Title: Improved n-butanol tolerance in *Escherichia coli* by controlling membrane related functions

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Abstract

As the increasing demand from both chemical and fuel markets, the interest in producing n-butanol using biological route has been rejuvenated to engineer an economical fermentation process, competing with the currently-dominant chemical synthesis. n-butanol has been traditionally produced from the ABE fermentation of *Clostridium acetobutylicum*. This system, however, is not economically feasible due to its limited efficiency and the lack of genetic modification tools for further improvements. Alternatively, n-butanol synthesis pathway was successfully transferred into *Escherichia coli* and rapidly improved to reach a level of production comparable to the native producer. Nevertheless, the toxicity of n-butanol has become a common issue that either approach has to deal with. Previously, we reported our success in improving n-butanol tolerance in *E. coli* by engineering an Artificial Transcription Factor (ATF) that can modify the expression level of multiple targets simultaneously and improved the n-butanol tolerance of MG1655 strain to 1.5% (vol/vol) n-butanol. However, it was observed that some possible n-butanol tolerance mechanisms did not occurred upon the ATF expression, especially the membrane-related functions such as the homeoviscous adaptation, iron uptaking system, and efflux pump system. In this work, we attempted to enhance the n-butanol tolerance associated with the ATF by combining it with the membrane-related functions in *E. coli*, including the overexpression of fatty acid synthesis genes, iron-uptaking protein FeoA, and introducing a SrpABC efflux pump from *Pseudomonas putida* into *E. coli*. The synergistic effect of this combinatorial approach led to 4, 5, and 9-fold improved growths in the cultures containing 1, 1.5, and 2% (vol/vol) n-butanol, respectively, of an MG1655 knockout strain engineered for n-butanol production, and expanded the tolerance limit to 2% (vol/vol) n-butanol.

Keywords: n-butanol, tolerance, ATF, cis-vaccenic acid, membrane, SrpABC

1. Introduction

n-butanol has been used widely in both chemical and fuel sections as a solvent, a chemical building block, and a gasoline additive and substitute. Even though the biological route for producing n-butanol, ABE fermentation, has been developed for almost a century, the efficiency is still below the economically feasible level for the industrial production. While n-butanol has been mostly produced by a chemical method called oxo process, interests in ABE fermentation has been recently rejuvenated in order to accommodate the growing demand from the chemical market, especially from the fuel market. n-butanol was shown to be superior to ethanol as an alternative fuel because it has higher energy content, less solubility in water, and can be manufactured based on the already-established infrastructures for ethanol production (Harvey and Meylemans, 2011; Mariano et al., 2013). Efforts have been made to engineer n-butanol synthetic pathway in advanced hosts such as *Escherichia coli* (Atsumi et al., 2008; Dellomonaco et al., 2011; Inui et al., 2008; Shen et al., 2011), yeast (Branduardi et al., 2013; Steen et al., 2008), and cyanobacteria (Lan and Liao, 2011, 2012) to overcome the limitations of the native host, *Clostridium acetobutylicum*, such as the anaerobic, heterologous fermentation, the complicated physiology and difficulty in genetic manipulation. Among the alternative hosts for n-butanol production, *E. coli* has shown the best proficiency that it can produce n-butanol titer at a comparable level to *Clostridium* (15 g/L) (Dellomonaco et al., 2011; Shen et al., 2011). However, *E. coli*, *C. acetobutylicum*, and other commonly used microorganisms are suffered from the toxicity of n-butanol, limiting the maximum titer of n-butanol accumulated in the medium. It has been known that most of studied microorganisms are severely inhibited in the presence of 1 - 2% (vol/vol) n-butanol (Lin and Blaschek, 1983), and particularly *E. coli* growth is completely stopped at 1.5% (vol/vol) or about 15 g/L n-butanol. In order to relieve the n-butanol toxicity, intensive studies have been done to identify the mechanism of cellular intoxication via transcriptional analysis, genome-wide functional study of n-butanol stressed bacteria (Rutherford et al., 2010; Tomas et al., 2004;

Winkler and Kao, 2011), genomic library enrichment (Reyes et al., 2011), membrane analysis (Ingram, 1986; Reyes et al., 2012) and visualization in real time of adaptive events (Reyes et al., 2012). From these researches, even though n-butanol was shown to be involved in a multi-target inhibitory and denaturing process, causing disruptions of the membrane, membrane related functions, and metabolic network, there are common groups of genes were found to be upregulated during n-butanol stress, including genes related to the cell wall and membrane biosynthesis, transcriptional regulation, multidrug resistance, stress response, and efflux pumps. In particular, the over-reactivity of membrane-related functions are very critical to maintain the vitality of the cells because damages to the membrane are widely considered as the initial and most detrimental effect caused by n-butanol and other solvents (Baer et al., 1987; Ingram, 1976; Isken et al., 1999). Previously, we engineered an Artificial Transcription Factor (ATF) to improve the n-butanol tolerance of the wild-type *E. coli* MG1655 to about 1.5% (vol/vol) n-butanol (Lee et al., 2011). This n-butanol tolerant ATF (BT) was screened from a library of fusions between an effective domain, cyclic AMP receptor protein (CRP), a transcriptional regulatory protein that can either activate or inhibit the expression of genes by interacting with the promoter sequence, and a DNA binding domain which is randomly assembled from 3 zinc finger motifs. With the ability to bind to a specific short 9-bp sequence, an ATF can simultaneously modify the expression of multiple targets and efficiently elicit the solvent tolerance phenotype. Interestingly, by transcriptional analysis of n-butanol tolerant BT cell, it was revealed that the most up-regulated proteins were membrane proteins (SdhCDAB), along with other proteins involved in the metabolism, but, surprisingly, there was not any change in the membrane fatty acid composition (Lee et al., 2011). Moreover, we found that the addition of ferrous ion (Fe^{2+}) to the medium can significantly improve the cell tolerance, but that cannot be applied to BT cell. Iron metabolism is of important factors limiting the growth of *E. coli* (Hartmann and Braun, 1981) and it depends on the activity of the iron uptaking system. Noticeably, the overexpression of an iron-transport related protein FeoA was

also found to be able to enhance n-butanol tolerance in *E. coli* (Reyes et al., 2013). These findings led us to hypothesize that the effectiveness of ATF on improving n-butanol tolerance can be further enhanced if it is engineered to work in concert with a group of proteins involving in controlling membrane-related functions such as membrane fatty acid synthesis and iron uptaking system. Besides, we are also interested in seeking an efflux system that can recognize n-butanol as its substrate, despite that an earlier effort was failed in screening an n-butanol efflux pump (Dunlop et al., 2011), to continuously relieve the internal stress caused by this solvent.

In this work, we selected several membrane related functions and analyzed their ability to assist the n-butanol resistance in *E. coli*, which was introduced by our BT ATF. First, we overexpressed genes in the Type II Fatty acid synthesis (FAS II) to modify the membrane fatty acid composition and screen for a construct that increases n-butanol toxicity. Second, we analyzed the impact of ferrous ion on *E. coli* cell and employed FeoA protein to enhance the iron uptaking system in BT cell, with the presumption that the reduction in Fe^{2+} uptake limited BT cell from improving the tolerance in the presence of Fe^{2+} . And lastly, we introduced the SrpABC efflux pump from *Pseudomonas putida* to *E. coli*, because this is the only system that was reported to have the promoter up-regulated in the presence of n-butanol, which hints for its ability to export n-butanol to the medium (Kieboom et al., 1998).

2. Materials and Methods

2.1 Bacterial strains

E. coli strains XL1-Blue (Stratagene, La Jolla, CA, USA) was used in this work as the transformation host and for propagating plasmids. *E. coli* K-12 strain MG1655, JM109 (Promega, Madison, WI, USA), and MS56, a reduced-genome derivative of MG1655 constructed in our lab with 56 genomic deletions (Park et al., 2014) were infected with λ DE3 prophage, using the Lysogenic Lambda Kit (Novagen, Madison, WI, USA), for the site-specific genomic integration of

DE3 region, generating MG1655(DE3), JM109(DE3), and MS56(DE3) strains, respectively, which allows the expression of genes under the control of a strong T7 promoter. BL21(DE3) (Novagen, Madison, WI, USA) and the aforementioned strains were employed for the n-butanol tolerance test.

For constructing knockout strains that have the n-butanol competing pathways (ethanol, lactate, acetate, succinate pathways) removed, MG1655(DE3) was subjected to P1 transductions. BW25113 strains with single knockouts ($\Delta adhE$, $\Delta ldhA$, Δpta , $\Delta yqhD$, and $\Delta frdA$) were obtained from Keio collection (Baba et al., 2006) and served as the donor strains for MG1655(DE3). P1 transduction was performed as described (Thomason et al., 2007) and colony PCR was employed to screen the deletion strains using a primer pair flanking the target DNA region. Afterward, the kanamycin resistance cassette was removed from the integrated region in the host genome via FLP recombinase expressed from pCP22 plasmid (Pichon et al., 2013). As such, the cells could be able to be infected by other P1 phages, creating MDB1 ($\Delta adhE$), MDB2 ($\Delta adhE \Delta yqhD$), MDB3 ($\Delta adhE \Delta yqhD \Delta ldhA$), MDB4 ($\Delta adhE \Delta yqhD \Delta ldhA \Delta pta$), and MDB5 ($\Delta adhE \Delta yqhD \Delta ldhA \Delta pta \Delta frdA$) strains, respectively.

2.2 Plasmid construction

To construct a high-copy number vector based on pACYCDuet-1 vector with two strong T7 promoters (Novagen, Madison, WI, USA), the high-copy number origin of replication (ColE1) from pUC19 (Invitrogen Corp., Carlsbad, CA, USA) was cloned by PCR and replaced the low-copy number origin of replication, p15A, between NheI and XbaI sites, generating pAUD vector. Alternatively, the chloramphenicol resistance gene on pAUD was replaced with kanamycin resistance cassette to generate pAUK vector, which shares the same origin of replication with pAUD.

For gene overexpression, *fabA*, *fabB*, *fabD*, *fabF*, *fabG*, *fabH*, *fabI*, *fabZ*, and *feoA* were amplified from genomic DNA of *E. coli* strain MG1655; *srpA*, *srpB*, and *srpC* were amplified from genomic DNA of *P. putida* P8 strain (ATCC 49451), which expresses the same SrpABC as

from S12 strain, as confirmed by sequencing; n-butanol tolerant ATF was amplified from pET_{lac}ATF plasmid (Lee et al., 2011) by PCR using Pfu Plus 5X Master Mix (ELPIS, Daejeon, South Korea) and primers listed in Table 1. PCR products were digested with their corresponding restriction enzymes (REs) as described in Table 1, then ligated into pACYCDuet-1 (in the cases of *srpA*, *srpB*, *srpC*) or pAUD (the rest of cloned genes) using T4 DNA ligase (ELPIS, Daejeon, South Korea). With most of the clonings, the REs used to digest the PCR products were also used to digest the corresponding vector. Except when AseI and BspHI were used to digest the PCR products, then NdeI and NcoI were selected to digest the vector, respectively.

In order to construct tandem copies of *fabD*, this gene was amplified using fabDAseF1 and fabDBglR primers (Table 1). Then, after being cloned into pAUD, fabDAseF2 and fabDBglR primers were used to re-clone *fabD*, covering the extra 5'UTR region to allow the expression of the added copies by the upstream Shine-Dalgarno sequence provided on pACYCDuet-1 vector. Since an NdeI recognition site was designed upstream the BglII recognition site on fabDBglR primer (Table 1), the re-cloned *fabD* can be ligated next to the previous one, generating pAD, pAD₂, pAD₃, and pAD₄ plasmids containing 1, 2, 3, and 4 copies of *fabD* (named as *fabD*, (*fabD*)₂, (*fabD*)₃, and (*fabD*)₄, respectively).

The genes encoding subunits of the efflux system SrpABC were assembled by subsequently ligating *srpB* to pACsA (between EcoRI and SacI sites), forming pACsAB, and *srpC* to the pACsAB plasmid (between NdeI and XhoI sites), creating pACsrp1. Alternatively, a second construct for the expression of this complex was generated by cloning the whole gene cluster including *srpA*, *srpB*, and *srpC*, using sANcoF and sCXhoR primers. The second plasmid, named pACsrp2, utilizes the native *P. putida* Shine-Dalgarno sequences of *srpB* and *srpC* for their expressions.

For a high expression level of n-butanol tolerant ATF (BT), a 5'UTR sequence was designed using *in silico* prediction on UTR Designer server (http://sbi.postech.ac.kr/utr_designer)

(Seo et al., 2013) and incorporated into the 5' termini of BTXhoF primer. While most of the gene expressions were verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the expression of *srp* genes were analyzed by reverse transcriptase – PCR (RT-PCR) following the protocol described in (Park et al., 2014).

2.3 Medium and culture conditions

Luria-Bertani (LB) medium (10 g tryptone, 5 g yeast extract, 10 g NaCl per 1 liter of water) was used for cell culture and tolerance tests. M9 minimal medium (200 ml 5x M9 minimal salts, 1 ml 1M MgSO₄, 50 µl 1M CaCl₂, 50 ml 40% glucose in 1 liter of water) was utilized in experiments identifying the optimal concentrations of supplementary chemicals for improving n-butanol tolerance. If needed, medium was supplemented with appropriate antibiotics (100 µg/ml ampicillin (Amp), 50 µg/ml kanamycin (Km), and 50 µg/ml chloramphenicol (Cm)). Cultures were incubated in a rotary shaker (200 rpm) with adjustable temperature control (at 30°C, unless otherwise specified).

2.4 Solvent tolerance test

Cells were incubated in 3 ml LB medium overnight at 30°C, supplemented with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and appropriate antibiotics. On the next day, cell density was measured by spectrophotometer at OD₆₀₀. Cells were harvested by centrifugation at 8,000 rpm for 5 min (RT), washed once and resuspended in fresh LB medium to OD₆₀₀ of 1, based on the recorded OD₆₀₀ value. Afterward, 300 µl of resuspended cells was added to 2,700 µL of LB medium containing 0.5 mM IPTG, appropriate antibiotics, and a toxic solvent stressor at a specific concentration, to be diluted to OD₆₀₀ of 0.1. By doing so, a uniform cell density of about 8×10^7 cells/ml among tested samples was used, with minimal differences in the starting media, which is normally caused by the carried-over metabolites from the pre-incubation cultures. The same

procedure was applied when the test was performed with other media. The tolerance was evaluated from the OD₆₀₀ value after challenging period of 24 h or otherwise noted.

2.5 Fatty acid analysis

Strains were grown in 100 ml LB medium, in the presence of 0.5 mM IPTG, antibiotics, and 0.5% n-butanol (vol/vol), at 30°C for 24 h and then harvested by centrifugation at 13,000 rpm for 15 minutes (RT). Fatty acid methyl esters (FAME) were prepared, separated and identified by Gas Chromatography – Mass Spectrometry (GC-MS) using the Sherlock Microbial Identification System (MIS) by MIDI, Inc. (Sasser, 1990).

3. Results and Discussion

3.1. Selection of an *E. coli* strain and temperature for n-butanol tolerance study

Four *E. coli* strains including MG1655, BL21(DE3), JM109, and a reduced-genome MS56 strain, were subjected to a growth test to evaluate their ability to tolerate to n-butanol at different temperatures. The strains were pre-incubated overnight in 3 ml LB at 18°C, 30°C, 37°C, and 42°C, respectively. The next day, cell cultures were diluted to OD₆₀₀ of 0.1 (as described in Materials and Methods) in 3 ml fresh LB medium, supplied with 0, 0.5, or 1% (vol/vol) n-butanol, respectively, and grown at the same temperatures as the pre-incubations for 24 h. Based on the result of OD₆₀₀ values measured after 24 h, the wild-type MG1655 showed the best n-butanol tolerance (Figure 1). The tolerance of MG1655 was slightly better than the minimized derivative MS56 at the commonly used temperatures (30°C and 37°C) and the difference was more significant at the lower and higher temperatures (18°C and 42°C). Even though BL21(DE3) is basically a wild-type strain, its tolerance was weaker than MS56 and very similar to the performance of JM109. Noticeably, all the tested strains exhibited a same pattern of temperature-dependent n-butanol tolerance that the cells showed the best survivability at 30°C and became weaker at both lower and higher temperatures. This behavior was observed in other reports on the temperature-dependent

1 tolerance toward n-butanol and other solvents of various microorganisms (Baer et al., 1987;
2 Georgieva et al., 2007; Li et al., 2010). It has been known that the optimal growth temperature for
3 *E. coli* is about 37°C, when the growth rate is maximized (White-Ziegler et al., 2007), thus it is not
4 likely that the improved n-butanol tolerance was due to an advantage of the normal cell growth.
5 Therefore, we decided to choose MG1655 as the main host to study the n-butanol toxicity and
6 performed all experiments at 30°C, except otherwise stated, to obtain the best n-butanol tolerance.

7 Apart from that, since it has been shown that the deletions of n-butanol competing
8 pathways is necessary to achieve high n-butanol titer (Atsumi et al., 2008; Bond-Watts et al., 2011;
9 Shen et al., 2011), we used a series of P1 transductions to remove the ethanol, lactate, succinate,
10 and acetate pathways and analyzed the n-butanol toxicity on the final knockout strain (MDB5).
11 Unfortunately, we found that MDB5 strain showed a weaker n-butanol tolerance, compared with
12 the wild-type MG1655, when BT ATF was expressed. To reveal the reason behind this, we
13 introduced BT ATF into all the intermediated knock out strains created by P1 transductions
14 (MDB1, MDB2, MDB3, MDB4) and compared their n-butanol tolerance with MG1655
15 (Supplementary Figure S1). As observed, all knock out strains tolerated to the same extent with the
16 wild-type MG1655 strain when BT ATF was not expressed in n-butanol tolerance test. However,
17 they failed to generate the high n-butanol tolerance phenotype observed in the wild-type cell when
18 BT ATF was expressed, and the most significant reduction was occurred in MDB1 with the
19 alcohol dehydrogenase *adhE* deleted. This alcohol dehydrogenase, even though was found to be
20 more than 2-fold up-regulated when BT ATF was activated (unpublished data), it was neglected
21 due to the level of up-regulation was not as high as the others which were reported in our previous
22 work (Lee et al., 2011). This data reflects a limitation of utilizing random approach to screen a
23 desired phenotype, which is the difficulty to figure out the differences in contribution to the
24 engineered phenotype from all the identified changes in the transcriptome. Nonetheless, because
25 the presence of the native alcohol dehydrogenases can degrade the alcohol products and increase

the alcohol tolerance, the deletion strain MDB5 with the knock-outs of *adhE* and *yqhD* was used as the main host for other tolerance test to obtain tolerance results closer to the n-butanol producing host.

3.2 Overexpression of several FAS genes improved n-butanol tolerance in *E. coli*

It was found that *E. coli* cell responded to the presence of short chain alcohols, including ethanol and n-butanol, by increasing the membrane unsaturated fatty acid (UFA) proportion, especially elevating the cis-vaccenic acid (C18:1) ratio in the expense of the saturated fatty acid (SFA) C16:0 (Ingram, 1976). However, one may concern if the change in the membrane fatty acid is an adaptive mechanism, because n-butanol is not a native metabolite produced in *E. coli*, or only a physically triggered phenomenon. As a hint for this question, a mutant of the *E. coli* fatty acid synthase II (FabF) was identified to overproduce cis-vaccenic acid, with a concomitant increase in n-butanol tolerance (Jeong and Han, 2010; Jeong et al., 2012), suggesting that this modification is adaptive, even though the mechanism is not yet revealed. Based on that finding, we decided to individually overexpress genes encoding enzymes involved in the Type II fatty acid synthesis (FAS II) to identify if other enzymes in this pathway can trigger the same phenotype. To this end, we induced the overexpression of FAS genes, including *fabA*, *fabB*, *fabD*, *fabF*, *fabH*, *fabG*, *fabI*, and *fabZ*, in MDB5 strain, and challenged the cells with different concentrations of n-butanol, from 0.5% to 1.5%. In addition, *fabD*, which encodes for malonyl-CoA:ACP transacylase, was cloned into pAUD vector as the tandem copies (Figure 2) for comparing n-butanol tolerance generated by different expression levels of this gene. FabD is the first enzyme to transfer the malonyl group from malonyl-CoA to the ACP in FAS cycle and is of the most important limiting factors in deciding the rate of the fatty acid synthesis (White et al., 2005), therefore, the expression level of FabD can significantly affect the rate of the fatty acid synthesis. As shown in the result, improvements of n-butanol tolerance at different levels were obtained with the *fabA*, *fabD* (all multiple-copies constructions), *fabB*, and *fabF* overexpressions. While *fabB*, *fabD*, and *fabF* were

1 reported to increase the cis-vaccenic acid **proportion** when being overexpressed (Demendoza et al.,
2 1983; Lee et al., 2013; Verwoert et al., 1992), *fabA* was arguably overproduced either SFA or UFA
3 depending on the experimental condition **(Cao et al., 2010; Luo et al., 2009)**. Nevertheless, by
4 analyzing fatty acid composition of these strains growing in medium containing n-butanol, it was
5 shown that the strains with increased cis-vaccenic acid, including FabA overexpression strain,
6 improved n-butanol tolerance (Figure 3). Interestingly, while n-butanol tolerance of the strains
7 overexpressing multiple copies of *fabD* increased accordingly with the number of *fabD* copy, from
8 1 to 3, the strain overexpressing **(*fabD*)₄** **reduced** the cis-vaccenic ratio and also lowered the n-
9 butanol tolerance. This could be explained that the translation of *fabD* gene cluster was probably
10 prone to produce aggregated proteins, reducing its overall catalytic activity. On the other hand, the
11 rest of the FAS genes showed no effect or even reduced n-butanol tolerance, according to the
12 lower level of cis-vaccenic acid they produced in the membrane. For further study, these FAS
13 strains were subjected to the challenges with other alcohols, including 3% ethanol, 1% isopropanol,
14 and 1% 2-butanol, under the similar experimental conditions (Supplementary Figure **S4**). Different
15 from n-butanol tolerance, only FabA, FabD construct-variants, and FabH showed increasing
16 ethanol tolerance; only FabD strains improved 2-butanol tolerance and most of the tested strains
17 enhanced isopropanol tolerance. This **dissimilarity** may be resulted from the difference in the
18 hydrophobicity of each specific molecular structure, even though these molecules have very close
19 carbon numbers.

20 All in all, the advantage of *fabD* overexpression, especially with high copy number, is the
21 ability to adapt to different alcohol stresses due to the position in FAS pathway that can control the
22 metabolic flux to form fatty acids in any regulatory direction affected by environmental conditions.
23 Also, the proportion of endogenous cis-vaccenic acid is critical to adjust the cell adaptation to
24 solvent stressors and that opens up more possibility to improve n-butanol tolerance of *E. coli* host.

3.3 n-butanol tolerance is affected by either endogenous or exogenous cis-vaccenic acid

After confirming the increased endogenous cis-vaccenic acid in several FAS strains improved n-butanol tolerance, we tested the cell response to n-butanol in the presence of cis-vaccenic acid. Interestingly, by either pre-growing the cells in the presence of cis-vaccenic acid or directly adding cis-vaccenic acid (30 mg/L) to the tested medium, the cells improved n-butanol tolerance (Supplementary Figure S5). Because free fatty acids can be incorporated directly into the membrane without going through the *de novo* endogenous synthesis, pre-treating the cells with free cis-vaccenic acid is a shorter way to adjust the membrane fatty acid composition. However, practically, pre-treatment of the cells with free fatty acid is more feasible with the industrial application of the procedure, since a large quantity of fatty acid will be needed to be added to a large fermentor, while incubating with small volume of strain inoculation is convenient and more economic. Moreover, the effect of cis-vaccenic acid retaining in the membrane will last rather long, as the advantage in term of cell survivability observed in the MDB5 strain pre-incubated with cis-vaccenic acid after 30 h of challenge with n-butanol (Supplementary Figure S6). It is noteworthy that the concentration of more than 30 mg/L with cis-vaccenic acid incubation did not improve further the n-butanol tolerance (Supplementary Figure S5). This result can be explained by the insolubility of cis-vaccenic acid in the water, so that it is easily to form floating pellets when the concentration is high enough. Therefore, only exogenous supply of cis-vaccenic acid maybe not efficient enough and hence the combination with the endogenous formation is needed.

For further improving n-butanol tolerance using the advantage of the direct fatty acid incorporation into the membrane and the homeoviscous adaptation of the cell to the temperature changes, we pre-incubated the cell, with cis-vaccenic acid, at the temperature as low as 4°C, and compared the tolerance with cells pre-incubated at higher temperatures. As expected, the advantage of pre-treatment with cis-vaccenic acid was observed at most of the conditions (Figure 4), even though the difference was more significant when the cells were incubated at the near-

1 optimal temperatures. When the cells were pre-incubated at the temperature higher than the
2 growing temperature in the test (37°C), the pre-treatment with cis-vaccenic acid seemed to have no
3 effect on the n-butanol tolerance (Figure 4). However, the direct adding of this fatty acid to the
4 testing medium improved the tolerance significantly and by doing so, the improvement in n-
5 butanol tolerance was more significant than the pre-treatment method. In addition, since the cells
6 were incubated at various temperatures before the tolerance test, they were exposed to the n-
7 butanol stressor at different stages of growth, and thus, the tolerance may be changed. Hence,
8 various pre-incubation times were used to examine cell tolerance. As shown in Figure 1B, even
9 though the cells at early stage were moderately less tolerant to n-butanol, the effect of pre-
10 incubation time is not notable.

11 3.4 The expression of the efflux pump SrpABC increased n-butanol tolerance in *E. coli*

12 Among the suggested approaches to improve the solvent tolerance in microorganisms, the
13 use of specific efflux pump system is promising because it reduces the cellular concentration of
14 solvent to relieve the stress on the metabolic network and cell membrane. On the other hand, it also
15 serves as an active exporter to secret metabolites to the medium. Therefore, efflux pump has been
16 acknowledged as a major mechanism for microbial resistance to toxic compounds (Fernandes et al.,
17 2003; Van Bambeke et al., 2000; Webber and Piddock, 2003). Following the successful
18 application of efflux pump in engineering microbial hosts to tolerate to various chemicals such as
19 nitrosyl indole (Zhang et al., 2011), n-octane and α -pinene (Foo and Leong, 2013), various efflux
20 pumps were tested for the ability to increase the n-butanol tolerance of *E. coli*. The result, however,
21 showed that none of the used efflux system could reduce n-butanol toxicity (Dunlop et al., 2011).
22 Therefore, up to now, there is no natural efflux system was successfully employed in *E. coli* to
23 reduce the internal concentration of n-butanol. In literature, there is only the efflux system named
24 SrpABC from *P. putida* S12 was reported to increase 6.6-fold the expression level in the presence
25 of n-butanol (Kieboom et al., 1998), which suggests the feasibility to use this system on other hosts

to pump out n-butanol. In this work, we cloned the SrpABC efflux system either by expressing each component (SrpA, SrpB, and SrpC) separately, or combining them together through sub-cloning (SrpABC1), or cloning and expressing the whole SrpABC operon (SrpABC2). Even though we successfully cloned these genes to the low-copy number vector pACYCDuet-1 and the medium-copy number vector pET16b, only the cells harboring the former construction grew after the IPTG induction, probably because of the toxicity caused by the overexpression of the transmembrane protein SrpB. Also, when the system was cloned to a pACYCDuet-1, we could not be able to detect their expressions by SDS-PAGE. RT-PCR was, hence, performed to verify the presence of *srpABC* transcripts. As a result, while *srpA* was expressed well by both the constructs, *srpB* mRNA was not detected when pAsABC1 was used. Only pAsABC2, with the natural *P. putida* ribosome binding site (RBS) upstream *srpB* and *srpC*, was able to express all the components of the efflux system together (Supplementary Figure S2B). On the other hand, we compared the n-butanol tolerance of these strains and found that the expression of SrpABC system using SrpABC2 construct improved about 20 to 35% the growth of the host in the presence of 0.5 - 1% (vol/vol) n-butanol, respectively (Figure 5). This increased tolerance is likely to attribute to SrpB activity, since the expression of only SrpB improved the tolerance to the similar level. Because SrpB is a transmembrane protein, its overexpression can help stabilizing the membrane and increase the resistance to solvent. Thus, to conclude whether this system actually recognizes n-butanol as a substrate, a synthetic pathway for n-butanol production expressed in *E. coli* and the monitor of endogenous versus exogenous n-butanol concentrations are needed. Nevertheless, SrpABC was chosen to combine with other genes for testing whether they have synergistic effects on n-butanol tolerance.

3.5 The Fe²⁺- dependent n-butanol tolerance in MDB5 strain

Similar to cis-vaccenic acid, the addition of ferrous iron to the medium also improved n-butanol tolerance. As determined from the n-butanol tolerance of MDB5 strain growing in M9

medium, the concentration of 75 mg/L of FeSO₄ in the medium was optimal for enhancing n-butanol tolerance of MDB5 strain (Supplementary Figure S5). It is interesting to find out that when low concentrations of cis-vaccenic acid were used for cell pre-treatment, ferrous iron enhanced the effect of cis-vaccenic acid. However, at high cis-vaccenic acid concentrations, Fe²⁺ negated that effect. Besides, when cis-vaccenic acid and ferrous ion were added together, the cell tolerance was even worse (Figure 4). Possible explanation for that is Fe²⁺ can interact and form toxic complexes with fatty acids when the fatty acids are abundant in the medium. By analyzing the cell growth in the presence of Fe²⁺ and n-butanol, it was shown that the improved n-butanol tolerance mediated by Fe²⁺ did not last long. The medium supplied with Fe²⁺ showed a significantly better growth in the presence of n-butanol within 24 h at 30°C and only within 15 h at 37°C, which is shorter compared to the effect caused by the pre-treatment with cis-vaccenic (Supplementary Figure S6).

Finally, BT ATF-containing MDB5 strain was tested for the n-butanol tolerance with the co-expression of *feoA* and the presence of Fe²⁺ in the medium. As observed, the n-butanol tolerance of BT was not improved when Fe²⁺ was added to the medium but when *feoA* was co-expressed with BT, n-butanol tolerance of BT expressing strain increased about 21% in the presence of 1% (vol/vol) n-butanol (Figure 6A). Even though the increase was not as much as the change in *FeoA* expressing strain when supplied with Fe²⁺ (about 40% increase at 1% n-butanol), this observation suggests that the inability of BT-containing strain to improve n-butanol tolerance in the presence of Fe²⁺ was due to the low activity of the iron uptaking system.

3.6 The synergistic effects of controlling membrane fatty acids and membrane related functions on improving n-butanol tolerance of *E. coli* strains co-expressing ATF with *FeoA* and *FabD*

When pre-treated with 30 mg/L cis-vaccenic acid, at 18°C for 24 h before undergoing the n-butanol challenge, M5D3BE strain (MDB5; (*fabD*)₃⁺ BT⁺ *feoA*⁺, as described in Table 2) improved about 6% n-butanol tolerance in the medium containing 1% (vol/vol) n-butanol,

1 compared with the previous experiment when the pre-treatment were neglected. Moreover,
2 compared with control strain growing under normal condition, M5D3BE strain with pre-treatment
3 and the addition of Fe^{2+} in the medium improved about 4, 5, and 9-fold cell growth in the presence
4 of 1, 1.5, and 2% (vol/vol) n-butanol, respectively. Unfortunately, when the SrpABC was co-
5 expressed with other genes such as (*fadD*)₃, severe cell toxicity was observed and negated the
6 improvement in butanol tolerance caused by FabD overexpression (Figure 6A). However, since
7 this efflux system was shown to improve n-butanol tolerance, a well-controlled expression system
8 should be designed for applying this system to n-butanol producing hosts.

9 Considering all the factors involving in enhancing the resistance of *E. coli* strain MDB5
10 against n-butanol, the constructed strain with the co-expression of BT ATF, FeoA, and 3 copies of
11 FabD, after the pre-treatment with cis-vaccenic acid and 18°C incubation, improved about 4-fold
12 the cell growth in the presence of 1% (vol/vol) n-butanol and extended the limit of n-butanol
13 tolerance to 2%, instead of 1.5% (vol/vol) when only BT ATF was expressed (Figure 6A).
14 Furthermore, the same approach was applied on the wild-type MG1655(DE3) strain, along with
15 MS56(DE3), BL21(DE3), and JM109(DE3) strains, to evaluate its effects on various *E. coli* strains.
16 As shown in Figure 6B, the improved n-butanol tolerance was observed in all of the tested strains,
17 to different extents. While the highest tolerance was observed when the wild-type MG1655(DE3)
18 host was used (MGD3BE), the improvement in n-butanol tolerance of BL21(DE3) and
19 JM109(DE3) strains (BLD3BE and JMD3BE, respectively) was just modestly better than the
20 knock-out strain MDB5 (M5D3BE). The main reason for this difference is most likely the
21 variation of tolerance phenotype generated by ATF in different strains of the same species and the
22 BT ATF is most effective in MG1655 and its close derivatives since it was screened through the
23 expression in this host (Lee et al., 2011). For applying ATF-based phenotype engineering on other
24 hosts, the same procedure should be performed to obtain specific ATFs with maximized activity in
25 these hosts.

1 Additionally, the growth profiles of MG1655(DE3) and its knock-out derivative MDB5
2 were measured in media containing 1% and 2% (vol/vol) n-butanol to analyze the changes in cell
3 proliferation when our combinatorial approach was applied. As a result, both strains showed
4 similar patterns with retarded growth and longer log phase in the high n-butanol concentration.
5 Specifically, while the maximum growth was achieved after around 15 h in the presence of 1%
6 (vol/vol) n-butanol, it was shifted to about 20 h in the presence of 2% (vol/vol) n-butanol (Figure 7).
7 Also, even though the survivability of MG1655(DE3) strain was higher than that of MDB5 strain,
8 their tolerances were limited to 2% (vol/vol) n-butanol since no significant growth was observed in
9 2.2% (vol/vol) n-butanol containing medium (Figure 6B). This level of tolerance may exceed the
10 current maximum titer of n-butanol that can be produced in fed-batch fermentation and
11 theoretically, this combinatorial approach can be applied for the production of n-butanol in *E. coli*
12 under prolonged fermentation process. However, further works to introduce the n-butanol pathway
13 to the n-butanol tolerance host is needed to determine the actual improvement that a host can
14 achieve in term of tolerance and the ability to increase the n-butanol tolerance with that advantage,
15 since many factors related to the fermentation process were neglected in a common solvent
16 tolerance study and the improvement in the tolerance to exogenous solvent is not always lead to
17 the increased production of that solvent (Atsumi et al., 2010).

18 4. Conclusion

19 This work represents an approach to combine membrane related functions in *E. coli*,
20 including controlling membrane fatty acid composition, activation of the iron uptaking system, and
21 a solvent efflux system, for improving n-butanol tolerance. It demonstrates that a high ratio of
22 membrane cis-vaccenic acid (C18:1) contributes a large part to the n-butanol tolerance, and can be
23 achieved by overexpressing fatty acid synthesis genes such as *fabD*, which encodes for the
24 malonyl-CoA:ACP transacylase, and other genes related to the unsaturated fatty acid synthesis,
25 including *fabA*, *fabB*, and *fabF*. As described and demonstrated, a simple pre-treatment such as the

adaptation to low temperature or the use of exogenous cis-vaccenic acid to increase the membrane C18:1 content is an efficient step to expand the limit of n-butanol tolerance, without the metabolic burden caused by introducing complex gene expressing systems into the host. Furthermore, the supplementation of ferrous ion to the medium can enhance the butanol tolerance of the host, which can be assisted by the co-expression of *E. coli feoA* gene for the activation of iron uptaking system. This is the first time the SrpABC efflux system from *P. putida* was applied for exporting intracellular metabolites in *E. coli* and was demonstrated to improved n-butanol tolerance. However, its application was limited due to the difficulty in expression and co-expression with other genes. In conclusion, our combinatorial strategy provides an effective method to optimize the n-butanol tolerance of an *E. coli* host and assists the development of the n-butanol production route via non-native producers.

Competing interests

The authors declare no competing financial interests.

Author contribution

L.M.B performed the major part of the experiments, created ideas, and prepared the manuscript

J.Y. L. constructed the BT ATF and provided guidelines for initial experiments

Z.R and A.G. did cloning and analysis works, and did proof reading for the manuscript

J.H.L. provided suggestions, guidelines, and planning the project

S.C.K. directed the project, provided ideas and suggestions to overcome problems

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Table 1. Genes, plasmids, and primers used to construct overexpression strains. The enzyme names indicated below protein names are the restriction enzymes used to digest the PCR amplified product of each gene. Sequences recognized and digested by REs are indicated by underlining.

With pADn, n means the number of *fabD* repeat.

Table 2. *E. coli* strains used in this study.

Figure 1. n-butanol tolerance of *E. coli* strains at different temperatures and solvent

concentrations. (A) Selected *E. coli* strains were challenged with 0, 0.5, and 1% n-butanol (vol/vol), respectively, for 24 h at 18°C, 30°C, 37°C, and 42°C, respectively. The temperatures used in the challenge were used to pre-incubate the cells for 12 h prior to the test. The initial OD₆₀₀ of the tested cultures were standardized to 0.1. (B) Effect of pre-incubation time on n-butanol tolerance test. MDB5 strain was pre-incubated for various time periods from 6 h to 24 h before the tolerance test in 1% n-butanol, at 18°C, 30°C, 37°C, and 42°C, respectively. The starting OD₆₀₀ of the tested cultures were uniformly diluted to 0.1. The temperatures used in the pre-incubation step were in resemblance with those in tolerance test. Error bars represent the standard deviation (SD) from the mean of three independent experiments.

Figure 2. Analysis of n-butanol tolerance of MDB5 strains overexpressing FAS genes at 30°C.

Strains were pre-incubated and induced with 0.5 mM IPTG at 30°C for 12 h prior to the test. Cell densities were standardized to OD₆₀₀ of 0.1 in 3 ml LB media containing 0.5 mM IPTG, Cm, and n-butanol (0.5, 1, and 1.5%, respectively). The cell survivability was measured by OD₆₀₀ after 24 h of n-butanol challenge at 30°C. Error bars indicate the SD from the mean of three independent experiments.

Figure 3. Fatty acid profiles of MDB5 strains overexpressing FAS genes in the presence of n-

butanol. Cell were grown for 24 h at 30°C in 100 ml LB medium, supplied with 0.5 mM IPTG, Cm, and 0.5% (vol/vol) n-butanol. Fatty acid methyl esters (FAME) were prepared, separated and

identified by GC-MS using the Sherlock Microbial Identification System (MIS) by MIDI, Inc. (Sasser, 1990). The fatty acid types showed in the figure are the major groups in *E. coli*.

Figure 4. Analysis of the synergistic effect on n-butanol tolerance of exogenous cis-vaccenic acid, pre-adaptation to low temperature, and ferrous iron. MDB5 strain was pre-incubated in LB medium at different temperatures, with or without the supplementation of 30 mg/L cis-vaccenic acid (C18:1) for 24 h prior to the test. P4, P18, P30, and P37 indicates the pre-incubation at 4°C, 18°C, 30°C, and 37°C, respectively. For 4°C incubation, the cells were grown at 18°C for 12 h, then switched to 4°C for another 12 h. Cis-vaccenic acid, if needed, was added to the culture when incubated at 18°C and described on the x-axis as +C18:1. For other temperatures, constant temperatures were kept through 24 h of incubation. Pre-incubated cells were diluted to OD₆₀₀ of 0.1 and challenged with 1% n-butanol for 24 h in LB medium at 30°C. The addition of 30 mg/L cis-vaccenic acid and/or 75 mg/L Fe²⁺ to the testing cultures is indicated in the legend. Error bars represent the SD from the mean of three independent experiments.

Figure 5. The effect of SrpABC efflux pump expression on the tolerance to exogenous n-butanol and the productions of metabolite in *E. coli*. (A) Three components of the efflux pump SrpABC were expressed individually or together using pACYCDuet-1 vector (low copy number). SrpABC1 (pAsABC1) and SrpABC2 (pAsABC2) are different in the Shine-Dalgarno sequences (SD) of *srpB* and *srpC* (*E. coli* SDs are utilized in the former and native *P. putida* SDs are used in the latter construct). Constructed strains were pre-grown at 30°C overnight in LB medium with 0.5 mM IPTG and Cm. The next day, they were subjected to the n-butanol tolerance test in LB medium with 0.5 mM IPTG, from OD₆₀₀ of 0.1, and challenged with 0.5, 1, and 1.5% (vol/vol) n-butanol, respectively. Different from other experiments, because *srp* genes were expressed on pACYCDuet-1, control cell harboring the empty plasmid pACYCDuet-1 was used, instead of pAUD. Error bars indicate the SD from the mean of three simultaneous experiments.

Figure 6. The synergistic effect on n-butanol tolerance by combining various factors into one

strain. (A) MDB5 strains containing different combinations of n-butanol tolerant genes were grown at 30°C and induced with 0.5 mM IPTG for 12 h before the test. For pre-treatment step, cell cultures were diluted 1/100 into 3 ml LB and grown at 18°C with 30 mg/L cis-vaccenic acid, 0.5 mM IPTG, Cm, and Km for 24 h. For n-butanol challenge, pre-treated cell cultures were diluted to OD₆₀₀ of 0.1 in 100 ml LB medium containing 0.5 mM IPTG, Cm, Km, and 1, 1.5, 2% (vol/vol) n-butanol, respectively. Fe²⁺ (75 mg/L) was added to the cultures as indicated on the figure. **(B)** MG1655(DE3), MDB5, MS56(DE3), BL21(DE3), and JM109(DE3) strains harboring the plasmids co-overexpressing (*fabD*)₃, BT ATF, *feoA* (Table 2) were pre-treated as described in (A) and challenged with 1, 1.5, 2, and 2.2% (vol/vol) n-butanol, respectively, in the presence of Fe²⁺ (75 mg/L) at 30°C for 24 h. Cell survivability was measured by OD₆₀₀ after 24 h of incubation at 30°C. Error bars indicate the SD from the mean of three independent experiments.

Figure 7. Growth profiles of MG1655(DE3) and MDB5 strains in the presence of n-butanol.

MGD3BE and M5D3BE strains were pre-treated with 30 mg/L cis-vaccenic acid, 0.5 mM IPTG and incubated at 18°C for 12 h prior to the n-butanol challenge. Cell cultures were uniformly diluted to OD₆₀₀ of 0.1 in 100 ml LB medium, supplied with 0.5 mM IPTG, 75 mg/L Fe²⁺, Cm, Km, and 1% (A) or 2% (vol/vol) (B) n-butanol, respectively. Growth profile was measured during 30 h of n-butanol challenge by spectrophotometer. MG1655(DE3) and MDB5 strains containing pACYCDuet-1 and pAUK were used as the controls. Both the pre-treatment step and the addition of Fe²⁺ to the testing culture were neglected in the experiments with control strains. Error bars indicate the SD from the mean of three simultaneous experiments.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Protein (RE sites)	Plasmid	Primers
FabA (NcoI/BamHI)	pAA (P _{T7} :: <i>fabA</i> ; ColE1 ori, Cm ^R)	fANcoF: CCTTAGCCATGGTAGATAAACGCGAATCCTATAC fABamR: CGGGATCCTCAGAAGGCAGACGTATCCTG
FabB (NcoI/BamHI)	pAB (P _{T7} :: <i>fabB</i> ; ColE1 ori, Cm ^R)	fBNcoF: CCTTAGCCATGGAAAAACGTGCAGTGATTACTGGC fBBamR: CGGGATCCTTAATCTTTCAGCTTGCGCAT
FabD (AseI/BglII)	pADn (P _{T7} ::(<i>fabD</i>) _n ColE1 ori, Cm ^R) pAKD3 (P _{T7} ::(<i>fabD</i>) ₃ ColE1 ori, Km ^R)	fabDAseF1: CCTTAGATTAATATGACGCAATTTGCATTTGT fabDAseF2: CCTTAGATTAATATCTTAGTATATTAGTTAAGTATAAGAAGG fabDBglR: GAAGATCTAGGCATATGTTATTAAGCTCGAGCGCCGCTG
FabF (NcoI/XhoI)	pAF (P _{T7} :: <i>fabF</i> ; ColE1 ori, Cm ^R)	fFNcoF: CCTTAGCCATGGAATCTAAGCGTCGTGTAGTTGTGAC fFXhoR: CCGCTCGAGTTAGATCTTTTTAAAGATCAAAGAACC
FabG (BspHI/XhoI)	pAG (P _{T7} :: <i>fabG</i> ; ColE1 ori, Cm ^R)	fGBspF: CCTTAGTCATGAATTTGAAGGAAAAATCGC fGXhoR: CCGCTCGAGTCAGACCATGTACATCCCCG
FabH (BspHI/XhoI)	pAH (P _{T7} :: <i>fabH</i> ; ColE1 ori, Cm ^R)	fHBspF: CCTTAGTCATGATGTATACGAAGATTATTGGTACTGGCAG fHXhoR: CCGCTCGAGCTAGAAACGAACCAGCGCG
FabI (NcoI/BamHI)	pAI (P _{T7} :: <i>fabI</i> ; ColE1 ori, Cm ^R)	fINcoF: CCTTAGCCATGGGTTTTCTTTCCGGTAAG fIBamR: CGGGATCCTTATTTTCAGTTCGAGTTCGTTTCATT
FabZ (NcoI/XhoI)	pAZ (P _{T7} :: <i>fabZ</i> ; ColE1 ori, Cm ^R)	fZNcoF: CCTTAGCCATGGAAACTACTAACAATCATACTCTGCAGATTG fZXhoR: CCGCTCGAGTCAGGCCTCCCGGCTA
SrpA (NcoI/EcoRI)	pACsA (P _{tac} :: <i>srpA</i> ; p15A ori, Cm ^R)	sANcoF: CCTTAGCCATGGCCAGACAGATACGATCCCCG sAEcoR: GGAATTCCTCAGTTAGCCGAGGCTTCTGT
SrpB (EcoRI/SacI)	pACsB (P _{tac} :: <i>srpB</i> ; p15A ori, Cm ^R)	sBEcoF: GGAATTCAGGAGATATACATATGTCTCGTTTCTTTATCGACAGG sBSacR: CGAGCTCTTAACTTCATGAGTCACCTCCTTG
SrpC (NdeI/XhoI)	pACsC (P _{tac} :: <i>srpC</i> ; p15A ori, Cm ^R)	sCNdeF: GGAATTCATATGAAGTTTAAGTCACTCCCCATG sCXhoR: CCGCTCGAGTTAGTTTTGACTCACGCTCCAG
SrpA + SrpB + SrpC (NcoI/XhoI)	pAsABC1 (P _{tac} :: <i>srpA-srpB-srpC</i> ; p15A ori, Cm ^R) pAsABC2 (P _{tac} :: <i>srpABC</i> , p15A ori, Cm ^R)	
FeoA (NcoI/BamHI)	pAFe (P _{T7} :: <i>feoA</i> ; ColE1 ori, Cm ^R)	feNcoF: CATTGCCATGGGCCAATACACTCCAGATACTGCGTG feBamR: CGGGATCCTTAACAGGAAACCGCTTCCA
BT-ATF (XhoI/PacI)	pABT (P _{T7} ::BT; ColE1 ori, Cm ^R)	BTXhoF: CCGCTCGAGCAATCTATAAAAAAGGAGCATCAAAAATGCCGACTCT CGAATGGTTCCTG BTPacR: AATATACCTTAATTAATTAACCGGTGTGAATCCGCTGGT
FeoA + BT-ATF	pAFeBT (P _{T7} :: <i>feoA</i> – BT, ColE1 ori, Cm ^R)	

Table 1

Strain	Relevant genotype	Reference
XL-1 Blue	<i>recA1 endA1 gyrA96 thi-1 supE44 relA1 lac</i> [F ⁺ <i>proAB lacF^r ZΔM15 Tn10</i> (Tet ^R)] <i>hsdR17(r^K m^{K+})</i>	Stratagene
MG1655	F- <i>λ- ilvG- rfb-50 rph-1</i>	(Blattner et al., 1997)
BL21(DE3)	F- <i>ompT gal dcm lon hsdSB(rB- mB-) λ</i> (DE3)	Novagen
JM109	<i>endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB⁺ Δ(lac-proAB)</i> e14- [F ⁺ <i>traD36 proAB⁺ lacF^r</i> <i>lacZΔM15</i>] <i>hsdR17(r^K m^{K+})</i>	Promega
MS56	MG1655 with 56 genome deletions	(Park et al., 2014)
MG1655(DE3)	MG1655 with <i>λ</i> (DE3)	This study
MS56(DE3)	MS56 with <i>λ</i> (DE3)	This study
JM109(DE3)	JM109 with <i>λ</i> (DE3)	This study
MDB1	MG1655(DE3) <i>ΔadhE</i>	This study
MDB2	MG1655(DE3) <i>ΔadhE ΔyqhD</i>	This study
MDB3	MG1655(DE3) <i>ΔadhE ΔyqhD ΔldhA</i>	This study
MDB4	MG1655(DE3) <i>ΔadhE ΔyqhD ΔldhA Δpta</i>	This study
MDB5	MG1655(DE3) <i>ΔadhE ΔyqhD ΔldhA Δpta ΔfrdA</i>	This study
M5D3	MDB5/pAKD3/pAUD	This study
M5D3S2	MDB5/pAKD3/pAsABC2	This study
M5D3BT	MDB5/pAKD3/pABT	This study
M5D3FE	MDB5/pAKD3/pAF _e	This study
M5D3BE	MDB5/pAKD3/pAF _e BT	This study
MGD3BE	MG1655(DE3)/pAKD3/pAF _e BT	This study
JMD3BE	JM109(DE3)/pAKD3/pAF _e BT	This study
MSD3BE	MS56(DE3)/pAKD3/pAF _e BT	This study
BLD3BE	BL21(DE3)/pAKD3/pAF _e BT	This study

Table 2

Supplementary File

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