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ORIGINAL RESEARCH PAPER

Improved ethanol tolerance in *Escherichia coli* by changing the cellular fatty acids composition through genetic manipulation

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Abstract To investigate the effect of cellular fatty acids composition on ethanol tolerance in *Escherichia coli*, we overexpressed either *des,* encoding fatty acid desaturase from *Bacillus subtilis,* or *fabA,* encoding *β*-hydroxydecanoyl thio-ester dehydrase from *E. coli,* or both genes together, into *E. coli*. Recombinant *E. coli* harboring *fabA* had elevated tolerance against ethanol compared to wild type strain. In contrast, *des* decreased resistance to ethanol. Co-expression of both genes together complemented ethanol tolerance of *E. coli*. This result indicates how to engineer bacterial strains to be resistant to higher concentrations of ethanol.

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Introduction

Changes in the membrane fatty acid composition in microbial cells occur in response to changes in environmental ethanol concentrations. In *Saccharomyces cerevisiae*, which is a traditional producer of ethanol, the amount of unsaturated fatty acid is directly correlated with tolerance against ethanol. You et al. (2003) showed that increasing the amount of oleic acid (18:1 *c* 9) in *S. cerevisiae* by expressing insect desaturases or supplementing the fatty acid in the media can enhance ethanol tolerance. Additionally, Kajiwara et al. (1996) reported that unsaturation of palmitoyl (16:1) and oleoyl fatty acyl residues of phospholipids in *S. cerevisiae*, which was achieved by overexpressing Δ12 fatty acid desaturase gene (*FAD2*) from *Arabidopsis thaliana* can result in increased resistance to high concentrations of ethanol (15%, v/v).

Other alcohol-tolerant microorganisms, such as *Zymomonas mobilis* has abundant *cis*-vaccenic acid (18:1 *c* 11; Carey and Ingram 1983) and *Lactobacillus heterohiochii* from sake, not only increase unsaturation in response to growth in ethanol but also increases the mean chain length of the fatty acids (Uchida 1974).

In *Escherichia coli*, the proportion of unsaturated fatty acids in the membrane lipid also increases when the cells are grown in the presence of ethanol (Ingram 1976). Therefore, increased levels of unsaturated fatty acids in *E. coli* might be important for sustaining cell growth in the presence of ethanol, as demonstrated in yeast. To evaluate the correlation between fatty acid composition and ethanol tolerance in *E. coli*, we constructed and analyzed recombinant strains with variations in cellular fatty acid composition brought about by cloning in genes encoding for fatty acid desaturase, *des*, from *Bacillus subtilis* and/or for *β*-hydroxydecanoyl thio-ester dehydratase, *fabA*, from *E. coli*.

Materials and methods

Bacterial strains, plasmids, and media

Escherichia coli DH5α was used for gene cloning and expression. *Bacillus subtilis* and *E. coli* W3110 were used as sources of the *des* and *fabA* genes, respectively. pGEM T Easy (Promega) was used for cloning and sequencing the amplified DNA fragment. pBR322 was used to express *des* and *fabA* gene in *E. coli*. For the construction of recombinant strains, microbial cells were grown in LB medium [yeast extract (Difco), 0.5%; Bacto-tryptone, 1%; and NaCl, 1%] supplemented with the appropriate antibiotics [ampicillin (50 μg ml^{-1}) or tetracycline (10 μg ml^{-1})].

Construction of recombinant plasmids

A schematic representation of the strategy used to construct the pBR-des, -fabA, and des-fabA is shown in Fig. 1. The 1.16 kb open reading frame (*orf*) of *des* (GenBank accession no. NC000964) was amplified from chromosomal DNA of *B. subtilis* using the following primers: Pdes-F (5′-*TCTAGA***ATG**ACTGA ACAAACCA-3′, italic letters indicate an *Xba*I site; bold letters indicate the start codon of *des*) and Pdes-R (5′-*GGATCC*CTCGAG**TCA**GGCATTCTTCCGC-3′, italic letters indicate a *Bam*HI; bold letters indicate the stop codon). The DNA sequence encoding FabA (0.88 kb) (GenBank accession no. NC000913) was amplified from chromosomal DNA of *E. coli* W3110 using the following primers, P3 (5′-*TCTAGA***ATG**G TAGATAAACGCG-3′, italic letters indicate an *Xba*I site; bold letters indicate the start codon of *fabA*) and P4 (5′-*GGATCC*GCTAGC**TCA**GGCATTCTTC CGC-3′, italic letters indicate a *Bam*HI; bold letters indicate the stop codon). The *lacZ* promoter sequences (P_{lacZ}-des for *des* and P_{lacZ}-fabA for *fabA*) were amplified using a set of primers; for PlacZ-des, PlacZ-des-F (5′-GCTAGCGCGCAACGCAAT-3′) and PlacZ-des-R (5′-*GGATCC*TCTAGAGCTGTTT CCTGTGT-3′, italic and underlined letters indicate a *Bam*HI and *Xba*I site, respectively) and for PlacZ-fabA, PlacZ-fabA-F (5′-*AGATCT*GTTAACGCGCA ACGCAAT-3′, italic letters indicate a *Bgl*II site) and PlacZ-fabA-R, respectively. The amplified DNA fragments were cloned into the pGEM TEasy (Promega) vector followed by nucleotide sequencing to confirm the absence of any error in the DNA polymerization reaction. The *Xho*I–*Bgl*II fragments including *des* or *fabA* were inserted into the corresponding restriction sites downstream of *lacZ* promoter sequences, respectively, and the *Eco*RI fragments transferred to the corresponding site of the pBR322 to create pBR-des and pBR-fabA. To

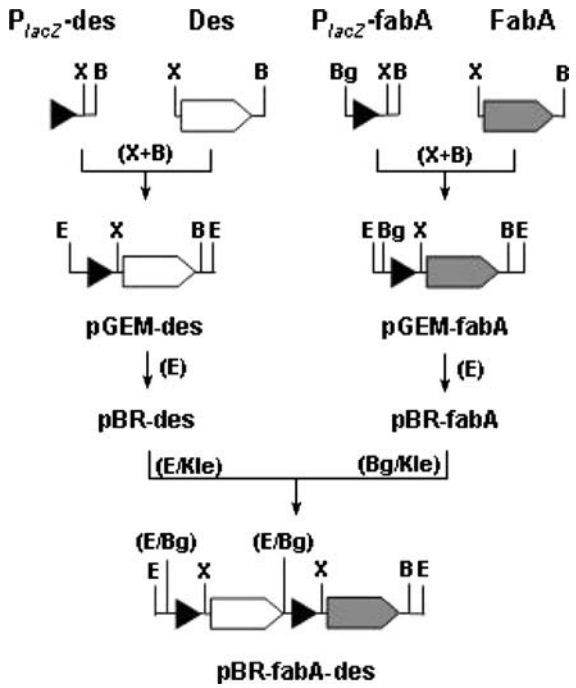

Fig. 1 Schematic representation of the strategies used for the construction of pBR-des, pBR-fabA and pBR-fabA-des. Abbreviations for restriction enzymes: *B Bam*HI, *Bg Bgl*II, *E Eco*RI on cloning vectors (pGEM T Easy and pBR322), *X Xba*I, *Kle* Klenow fragment

generate pBR–des–fabA, the *Eco*RI fragment including *des* was inserted into *Bgl*II site of pBR–fabA after treatment of Klenow fragment.

Cultivation of recombinant *E. coli* strains

Escherichia coli clones harboring pBR-des, pBR-fabA, or pBR-des-fabA were cultivated in 50 ml of LB medium supplemented with tetracycline (10 μg/ml) and 0.5 mM IPTG at 37°C with shaking at 120 rpm. Growth of the cells was monitored from the OD_{600}. To examine effect of ethanol on the cell growth, ethanol was added to the medium at 1, 2, and 3% (v/v).

Analysis of fatty acid composition

Cells were harvested from 50 ml of culture broth by centrifugation at 10,000*g* for 10 min at 4°C and washed twice with 1 M sorbitol. They were dried, mixed with 3 ml 5% (v/v) methanol/sulfuric acid and heated at 90°C for 1 h in sealed tubes. Fatty acid methyl esters (FAMEs) were extracted from the cells with 0.2 ml hexane and analysed by gas GC equipped with a flame-ionized detector (FID) and a HP-5 (30 m × 0.25 mm, 0.25 μm, Agilent Technologies, USA) capillary column. The column temperature was raised from 150°C (2 min) to 270°C (2 min) at 7°C per min. All the results were confirmed by at least three independent experiments. The FAMEs were also subjected to GC–MS analysis using a Hitachi GC-mass spectrometer (M-80A) with a capillary column (DB-WAX, 0.32 mm × 30 m, J & W Scientific).

Results and discussion

E. coli recombinant strains having variations in cellular fatty acids composition

Expression of *B. subtilis* desaturase gene (*des*) produces novel unsaturated fatty acids such as 5-*cis*-palmitoleic acid (16:1, Δ5) in *E. coli* (Aguilar et al. 1998; Bonamor et al. 2006). Based on these reports, we constructed a recombinant strain harboring multiple copies of *des* gene under the direction of a strong *lacZ* promoter on the cloning vector pBR322 (called pBR-des). An increase of unsaturated 5-*cis*-palmitoleic acid was observed in *E. coli* harboring pBR-des, whereas saturated palmitic acid (16:0) was reduced (Supplementary Table 1). On the whole, the equilibration of fatty acid composition in the recombinant strain has a bias toward to unsaturated fatty acids.

FabA, β-hydroxydecanoyl thio ester dehydrase, which catalyzes reversible conversion of 3-hydroxydecanoyl-acyl carrier protein (ACP) to *trans*-2-decenoly-ACP and subsequent isomerization to *cis*-2-decenoly-AC participates in production of unsaturated fatty acids in *E. coli* in combination with β-ketoacyl-acyl-ACP synthase dehydrase (I) (FabB). However, Clark et al. (1983) found that overexpression of *fabA* alone resulted in an increase in the amount of saturated fatty acids with a decrease in the amount of unsaturated fatty acids in *E. coli*, even though the biochemical mechanism was not clearly understood. We also prepared a recombinant *E. coli* strain harboring multiple copies of *fabA* using a cloning vector pBR322 (called pBR-fabA). As expected, the equilibration of fatty acids composition in *E. coli* harboring pBR-fabA strongly inclined to saturated fatty acids (Supplementary Table 1).

When *des* and *fabA* was coexpressed (pBR-des-fabA), equilibration of fatty acids composition was restored to a certain degree to that of the wild type strain due to the conversion of increased palmitic acid (16:0) to 5-*cis*-palmitoleic acid (16:1, Δ5) by the action of desaturase (Supplementary Table 1).

Effect of ethanol on cell growth of recombinant *E. coli* strains

All of the recombinant strains showed similar cell growth in the absence of ethanol despite the dissimilar fatty acid composition (Fig. 2). However, with increased ethanol concentration in the culture medium, the cells showed different responses against ethanol. *E. coli* harboring pBR-des had slower growth with ethanol at 2% (v/v) or above as compared to wild type (pBR322), whereas *E. coli* harboring pBR-fabA had higher growth compared to wild type (Fig. 2). This was most likely to be related to the dissimilar equilibration of fatty acid composition, i.e., a bias toward to saturated (pBR-fabA) or unsaturated fatty acids (pBR-des) as shown above. The prediction was supported by complementation of cell growth in *E. coli* coexpressing *des* and *fabA* to the similar level of the wild type. Interestingly, this result contrasts with the previous report that an increase of unsaturated fatty acids stimulates ethanol tolerance in yeast *S. cerevisiae*

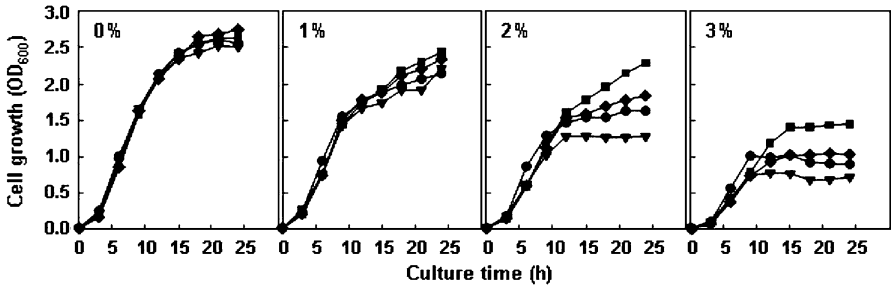

Fig. 2 Ethanol tolerance of *E. coli* strains harboring pBR322 (control, *circle*), pBR-des (*triangle*), pBR-fabA (*square*), and pBR-fabA-des (*diamond*). The cells were grown in LB medium supplemented with ethanol (0, 1, 2, or 3%) and 0.5 mM IPTG

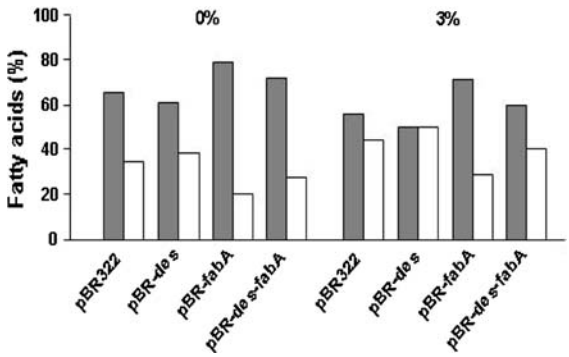

Fig. 3 Analysis of fatty acids composition of recombinant *E. coli* strains in the absence and presence of ethanol. Saturated fatty acids, *closed bar*; unsaturated fatty acids, *open bar*

(Kajiwara et al. 1996). The different effect of fatty acid composition on ethanol tolerance might be due to the dissimilar membrane structures, with membranes of eukaryotic cells consisting of more complicated components than those of prokaryotic cells.

Analysis of fatty acids composition of recombinant *E. coli* strains in the presence of ethanol

As in an earlier report (Ingram 1976), equilibration of fatty acid composition shifted to unsaturated fatty acids in all of the recombinant cells in proportion to increase of ethanol concentration. However, general fatty acid compositions were still retained in the recombinant strains at high concentrations of ethanol (Fig. 3). *E. coli* wild type and recombinant strain coexpressing *des* and *fabA* that showed similar cell growth were found to have similar fatty acid compositions.

In this study, genes related to fatty acid composition were expressed in the bacterium *E. coli* to alter the properties of the cell membrane that afford the cells a greater tolerance toward ethanol. The results were in agreement with the previous reports on response to elevated levels of ethanol, where the composition of the fatty acids in the cell membrane in individual strains changed with an increase in the proportion of unsaturated long-chain fatty acids. However, the strain with increased unsaturated fatty acids exhibited lower ethanol tolerance and the strain with increased saturated fatty acids showed elevated ethanol tolerance. This study may be of practical value for engineering strains with high ethanol tolerance.

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