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Enhanced Organic Solvent Tolerance of *Escherichia coli* by 3-Hydroxyacid Dehydrogenase Family Genes

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Abstract A 3-hydroxyisobutyrate dehydrogenase-encoding gene *mmsB* has been identified as one of the key genes responsible for the enhanced organic solvent tolerance (OST) of *Pseudomonas putida* JUCT1. In this study, the OST-related effect of two 3-hydroxyacid dehydrogenase family genes (*mmsB* and *zwf*) was investigated in *Escherichia coli* JM109. It was noted that the growth of *E. coli* JM109 was severely hampered in 4 % decalin after *zwf* knockout. Additionally, its complementation resulted in significantly enhanced solvent tolerance compared with its parent strain. Furthermore, *E. coli* JM109 carrying *mmsB* showed better OST capacity than that harboring *zwf*. To construct *E. coli* strains with an inheritable OST phenotype, *mmsB* was integrated into the genome of *E. coli* JM109 by red-mediated recombination. Using *E. coli* JM109(DE3) (Δ*endA*::*mmsB*) as host strain, whole-cell biocatalysis was successfully carried out in an aqueous/butyl acetate biphasic system with a remarkably improved product yield.

Keywords Organic solvent tolerance · *mmsB* · *zwf* · 3-Hydroxyacid dehydrogenase family · *Escherichia coli* · Whole-cell biocatalysis

Introduction

Organic solvents are toxic to microbial cells even at concentrations as low as 0.1 % [1]. The toxicity of organic solvents is correlated to their log *P* value; the lower the value is, the higher the toxicity to microbes [2, 3]. Log *P* is defined as the common logarithm of the partition coefficient of a particular solvent between *n*-octanol and water phases [4, 5]. As the most commonly used industrial host strain, *Escherichia coli* barely tolerates organic solvents with log *P* values greater than 3.4–3.8 [6]. Therefore, the poor organic solvent tolerance (OST) property of *E. coli* has become a major limitation to its potential application in nonaqueous whole-cell biocatalysis, alcohol production, etc.

Whole-cell biocatalysis has a number of advantages as compared with that of isolated enzymes. It could not only achieve enzymatic cascade reactions with enhanced product yield, but also reduce the cost of enzyme preparation and purification [7–10]. In many biocatalytic

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reactions, it is often necessary to introduce organic solvents as nonaqueous media to facilitate the solubility of hydrophobic substrates and products. However, poor biocompatibilities of organic solvents always hinder their application in microbial-catalyzed reactions. Therefore, the availability of general host strains (such as *E. coli*) with excellent OST properties could be of significant importance for whole-cell biocatalysis in nonaqueous media.

Several mechanisms of microbial OST have been elucidated in the past two decades. Sardessai and Bhosle investigated the OST mechanisms of Gram-negative bacteria including *Pseudomonas* and certain *E. coli* mutants [1]. When challenged by organic solvents, cell membrane rigidity increases along with a decrease in permeability. Also, various efflux pumps (*tolC*/*mar*/*rob*/*soxS*/*acrAB*) have been identified to be responsible for the active expelling of solvents. Additionally, some microbes possess specific enzymes capable of degrading certain organic solvents. Furthermore, stress response is another important OST-related mechanism, in which a number of energy-producing and regulator proteins are involved.

In our previous study, an OST strain (*Pseudomonas putida* JUCT1), capable of growing in 60 % (*v*/*v*) cyclohexane, was isolated following several rounds of solvent adaptation. Proteomics and MALDI-TOF/TOF analysis have revealed that several high-abundance protein spots such as 3-hydroxyisobutyrate dehydrogenase (*mmsB*) showed an over 60 % discrepancy under different solvent conditions. The prominent OST-related effect of *mmsB* has been further confirmed by its recombinant expression in a non-OST *E. coli* strain [11].

Glucose-6-phosphate dehydrogenase-encoding gene *zwf* from *E. coli* shares a similar evolutionary origin and enzymatic mechanism with 3-hydroxyisobutyrate dehydrogenase. Both enzymes belong to the 3-hydroxyacid dehydrogenase family [12]. In this study, the effect of *zwf* on the OST properties was evaluated using gene knockout mediated by red recombination and its complementation in *E. coli*. Compared with *zwf*, *mmsB* transformants showed higher solvent tolerance. Furthermore, *mmsB* was integrated into the genomic DNA of an *E. coli* strain to generate JM109(DE3) (Δ*endA*::*mmsB*) with an improved OST phenotype, which was successfully utilized in a nonaqueous whole-cell biocatalysis for the synthesis of chiral alcohols.

Material and Methods

Strains and Media

Strains and plasmids used in this study are shown in Table 1. Primers used in this study are shown in Table 2. *P. putida* JUCT1 was cultivated at 30 °C for 12 h in a nutrient broth medium

Table 1 Strains and plasmids used in this study

Strains and plasmids	Description	Source
Pseudomonas putida JUCT1	Adapted OST strain	Ni et al. [11]
Escherichia coli JM109(Δ*zwf*)	Knockout strain	In this study
E. coli JM109 (Δ*zwf*)/pQE-*zwf*	Knockout strain	In this study
E. coli JM109(DE3) (Δ*endA*::*mmsB*)	Insertion strain	In this study
BW25141, BW25113, and BT340	Gene disruption set	Datsenko and Wanner [15]
pKD13, pKD46, and pCP20	Gene disruption set	Datsenko and Wanner [15]
pQE80L	Expression vector	QIAGEN
pET20b	Expression vector	Novagen

Table 2 Primes used in this study

Purpose	Prime	Sequence (5' → 3')
Amplification of FRT-*Kan*-FRT	F-*Bgl*II	CAACAGATCTATTCCGGGGATCCGTCGACC (*Bgl*II)
	RM-*Bgl*II	CTGTCTCTTGATCAGTTCTTGATCCCCTGC
	FM-*Bgl*II	GCAGGGGGATCAAGAACTGATCAAGAGACAG
	R-*Bgl*II	CCAACAGATCTGTAGGCTGGAGCTGCTTCG (*Bgl*II)
Amplification of *mmsB* for pET20b-*mmsB* construction	F-*mmsB*	GGAATTCCATATGCGTATTGCATTCATCATTGG (*Nde*I)
	R-*mmsB*	CCCCAAGCTTTCAATCCTTCTTGCGATACC (*Hin*dIII)
Amplification of *mmsB* and *zwf* for pQE-*mmsB* and pQE-*zwf* constructions	*mmsB*-F	ATCGGGATCCATGCGTATTGCATTCATTGG (*Bam*HI)
	mmsB-R	CCCCAAGCTTTCAATCCTTCTTGCGATACC (*Hin*dIII)
	zwf-F	TATCGGATCCATGGCGGTAACGCAAACAG (*Bam*HI)
	zwf-R	CGCCCAAGCTTTTACTCAAACTCATTCCAGG (*Hin*dIII)
Knockout of *zwf*	Δ*zwf*-F	CGATGATTTTTTTATCAGTTTTGCCGCACTTTGCGCGC
		TTTTCCCGTAATCGCACGGTGTAGGCTGGAGCTGCTTC
	Δ*zwf*-R	TCGTGGGTGAATCCGCACGAGGCCTGAAAGTGTAAAA
		ATTGTTCTACAATCTGCGCATTCCGGGGATCCGTCGACC
Check for knockout	C-*zwf*-F	TGTCAGATATTACGCCTGTGTGC
	C-*zwf*-R	GTATGACTGAAACGCCTGTAACC
	K2	CGGTGCCCTGAATGAACTGC
	Kt	CGGCCACAGTCGATGAATCC
Insertion of *mmsB* at *endA* site	Δ*endA*-F	CCAAAACAGCTTTCGCTACGTTGCTGGCTCGTTTTAAC ACGGAGTAAGTGAGATCTATTCCGGGGATCCG
	Δ*endA*-R	GGTTGTACGCGTGGGGTAGGGGTTAACAAAAAGAAT CCCGCTAGTGTAGGCAAAAAACCCCTCAAGACCC
Check for insertion	C*endA*-F	CTTTCCTGATCTGGCTGATTGCATA
	C*endA*-R	AAAAATCCGCGTCGTCTCCCCACGC
	Mid-R	GTCGCCCATGTCCACACCCTTGGC

The underlined nucleotide sequences indicate the restriction sites

(*w*/*v*) consisting of 1 % peptone, 0.3 % beef extract, and 0.5 % NaCl, pH 7.0. *E. coli* JM109 and JM109(DE3) were grown at 37 °C in Luria-Bertani (LB) medium. For recombinant *E. coli* strains, antibiotics were supplemented as necessary.

Construction of pQE-*mmsB* and pQE-*zwf*

DNA sequences of *mmsB* and *zwf* were amplified by PCR using the genomic DNA of *P. putida* JUCT1 and *E. coli* JM109 as templates, respectively. The PCR fragments were double-digested with *Bam*HI and *Hin*dIII, then ligated into the corresponding sites of pQE to generate pQE-*mmsB* and pQE-*zwf*.

Chromosomal Integration of FRT-T7-*mmsB*-Tet by Red Recombination

DNA fragments of *mmsB* and flippase recognition target-kanamycin resistance (FRT-Km^R) cassette were inserted into pET20b. The resultant FRT-Km^R-T7-*mmsB*-Tet fragment was integrated into the chromosomal DNA of *E. coli* following a method described by Koma et al. [13].

Kanamycin resistance (Km^R) from pKD13 was used as a selective marker in this study. The FRT-Km^R-T7-*mmsB*-Tet cassette (2,490 bp) was constructed using pET20.

It comprises *mmsB* gene (888 bp), FRT-Km^R (1,350 bp), T7 promoter (152 bp), T7 terminator, and 100-bp flanking sequence homologous to the adjacent regions of *endA*. The total 2,490-bp fragment was inserted into the target loci *endA* [14] of JM109(DE3) genomic DNA by red recombination [15]. Successful integration of FRT-Km^R-T7-*mmsB*-Tet was verified by PCR using primer pairs of C*endA*-F and C*endA*-R, and C*endA*-F and Mid-R, which yielded PCR fragments of 2,590 and 1,800 bp, respectively. The Km^R fragment was further excised by flippase (FLP)/FRT recombination using plasmid pCP20. The residual segment was about 600 bp as confirmed by PCR with primer pairs of C*endA*-F and Mid-R.

The method for red recombination for *zwf* was the same as described above except that a Km cassette fragment was used.

OST Assay

The OST effect of *zwf* was studied using its knockout and complementation *E. coli* strains. JM109(Δ*zwf*)/pQE-*zwf*, JM109(Δ*zwf*), and JM109 were cultured in LB/Kan medium at 37 °C. When OD_{660} of 0.2 was reached, 4 % (*v*/*v*) decalin was added for all strains. Meanwhile, 0.6 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was also added to the complementation strain (JM109(Δ*zwf*)/pQE-*zwf*) to initiate induction. Cell growth was monitored each hour.

For OST studies on *mmsB*, JM109(DE3) and JM109(DE3)/pET20-*mmsB* were regarded as negative and positive controls, respectively. The OST of JM109(DE3) (Δ*endA*::*mmsB*) was compared with two control strains in different organic solvents. Decalin (log*P* = 4.8), cyclohexane (log*P* = 3.2), butyl acetate (log*P* = 1.7), and butanol (log*P* = 0.9) were chosen as solvent stresses based on their distinct log *P* values. The growth of the above three strains was monitored every hour in the presence of cyclohexane (4 %, *v*/*v*), decalin (4 %, *v*/*v*), butyl acetate (0.4 %, *v*/*v*), and butanol (0.1 %, *v*/*v*).

As a matter of fact, the growth of knockout strains was slightly slower than their parents in this study. To better illustrate the growth kinetic of different strains, organic solvents were added when cell density of 0.2 OD_{660} was reached for all strains investigated. In our study, the cell density of various strains reached 0.2 OD_{660} within 2 h, and the solvent addition at 0.2 OD_{660} was defined as the starting time point. Data represent mean ± standard deviation of three independent biological experiments.

Whole-Cell Biotransformation

Recombinant plasmid pET20-*kmCR* harboring a carbonyl reductase from *Kluyveromyces marxianus* had been previously constructed in our laboratory [16], and it was transformed into JM109(DE3) (Δ*endA*::*mmsB*) and JM109(DE3). The resultant recombinant *E. coli* strains were utilized in the asymmetric synthesis of ethyl (*R*)-2-hydroxy-4-phenylbutyrate [(*R*)-HPBE] in an aqueous/butyl acetate (1:1) biphasic system. The reaction mixture contained 1 g of wet cells, 5 g/L ethyl 2-oxo-4-phenylbutyrate (OPBE), 5.0 mmol/L NADPH, 5 mL of 100 mmol/L phosphate buffer (pH 6.0), and 5 mL butyl acetate. The reaction was incubated at 30 °C and 220 rpm in a 50-mL flask for 12 h.

One unit of activity was defined as the amount of enzyme required for catalyzing the oxidation of 1 μmol of NADPH per minute. The protein concentration was measured by the Bradford method using bovine serum albumin as the standard [17].

Results and Discussion

Effect of *zwf* Knockout on OST of *E. coli*

In our previous study, *mmsB* was identified as one of the 22 key genes responsible for the enhanced OST phenotype of *P. putida* JUCT1 using 2D-PAGE combining MALDI-TOF/TOF analysis [11]. Glucose-6-phosphate dehydrogenase and 3-hydroxyisobutyrate dehydrogenase are encoded by *zwf* and *mmsB*, respectively. Both enzymes belong to the 3-hydroxyacid dehydrogenase family and share a similar function and catalytic mechanism. Our results also indicate that *mmsB* could improve the solvent tolerance of *E. coli*. To evaluate the OST-related function of *zwf*, a knockout strain JM109(Δ*zwf*) was prepared.

The OST experiments on the *zwf* knockout and complementation strains were conducted in LB medium by addition of 4 % (*v*/*v*) decalin when OD_{660} reached 0.2 (Fig. 1). For JM109(Δ*zwf*), cell density hardly increased after 6 h of incubation. A maximum OD_{660} of 0.23 was reached, indicating that the solvent tolerance capacity of *E. coli* was severely damaged by *zwf* knockout. In contrast, cell growth of the parent strain (JM109) was much better than that of JM109(Δ*zwf*), giving a final OD_{660} of 0.62. To further confirm the function of *zwf*, a complementation test was carried out using strain JM109(Δ*zwf*)/pQE-*zwf*. The recombinant expression of *zwf* was validated by a 52-kDa band on SDS-PAGE, corresponding to the correct size of glucose-6-phosphate dehydrogenase (Fig. 2). As expected, JM109(Δ*zwf*)/pQE-*zwf* strain showed remarkably faster growth compared with both JM109(Δ*zwf*) and JM109. The cell density of the *zwf* complementation strain reached 1.24 OD_{660} after 6 h, while that of JM109 and JM109(Δ*zwf*) was 0.62 and 0.23, respectively (Fig. 1). The results indicate that 3-hydroxyacid dehydrogenase family genes (*zwf* and *mmsB*) play critical roles in improving the OST-related properties of *E. coli*.

Effect of 3-Hydroxyacid Dehydrogenase Family Genes on OST of *E. coli*

Our previous studies show that 3-hydroxyisobutyrate dehydrogenase (encoded by *mmsB*) could help keep a relatively lower intracellular solvent concentration by affecting cell

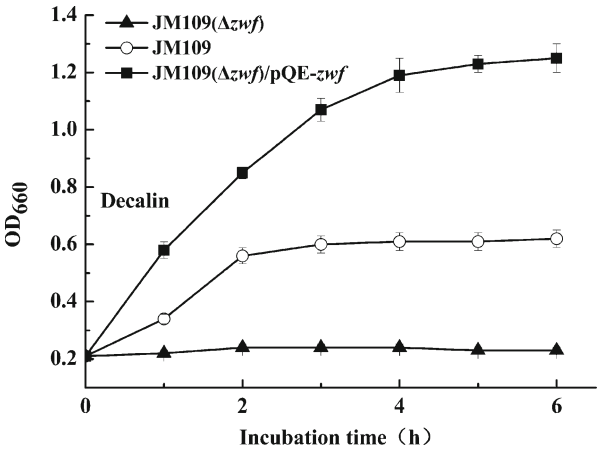

Fig. 1 Cell growth of *E. coli* strains JM109, JM109(Δ*zwf*), and JM109(Δ*zwf*)/pQE-*zwf* in the presence of 4 % (*v*/*v*) decalin

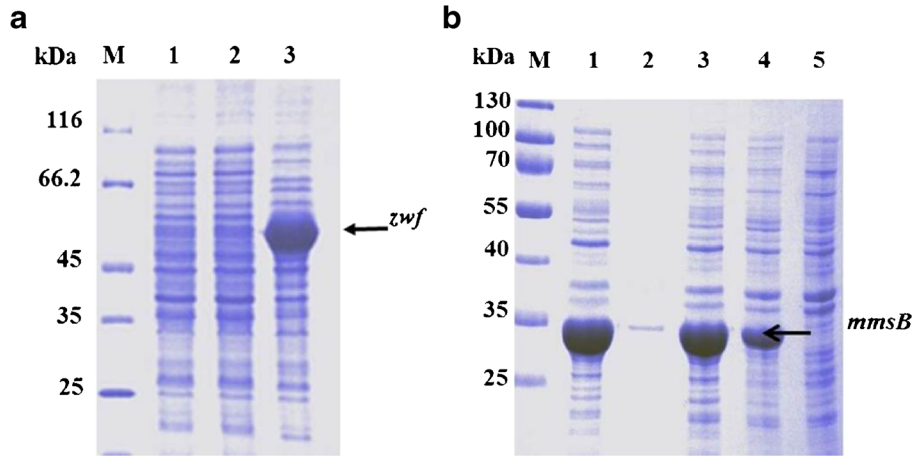

Fig. 2 SDS-PAGE analysis of recombinant expression of *zwf* (**a**) and *mmsB* (**b**). **a** *M*, marker; *1–3*, soluble protein of *E. coli* JM109, JM109/pQE-*zwf* without induction, and JM109/pQE-*zwf* with 1 mM IPTG induction. **b** *M*, marker; *1–2*, soluble and insoluble protein of JM109(DE3) (Δ*endA*::*mmsB*) with 0.1 mM IPTG induction; *3–4* total protein of JM109(DE3) (Δ*endA*::*mmsB*) with 0.1 mM IPTG induction and without induction; *5*, total protein of JM109(DE3)

membrane composition and accelerating organic solvent extrusion [11]. Gene *zwf* is a member of *mar-sox* regulon genes, which are responsible for the regulation of various stress response genes [18]. It is therefore speculated that the function of *zwf* could also be related to OST mechanisms such as solvent efflux pumps. On the other hand, glucose-6-phosphate dehydrogenase (encoded by *zwf*) catalyzes the first step in the pentose phosphate pathway (PPP). It is a process that generates NADPH and a five-carbon sugar and its derivatives which are important starting materials for the biosynthesis of membrane fatty acids. Furthermore, Heipieper et al. reported that the saturation degree and average chain length of fatty acids were increased when bacteria cells were challenged with organic solvents [19]. Wittmann and coworkers confirmed that overexpression of *zwf* resulted in a significantly improved lysine production as well as other amino acids [20]. Kao et al. reported that the expression of genes involved in amino acid transportation (such as *lysC*, *LeuA*, and *LeuB*) is enriched to satisfy the higher energy requirement under solvent challenge [21]. Consequently, the superior OST-related function of *zwf* could also be attributed to the fortified PPP flux in the *zwf* complementation strain, which in turn regulates the structure and composition of fatty acids in the cell membrane.

In order to further compare the impact of *zwf* and *mmsB* on the OST phenotype of *E. coli* strains, cell growth of four recombinant strains JM109/pQE, JM109(Δ*zwf*)/pQE-*zwf*, JM109/pQE-*zwf*, and JM109/pQE-*mmsB* was monitored in LB medium supplemented with 3 % (*v*/*v*) cyclohexane (log*P* = 3.7), which is more toxic than decalin (log*P* = 4.8) (Fig. 3). A stunning cell density of 1.63 OD_{660} was reached by JM109/pQE-*mmsB* after 6 h, while OD_{660} of 1.12 and 1.32 were attained by JM109(Δ*zwf*)/pQE-*zwf* and JM109/pQE-*zwf*, respectively. The results suggest that *mmsB* is more potent than *zwf* on boosting the solvent tolerance of *E. coli*. Since 3-hydroxyisobutyrate dehydrogenase plays an essential role in the catabolism of various amino acids such as valine, leucine, and isoleucine, its enhanced expression level under solvent challenge could help to generate more energy for extruding toxic organic solvents [22].

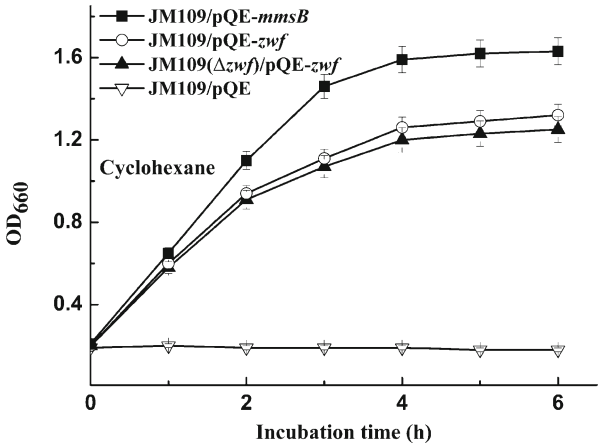

Fig. 3 Cell growth of *E. coli* strains JM109/pQE, JM109(Δ*zwf*)/pQE-*zwf*, JM109/pQE-*zwf*, and JM109/pQE-*mmsB* in the presence of 3 % (*v*/*v*) cyclohexane

OST Properties of *E. coli* JM109(DE3) (Δ*endA*::*mmsB*)

Our results indicate that recombinant *E. coli* carrying pQE-*mmsB* could have improved solvent tolerance properties. However, plasmid burden and its instability would affect recombinant protein expression as well as cell growth [23–28]. To construct plasmid-free *E. coli* strains with an inheritable OST phenotype, we attempted to integrate *mmsB* into the genome of JM109(DE3) using a modified method based on one-step chromosomal gene disruption [13, 15]. A 2,490-bp FRT-Km^R-T7-*mmsB*-Tet fragment comprising *mmsB*, FRT-Km^R, and T7 promoter was successfully inserted into the target locus *endA* by homologous recombination. Then, the kanamycin resistance gene was excised by FLP recombinase, leaving behind a T7-*mmsB*-Tet cassette in the *endA* region. The resulted strain was designated as JM109(DE3) (Δ*endA*::*mmsB*). As confirmed by SDS-PAGE, the *mmsB* gene was successfully expressed in the genomic DNA of *E. coli* without induction, exhibiting a 32-kDa band (Fig. 2).

The growth of JM109(DE3) (Δ*endA*::*mmsB*) and two control strains, JM109(DE3) (negative control) and JM109(DE3)/pQE-*mmsB* (positive control), was monitored in LB medium supplemented with solvents (*v*/*v*) of different toxicities, including 4 % decalin (log*P* = 4.8), 4 % cyclohexane (log*P* = 3.7), 0.4 % butyl acetate (log*P* = 1.7), and 0.1 % butanol (log*P* = 0.8). Among the three strains, JM109(DE3) (Δ*endA*::*mmsB*) showed the best growth in the presence of all solvents investigated (Fig. 4).

In 4 % decalin, a solvent with relatively low toxicity, cell growth of JM109(DE3) (Δ*endA*::*mmsB*) did not show remarkable superiority than the other two strains. After 8 h, the cell density of JM109(DE3) (Δ*endA*::*mmsB*) was 0.638 OD_{660} higher than that of JM109(DE3) and 0.116 OD_{660} higher than that of JM109(DE3)/pQE-*mmsB* (Fig. 4a). In 4 % cyclohexane, JM109(DE3) (Δ*endA*::*mmsB*) showed a notably better growth than the control strain JM109(DE3) and slightly a higher cell density than JM109(DE3)/pQE-*mmsB*, giving final OD_{660} values of 1.59, 1.452, and 0.56, respectively, after 9 h (Fig. 4b). In 0.4 % butyl acetate, JM109(DE3) showed no appreciable growth after 7 h, while the cell density of JM109(DE3) (Δ*endA*::*mmsB*) reached 1.495 OD_{660}, representing approximately 1.5- and 8.5-fold of the two control strains (Fig. 4c). In 0.1 % butanol, a highly toxic solvent, JM109(DE3) (Δ*endA*::*mmsB*) showed distinct growth superiority over the two control strains. A high cell density of 1.75 OD_{660} was attained by JM109(DE3) (Δ*endA*::*mmsB*) after 8 h,

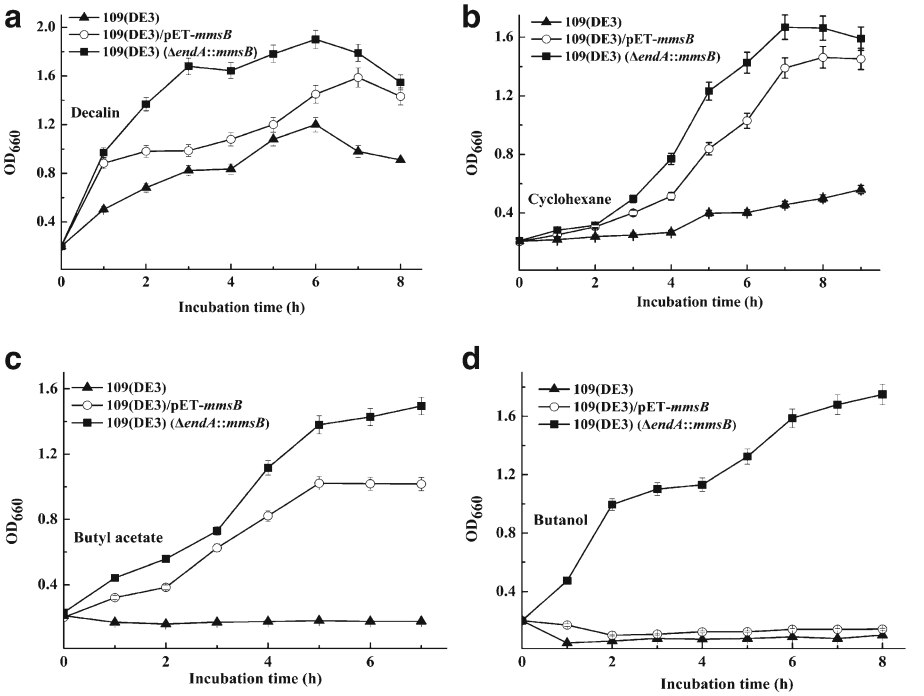

Fig. 4 Cell growth of *E. coli* strains JM109(DE3), JM109(DE3)/pET-*mmsB*, and JM109(DE3) (Δ*endA*::*mmsB*) in the presence of 4 % (*v*/*v*) decalin (**a**), 4 % (*v*/*v*) cyclohexane (**b**), 0.4 % (*v*/*v*) butyl acetate (**c**), and 0.1 % (*v*/*v*) butanol (**d**)

whereas the two control strains showed decreased OD_{660} values (possibly cell lysis) due to the high toxicity of butanol (Fig. 4d).

Hence, JM109(DE3) (Δ*endA*::*mmsB*) is a more advantageous strain even when grown in solvents with high toxicity such as butanol and butyl acetate and displayed a drastic growth superiority over the control strain JM109(DE3). Compared with recombinant expression using pQE-*mmsB*, chromosomal integration of *mmsB* apparently eliminates the "metabolic burden" that arises from plasmid maintenance and replication and therefore contributes to an exceptional OST phenotype in *E. coli*.

Application of JM109(DE3) (Δ*endA*::*mmsB*) in Whole-Cell Biotransformation

To evaluate the potential of JM109(DE3) (Δ*endA*::*mmsB*) as an OST biocatalyst host, pET-*kmCR* carrying a carbonyl reductase from *K. marxianus* was transformed into JM109(DE3) (Δ*endA*::*mmsB*) and JM109(DE3) (as control). Compared with the control strain, the activity of carbonyl reductase in JM109(DE3) (Δ*endA*::*mmsB*) (1.09 U/mg) was a bit lower than that of the control (1.25 U/mg) (Table 3). The strains were applied in the bioreduction of OPBE to (*R*)-HPBE in an aqueous/butyl acetate (1:1) biphasic system. In this reaction, butyl acetate was adopted as the organic phase. It has a relatively low log *P* value of 1.7 and thus extremely toxic to *E. coli* cells. After 12 h of the reaction, (*R*)-HPBE of 99.5 % enantiomeric excess (*ee*) was achieved in a yield of 67.1 % at 5 g/L OPBE with the *mmsB* integration strain, while the yield of the control strain was merely 36.7 % (Table 3). Therefore, an *E. coli* strain carrying *mmsB* integration conduced to a significantly higher product yield in biphasic reaction. The results suggest that *mmsB*

Table 3 Asymmetric synthesis of ethyl (*R*)-2-hydroxy-4-phenylbutyrate in an aqueous/butyl acetate (1:1) biphasic system

Strains	*kmCR* activity (U/mg)	Yield (%)	*ee* (%)
JM109(DE3) (Δ*endA*::*mmsB*)/pET-*kmCR*	1.09	67.1	>99.5
JM109(DE3)/pET-*kmCR*	1.25	36.7	>99.5

The reaction mixture contains 1 g of wet cells, 5 g/L OPBE, 5.0 mmol/L NADPH, 5 mL of 100 mmol/L phosphate buffer (pH 6.0), and 5 mL butyl acetate, which was incubated at 30 °C and 220 rpm in a 50-mL flask for 12 h

chromosomal integration could potentially be applied in construction of microbial strains for nonaqueous biocatalysis and biofuel production.

Conclusion

In this study, two 3-hydroxyacid dehydrogenase family genes, *mmsB* from *P. putida* and *zwf* from *E. coli*, were proven to enhance the solvent tolerance of *E. coli* JM109 strains by recombinant expression, gene knockout, and its complementation. It is presumed that the OST-related function of *zwf* is related to its regulatory effects on the solvent response genes such as efflux pumps, as well as its critical roles in membrane fatty acid synthesis as the first enzyme of PPP. Previously, enhanced expression level of *mmsB* (encoding 3-hydroxyisobutyrate dehydrogenase) has been identified to contribute to the enhanced OST phenotype of *P. putida* JUCT1. Due to the key roles of *mmsB* in catabolism of amino acids, enhanced expression of 3-hydroxyisobutyrate dehydrogenase could render a higher energy production which is essential for solvent expulsion. An *E. coli* strain with chromosomal integration of *mmsB* showed remarkable advantages when grown in the presence of various solvents, especially highly toxic solvents like butanol. The *mmsB* integration strain was successfully applied as an OST host strain for biocatalysis in an aqueous/butyl acetate biphasic system, resulting in an almost doubled product yield. This study could provide useful knowledge for the genetic engineering of microbial strains with excellent OST phenotypes for industrial applications in nonaqueous biocatalysis and biofuel production.

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References

1. Sardessai, Y., & Bhosle, S. (2002). Tolerance of bacteria to organic solvents. *Research in Microbiology, 153*, 263–268.
2. Aono, R., Aibe, K., Inoue, A., et al. (1991). Preparation of organic solvent-tolerant mutants from *Escherichia coli* K-12. *Agricultural and Biological Chemistry, 55*, 1935–1938.
3. Inoue, A., & Horikoshi, K. (1989). A *Pseudomonas* thrives in high concentrations of toluene. *Nature, 338*, 264–266.
4. Hansch, C., & Fujita, T. (1964). p-σ-π Analysis. A method for the correlation of biological activity and chemical structure. *Journal of the American Chemical Society, 86*, 1616–1626.

5. Hansch, C., Muir, R. M., Fujita, T., et al. (1963). The correlation of biological activity of plant growth regulators and chloromycetin derivatives with Hammett constants and partition coefficients. *Journal of the American Chemical Society, 85*, 2817–2824.
6. Inoue, A., & Horikoshi, K. (1991). Estimation of solvent-tolerance of bacteria by the solvent parameter log P. *Journal of Fermentation and Bioengineering, 71*, 194–196.
7. Gokhale, D. V., Bastawde, K. B., Patil, S. G., et al. (1996). Chemoenzymatic synthesis of D(−)phenylglycine using hydantoinase of *Pseudomonas desmolyticum* resting cells. *Enzyme and Microbial Technology, 18*, 353–357.
8. Wagner, T., Hantke, B., & Wagner, F. (1996). Production of L-methionine from D,L-5-(2-methylthioethyl) hydantoin by resting cells of a new mutant strain of *Arthrobacter* species DSM 7330. *Journal of Biotechnology, 46*, 63–68.
9. Shu, Z. Y., Wu, J. G., Cheng, L. X., Chen, D., Jiang, Y. M., Li, X., et al. (2012). Production and characteristics of the whole-cell lipase from organic solvent tolerant *Burkholderia* sp. ZYB002. *Applied Biochemistry and Biotechnology, 166*, 536–548.
10. Gu, M. Z., Wang, J. C., Liu, W. B., et al. (2013). Expression and displaying of β-glucosidase from *Streptomyces coelicolor* A3 in *Escherichia coli. Applied Biochemistry and Biotechnology, 170*, 1713–1723.
11. Ni, Y., Song, L., Qian, X., & Sun, Z. (2013). Proteomic analysis of *Pseudomonas putida* reveals an organic solvent tolerance-related gene *mmsB. PloS one, 8*, e55858.
12. Hawes, J. W., Harper, E. T., Crabb, D. W., & Harris, R. A. (1996). Structural and mechanistic similarities of 6-phosphogluconate and 3-hydroxyisobutyrate dehydrogenases reveal a new enzyme family, the 3-hydroxyacid dehydrogenases. *FEBS Letters, 389*, 263–267.
13. Koma, D., Yamanaka, H., Moriyoshi, K., et al. (2012). A convenient method for multiple insertions of desired genes into target loci on the *Escherichia coli* chromosome. *Applied Microbiology and Biotechnology, 93*, 815–829.
14. Baba, T., Ara, T., Hasegawa, M., et al. (2006). Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Molecular Systems Biology, 2*.
15. Datsenko, K. A., & Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proceedings of the National Academy of Sciences, 97*, 6640–6645.
16. Li, H., Sun, Z., & Ni, Y. (2013). Novel stereoselective carbonyl reductase from *Kluyveromyces marxianus* for chiral alcohols synthesis. *Chemical Research in Chinese Universities, 29*, 1140–1148.
17. Walker, J. M. (1996). *The protein protocols handbook* (2nd ed.). Totowa, NJ: Humana.
18. Aono, R., Tsukagoshi, N., & Yamamoto, M. (1998). Involvement of outer membrane protein TolC, a possible member of the mar-sox regulon, in maintenance and improvement of organic solvent tolerance of *Escherichia coli* K-12. *Journal of Bacteriology, 180*, 938–944.
19. Heipieper, H. J., Weber, F. J., Sikkema, J., Keweloh, H., & de Bont, J. A. (1994). Mechanisms of resistance of whole cells to toxic organic solvents. *Trends in Biotechnology, 12*, 409–415.
20. Becker, J., Klopprogge, C., Herold, A., Zelder, O., Bolten, C. J., & Wittmann, C. (2007). Metabolic flux engineering of L-lysine production in *Corynebacterium glutamicum*—over expression and modification of G6P dehydrogenase. *Journal of Biotechnology, 132*, 99–109.
21. Reyes, L. H., Almario, M. P., & Kao, K. C. (2011). Genomic library screens for genes involved in *n*-butanol tolerance in *Escherichia coli. PloS one, 6*, e17678.
22. Chowdhury, E. K., Akaishi, Y., Nagata, S., & Misono, H. (2003). Cloning and overexpression of the 3-hydroxyisobutyrate dehydrogenase gene from *Pseudomonas putida* E23. *Bioscience, Biotechnology, and Biochemistry, 67*, 438–441.
23. Peredelchuk, M. Y., & Bennett, G. N. (1997). A method for construction of *E. coli* strains with multiple DNA insertions in the chromosome. *Gene, 187*, 231–238.
24. Bailey, J. E., Da Silva, N. A., Peretti, S. W., et al. (1986). Studies of host–plasmid interactions in recombinant microorganisms. *Annals of the New York Academy of Sciences, 469*, 194–211.
25. Diaz Ricci, J. C., & Hernández, M. E. (2000). Plasmid effects on *Escherichia coli* metabolism. *Critical Reviews in Biotechnology, 20*, 79–108.
26. Jones, K. L., & Keasling, J. D. (1998). Construction and characterization of F plasmid-based expression vectors. *Biotechnology and Bioengineering, 59*, 659–665.
27. Jones, K. L., Kim, S. W., & Keasling, J. D. (2000). Low-copy plasmids can perform as well as or better than high-copy plasmids for metabolic engineering of bacteria. *Metabolic Engineering, 2*, 328–338.
28. Wang, Z., Xiang, L., Shao, J., et al. (2006). Effects of the presence of ColE1 plasmid DNA in *Escherichia coli* on the host cell metabolism. *Microbial Cell Factories, 5*, 34.