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Alcohol tolerance of *Escherichia coli acrR* and *marR* regulatory mutants

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ABSTRACT

We investigated the alcohol tolerance of *Escherichia coli acrR* and *marR* regulatory mutants. The local repressor gene *acrR* and the global repressor gene *marR* were deleted either separately or together from the genomic DNA of *E. coli* for overexpression of AcrAB-TolC. Mutants with Δ*acrR* background indicated no growth difference in the exponential growth phase and modestly better growth in the stationary growth phase than the parental strain under various concentrations of alcohol. However, single *marR* mutant did not show any better growth than the parental strain in the presence of butanol and isobutanol although the efflux pump AcrAB-TolC was upregulated.

1. Introduction

The global energy crisis and an increasing awareness of the environment have led to a growing interest in developing alternatives for fossil fuels. Due to their renewable features, biofuels are potential candidates for partially or completely replacing crude oil. As hosts for biofuel production, microorganisms play an important role. Although a selection of microorganisms were engineered for such a purpose, *Escherichia coli* and *Saccharomyces cerevisiae* are the major hosts due to their well-documented genetic background and well-developed genetic tools [1–4]. In order for these microorganisms to produce biofuels viably, they must be able to survive under certain concentrations of those biofuels and other major inhibitors in the hydrolysate of lignocellulosic biomass. Unfortunately, their natural tolerance level to those alcohols and other inhibitors is not sufficient. Thus, it is essential to engineer those microorganisms to render them more resistant to higher levels of alcohols and other inhibitors.

E. coli has a natural bacterial efflux system consisting of single or multi-component drug transporters which secrete toxins, antibodies and foreign compounds to enable it to survive under harsh environments [5–7]. Those transporters can either hydrolyze ATP as primary transporters or as secondary transporters, use the proton gradient as a source of energy. There are five different classes of bacterial drug transporters [5,6]. The RND family of secondary multidrug transporters is important to the intrinsic resistance of Gram-negative bacteria to various anti-microbial compounds. It is typically encoded by chromosomal genes. This family of transporters recognizes and exports the widest range of toxic chemicals including biocides and organic solvents, contributing significantly to the antibiotic resistance of *E. coli* [7]. The AcrAB-TolC efflux system is the best-studied member of the RND family, and is also a major protein transporter involved in the survival of *E. coli* in alcohols and the inner membrane to the outer membrane of the cell [7,8].

The expression of efflux pumps is controlled by different regulators. The *acrAB* genes are regulated by a local transcriptional repressor, AcrR, and global transcriptional activator, MarA. The major role of AcrR is to repress the expression of *acrAB* genes [9]. The *E. coli mar* regulon consists of the *marRAB* operon and is responsible for the *mar* phenotype [10]. In this regulon, repressor MarR plays a key role in regulating the *mar* regulon. Overall, MarA activates the expression of the *mar* regulon, including *acrAB*, *tolC* and *marRAB* genes, while MarR represses the *mar* regulon by repressing the synthesis of MarA.

Recent studies demonstrated that AcrAB was induced by *n*-butanol [11] and a number of pumps were heterologously expressed in *E. coli* for the improved survival under stress conditions [12]. This prompted us to examine the effect of efflux pump AcrAB-TolC in *E. coli* on its tolerance to those alcohols. Using the P1 transduction method, we deleted the local transcriptional repressor gene *acrR* of AcrAB-TolC and the global transcriptional repressor gene *marR* and found that *E. coli* Δ*acrR* mutant with the upregulated AcrAB-TolC efflux pump were slightly more tolerant to various alcohols. However, Δ*marR* mutant did not show increased tolerance to butanol and isobutanol relative to the parental strain.

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Table 1
Strains and plasmids used in this study.

Strain or plasmid	Relevant genotype	Source
Strains		
E. coli MC4100 *Δara714*	*Δara714 ΔlacZ*	[13]
E. coli MC4100 *Δara714 ΔacrR*	*Δara714 ΔlacZ ΔacrR*	This study
E. coli MC4100 *Δara714 ΔmarR*	*Δara714 ΔlacZ ΔmarR*	This study
E. coli MC4100 *Δara714 ΔacrR ΔmarR*	*Δara714 ΔlacZ ΔacrR ΔmarR*	This study
Plasmids		
pKD04	*kan*	[14]
pKD46	*Amp*	[14]
pCP20	*Amp*	[14]

2. Materials & methods

2.1. Microorganisms, plasmids and culture medium

E. coli MC4100 Δ*ara714* was the parental strain [13] used in this study. Plasmid pkD46 containing the phage lambda red recombinase was used for integration of gene cassettes into the chromosome, while plasmid pkD4 was used to amplify the kanamycin marker gene. Plasmid pCP20 containing FLP-mediated recombinase was used for the removal of the antibiotic marker from the genomic DNA of the integrants [14]. *E. coli* cells were cultivated in LB broth and appropriate antibiotic was supplied where necessary. Other *E. coli* mutants created in this study were listed in Table 1.

2.2. Reagents

Isobutanol was purchased from Sigma Aldrich, while ethanol and butanol were from Fisher.

2.3. DNA manipulation

Deletion of *E. coli* genes from their chromosomal locations and subsequent curing of the antibiotic-resistant marker at the deletion sites were done using the λ-red mediated gene deletion method as described elsewhere [14]. Briefly, the gene cassette for deletion of *acrR* was PCR-amplified from *E. coli* containing pKD4 using oligo pair *del-acrR-Fwd* and *del-acrR-Rev* (Supplemental Table S1) by colony PCR. The PCR product (kanamycin resistant gene flanked by FRT sites) was electroporated into *E. coli* strain bearing pKD46 which carried genes encoding lamda Red recombinase. The resulting kanamycin resistant colonies were purified and the integration of Kan marker gene cassette was verified by colony PCR. With this strain, phage P1 lysate was created and deletion of *acrR* was transduced into *E. coli* MC4100 *Δara714* background which resulted in *E. coli* MC4100 *Δara714 ΔacrR*::*kan*. Transformation of plasmid pCP20 containing FRT-mediated recombinase into this strain looped out the kanamycin resistant gene, leaving a copy of FRT sequence in the genome of this strain which was designated as *E. coli* MC4100 *Δara714 ΔacrR*. By applying the same procedure, we further deleted *marR* gene in *E. coli* and created two other mutants which were designated as *E. coli* MC4100 *Δara714 ΔmarR* and *E. coli* MC4100 *Δara714 ΔacrR ΔmarR*.

Colony PCRs were performed in 50 μl of 1× High Fidelity Buffer (Thermo Fisher Scientific Inc., Finland) and 20 pmol of each primer, 10 μmol of each dNTP, *E. coli* cells from a colony as templates, and 2.5 units Phusion DNA polymerase (Thermo Fisher Scientific Inc., Finland) for 30 cycles on a thermal cycler (BioRad). Each cycle consisted of 10 s at 98 °C, 30 s at 56 °C and 20–70 s at 72 °C, with a final extension of 5 min. The resulting PCR products were either gel-purified for further transformation into *E. coli* for deletion of target genes or their sizes verified to confirm the deletion of the target genes.

2.4. Profiling gene expression by RT-qPCR

Overnight cell culture of *E. coli* was subcultured at 37 °C until OD_{600} reached ca. 0.6. Cells were harvested and total RNA was isolated by following the protocol for total RNA isolation from Invitrogen (Carselberg, CA, USA) with TRIzol reagents (Cat#16096020). The isolated mRNAs were reversely transcripted to cDNAs using random hexamers and the SuperScript® III Reverse Transcriptase from Invitrogen (Cat# 18080-044) at 50 °C for 1 h.

Relative quantification of the target gene expression was performed by real-time PCR on the StepOne real time system (Applied Biosystems) using Power SYBR Green. The comparative C_T method was chosen and a housekeeping gene (*ftsL*) was included for comparison. Primers for the target genes (Table S1) were designed with the Primer Express Software (Applied Biosystems) to give amplicons of 150 bp. The PCR mixture consisted of 2× Power SYBR Green PCR Master Mix (SYBR Green I Dye, AmpliTaq Gold DNA Polymerase, LD, dNTPs, passive reference and optimized buffer components), 0.25 μM of each primer, and 5 ng of cDNA template. Water was added to make up a total volume of 20 μl per well. The thermal cycling conditions were as default on the StepOne system: 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The runs were conducted with negative controls without template, and all reactions were performed in triplicate to ensure accuracy. As real time PCR using SYBR Green simply measures the increased fluorescence due to the dye binding to amplified DNA, a melt analysis was done to determine the melting temperature of the amplified DNA, and hence the specificity of the primers to our target genes. All products for a primer pair should have the same melting temperature, unless primer–dimer artifacts or other unspecific products were present. The results of the real-time PCR including the fold change in gene expression measured by the comparative C_T method were analyzed by the system.

2.5. Alcohol tolerance testing

The selected alcohols for this project were ethanol, butanol, and isobutanol. We previously performed single alcohol tolerance testing with the parental *E. coli* strain used in this study and the testing indicated that cells were not able to grow under the alcohol concentrations higher than 40 g/L ethanol, 5 g/L butanol and 5 g/L isobutanol, respectively. Based on that, we selected those alcohol concentrations as the highest ones for the tolerance testing for the mutants. Another concentration below the threshold concentration was selected as well for comparison. LB media containing different concentrations of those chemicals were prepared by serial dilution and each mutants created previously was tested for growth in the resulting media. Briefly, the overnight cell culture of those mutants was inoculated into 20 ml of LB containing the individual alcohols and inhibitors at various concentrations in 50 ml Eppendorf tubes. To minimize the evaporation of alcohols, the tubes were tightly capped post inoculation. The growth of *E. coli* mutants was monitored by analysis of OD_{600} of the resulting cell culture. Each trial was repeated thrice and the parental *E. coli* strain MC4100 *Δara714* was used as control.

3. Results and discussion

To examine the tolerance of *E. coli* with the up-regulation of AcrAB-TolC efflux pump to various alcohols, we deleted the local repressor gene *acrR* and the global repressor gene *marR* from the genomic DNA of *E. coli*. Using chromosomal gene deletion method in *E. coli* [14], we created two single mutants with Δ*acrR* and Δ*marR*

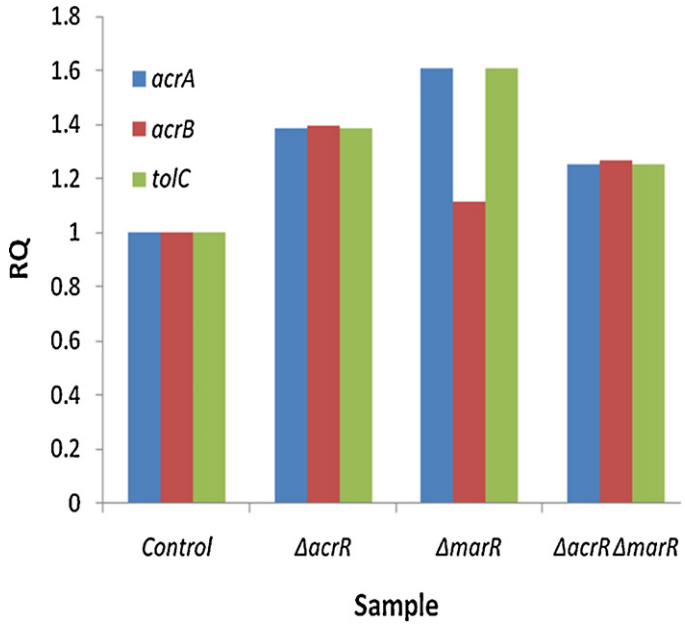

Fig. 1. RT-qPCR analysis of *acrA*, *acrB* and *tolC* transcripts in the *acrR* and *marR* mutants listed. RQ indicates relative quantity.

background respectively, and a double mutant in which both *acrR* and *marR* were deleted from its genome. The deletion of those genes from *E. coli* genome was verified by PCR amplification of the corresponding locus (supplementary Fig. S1) in its genome and further confirmed by RT-qPCR analysis (supplementary Fig. S2).

Using *ftsL* as a housekeeping gene, quantitative PCR analysis of cDNA produced from mRNA transcript from those mutants as well as the parental *E. coli* strain was performed to analyze the activation of AcrAB-TolC efflux pump. Overnight cultures grown at 37 °C were subcultured (1:100) and grown to an $OD_{600} \approx .6$ at 37 °C. Total RNA was isolated and mRNA was reversely transcribed to cDNA and subjected to RT-qPCR [15]. We examined *acrA*, *acrB* and *tolC* expression in those mutants relative to the parental strain. As indicated in Fig. 1, both *acrA* and *acrB* transcripts indicated an increase in all the mutants relative to the parental strain. As expected, RT-qPCR analysis also revealed that *tolC* transcripts were overexpressed among those mutants compared to the parental strain. Taking together, RT-qPCR analysis of *acrA*, *acrB* and *tolC* clearly demonstrated that AcrAB-TolC efflux pumps were upregulated in those mutants relative to the parental strain.

Using mutants with various Δ*acrR* and Δ*marR* backgrounds, we performed tolerance testing to the selected alcohols under two different concentrations. As indicated in Fig. 2, both the *acrR* and *marR* single mutants indicated no growth difference in the exponential growth phase with the parental strain. However, the single mutants showed slightly better growth than the parental strain in the stationary growth phase under both 30 and 40 g/L ethanol in LB medium. At 30 g/L ethanol, the highest OD_{600} after 6 days' culture reached up to 0.991 for Δ*marR* mutant and 1.131 for Δ*acrR* mutant, which were 1.1- and 1.2-fold higher than the parental strain respectively. In addition, the single mutant Δ*acrR* indicated better growth than the single mutant with Δ*marR* background (Fig. 3A) under 30 g/L ethanol. Under 40 g/L ethanol in medium, although no significant difference in growth was detected among those mutants and the parental strain (Fig. 3B), we did observe that all the mutants indicated a slightly higher OD_{600} than the parental strain during the testing.

In terms of butanol and isobutanol tolerance testing, we observed the similar phenomena with the ethanol tolerance testing with *acrR* mutants. Both the single and double mutants with Δ*acrR* background indicated a slightly better growth in the stationary growth phase than the parental strain at both 2 g/L and 5 g/L butanol and isobutanol respectively (Figs. 3 and 4). In addition, a further deletion of the global transcription repressor MarR in Δ*acrR* strain did not demonstrate a combined effect on cells' tolerance to those compounds. However, either no difference or slight decrease in growth was observed for the single Δ*marR* mutant relative to the parental strain under those testing conditions (Figs. 3 and 4) although the AcrAB-TolC efflux pump was upregulated. This implies that the mechanism of butanol and isobutanol stress response is different from that of ethanol.

The interest in biofuel technology has resulted in a need to develop microorganisms with higher tolerance to alcohols and the major inhibitors present in the hydrolysate of lignocellulosic biomass. For most biofuel programs, a host microorganism with higher tolerance to a selected alcohol and other inhibitors is essential for an economically viable process. Such a trait can be engineered by either adaptive evolution [16] or direct

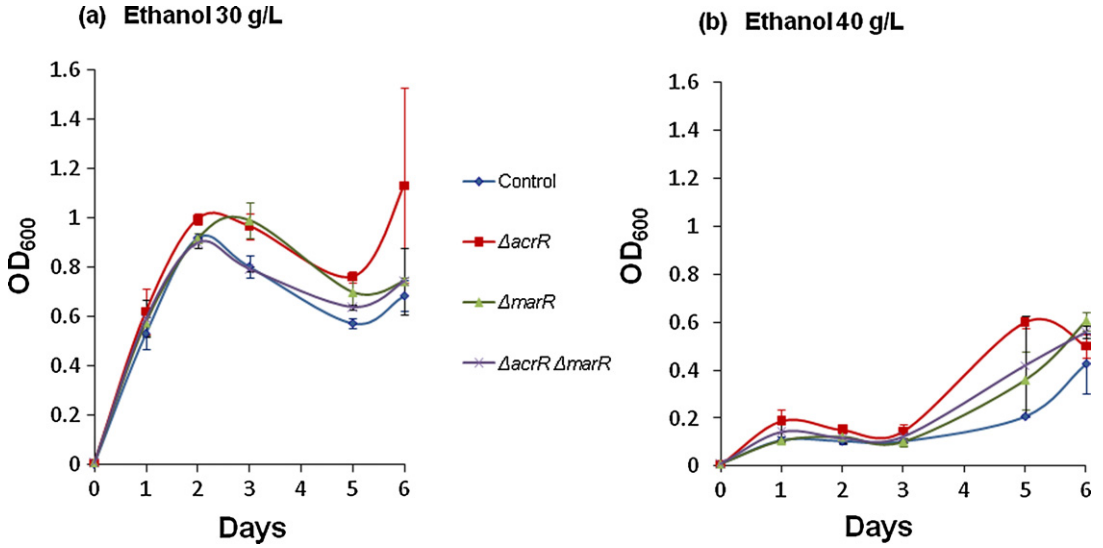

Fig. 2. Ethanol tolerance testing of the *E. coli* mutants. The growth of the *ΔacrR*, *ΔmarR*, *ΔacrR ΔmarR* mutants and the parental *E. coli* strain under 30 g/L (a) and 40 g/L (b) ethanol in LB medium.

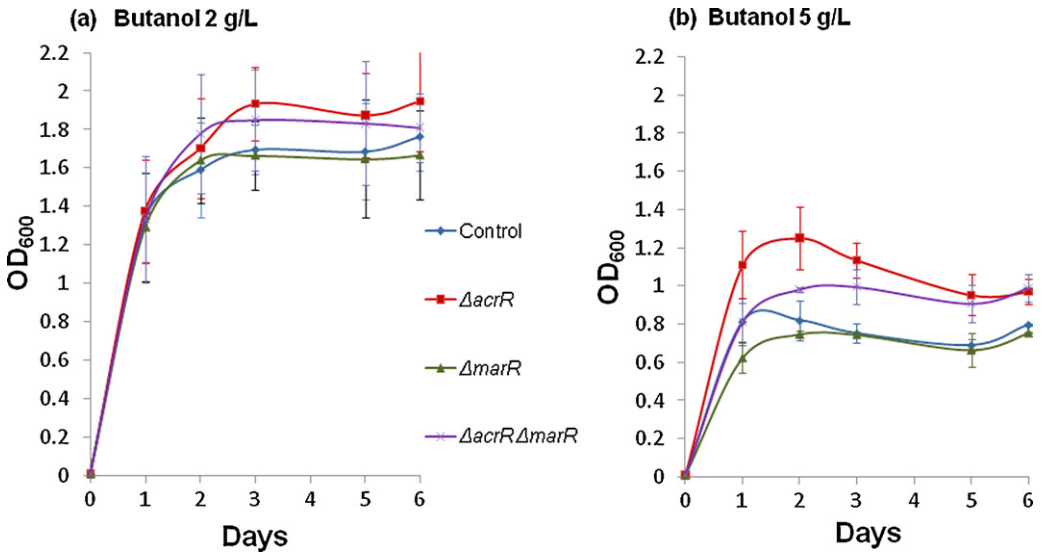

Fig. 3. Butanol tolerance testing of the *E. coli* mutants. The growth the *ΔacrR*, *ΔmarR*, *ΔacrR ΔmarR* mutants and the parental *E. coli* strain under 2 g/L (a) and 5 g/L (b) butanol in LB medium.

molecular manipulation such as genome shuffling [17] and gTME [18]. Here we investigated the effect of overexpression of AcrAB-TolC efflux pump in *E. coli* on its alcohol tolerance. As expected, deletion of the transcription repressor gene upregulated the expression of AcrAB and TolC, which was verified by RT-qPCR analysis (Fig. 1). However, no significant increase of alcohol tolerance was observed in spite of a modest increase in *acrR* mutants. Furthermore, we observed that deletion of the global transcription repressor gene *marR* had no positive contribution to cells' tolerance to butanol and isobutanol (Figs. 3–4). As a repressor, MarR is known to plays an important role in regulation of AcrAB-TolC efflux pump through MarA. Although deletion of *marR* leads to the overexpression of AcrAB-TolC, it might negatively affect either the expression of other members of MarA regulton or imply the stress response of *E. coli* to C4 alcohols is different with ethanol as suggested by Atsumi et al. [19].

In agreement with the recent studies by other groups [19,20], our *marR* mutant with the upregulated AcrAB-TolC efflux pump was less tolerant to butanol and isobutanol than the parental *E. coli* strain. However, the *acrR* mutants in this study indicated a modest increase in tolerance to butanol and isobutanol. The tolerance difference between *marR* and *acrR* mutants may suggest other factors contributing to the alcohol tolerance phenotype [21] in addition to the AcrAB-TolC efflux pump. In consideration of butanol stress affecting the membrane stability of microorganisms [24], we then further analyzed the gene expression level of a few membrane proteins including the periplasmic chaperon SurA, the outer membrane protein OmpA and the β-barrel assembly machinery BamA in those two mutants. The RT-qPCR profiling indicated that there was no significant difference in the transcription levels of *surA* and *bamA* between the *acrR* and the *marR* mutants (supplementary Fig. S3). However, the transcription level of *ompA* in the *acrR* mutant

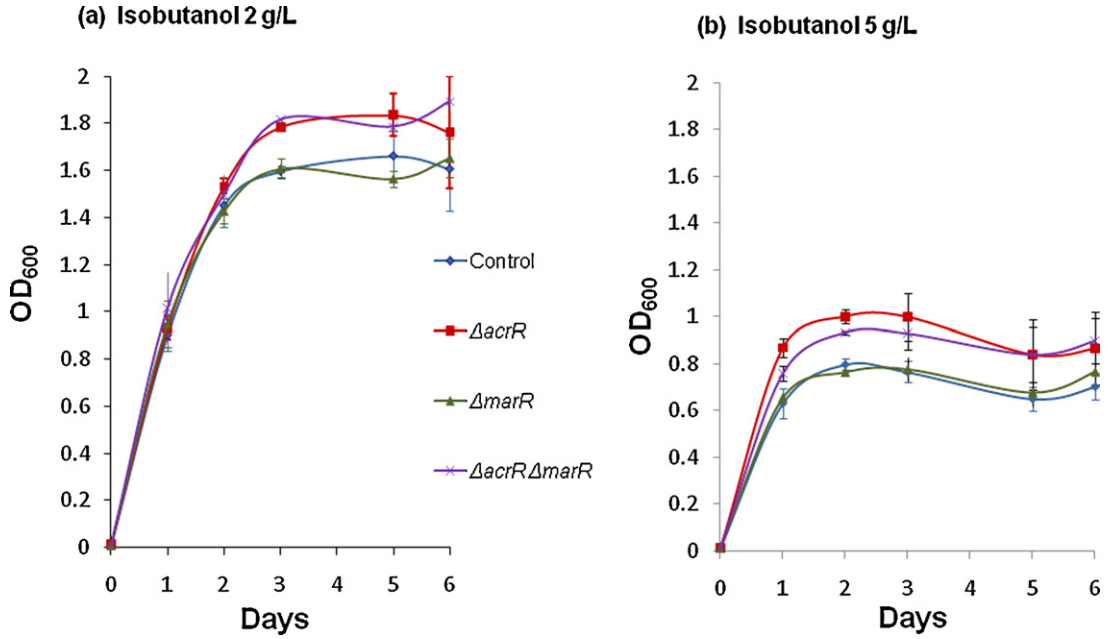

Fig. 4. Isobutanol tolerance testing of the *E. coli* mutants. The growth of *ΔacrR*, *ΔmarR*, *ΔacrR ΔmarR* mutants and the parental *E. coli* strain under 2 g/L (a) and 5 g/L (b) isobutanol in LB medium.

was slightly higher than that in the *marR* mutant. OmpA is known to have a large C-terminal soluble domain in the periplasm which is thought to interact with the peptidoglycan layer for membrane stability [25,26]. The slightly higher level of *ompA* transcripts in the *acrR* mutant might explain the modest increase of C4 alcohol tolerance of the *acrR* mutant as compared to the *marR* mutant.

E. coli is known to have approximately 37 putative efflux systems in its genome which are able to transport a variety of structurally-unrelated toxic compounds. Among those efflux pumps, the AcrAB-TolC efflux pump contributes to the natural resistance of the bacterium to inhibitors [22]. Similar to many RND pumps characterized in other Gram-negative species, AcrAB-TolC is able to transport a wide range of compounds including oxacillin, chloramphenicol, tetracycline, erythromycin, etc. [23]. A recent study also demonstrated that mutants SA481 and TW306 with the downregulated AcrAB-TolC efflux pump did not show increased tolerance to ethanol [19]. Considering the many factors involved in cell's response to stress, its effect on the tolerance of *E. coli* to alcohols will need to be further investigated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2011.11.013.

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