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*Full Length Research Paper*

# Metabolic engineering of an ethanol-tolerant *Escherichia coli* MG1655 for enhanced ethanol production from xylose and glucose

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**Efficient ethanol production will require a recombinant to able to ferment a variety of sugars (pentoses, and hexoses), less formation of by-products, as well as to tolerate high ethanol stress. In this study, a mutant (MGE) that can grow in 60 g ethanol/l was selected from *Escherichia coli* MG1655 by enrichment method with increasing concentrations of ethanol. The ethanol-tolerant mutant was used as the host to develop the ethanologenic recombinant by knockout of pyruvate formate lyase (*pflB*) and lactate dehydrogenase (*ldhA*) genes, and expression of *Zymomonas mobilis* alcohol dehydrogenase and pyruvate decarboxylase genes in plasmid pZY507bc. The resultant recombinant (GMEPLbc) showed the genetic stability of *Z. mobilis* genes in glucose medium without antibiotics under anaerobic conditions, and generated little acetic acid (3.6 mM), no formic acid and lactic acid. The ethanol production by GMEPLbc were 41.6 and 35.8 g ethanol/l from 100 g/L glucose and 100 g/L xylose during fermentation in M9 mineral medium, 37.0 and 36.5% more than that of the ethanol-sensitive strain carrying pZY507bc alone, respectively. Our results indicated that enhancement of ethanol tolerance and inactivation of *pflB* and *ldhA* are advantageous in the production of ethanol.**

**Key words:** *Escherichia coli*, ethanol production, ethanol-tolerance, Gene knockout, metabolic engineering.

## INTRODUCTION

Hexose and pentose sugars, as major components of lignocellulosic biomass, are abundant in nature (Deanda et al., 2003), and represent an inexpensive and readily available source for ethanol production. Efficient conversion of biomass to ethanol requires the development of microorganisms capable of fermenting the components in lingocellulosic materials and that tolerating high concentrations of ethanol (Zaldivar et al., 2001). *E. coli* is widely recognized as the modern workhorse for industrial bio-technology. Strains of this organism can naturally metabolize all the sugars present in lignocellulosic hydrolysates, but normally produce only small amounts of ethanol.

*Zymomonas mobilis* efficiently ferment carbohydrates using the Enter-Doudoroff pathway and pyruvate decarboxylase (PDC; *pdc*), alcohol dehydrogenase II (ADHII; adhB) are the key enzymes in the formation ethanol. PDC catalyses the decarboxylation of pyruvic acid to acetaldehyde, then ADHII catalyses the conversion between alcohols and aldehydes. Introduction of *Z. mobilis* alcohol dehydrogenase (*adhB*) and pyruvate decarboxylase (*pdc*) genes into *E. coli* has significantly improved ethanol productivity (Ingram et al., 1987). *E. coli* strains have been constructed, which feature traits that are advantageous in the production ethanol using lignocellulose sugars, however, they lack the high ethanol tolerance as *S. cerevisiae* (100 g/L) or *Z. mobilis* (110 g/L) (Ghareib et al., 1988; Rogers et al., 2007). Since the molecular basis for ethanol tolerance is not clearly understood, and the tolerance is a multigenic trait, underlying mechanisms are best explored using a variety of approaches (Alper and Stephanopoulos, 2007). Classic random mutation techniques have been proven helpful

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random mutation techniques have been proven helpful in isolating 35 g/L ethanol-tolerant mutants (Yomano et al., 1998). Ethanol-resistant mutants of *E. coli* that can grow in 38 g ethanol/l have been isolated after nitrosoguanidine-mediated mutagenesis (VA and Novick, 1973).

The recombinant *E. coli* strains, containing *Z. mobilis* genes for the ethanol pathway, was developed for the fermentation of sugars from cellulose and hemicellulose into ethanol, but ethanologenic strains should be further improved by enhancing the ethanol-toloerance, reducing the by-product formation and increasing the phenotypic stability. In this study, we used an ethanol tolerant mutant (MGE), tolerant to 60 g ethanol/l, as the cloning host, which was selected by enrichment method with increasing concentrations of ethanol. To redirect the energy and carbon flux toward ethanol metabolism, two genes encoding lactate dehydrogenase and pyruvate formate lyase were knocked out by homologous recombination and genes encoding enzymes of the ethanol pathway from *Z. mobilis* were incorporated into the mutants. The recombinants were investigated by measuring the intracellular metabolite concentrations and ethanol production from hexose and pentose sugars.

## MATERIALS AND METHODS

### Strains, plasmids, and culture conditions

The strains and plasmids used in this study are listed in Table 1. The *E. coli* strains were grown at 37ºC in Luria-Bertani medium (LB). The pZY507bc plasmid containing *Z. mobilis* pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adhB*) genes under the control of the *lac* promoter was constructed in our previously study (Wang et al., 2008). For the fermentation experiments, the strains anaerobic cultured at 37℃ in mineral medium M9 ($Na_2HPO_4$ 64 g/l, $KH_2PO_4$ 15 g/l, $NH_4Cl$ 5 g/l, $MgSO_4$ 0.24 g/l, $CaCl_2$ 0.011 g/l) containing 100 g/L glucose or xylose in 100 ml volumetric flasks containing 80 ml broth and fitted with drilled rubber septa to allow gas to escape. Fermentation flasks were immersed in a temperature-controlled water bath and stirred by a magnetic stirrer at 100 rpm. When required, the medium was supplemented with 50 µg kanamycin (Kan)/ml, or 34 µg chloramphenicol (Cm)/ml. Cell growth was measured as optical density at 600 nm (OD600) after 24 - 96 h of incubation.

### Selection and isolation of ethanol-tolerant mutants

A combination liquid and solid medium was used to successfully enrich the strains with mutations, which increase ethanol tolerance and maintain the genetically engineered trait of efficient ethanol production (Yomano et al., 1998). Strains were tested for their tolerance to ethanol (30 g/l) in M9 medium containing 100 g/L glucose. MG1655 was chosen for further improving the ethanol-tolerance. The strain was inoculated and transferred serially into LB medium containing increasing concentrations of ethanol (30 - 60 g/l). After every 3 - 5 liquid transfers, the cultures were diluted and spread on solid medium and the single colonies were re-selected in higher concentration of ethanol, and tested for their ability to grow in the presence of ethanol. One of the best clones (designated MGE) was selected to develop for enhancement of ethanol production.

### Construction of strains in *E. coli*

A DNA fragment (1.0 kb) containing part of the *pflB* was amplified by polymerase chain reaction with PFLF (5'-GTCCGAGCTT AATGAAAAG-3') and PFLR (5'-AAGTCCACTG GATAGCTT-3') primers. A $Kan^R$ gene was inserted in the *Eco*RI site within *pflB*. The disrupted *pflB* gene fragment with its flanking sequences was electroporation transferred into MGE to generate the *pflB* mutant (MGEP).

The 5'-flanking region of *ldhA* was amplified with primers LdhaF1 (5'-ATTCATTAAA TCCGC CAGCT TATAAG-3') and LdhaR1 (5'-GAAGCAGCTC CAGCCTACAG AAAGTAGCCG CGTTTGTTGC-3')-3'); the 3'-flanking region of *ldhA* gene was amplified with primers LdhaF2 (5'-GGACCATGGC TAATTCCCAT GGCGCAACCT TCAACTGAA C-3') and LdhaR2 (5'-ATCCAGGTGT TAGGCAGCAT G-3'). The $Cm^R$ cassette fragment from PKD3 was amplified with oligonucleotide primers CmrF (5'-GTGTAGGCTG GAGCTGCTTC-3', complementary to LdhaR1) and CmrR (5'-ATGGGAATTA GCCATGGTCC-3', complementary to LdhaF2). The $Cm^R$-marked *ldhA* deletion fragment connected by overlap extension PCR was transformed into MGEP. The $Cm^R$ gene and one of the FRT sequences from the deletion strains constructed by this method were removed as described by Datsenko and Wanner (2000) using the yeast FLP recombinase. The resulting double deletion mutant was designated MGEPL. The mutants (MGEP and MGEPL) were validated by nested PCR.

The plasmid pZY507bc carrying *Z. mobilis pdc* and *adhB* genes was transformed into MG1655, MGE, MGEP and MGEPL to yield MG1655bc, MGEbc, MGEPbc and MGEPLbc, respectively. Strains were serially transferred to LB medium containing 100 g/L glucose without antibiotics for more than 60 generations at 37℃. Appropriate dilutions of cultures were plated containing 2% glucose (without antibiotics) after 48 and 120 h. Colonies were screened on 2% glucose-LB plates containing 34 µg Cm/ml and on aldehyde indicator plates.

### Analysis of carbon substrate utilization

Strains were tested for their ability to utilize carbon substrates using Biolog GN2 plates (BIOLOG Inc., Hayward, CA, USA). GM1655 and GME strains were grown overnight in LB medium plate at 37℃. Cells were scraped from the surface of the plate using a cotton swab, and suspended in GN/GP-IF inoculating fluid (BIOLOG Inc., Hayward, CA, USA) at a cell density equivalent to 61% transmittance on a Biolog turbidimeter. Suspensions with an $OD_{600}$ of 0.2 to 0.3 were transferred to the GN2-Plate (150 µl per well) at 37℃ for 24 h. The amount of tetrazolium violet dye in each well was determined spectrophotometrically at 590 nm with subtraction of a 750 nm reference reading (A590-750) using a Biolog plate reader.

### Fermentation

Cells were first grown in 100 ml LB medium at 37℃ and shaking until the $OD_{600}$ reached 1.0. After centrifuging and washing twice with M9 medium, seed cultures were resuspended in 100 ml M9 medium containing 100 g/L glucose or xylose. Ethanol was assayed using gas chromatography (GC) with a glass column (0.26 × 200 cm) filled with Porapak Type QS (80 - 100 mesh, Waters, Milford, MA) at 180℃; nitrogen was the carrier gas (40 ml/min), and an FID detector was used (Lapaiboon et al., 2007).

Concentrations of glucose, xylose, and organic acids were determined by high- pressure liquid chromatography (HPLC) (Agilent Technologies Palo, Alto, CA, USA) (Hespell et al., 1996) equipped with a refractive index detector, using an Aminex 87H column (Biorad) maintained at 65℃. The mobile phase used was 0.5 mM H2SO4 at a flow rate of 0.6 ml/min. The standard components used

**Table 1.** *E. coli* strains and plasmids used in this study.

| Strains | Relevant characteristics | References or resources |
|---|---|---|
| ***E. coli*** | | |
| JM109 | *recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi*Δ(*lac-proAB*)F' [*traD*36 *proAB*+ *lacIq* *lacZΔM*15] | TransGen Biotech |
| MG1655 | Wild type | Novagen |
| MGEP | Δ(*pflB*)- $Kan^R$ | This work |
| MGEPL | Δ(*pflB*)- $Kan^R$, Δ*ldhA* | This work |
| Plasmid | | |
| pKD46 | Phage λ red recombinase expression vector, $Kan^R$, help recombination | Gift from Yale University |
| pKD3 | Template plasmid for gene disruption, $Cm^R$ gene is flanked by FRT sites | Gift from Yale University |
| pCP20 | $Ap^R$, $Cm^R$, FLP recombinase expression | Gift from Yale University |
| pZY507bc | Cloning vector, $Cm^R$, carrying *pdc* and *adhB* of *Z. mobilis* | Wang Zc *et al*., 2008 |
| pMD18-T simple | Cloning vector, $Amp^R$ | Takara |
| pGEM-T | Cloning vector, $Amp^R$ | Promega |

nents used for calibration were glucose, xylose, formic acid, acetic acid and lactic acid. These were purchased from Sigma (St. Louis, MO). The chromatograms obtained had the results in terms of micro-refractive index units. Triplicate analyses were performed on each sample to guarantee the reliability of the analysis.

## RESULTS

### Selection and isolation of ethanol-tolerant mutants of *E. coli*

To choose an ethanol tolerant *E. coli* strain as the starting strain for ethanol production, we compared 12 *E. coli* strains for their ability to tolerate ethanol. The strain MG1655 was superior to others *E. coli* strains examined. Enrichment method with increasing ethanol concentrations was performed to further improve the tolerance of MG1655. After a further series of transfers, an ethanol-tolerant mutant, MGE, was isolated. MGE showed the normal growth in presence of 50 g ethanol/l (Figure 1), as compared with MG1655 or MGE in M9 medium lacking ethanol (control). With 60 g ethanol/l, MGE maintained growth to reach an OD600 value of 2.47 ± 0.13 (about 80% of the OD600 value of control (3.05 ±0.07)). While MG1655 displayed only 50% relative growth rate in 30 g ethanol /l, and failed to grow in 50 g ethanol /L (Figure 1).

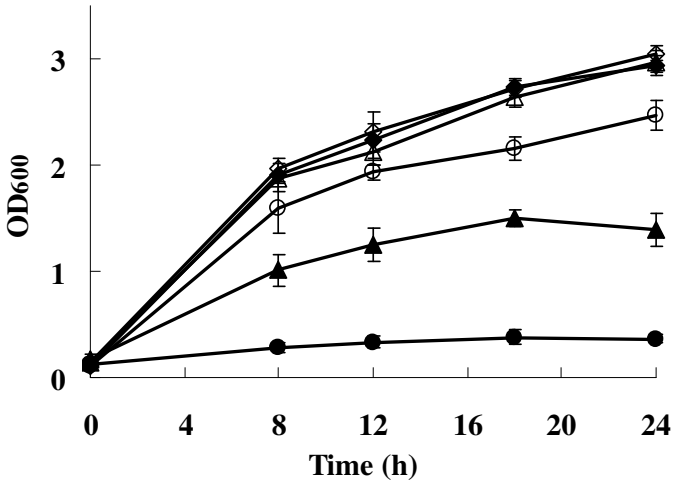


**Figure 1.** Effect of ethanol on growth. Standard deviations for three experiments are represented by error bars. ◆, ▲, and ● represent the growth of MG1655 in absence of ethanol, 3% and 5% of ethanol respectively; ◇, △, and ○ show the growth of GME in 0%, 5%, and 6% ethanol.

### Plasmid stability

The strains of *pflB* mutant (MGEP), *pflB*/*ldhA* double mutant (MGEPL) grew as well as their parent strain MGE in M9 medium containing glucose under aerobic conditions. Under anaerobic conditions, however, the growth rate of MGEP was only approximately 50% of that of MGE, while the MGEPL strain was incapable of anaerobic growth (Figure 2). The results showed that both *pflB* and *ldhA* genes are essential for anaerobic growth of *E. coli*. Transforming the mutants with plasmid pZY507bc containing *Z. mobilis pdc* and *adhB* genes totally restored the fermentative growth under anaerobic conditions (Figure 2). The expression of these ethanologenic enzymes caused dramatic increase in final cell density (about 2-fold increase compared with the parent strain MGE).

During anaerobic growth, the mutants were fully complemented by pZY507bc. We therefore, examined plasmid stability after growth in glucose medium without antibiotics for 60 generations under anaerobic conditions.

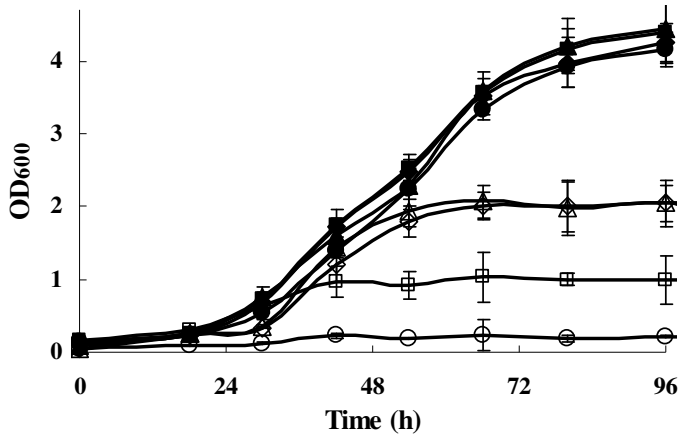


**Figure 2.** The growth of *E. coli* strains in M9 medium with 100 g/L glucose under anaerobic conditions. Standard deviations for three experiments are represented by error bars. Symbols: ◇, MG1655 ; △, MGE ; □, MGEP ; ○, MGEPL ; ◆, MG1655bc ; ▲, MGEbc ; ■, MGEPbc ; ●, MGEPLbc.

The plasmid pZY507bc was well maintained and showed negligible loss in the MEPLbc strain. In MGEPbc, the plasmid pZY507bc was relatively stable, with 98% of the population retaining both the antibiotic resistance gene and the genes from *Z. mobilis*.

## Production of organic acids

The *E. coli* strain ferments a wide range of sugars, including pentoses and hexoses, and in doing so yields a variety of organic acids. After a 96 h fermentation of 100 g/L glucose, MGE generated less organic acids than the ethanol sensitive strain MG1655 (Table 2). Inactivation of *pflB* clearly resulted in a decrease in the production of acetic acid (5.4 ± 0.6 mM), and loss of formic acid production, but the lactic acid concentration (102.4 ± 10.1 mM) in MGEP was higher than that of MGE (82.5 ± 7.4 mM).

The expression of *Z. mobilis pdc* and *adh* genes in *E. coli* resulted in the production of ethanol as the primary fermentation product during anaerobic growth. MGEPbc produced less acetic acid (2.7 ± 0.2 mM) and lactic acid (40.3 ± 6.3 mM) than the MGE or MGEP strains. Inactivation of *ldhA* further reduced the formation of organic acids. MGEPLbc generated relatively small amounts of acetic acid (3.6 ± 0.5 mM), and no formic acid or lactic acid were detected (Table 2). The data indicate that metabolic engineering of *E. coli* resulted in a redirection of the energy and carbon flux to ethanol biosynthesis.

## Ethanol production

The efficiency of fermentation in M9 medium with glucose or 100 g/L xylose at pH 7.0 and 37°C is presented in Figure 3. In spite of the fact that the growth rates, glucose-consumption rates and xylose-consumption rates were similar, clear differences in ethanol production in all the ethanolgenic *E. coil* strains were observed. The final concentrations of ethanol in MGEbc cultures grown with 100 g/L glucose or xylose were 33.8 and 28.9 g/L, a 100 g/L increase compared with the ethanol-sensitive strain MG1655bc (30.3 and 26.2 g ethanol/l). Inactivation of the *pflB* and *ldhA* genes blocks the main pyruvate assimilation pathway under anaerobic conditions (Clark, 1989; Mat-Jan et al., 1989). The growth defects of strain MGEPL were compensated for by the introduction of pZY507bc (Figure 3), implying the redirection of energy and carbon flux to pyrvate-to-ethanol pathways. The maximum ethanol levels produced by MEPbc and MGEPLbc were 35.4 and 41.6 g/L from 100 g/L glucose, 30.5 and 35.8 g/L from 100 g/L xylose in M9 medium. Disrupting *pflB* and *ldhA* genes in the pyruvate assimilation pathway resulted in a significant decrease in the formation of formic acid, acetic acid and lactic acid, and a concomitant increase in ethanol levels (16 and 37% more than that of MG1655bc from glucose and xylose, respectively).

**Table 2.** Organic acids formed during fermentation of 100 g/L glucose by *E. coli* strains after 96 h.

| Strains | Organic acids (mM) | | |
|---|---|---|---|
| | Formic acid | Acetic acid | Lactic acid |
| MG1655 | 14.2 ± 5.2 | 60 ± 8.1 | 460 ± 37.4 |
| MGE | 12.4 ± 1.5 | 24.6 ± 2.4 | 82.5 ± 7.4 |
| MGEP | ND | 5.4 ± 0.6 | 102.4 ± 10.1 |
| MGEPbc | ND | 2.7 ± 0.2 | 40.3 ± 6.3 |
| MGEPLbc | ND | 3.6 ± 0.5 | ND |

ND = not detected. The values shown represent the mean ± standard deviations for 3 independent measurements

## DISCUSSION

It is highly desirable to increase the ethanol-tolerance of *E. coli* strains undergoing ethanologenic cell growth activity and ethanol production. Several approaches such as mutagenesis and genetic modifications have been used to develop ethanol-tolerant strains (Alper and Stephanopoulos, 2007; Yomano et al., 2001). Perhaps the best example of increased ethanol tolerance of E. coli strain was described by Yomano (1998). The ethanol tolerant mutants were successful generated by enrichment method. In this study, we used a similar approach to generate the ethanol-tolerant mutants. However, in considering selection of the best candidates with tolerance to harsh conditions (e.g. high ethanol concentration) for enhancement of ethanol production by

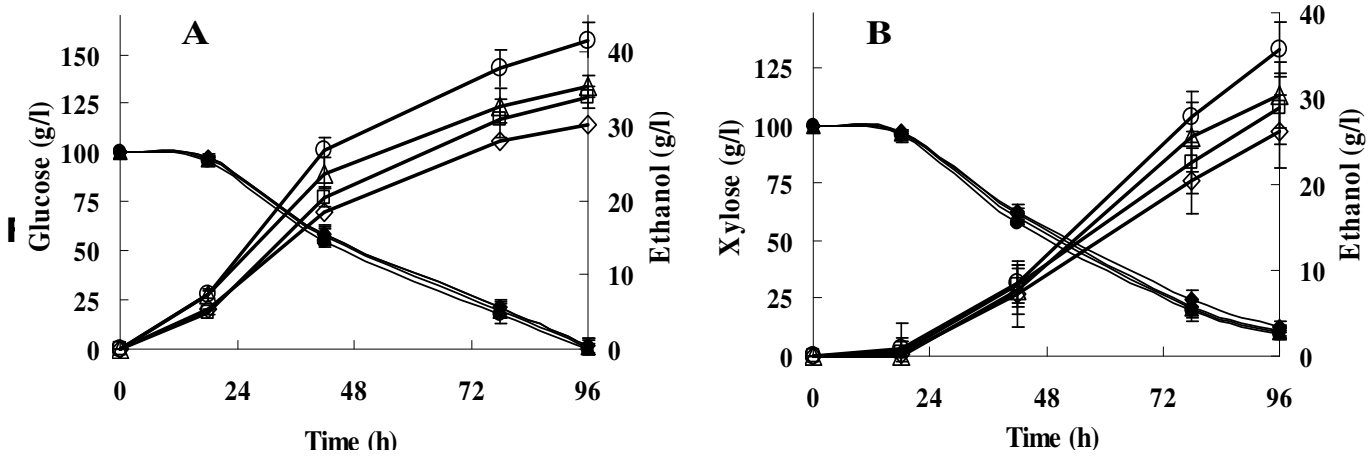


**Figure 3.** Fermentation of *E. coli* strains in M9 medium with 100 g/L glucose (A) and xylose (B). Vertical bars represent standard deviations for three experiments. Solid symbols represent the sugar concentrations in the cultures, and open symbols represent ethanol levels in the cultures. Diamond symbols (◆ and ◇), MG1655bc, square symbols (■ and □), MGEbc; triangle symbols (▲ and △), MGEPbc; cycle symbols (● and ○), MGEPLbc.

metabolic engineering, we tested the ethanol tolerance of different *E. coli* strains, and selected MG1655 to develop ethanol-tolerant mutants by enrichment method.

Ethanol tolerance is a multigenic trait involving a number of biochemical reactions and physiological processes. Adaptive responses of bacteria to ethanol stress correlates with increases in fatty acid chain length (Ingram et al., 1987), the levels of *trans*-fatty acids (Heipieper and Bont, 1994; Pinkart and White, 1997), and changes in phospholipid composition (Clark and Beardl, 1979). In this study, the ethanol-tolerant strain *E. coli* MGE showed 100 and 80% relative growth rate at 50 and 60 g ethanol/l by serial transfers into increasing concentrations of ethanol, respectively. Compared with the MG1655bc ethanol-sensitive strain, the engineered ethanol-tolerant mutant (MGEPLbc), had a higher rate of ethanol production and higher ADHB and PDC activities, especially in high concentrations of ethanol. Our results show that directed evolution of a bacterial train is an effective means of eliciting a desired trait, the result is in agreement with previous observations (Ingram, 1986; Yomano, *et al.,* 2001).

To further improve ethanol production and to block undesirable metabolic pathways, the *pflB* and *pflB*/*ldhA* double mutants of *E. coli* were created. These mutants were incapable of anaerobic growth because of deficiencies in the fermentative lactate dehydrogenase and pyruvate formate lyase (Clark, 1989; Hasona et al., 2004). The plasmid containing *Z. mobilis adhB* and *pdc* genes can complement the mutants for anaerobic growth (Hespell et al., 1996).

Therefore, the plasmid in the mutants was quite stable and should not require antibiotics in the medium to maintain genetic stability and high ethanol production traits (Ohta et al., 1991; Yomano et al., 2001). Elimination of antibiotics requirement for recombinants to carry the *Z. mobilis* genes, now provides additional opportunities for the commercial application of ethanologenic *E. coli*. Our results showed that the plasmid pZY507bc was quite stable in MGEPbc and MGEPLbc and diverted the carbon flow of pyruvate from organic acids to ethanol production (Jarboe et al., 2007). Inactivation of *pflB* and *ldhA* genes resulted in a significant decrease of the formation of organic acids, and a concomitant increase of the ethanol levels.

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