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N-ALKANOL STRESS-INDUCED CELL ENVELOPE INJURY OF σ^E PROMOTER IN *Escherichia coli*

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ABSTRACT

To characterize the cellular stress by *n*-alkanols with different alkyl chain lengths in *Escherichia coli*, we investigated how *n*-alkanols damage cell envelope permeability and whether they enhance the promoter activity of the envelope stress response regulator, σ^E, by using variants of green fluorescent protein (GFP). By using *E. coli* cells having GFPuv expressing and localizing in the cytoplasm, the inner membrane, and the periplasm, after exposure to *n*-alkanols, the fluorescent intensity of GFPuv released from cells was examined. Our data showed that at the similar levels of cell death of about 90–97%, ethanol, a short-chain alkanol, at a concentration of 20% damaged the outer membrane more greatly than the inner membrane, whereas a longer-chain alkanol of pentanol at a concentration of 1.125% damaged both of the outer and inner membranes. Then we investigated the envelope stress response to *n*-alkanols by σ^E factor by ratiometric analysis of *rpoE* promoter activity for the downstream GFPuv expression referenced to that of housekeeping sigma 70 (σ^{70}) recognizing *lacUV5* promoter for red fluorescent protein (RFP) expression. The results indicated that the relative activity of *rpoE* promoter by pentanol was much greater than that of ethanol. The degree of its sensitization by *rpoE* deficiency was much more remarkable for cells treated with pentanol than for those with ethanol. The results suggest that the response of the σ^E plays a significant role in the membrane integrity and survival of *E. coli* cells treated with n-alkanols depending on the alkyl chain length of the molecule.

Keywords: *n*-alkanols, membrane injury, envelope stress response, sigma E, *Escherichia coli.*

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INTRODUCTION

Pathogenic *E. coli* strains contaminating foods, feeds or fruits can cause serious diseases for humans and animals. *n*-alkanols have been known to possess antibacterial activity. In particular, ethanol has substantial killing activity on bacteria cells and therefore it has been extensively used for sanitation disinfection and cleaning in food manufacturing, medical instruments and equipment, the environmental surface of materials and space, and human skin in solution or vapor. Like other disinfectants and sanitizers, the killing action of alcohol is nonspecific as compared with antibiotics, and therefore many targets of the action such as nucleic acids, proteins, intracellular enzymes, and cell membranes have been proposed, although the membrane damage in alkanol stressed cells of *E. coli* have been investigated and studied (Tsuchido et al., 1985; Ingram et al., 1980; Fried et al., 1973; Harold et al., 1970; Hugo et al., 1967). The detailed mechanism remains unclear. The response of *E. coli* cell envelope to *n*-alkanols stress has been investigated for the composition of fatty acids in the membrane and membrane fluidity (Ingram et al., 1976), lipid solubility (Jain et al., 1977; MacDonald et al., 1978; Paterson et al., 1972) and the changes of lipid composition in growing cells (Silbert et al., 1970). Sullivan et al. (1979) indicated that short-chain alcohols of up to four carbons in length induce large changes in the fatty acid composition, which is in contrast to those induced by the longer-chain alcohols. As well as ethanol and other alcohol, benzyl alcohol has been also shown to directly affect membrane fluidity (Jain et al., 1977; Ingram et al., 1976; Hubbel et al., 1970; Paterson et al., 1972; Grisham et al., 1973). However, the stresses by short and long-chain *n*-alkanols on the cellular inner and outer membrane, in gram-negative bacteria and the role of the sigma E factor for cellular response and survival under those *n*-alkanols have not been characterized.

It has been known that the σ^E factor plays a very important role for cell response and growth under ethanol and heat stresses (Keichiro Hiratsu et al., 1995). The σ^E - directed envelope stress response maintains outer membrane homeostasis is an important virulence determinant upon host infection in *E. coli* and related bacteria (Makinoshima et al., 2002; Mutalik et al., 2009). Stress activates the response to intracellular signals related to the growth of phase and nutrient availability (Silbert et al., 1970; Sullivan et al., 1979; Mutalik et al., 2009).

The model for cell regulation and response to heat or alkanol stress has been described (Ingram et al., 1976; Hubbel et al., 1970). Although, under non-stress conditions, σ^E is inactivated with anti-sigma factor RseA, and a periplasmic protein, RseB. Heat and alkanol cause the release of σ^E through cleavage of RseA. Resultantly, σ^E binds to RNA polymerase to activate target promoters (such as P*rpoE* and P*degP*) for the expression of envelope homeostasis proteins. Furthermore, σ^E also contributes to cytoplasm homeostasis of the cell responding to the stress by activation of the transcription of the *rpoH* gene encoding σ^{32} to produce cytoplasmic heat shock proteins. The activity of *rpoE* promoters recognized by σ^E was indicated even under normal conditions of cell cultivation (Mutalik, 2009). Under ethanol stress conditions, *Salmonella* cell with the *rpoE* gene-deficient is dramatically reduced (Gabriela et al., 2002). Although the important role of σ^E for cellular response to heat and ethanol stress was indicated, the promoter activity of *rpoE*-regulated genes has not been evaluated in the presence of alkanols, especially a longer alkyl chain type.

In the present work, we analyzed and compared the cell membrane injury in *E. coli* cells exposed to short and long-chain *n*-alkanols by using plasmids for expression of reporter GFPs localizing at periplasm and cytoplasm as well as the inner membrane. The future application of this model was also suggested.

MATERIALS AND METHODS

Strains and cultivation

E. coli KP7600 (no *lac*I^q repressor, a W3110 derivative) and BL21 were used in this study. These *E. coli* cell strains were cultivated in LB medium (components: yeast extract 5 g, NaCl 10 g, Tripton 10 g, distilled water 1 L) with shaking at a constant rate (120 rpm) at 30 ^oC. Overnight pre-cultures at a final OD_{650} of 0.3 were inoculated into fresh medium. Ampicillin sodium salt 100 $\mu g/mL^{-1}$ was added to the growth medium for plasmid selection. IPTG (0.1 mM) was added for promotion of GFP expression if necessary Pcp and PIN. Chloramphenicol (0.05 mM) was added to stop gene expression if necessary. Cell growth was monitored by measuring the turbidity at $OD_{650\ nm}$.

Plasmid construction

Vector plasmids for GFP expression at various locations of the cell envelope

The following vectors were previously constructed and given by Testsuaki Tsuchido Laboratory, Kansai University, Japan. Vector Pcp: pGFP-uv-His without signal peptides was used for expression of GFP located almost in the cytoplasm. Vector PIM: pEF-2la-qcrA (in) GFPuv-His-TAT, harboring T7-*lac* promoter and GFP variant gene fused with a specific sequence of signal peptide harboring twin arginines and lipobox carrying the terminal amino acid sequences with cysteine of LAACD, that will drive the expressed GFP to be transported and located in inner membrane by TAT pathway. Vector Ppp: pMal-p2-GFPuv harboring MalE (signal peptidase I) that will drive the expressed GFP to be transported and located in the periplasm.

GFPuv as a green fluorescent protein variant expression from vector Pcp (GFP expressed almost in the cytoplasm and a small part in periplasm) for analysis of cell envelope injury by *n*-alkanols. *E. coli* KP7600 without *lac*I^q, could not be used for GFP expression from vector PIM (GFP expression in the inner membrane) and vector Ppp (GFP expression in periplasm), and this strain was replaced by *E. coli* BL21 for GFP expression in the inner membrane from vector PIN and in periplasm from vector Ppp. *E. coli* KP7600 was used for GFP expression from vector Pcp: pGFP-uv-His without signal peptides, expression of GFP, and RFP (red fluorescent protein) from promoter assay vector pTwoFP.

Table 1. Vector plasmid for GFP expression at various sites of *Escherichia coli* cells

Plasmid	Signal peptide parts preceding to GFPuv	Signal peptide sequence	Localization in *E. coli* BL21
Pcp: pGFPuv-His	None	None	Cytoplasm
PIM: pEF-2la-qcrA1 (IN)-GFPuv-His	QcrA plus YokF (Lipobox, S2D)	MGGKHDISRRQFLNY TLTGVGGFMLAACD	Inner membrane
Ppp: pMal-p2-GFPuv	MalE (signal peptidase I)	MKIKTGARILALSALT TMMFSASA	Periplasm

Vector Pcp and PIM and Ppp were used for GFP expression in the cytoplasm, inner membrane, and periplasm, respectively, as reporter sensors for monitoring the injury of the cell envelope by *n*-alkanol stress.

Construction of promoter assay vectors for the measurement of the specific rpoE promoter activity to σ^E

For quantitative measurement of the promoter activity *in vivo*, two vector plasmids pTwoFP1 and pTwoFP2 were prepared, which consisted of two types of fluorescent protein genes, one for the red fluorescent protein (RFP) and the other for the green fluorescent protein (eGFP) in the downstream of either a *lacUV5* reference promoter or target *rpoE* promoter (Fig. 1). Plasmid pTwoFP1 contains egfp and rfp genes both directed by *lacUV5* promoter, whereas plasmid PTwoFP2 does RFP gene directed by the reference promoter *lacUV5*, and

GFP gene directed by the test promoter of *rpoE*, a target and specific promoter of σ^E.

The test *rpoE* promoter sequence was amplified from the DNA genome of *E. coli* cell OW6 strain, using primer pairs 5'-GAT CCGTCTACAGCATGACAAA-3' (forward) and 5'-CCGAGGTAAAGGATCCCCAAAC-3' (reverse), introducing a *Bam*HI site in the reverse primer. The protocol from the Kits of iProof™ High Fidelity DNA polymerase (BIO-RAD) was used. The application temperature program was as follows: 30s at 98 °C, 35 cycles including denaturation 10 s at 98 °C, annealing 15s at the primer's melting temperature of 59.5 °C, elongation 4 s at 72 °C, and finally prolonged elongation 10 min at 72 °C. The PCR product of the amplified fragment was purified from 1% agarose gel. The purified fragment digested by *Bam*HI was ligated into multi cloning site (MCS) of vector pTwoFP which was previously digested with *Bam*H1 and *Sma*I, resulting in a recombinant vector plasmid pTwoFP2 for GFP expression directed the test *rpoE* promoter.

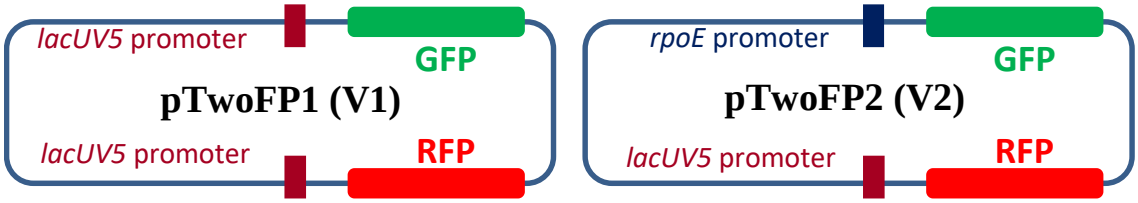


Figure 1. The control vector pTwoFP1 (V1) harbored two reference *lacUV5* promoters for control GFP and RFP expression. The constructed vector pTwoFP2 (V2) harbored the test *rpoE* promoter and *lacUV5* promoter for control of GFP and RFP expression, respectively

_n_-Alkanols stress treatments

E. coli KP7600 harboring vector Pcp, *and E. coli* BL21 harboring vector PIM, were cultured for 24 hours in LB medium at 30 °C by shaking at 120 rpm. Cells were washed and re-suspended in KPB buffer 50 mM at an OD_{650} of 3, and then exposed to various concentrations of ethanol (10%, 15%, 20%, 25%) and pentanol (0.75%, 0.875%, 1%, 1.125%, 1.25%, 1.375%) with shaking at 30 °C and 120 rpm for 1hr. GFP fluorescent distribution in the inner membrane, extracellular supernatant, periplasm, and cytoplasm fractions was evaluated after n-alkanol stress.

Viability measurement

The survival of *n*-alkanol treated cells was determined by the growth-delayed analysis method using a Bio-scanner (Takano & Tsuchido 1982): 0.4 ml of cell suspension was added to 3.6 ml of LB medium, and then cultivated at 37 °C with shaking. Besides this, the conventional colony count was also used by using LB agar medium (components: yeast extract 5 g, NaCl 10 g, Tripton 10 g, agar 20 g, distilled water 1 liter).

Analysis of cell envelope injury by *n*-alkanol stress

Fractionation of periplasm and cytoplasmic membrane

The transformed cells of *E. coli* KP7600 harboring vector Pcp which expresses GFP in the cytoplasm were cultivated, 1ml of the culture (OD_{650} = 3) was washed 3 times with 20 mM Tris-HCl buffer (pH: 7.5). Resuspending the cells in 1ml of hypertonic buffer containing 100 mM Tris-HCl at pH: 8, 15% sucrose, and 1 mM dithiothreitol (DTT). The cell suspension was incubated on ice for 10 min, followed by centrifugation at 12,000 rpm for 10 min at 4 °C. The pellet was gently resuspended in deionized and sterilized H_2O, followed by incubation on ice for 10 min, centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant contained only periplasmic protein and the resultant pellet was gently resuspended in deionized and sterilized water, and the cell suspension was incubated on ice

for 10 min and then centrifuged at 12,000 rpm for 10 min at 4 ^oC. The supernatant contained substantially periplasmic proteins and the pellets contained the cytoplasmic proteins (Paterson et al., 1972). The GFP content in the cytoplasm and periplasm of the control sample was calculated as 100%. All other values in treated samples were also calculated based on the value from the control sample.

Fractionation of outer and inner membrane GFP

Outer membrane protein (OM-protein) and inner membrane protein (IM-protein) were separated and recovered by the lysozyme-EDTA spheroplast method (Grisham et al., 1973).

The transformed *E. coli* BL21 harboring vector PIN was cultivated for 4 hrs and followed by the addition of IPTG 0.1 mM, for GFP expression in the inner membrane for 24 ours, in LB medium at 30 ^oC. Ethanol and pentanol were also applied as the mentioned method. IM and OM protein were recovered by the TSD method (Hernandez et al., 2008). 10 mL of cells (OD_{650} = 3) were recovered by centrifugation at 10,000 rpm for 5 min and then rapidly re-suspended in 10 mL of cold buffer solution of 0.75 M sucrose and 10 mM Tris-HCl buffer, pH 7.8. Lysozyme was added at a final concentration of 100 μg/mL, and incubation on ice for 2 minutes. Then cells were slowly diluted at a constant rate for 8 to 10 minutes by using the peristaltic pump with 1V (10 mL) of cold 1.5 mM EDTA (Na^+), pH 7.5, for conversion to spheroplasts. Spheroplasts were lyzed by sonication for 5 minutes at 4 ^oC. The solution of lyzed spheroplasts was centrifuged for 20 minutes at 1200 g for recovery of supernatant solution of the total of IM, OM, PP, and CP protein in the upper phase. Centrifugation at 50,000 rpm for 2 hours at 4 ^oC was carried out to recover total OM and IM -GFP in the pellets. OM-GFP and IM-GFP were separated by sarkosyl solution (0.5% sarkosyl in 10 mM Na_2HPO_4, pH 7.2) by centrifugation at 50,000 rpm for 2 ours at 4 ^oC. The sarkosyl solution only dissolves IM-protein, so that, IM-protein and OM-protein will be located in the supernatant and the pellets, respectively. Besides this, to confirm the accurate results of separation of OM and IM-protein as well as OM-GFP and IM-GFP from the sarkosyl solution, the solution of the total of IM-protein and OM-protein was separated and recovered by sucrose density gradient that was prepared by layering of 55, 50, 45, 40, 35, 30 and 25% sucrose solutions (w/w), in 5 mM EDTA, pH 7.5. Solution of total OM and IM protein were added and layered on top 50,000 rpm for 16 hrs at 4 ^oC was carried out in the SW rotor. OM-protein and IM-protein were separated into heavy fragments and light fragments. In each fragment, FI of GFP and activity of IM-enzyme of D-lactate dehydrogenase and OM-enzyme of phospholipase were also assayed and measured to confirm the results.

Promoter activity assay

The response and resistance of *E. coli* cells to various n-alkanol concentrations were investigated based on the response of envelope stress of σ^E. Cells grown in LB medium with and without alkanol were harvested by centrifugation, re-suspended in KPB buffer, and diluted to obtain OD_{650} of 0.4, for analysis FI of all samples. For quantitative measurement of the promoter activity *in vivo* with various *n*-alkanol concentrations, the measurements of the fluorescent GFP and RFP expressed in *E. coli* were carried out at the excitation wavelength of 474 nm and 573 nm, at the emission at wavelengths of 509 nm and 610 nm, respectively. The operation of σ^E was evaluated based on the activity of its specific and target *rpoE* promoter in comparison with the housekeeping *lacUV5* promoter. The strength of the *rpoE* promoter was evaluated based on the ratios of fluorescent intensity of GFP-*rpoE*/RFP-*lacUV5* from vector V2 divided to the value of the fluorescent intensity of GFP-*lacUV5*/RFP-lacUV5-from vector V1. The operation and activity of σ^E to respond to *n*-alkanols were evaluated based on the strength of the test rpoE promoter.

RESULTS

Characteristics of short and long-chain *n*-alkanols of ethanol and pentanol stress treatment-induced cell death and cell envelope injury

The effect of ethanol and pentanol stress treatments on the cell death

We examined the survival of *E. coli* KP7600 cells transformed with Pcp plasmid after ethanol and pentanol treatments. After cell cultivation in LB medium for 24 hours, cells were washed in KPB buffer at pH 7.5 and then treated with *n*-alkanols for 1 hour. The cell survivals were analyzed by Bio-scanner, and counting colonies in LB agar medium.

We investigate the cell survival after stress treatment of ethanol (C2) with various concentration of 10%, 15%, 20% and 25%; and pentanol (C5) with various concentration of 0.75%, 0.875%, 1%, 1.125%, 1.25% and 1.375%. The results are in Figure 2. demonstrated that a similar level of cell death reached 90–97% when the cells were treated with 20% of ethanol, and 1.125% of n-pentanol at 30 ^{o}C. Cells were much more sensitive to long-chain *n*-alkanol of pentanol than ethanol. The treatment with both 25% ethanol and 1.375% pentanol at this temperature caused the death of almost 100%, the experiment was repeated 3 times and calculated for an average value.

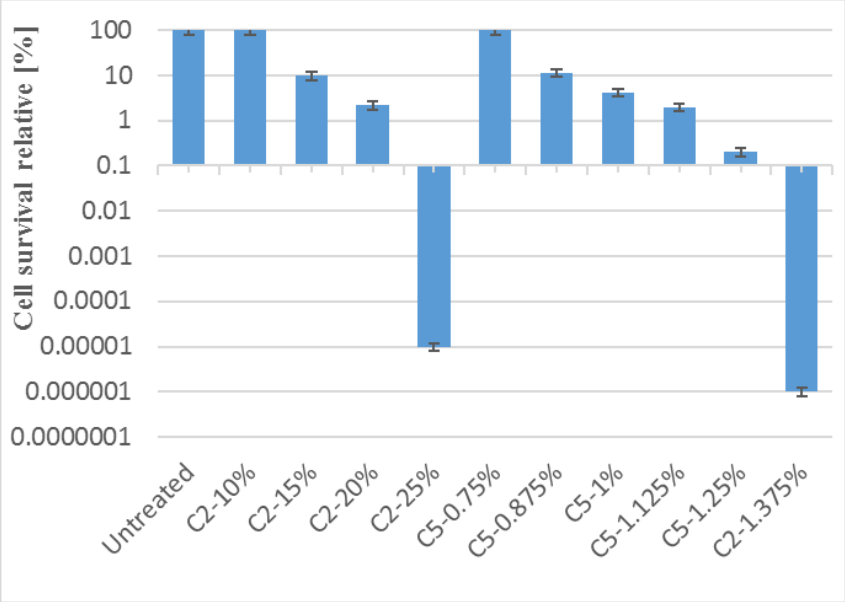


Figure 2. Cell survival of *E. coli* KP7600 after ethanol (C2) and pentanol (C5) stress treatments at various concentrations

The effects of ethanol and pentanol on the release of GFP and A260 from cells

From the above-treated cells with ethanol and pentanol, the periplasm and cytoplasm fractions were obtained. Levels of a large molecular GFPuv and small molecules of A260 materials including ATP, AMP, ADP, TTP, CTP, and UTP,… were measured from each fraction, and the experiment was repeated 3 times and calculated for an average value. The results are indicated in Figures 3, 4.

For comparison of the effect of ethanol and pentanol on the possible injury of the inner membrane and outer membrane at a similar level of cell death of 90−97%, the data of the treatment of 20% ethanol and 1.125% pentanol were summarized in Table 2.

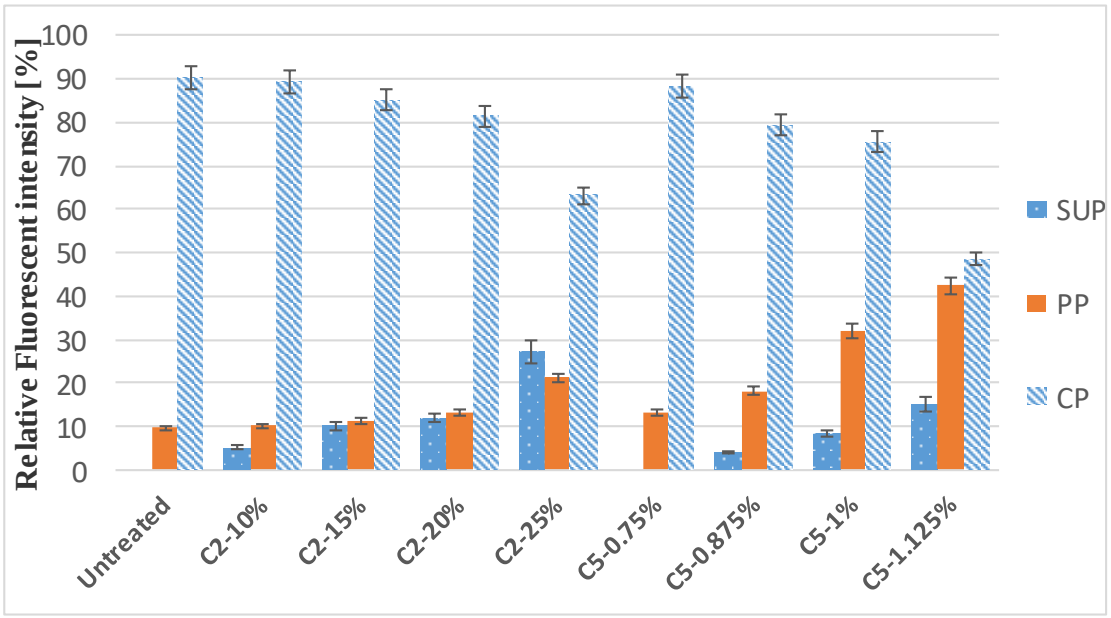


Figure 3. The fluorescent intensity of GFP from recovered fragments in the cytoplasm (CP), periplasm (PP), and supernatant (SUP) fractions, was obtained from cells treated with and without stress treatment of ethanol (C2) and pentanol (C5)

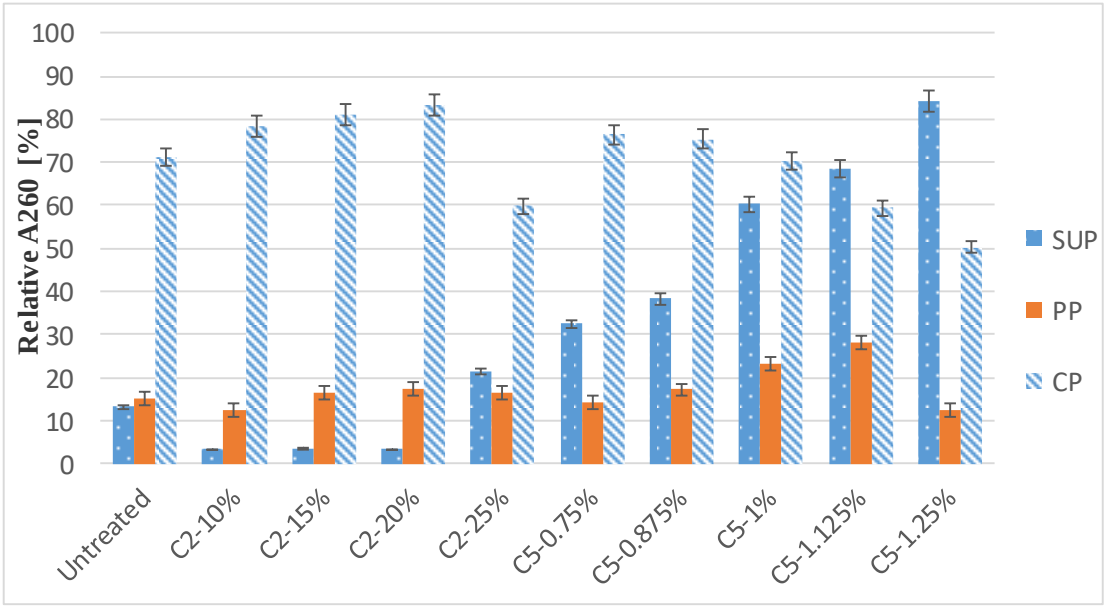


Figure 4. The absorbance at A260 nm of the cytoplasm, periplasm, and supernatant fractions, obtained from cells with and without stress treatment of ethanol (C2) and pentanol (C5)

We investigated the released level of GFP and A260 materials in recovered fractions of the cytoplasm, periplasm, and supernatant from stressed cells treated with ethanol and pentanol for 1 hour at 30 ^{o}C, the experiment was repeated 3 times and calculated for an average value. The results indicated in Table 2, in the supernatant

fraction, there were 13% and 11% of released PP-GFP, respectively, in comparison with the control sample of 0%. However, there are 3% and 70% of released A260 materials, respectively, in comparison with the control sample of 13%. In the periplasm fraction of the control and ethanol samples, the released levels amount to 10% and 14% for PP-GFP, 15% and 17% for A260 respectively, in comparison with 31% PP-GFP and 28% PP-A260 in the pentanol sample. In the cytoplasm fraction, the control and ethanol samples, there are 90% and 83% of remained CP-GFP, 74%, and 85% CP-A260 respectively, in comparison with 63% CP-GFP and 60% CP-A260 in the pentanol sample. The bioassay experiment of cytoplasm enzyme β-galactosidase was also analyzed, cytoplasmic enzyme β-galactosidase was only recovered from the cytoplasm fraction and not detected in periplasm and supernatant (data not shown).

Table 2. Comparison of the release of GFP and relative level of A260 in recovered fractions of the cytoplasm, periplasm, and supernatant from stressed cells treated with ethanol and pentanol for 1 hr at 30 ^{o}C

Fraction	Control sample		By ethanol		By pentanol	
	GFP	A260	GFP	A260	GFP	A260
Supernatant	0	13	13	3	11	70
Periplasm	10	15	14	17	31	28
Cytoplasm	90	74	83	85	63	60
Total (%)	100	102	110	105	105	158

These results demonstrated that both ethanol and pentanol damaged the outer membrane indicated by the release of PP-GFP into the supernatant. However, pentanol strongly destabilized the inner membrane for release at the high level of A260 materials through the inner membrane and the outer membrane. On the contrary, ethanol did not so much affect the inner membrane, it prevented the release both of CP-GFP and CP-A260 materials from the cytoplasm through the inner membrane in comparison with the control sample.

The effect of ethanol and pentanol on the release of GFP from its location at the inner membrane

Fractionation of inner membrane and outer membrane proteins and GFP: The plasmid PIM (pEF-2la-qcrA (in) GFPuv-His-TAT) harboring *lac* promoter and GFP gene carrying specific signal peptide sequence in lipobox with two cysteine amino acids, was used for the expression of GFP locating at the inner membrane of *E. coli* BL21. *E. coli* KP7600 without *lac*I^q repressor so that lac promoter from vector PIM cannot be expressed in this strain. *E. coli* BL21 was used and replaced with *E. coli* KP7600 for GFP expression in the inner membrane to analyze the release of IM-GFP. To know how ethanol and pentanol destabilize the inner membrane, we measure the amount of GFP released from its localizing inner membrane. The sucrose density gradient centrifugation analysis showed that both IM-GFP and D-lactate dehydrogenase were successfully recovered in the light fraction layers from fractions 2 to 5 from a total of 24 fractions of sucrose gradients. Whereas, in the heavy fractions, only an outer membrane enzyme phospholipase was recovered from fraction15 to 19 of sucrose gradients although no OM-GFP was detected (data not shown). These results confirmed that there is no OM-GFP, only 10% IM-GFP was successfully expressed in *E. coli* BL21. As shown in Figure 5, after treatment with ethanol and pentanol, the released IM-GFP were 35% and 65% respectively, at a similar level of cell death of 90−97%, the experiment was repeated 3 times and calculated for an average value.

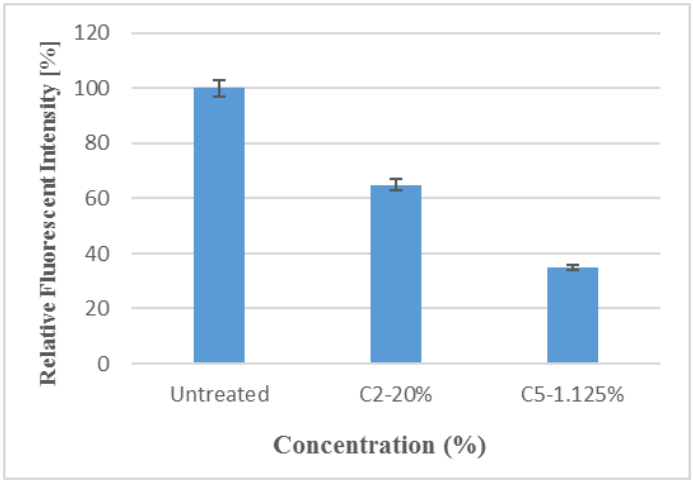
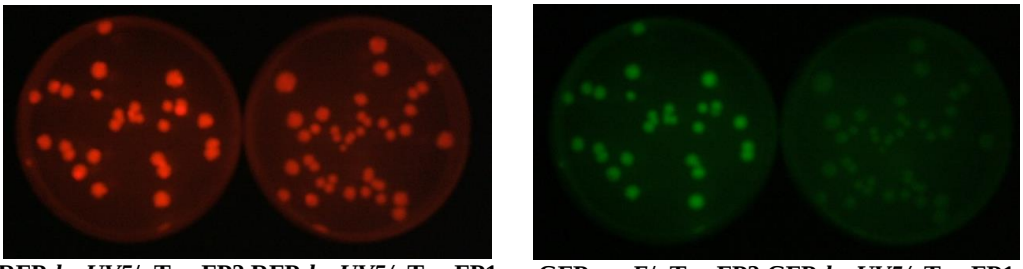


Figure 5. The fluorescent intensity of GFP from recovered fragments of the inner membrane with and without stress treatment of ethanol (C2) and pentanol (C5)

Investigation of the significant operation and activity of σ^E response to *n*-Alkanols induced activation of *rpoE* promoter

To investigate the activity of σ^E after exposure to ethanol and pentanol stress, the plasmid pTwoFP2 harboring *rpoE* promoter specific to σ^E for GFP expression and a reference *lacUV5* promoter for RFP expression; together with control plasmid pTwoFP1 harboring two *lacUV5* promoters for reference of GFP and RFP expression, were transformed into *E.coli* cells described above. The expression of GFP and RFP in *E. coli* cell colonies were shown in Figure 6. The result indicated that the fluorescent intensity of GFP controlled by the *rpoE* promoter was much higher than that controlled by the housekeeping *lacUV5* promoter.



RFP-*lacUV5*/pTwoFP2 RFP-*lacUV5*/pTwoFP1 (a) **GFP-*rpoE*/pTwoFP2 GFP-*lacUV5*/pTwoFP1 (b)**

Figure 6. Fluorescent intensity of RFP expression (**a**) and GFP expression (**b**) was controlled by *lacUV5/lacUV5* and *rpoE/lacUV5* promoter, respectively in *Escherichia coli* cells

The response and resistance of *E. coli* cells to various n-alkanol concentrations were investigated based on the response of envelope stress of σ^E. The strength of the *rpoE* promoter controlled by σ^E in comparison with the *lacUV5* promoter (house-keeping promoter), was evaluated and based on the ratios of fluorescent intensity of GFP-*rpoE*/RFP-*lacUV5* from vector V2 divided to the fluorescent intensity of GFP-*lacUV5*/RFP-*lacUV5* from vector V1. The results in Figures 7, 8 indicated that the fluorescent intensity of GFP expressed by *the rpoE* promoter was substantially much higher than that of GFP and RFP expression directed by *lacUV5* promoter from both

pTwoFP1 and pTwoFP2. Ethanol and pentanol activated *rpoE* promoter, the ratios of *rpoE*/*lacUV5* were 4.0 times and 4.5 times, respectively, in comparison with the control sample without stress treatment of n-alkanols of 3.0 times, the experiment was repeated 3 times, and calculated for an average value.

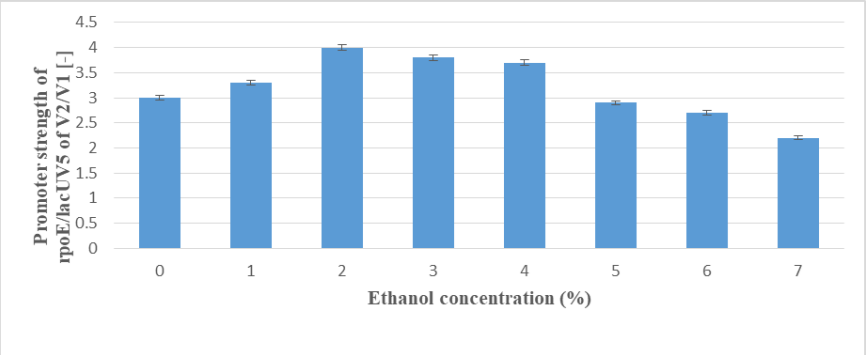


Figure 7. The effect of various concentrations of ethanol on the gene expression of *rpoE* promoter in reference to *lacUV5* promoter

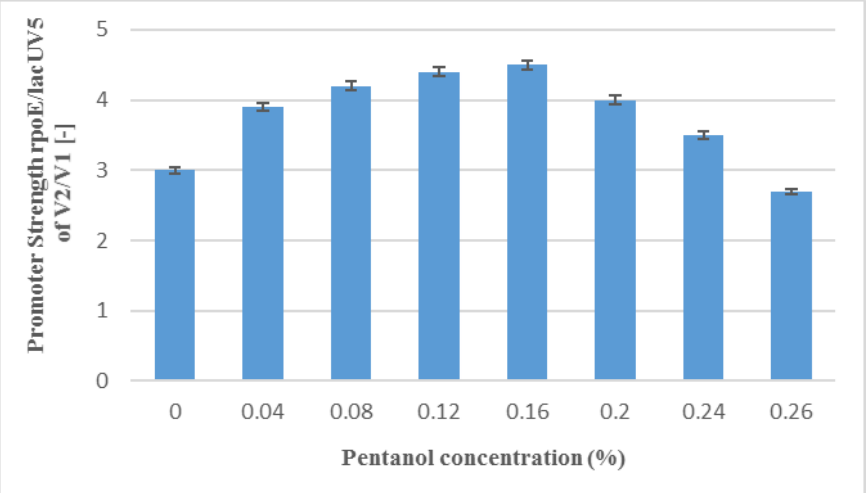


Figure 8. The effect of various concentrations of pentanol on the gene expression activity of test *rpoE* promoter in reference to *lacUV5* promoter

The activation of the *rpoE* promoter by *n*-alkanol contributed a significant role in the resistance and survival of *E. coli* cells. The cell was more sensitive to pentanol than to ethanol. Pentanol also activated the *rpoE* promoter stronger than ethanol.

DISCUSSION

At concentrations causing similar cell death, pentanol damaged both OM and IM, whereas ethanol damaged only OM and denature IM for destabilization of IM protein

Short-chain alcohols such as ethanol are compatible with both hydrophobic and hydrophilic environments as evidenced by their miscibility with both diethyl ether and water. Long-chain alcohols of hexanol increase membrane fluidity, whereas ethanol has little effect. Quantitatively, the effect of hexanol on membrane fluidity is 10-fold that of ethanol when compared at concentrations

causing a similar inhibition of growth in *E. coli* (Ingram et al., 1980; Ingram et al., 1976). Our results indicated that, at the same level of cell death of 98%, pentanol with a long carbon chain with hydrophobicity linkage at a higher level than that of ethanol, can easily interact with the hydrophobic region in both the outer and inner membrane, to highly enhance membrane fluidity and dis-stability of the membrane. As a consequence, pentanol can easily damage both inner and outer membrane to cause cell death at a high level and also strongly denatured and destabilize inner membrane protein indicated by the reduction of IM-GFP (Figs. 2–5). On the contrary, ethanol with a short carbon chain that is compatible with both hydrophobic and hydrophilic environments, for its miscibility with both diethyl ether and water of outer membrane, so that ethanol can easily interact with the outer membrane and reduce membrane fluidity to damage it. However, the inner membrane having hydrophobicity linkage at a high level was also protected by a peptidoglycan layer, so that ethanol having a short carbon chain and lower hydrophobicity linkage, is weakly interacting with the inner membrane to increase membrane fluidity as well as damaging inner membrane as pentanol. Therefore, ethanol only mainly damaged the outer membrane and did not damage the inner membrane to control cell death. It also denatured the inner membrane to destabilize inner membrane protein at a low level, indicated by the reduction of GFP located in the inner membrane, but it still did not damage the inner membrane. These results also clarified that *E. coli* cell is much more sensitive to pentanol than ethanol.

These results indicated that both ethanol and pentanol damaged the outer membrane indicated by the release of Sup-GFP (Fig. 3). However, pentanol strongly destabilized the inner membrane for release at the high level of total RNAs through the inner membrane and outer membrane. On the contrary, ethanol prevented the release of total RNAs from cytoplasm through the inner membrane in comparison with the control sample (Fig. 4).

These results reinforced that pentanol destabilized and damaged the inner membrane for the release both of CP-GFP and RNAs through the inner membrane, whereas ethanol did not. These results confirmed that at a similar level of more than 90% of cell death by the treatment of *n*-alkanol stress, pentanol damage the outer membrane, injury and destabilize the inner cell membrane for release of both small molecular of CP-RNAs at a high level and big molecular of CP-GFP from the cytoplasm. On the contrary, ethanol only damages the outer membrane, it was seemingly inserted into the inner cell membrane and weakly interacts with it to reduce the membrane fluidity and prevent the release both of CP-RNAs and CP-GFP from the cytoplasm through the inner membrane to the outside.

We hypothesized that ethanol with a short carbon chain and low hydrophobicity can be easily inserted into the inner membrane and reduce membrane fluidity so that the inner membrane became more tightly to prevent the release both of big and small molecules from the cytoplasm through the inner membrane to the outside. On the contrary, pentanol with a long carbon chain and high hydrophobicity can strongly increase the fluidity and destabilize the inner membrane for the release both of small molecules such as CP- RNA and big molecules such as CP-GFP from the cytoplasm through the inner membrane to the outside.

To confirm this hypothesis, some more experiments were carried out: (1) the effect of ethanol and pentanol on the folding of GFP as the fluorescent intensity was investigated. We indicated that both ethanol and pentanol do not affect the fluorescent intensity of GFP. However, when the cell membrane was damaged, the fluorescent intensity of GFP was increased from 135–150% in comparison with that inside the cell (data not shown). The effect of ethanol and pentanol on the absorbance of A260 was also investigated and indicated that ethanol and pentanol enhance A260 by 10% and 20%, respectively. Besides this, to confirm the accurate results of fragmentations of PP-

GFP and CP-GFP, the bioassay of the cytoplasmic β-galactosidase enzyme was also carried out by using *E. coli* BL21 to replace *E. coli* KP7600 without β-galactosidase. The results indicated that by using the TSD method for recovery of periplasm and cytoplasm fragmentations (Tsuchido et al., 1985), the β-galactosidase enzyme was not detected in the periplasm fragment, it was only detected at the level of 80 units/ml cell in cytoplasm fragment (data not shown). These results confirmed that PP-GFP and CP-GFP were successfully recovered.

The above results clarified that at the same level of 90−97% of cell death caused by ethanol and pentanol treatment, the long *n*-alkanol carbon chain of pentanol damaged the outer membrane and strongly destabilized the inner membrane to release a high level of IM-protein and small molecule of RNAs, and a lower level of the big molecule of GFPs from cytoplasm through the inner membrane to outside of the cell to control cell death (Figs. 3–5 and Table 2). On the contrary, ethanol damaged only the outer cell membrane to release molecular in the periplasm including PP-GFP, it was inserted into the inner membrane and released a part of 35% of IM-GFP, but it prevented the release of small and big molecules in the cytoplasm through inner membrane to the outside of the cell. Ethanol seems to have been inserted into the inner membrane and reduce the membrane fluidity to prevent the release both of big molecular of CP-GFP and small molecular of CP-RNAs from the cytoplasm through the inner membrane to the outside. 35% IM-GFP was also released from the inner membrane by ethanol stress in comparison with the control sample without the stress of 0%.

The effect of n-alkanol on the activation of σ^E operation contributed a significant role to cell resistance and survival to alkanol stress

It has been indicated that the short and long-chain alkanol stresses of ethanol and pentanol injured *E. coli* cell envelope of inner and outer membrane with different characteristics, which will also affect the control operation of σ^E at different levels.

Normally, in the absence of inducing signals, σ^E is held at the cytoplasmic side of the inner membrane by the anti-sigma factor RseA, a single-pass membrane protein. Besides this, a periplasmic protein, RseB, binds to the periplasmic domain of RseA, creating a complex of RseA/SigmaE/RseB to enhance the inhibition of σ^E. When the short-chain alkanol of ethanol and long-chain alkanol of pentanol are attached to the cell, they firstly disrupted and denatured the folding of outer membrane porins (OMPs) that will rapidly interact with RseA in complex RseA/SigmaE/RseB in the inner cell membrane, resulting in releasing free σ^E and activation of the alternative and operation of σ^E (Tsuchido et al., 1985; Sullivan et al., 1979). Free σ^E will bind specifically to RNAP (RNA polymerase) and then guide this complex to bind and activate target promoters, such as P*rpoE*, PdegP, or *rpoH*3 promoter for expression of HSPs having chaperon function for refolding proteins in OM, IM, PP, and CP for protection and protect the cell under stress conditions (Tsuchido et al., 1985). Therefore, the activity and operation of σ^E that can be indirectly evaluated by the activity of test *rpoE* promoter to control GFP expression in comparison with the housekeeping reference promoter *lacUV5* to control RFP expression were investigated in our study. The results indicated that the presence of long-chain alkanol of pentanol can enhance membrane fluidity at a much higher level than that of short-chain alkanol of ethanol (Ingram et al., 1980; Ingram et al., 1976). As shown in our study, we supposed that pentanol stress can affect both of outer and inner membrane, so, it can easily and strongly affect the stability of complex RseA/SigmaE/RseB to release σ^E at a higher level than that of ethanol. This resulted in the operation of σ^E activated by pentanol having higher activity than that activated by ethanol. Our data indicated that pentanol is much more sensitive to killing the cell than ethanol under stress treatment (Fig. 2). So, at 30 ^oC of cultivation with ethanol and

pentanol, *rpoE*/*lacUV5* were 4,0 times and 4.5 times, respectively, in comparison with that of the control sample of 3.0 times (Figs. 7, 8). And then, it was finally indicated that the enhancement of operation of σ^E by n-alkanol promoted the enhancement of cell resistance and survival. Finally, the results clarified that the existence of the *rpoE* gene encoding its *rpoE* promoter, and its operation enhanced by n-alkanol plays significant roles in the enhancement of *E. coli* cell resistance and survival under n-alkanol stress treatment.

CONCLUSIONS

Here we showed that short and long-chain alkanol stress of ethanol and pentanol injured *E. coli* cells, in which the cell was more sensitive to pentanol than ethanol. Ethanol only mainly damaged the outer membrane and did not damage the inner membrane to control cell death. However, pentanol damaged both of inner and outer membrane to control cell death. The existence of the *rpoE* gene encoding the *rpoE* promoter, and its operation enhanced by n-alkanol plays significant roles in the enhancement of *E. coli* cell resistance and survival under n-alkanol stress treatment.

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REFERENCES

Tsuchido T., Katsui N., Takeuchi A., Takano M., Shibasaki I., 1985. Destruction of the outer membrane permeability barrier of *Escherichia coli* by heat treatment. *Applied and Environmental Microbiology*, 50(2): 298–303.

Ingram O. L., Vereeland S. N., 1980. Different Effects of Ethanol and Hexanol on the *Escherichia coli* cell envelope. *Journal of Bacteriology*, 144(2): 481−488.

Fried V. A., Novick A., 1973. Organic solvents as probes for the structure and function of the bacterial membrane: effects of ethanol on the wild type and an ethanol-resistant mutant of Escherichia coli K-12. *J. Bacteriol,* 114: 239−248.

Harold F. M., 1970. Antimicrobial agents and membrane function. *Adv.Microb. Physio*, 4: 45−104.

Hugo W. B., 1967. The mode of action of antibacterial agents. *J. Appl. Bacteriol*; 30: 17−50.

Lee A.G., 1976. Interaction between anesthetics and lipid mixtures: normal alcohols. *Biochemistry,* 15: 2448−2454.

Jain M. K., Wu N. M., 1977. Effect of small molecules on the dipalmitoyl lecithin liposomal layer. III. Phase transition in the lipid bilayer. *J. Membr. Biol.*, 34: 157−201.

MacDonald A. G., 1978. A dilatometric investigation of the effects of general anesthetics, alcohols, and hydrostatic pressure on the phase transition in smectic mesophases of dipalmitoyl phosphatidylcholine. *Biochim. Biophys. Acta*, 507: 26−27.

Paterson S. J., Butler K.W., Huang P., Labelle J., Smith I. C. P., Scheneider H., 1972. The effects of alcohols on lipid bilayers: a spin label study. *Biochim. Biophys. Acta*, 266: 597−602.

Silbert D. F., 1970. Arrangement of fatty acids groups in phosphatidylethanolamine from fatty acid auxotroph of *Escherichia coli*; *Biochemistry*, 9: 3631−3640.

Sullivan K. H., Hegeman G. D., Cordes E. H., 1979. Alteration of the fatty acid composition of *Escherichia coli* by growth in the presence of normal alcohols. *J. Bacteriol.*, 138: 133−138.

Ingram L. O., 1976. Adaptation of membrane lipids to alcohols. *Journal of Bacteriology*, 125(2): 670−678.

Hubbel W.L., Metcalfe J. C., Metcalfe S. M., McConnell H. M., 1970. The interaction

of small molecules with spin - labeled erythrocyte membrane-active agents. *Biochim. Biophys. Acta*, 219: 415−427. doi: 10.1016/0005-2736(70)90219-1

Paterson S. J., Butler K. W., Huang P., Labelle J., Smith I. C. P., Schneider H., 1972. The effects of alcohols on lipid bilayers: a spin-label study. *Biochim. Biophys. Acta*; 266: 597−602.

Grisham C. M; Barnett R. E., 1973. The effects of long-chain alcohols on membrane lipids and the (Na^+ + K^+)-ATPase. *Biochim. Biophys. Acta*, 311: 417−422.