were grown in 250 ml Erlenmeyer flasks containing 50 ml lysogeny broth (LB) medium at 37 °C and 190 rpm for 12 h. The culture was then inoculated into 100 × 15 mm test tubes containing 5 ml LB medium and the associated SMCA to an initial optical density (OD600) of 0.1. Culturing was carried out at 37 °C on a rotary shaker at 200 rpm for 12 h. Kanamycin (50 μg/ml) was added when required. Growth inhibition rate (GIR) was defined as the relative change in cell mass as a percentage of that of the control, and was calculated using Eq. (1) [13].

$$\text{The growth inhibition rate} = \left(1-\frac{OD_{600\ \text{without alcohol}}}{OD_{600\ \text{without alcohol}}}\right) \times 100 \quad (1)$$

A range of SMCA concentrations were used to establish the concentration to elicit a 50% inhibition of growth of *E. coli* BW25113 (Table S2). We compared GIR of single-EP gene deleted derivatives of *E. coli* BW25113 with control strain to identify the capability of EPs to export SMCAs.

2.3. Cell viability assay

Colony-forming units (CFU) were used as a measure of cell viability [7,27]. Briefly, a secondary culture of *E. coli* grown under the same culture conditions as described above was serially diluted in tenfold steps in LB media without addition of antibiotics. 100 μl of serially diluted *E. coli* was spread on the surface of an LB agar plate with addition of 25 μg/ml chloramphenicol, incubated for 12–16 h, and cell counts were performed. The CFU of *E. coli* exposed to the alcohols was compared to *E. coli* grown on LB agar plates. Each experiment was duplicated.

Table 1
Strains and plasmids used in this study.

Strains	Description	Sources
E. coli DH5α	F⁻ Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recA*1 *endA*1 *hsdR*17 (r_K^-, m_K^+) *phoA supE*44 λ⁻ *thi*⁻1 *gyrA*96 *relA*1	Lab stock
E. coli BW25113	Δ(araD-araB)567ΔlacZ4787(:rrnB-3) λ- rph-1Δ(rhaD-rhaB)568 hsdR514	CGSC
MJ133	MG1655 Δ*yjhH* Δ*yagE* Δ*xylA*	[39,40]
MJ133K-1	MJ133 carrying pTMX with BT producing capability.	[39,40]
BWpBB	BW25113 carrying pBBR1MCS	This work
BWmdtJ	BW25113 carrying pBBmdtJ	This work
BWDmdtJ	BW25113 Δ*mdtJ*	CGSC JW1592-1
BWbcr	BW25113 carrying pBBbcr	This work
BWDbcr	BW25113 Δ*bcr*	CGSC JW5363-1
BWmdtH	BW25113 carrying pBBmdtH	This work
BWDmdtH	BW25113 Δ*mdtH*	CGSC JW1052-1
BTmdtH	MJ133K-1 carrying pBBmdtH	This work
BTydeA	MJ133K-1 carrying pBBydeA	This work
BTpBB	MJ133K-1 carrying pBBR1MCS	This work
BTDacrB	MJ133K-1 *ΔacrB*	This work
pBBR1MCS	expression vector, Cm resistant	Lab stock
pTMX	pTrc99A harboring *mdlC* and *xdh*	[39,40]
pBBmdtJ	pBBR1MCS harboring *mdtJ*; Cm resistant	This work
pBBbcr	pBBR1MCS harboring *bcr*; Cm resistant	This work
pBBmdtH	pBBR1MCS harboring *mdtH*; Cm resistant	This work
pBBydeA	pBBR1MCS harboring *ydeA*; Cm resistant	This work
pKD4	$oriR_{\gamma R6k}$, FRT:kan:FRT; Ap resistant	Lab stock
pkD46	repA101(ts), oriR101, *araBp-gam-bet-exo*; Ap resistant	Lab stock
pCP20	Rep(Ts); cI857λ(Ts), FLP; Ap resistant, Cm resistant	Lab stock

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