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Development of a Suicidal Vector-Cloning System Based on Butanal Susceptibility Due to an Expression of YqhD Aldehyde Reductase

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Previously, we observed butanal/propanal sensitivity of *Escherichia coli K-12* when cells overexpress YqhD protein, a NADPH dependent aldehyde reductase, possibly due to an accumulation of butanol/propanol *in vivo* as the reaction products. Based on this finding, we developed a suicidal vector-cloning system derived from pUC19, in which *lacZ* was substituted with the *yqhD* gene. As a result, when foreign DNA was inserted into its multiple cloning sites by disrupting an expression of YqhD, the recombinants survived on butanal/propanal containing plate, whereas cells containing the YqhD vector died because of the alcohol production by YqhD. The cloning efficiency, estimated based on colony PCR and enzyme digestion, was achieved more than 90% when the suicidal vector system was used. Moreover, the plasmid vector itself was stably maintained in the cell, presumably due to its ability to remove toxic aldehydes being accumulated in *E. coli* cell by metabolic stress.

Keywords: YqhD, suicidal vector, butanal, propanal

Introduction

A number of strategies have been adopted to test or select for transformants that acquire plasmids containing a desired insert. Typically, one has to rely on an indicator or a suicidal selection system. Because of its availability, the indicator system has been widely used to distinguish positive clones that contain a foreign gene, e.g. β-galactosidase (β-Gal), green fluorescent protein (GFP), and acid phosphatase dioxygenase (Clark and Cirvilleri, 1994; Burioni *et al.*, 1995; Inouye *et al.*, 1997; Riccio *et al.*, 1997; Chaffin and Rubens, 1998). However, the caveats are in the costly usage of reagents, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and isopropyl-β-D-thiogalactopyranoside (IPTG), used for the detection of β-Gal activity in the screening for recombinants based on α-complementation (Sambrook and Russel, 2001). Thus, the indicator method might be disadvantageous over the system using non-induced auto-catalytic proteins, e.g. GFP (Inouye *et al.*, 1997). In addition, the cloning host of *Escherichia coli* must be engineered to contain *lacZ*⁻ mutation and some other regulatory changes. In the case of GFP, it requires relatively long (18–19 h) maturation time for fluorophore development, although it is unnecessary to consider any cofactor/substrate or genotype of the host strain. If an expression level of target protein is low, strong and toxic UV light should be illuminated to excite the cells.

A group of cloning vectors was designed to screen for recombinants by selecting against non-recombinants (Henrich and Schmidtberger, 1995; Schlieper *et al.*, 1998; Yazynin *et al.*, 1999). They exploited genes that are toxic to host, e.g. the *ccdB*- or *sacB*-based systems (Gay *et al.*, 1985; Bernard *et al.*, 1994; Gabant *et al.*, 1998). The *ccdB* selection system utilizes the property of insertional inactivation of the cytotoxic *ccdB* gene (Bernard *et al.*, 1994; Gabant *et al.*, 1998) that is involved in positioning the DNA-gyrase cleavable complexes responsible for DNA breakage and activation of SOS system (Bernard and Couturier, 1992; Bernard *et al.*, 1993; Van Melderen *et al.*, 1994). The multiple cloning sites are located in the 5′ region of *ccdB* gene (Bernard *et al.*, 1994; Gabant *et al.*, 1998). Because of cytotoxic activity of the *ccdB*-based selection plasmid, the plasmid has to be maintained in a gyrase (*gyrA*) mutant (Bernard *et al.*, 1994), and an IPTG induction is necessay for positive selection (Bernard *et al.*, 1994).

The *sacB* gene of *Bacillus subtilis* encodes levansucrase (sucrose: 2,6-β-D-fructan 6-β-D-fructosyltransferase) that synthesizes levans, high-molecular-weight fructose polymers, by hydrolyzing sucrose (Dedonder, 1966; Gay *et al.*, 1983; Steinmetz *et al.*, 1983). An expression of SacB in Gram-negative bacteria is lethal in media containing 5% sucrose, causing cell lysis or growth inhibition, thereby providing a basis for a suicidal selection system (Gay *et al.*, 1983, 1985; Steinmetz *et al.*, 1983). The toxic mechanism in *E. coli* is unclear but suspected due to an accumulation of levans in the periplasm, causing a damage because of their size or transfer of fructose residues to inappropriate acceptor molecules (Steinmetz *et al.*, 1983). Since Gram-positive bacteria do not have an outer membrane, it was thought that sucrose selection does not work for these species. However, sucrose sensitivity was observed in some Gram-positive species, such as *Corynebacterium glutamicum*, whose lethality was explained by the presence of characteristic cell wall, in which the mycolic acids form an outer membrane-like structure (Jager *et al.*, 1992). As mentioned above, the *sacB* suicidal system can be generally used in Gram-negative species, but not in Gram-positive species. The system does not work in *B. subtilis*, because of the presence of wild-type *sacB* gene in its genome (Pelicic *et al.*, 1996).

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Limitations may also exist in the availability of indicator compound or suicidal selection itself, e.g. costly medium or requirement of a specific mutation in host strain, respectively. Furthermore, the plasmids are difficult to be propagated because of its cytotoxicity as well as limited numbers of restriction sites, making it inadequate for cloning.

YqhD has been characterized in *E. coli* as a NADPH dependent aldehyde reductase with specificities to various aldehyde compounds including glyoxal, glycolaldehye, and methylglyoxal (Lee *et al.*, 2010), as well as propanal, butanal, and acrolein, which are formed by lipid peroxidation (Perez *et al.*, 2008). Interestingly, we observed that over-expression of YqhD makes cells highly susceptible to propanal and butanal (Lee *et al.*, 2010). It was thought that the susceptibility is due to a production of alcohols, i.e. propanol and butanol, by YqhD or due to an intracellular depeletion of NADPH (Jean *et al.*, 1987; Anne *et al.*, 1989; Miller *et al.*, 2009). By exploiting this property, we designed a suicidal system using the pUC19 vector containing *yqhD* gene, whose insertional inactivation restores cell growth in butanal/propanal-containing medium. The system is simple, convenient, and easily applicable to any cloning purpose.

Materials and Methods

Bacterial strain, plasmid, and growth conditions

E. coli DH10β strain (Invitrogen, USA) and pUC19 plasmid (New England Biolabs, USA) were used. Cells were grown at 37°C in LB (Difco, France) containing ampicillin (100 μg/ml, USB, USA) with agitation. Butanal (Fluka, USA) and propanal (Sigma, USA) were reagent grades.

Construction of pYqhD

The pYqhD plasmid was derived from pUC19 (New England Biolabs, USA), in which the *yqhD* gene amplified from *E. coli* K-12 (MG1655) genomic DNA using the primers of EcoRI-yqhD-F and NarI-yqhD-R (Table 1) was inserted into the *Eco*RI and *Nar*I sites in the *lacZα* gene by the restriction enzymes and T4 DNA ligase (Fermentas, USA). The plasmid clone was maintained in DH10β for further experiments. The region near the multiple cloning sites (MCS) contained in *yqhD* was confirmed by DNA sequencing. The expression of YqhD protein was observed by 12% SDS-PAGE analysis.

Cell viability assay for determining lethal concentrations of aldehyde compounds (Spot assay)

Cell viability after exposure to aldehyde compounds was determined by growing cells on LB plates containing different concentrations of aldehyde compounds. Fresh colonies of wild-type and mutant strains were grown overnight in LB broth, diluted 100-fold in the same medium, and incubated with shaking until the OD_{600} reached 1.0. The cells were diluted from 10^{-1} to 10^{-5} and spotted (4 μl) onto LB plates containing different concentrations of aldehyde compounds. Growth was assessed after 12–14 h of incubation at 37°C.

Measuring inhibitory concentrations (IC_{50}) of aldehyde compounds

To compare IC_{50} among strains, we measured optical densities of cells growing in media containing different concentrations of aldehyde compounds. A 96-well culture plate containing 180 μl LB media per well was treated with different concentrations of aldehyde compounds. Twenty microliters of inoculum containing cells at an OD_{600} of 1 were then added to each well. The 96-well plate was sandwiched between two acrylic plates (preheated) for efficient temperature equilibration and then incubated at 37°C with shaking for 6 h. OD_{595} was measured using a microplate reader (Model 680, Bio-Rad, USA). IC_{50} was estimated using Sigmaplot (SPSS Inc., USA) by fitting to the sigmoidal dose-response equation.

Measurement of growth rate

Fresh colonies of transformants were seeded in 3 ml of LB broth and cultured overnight. The cells were then inoculated into 10 ml of LB broth and incubated for fixed time intervals, and the growth rate was monitored by measuring OD_{600} with Beckman DU_{700} spectrophotometer, which was plotted using Sigmaplot (SPSS Inc., USA).

Table 1. Strain, plasmids, and primers used

Strain/plasmids/primers	Genotype or description[a]	Source or references
Strain		
DH10β	F⁻ mcrA Δ(*mrr-hsd*RMS-*mcr*BC) Φ80d*lac*ZΔM15 Δ*lac*X74 *end*A1 *rec*A1 *deo*R Δ(*ara*,*leu*)7697 *ara*D139 *gal*U *gal*K *nup*G *rps*L λ⁻	Invitrogen
Plasmids		
pUC19	ColE1 Ap^r	New England Biolabs
pYqhD	Derived from pUC19 (Substitution of 235–400 bases of pUC19 with *yqhD*)	This work
Primers		
EcoRI-yqhD-F	5′-GCGAATTCATGAACAACTTTAATCTGCACAC-3′	This work
NarI-yqhD-R	5′-CGGGCGCCTTAGCGGGCGGCTTCGTATATACG-3′	This work
BamHI-sodB-F	5′-CGCGGATCCATGTCATTCGAATTACCTGC-3′	This work
BamHI-sodB-R	5′-GCGGGATCCTTATGCAGCGAGATTTTTCG-3′	This work
pUC19-F	5′-GCTGTTTCCTGTGTGAAATTG-3′	This work

[a] Ap^r, ampicillin resistance

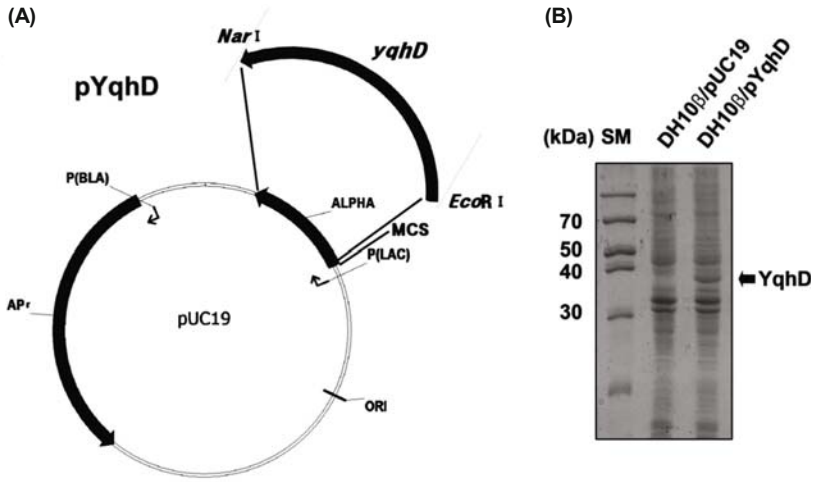

Fig. 1. Vector construction and expression of YqhD. (A) pYqhD was derived from pUC19, in which *lacZ*α was replaced with *yqhD*, a NADPH dependent aldehyde reductase, using *Eco*RI and *Nar*I restriction sites. The multiple cloning site (MCS) of pUC19 was retained; the *bla* gene in pUC19 confers ampicillin resistance. (B) Expression of YqhD protein in DH10β/pYqhD. The protein band migrates at around 40 kDa region on 12% SDS-PAGE.

Determination of plasmid abundance and stability

To determine plasmid abundance, we compared the band intensities of genomic DNA (gDNA) to plasmid DNA (pDNA) on 1.2% agarose gel (Friehs, 2004; Goh and Good, 2008). Total DNA that includes both gDNA and pDNA was isolated from five different clones of pUC19 and pYqhD transformants. The cells were grown overnight (18 h) in LB broth with 100 μg/ml of ampicillin. Band intensities were measured by using ImageJ program (http://rsb.info.nih.gov/ij/). For determining plasmid stability, cells containing pUC19 or pYqhD were cultured overnight in LB media containing ampicillin. 10^{-2} volume of the overnight culture was inoculated into 10 ml of LB media without ampicillin. After incubating 25 and 50 h at 37°C, the cells were diluted and plated onto LB plate to count cell number. The colonies from LB plate were replica plated onto ampicillin containing plate, from which ampicillin-resistant colonies were counted.

Positive selection of recombinant plasmid

As a DNA insert for cloning, the *sodB* gene was amplified from *E. coli* K-12 (MG1655)genomic DNA by PCR using primers, BamHI-sodB-F and BamHI-sodB-R (Table 1). The vector and insert DNAs were digested with *Bam*HI, which were purified by gel electrophoresis, and the insert was mixed and ligated with the digested pUC19 or pYqhD vector. Subsequently, the ligated samples were used to transform DH10β cells. The plates containing ampicillin or plus 90 mM propanal/butanal were used for selection. A colony PCR was performed to confirm positive clones by using primers that recognize *yqhD* or *sodB* NarI-yqhD-R, BamHI-sodB-F, and BamHI-sodB-R for pYqhD. The primers that bind to the upstream region of MCS and *sodB*, pUC19-F, BamHI-sodB-F, and BamHI-sodB-R were used for con-

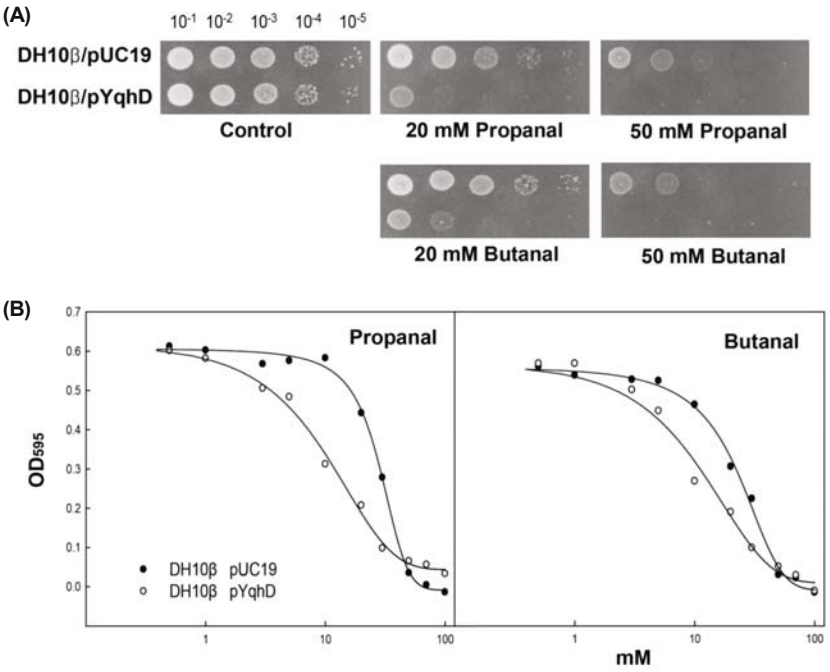

Fig. 2. Susceptibility to propanal and butanal. (A) Spot assay exhibits susceptibility to propanal/butanal in DH10β/pYqhD strain. (B) Growth inhibitions of propanal and butanal were monitored by measuring IC_{50}s. The IC_{50}s of DH10β/pUC19 and DH10β/pYqhD transformants for propanal/butanal were 28.3/9.7 mM and 20.2/9.2 mM, respectively.

Table 2. Efficiency of positive selection

	pUC19	pYqhD	pYqhD	pYqhD
Selection[a]	Ap	Ap	Ap, Propanal (90 mM)	Ap, Butanal (90 mM)
Cloned/total number of colonies	9/55	9/65	64/70	73/77
Percentage (%)	14.5	13.8	91.4	94.8

[a] Selection was performed with 100 μg/ml of ampicillin (Ap) or plus 90 mM of propanal/butanal.

firming positive clones of pUC19. In order to verify the positive clones, plasmids were prepared, digested with *Bam*HI, and analysed.

Results

A plasmid containing *yqhD* makes cells sensitive to butanal/propanal

Previously, we isolated glyoxal-resistant mutants constitutively over-expressing YqhD, a NADPH-dependent aldehyde reductase. The phenotype resulted from a constitutively activating missense mutation in *yqhC*, encoding an AraC-type transcriptional activator for *yqhD* (Lee *et al*., 2010). We observed that YqhD catalyzes reductions of various aldehydes including glyoxal, glycolaldehyde, propanal, and butanalusing NADPH as a cofactor. The aldehydes also enhance an expression of *yqhD* through *yqhC* (Lee *et al*., 2010). While an over-expression of YqhD confers glyoxal resistance, the cells became sensitive to propanal or butanal (Lee *et al*., 2010). It is likely that YqhD converts propanal or butanal to toxic alcohols, i.e., propanol or butanol, respectively, affecting cell viability (Jean *et al*., 1987; Anne *et al*., 1989). Alternatively, as reported recently (Miller *et al*., 2009), an over-expression of YqhD may deplete intracellular NADPH by facilitating reductions of aldehydes, thereby deactivating cellular metabolism.

A suicidal vector, named pYqhD, was designed using the property of propanal or butanal susceptibility by an over-expression of YqhD. The vector itself was derived from pUC19, in which the *lacZα* gene was engineering to contain the *yqhD* gene (Fig. 1A). *E. coli* DH10β strain was transformed with pUC19 and pYqhD, which were selected on LB plates containing ampicillin. Since pYqhD highly expresses YqhD (Fig. 1B), cells containing pYqhD became susceptible to propanal or butanal (Fig. 2). Inhibitory concentrations (IC_{50}) of the pUC19 and pYqhD containing cells to propanal/butanal were 28.3/20.2 and 9.7/9.2 mM, respectively (Fig. 2B), enabling us to use pYqhD as a suicidal vector system.

The pYqhD vector permits a positive selection of recombinant plasmid

The suitability of pYqhD as a suicidal vector lies in its property of sensitivity to propanal or butanal. Thus, an insertion of foreign DNA into the MCS of pYqhD, located downstream of its ribosome binding sequence, allows cells to survive on propanal/butanal containing plate by blocking an expression of *yqhD*. In order to assess fidelity and efficiency of positive selection, we performed cloning experi-

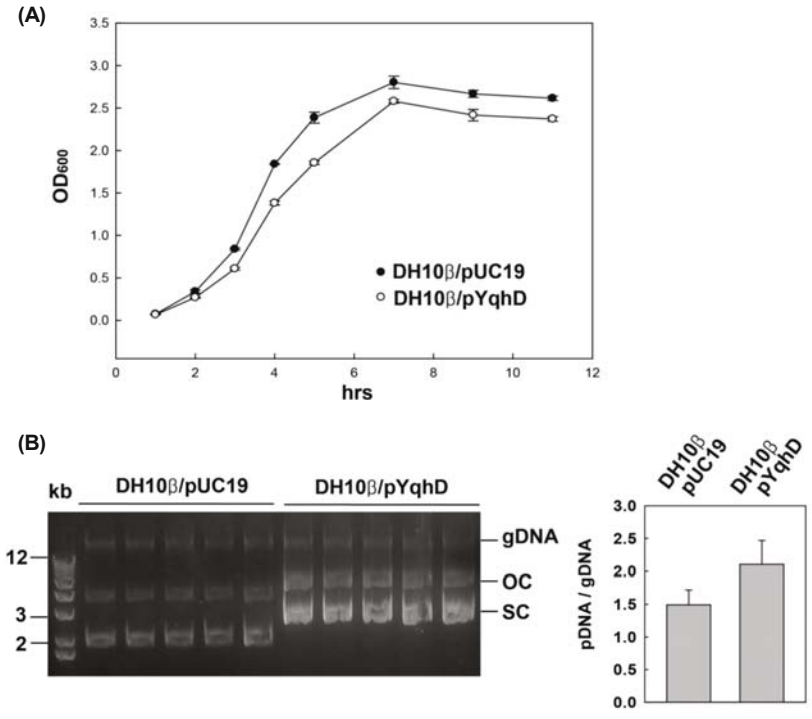

Fig. 3. Effect of pYqhD on growth rate and estimation of plasmid amount. (A) The DH10β/pUC19 and DH10β/pYqhD transformants were grown in LB broth with 100 μg/ml of ampicillin. The growth rates were estimated by measuring OD_{600} for 12 h. (B) Total DNA extracted from five clones, each from DH10β/pUC19 and DH10β/pYqhD, were used for agarose gel electrophoresis. The band intensities of genomic DNA (gDNA) and plasmid DNA (pDNA) were quantified by using Image J program. The ratio between pDNA and gDNA was calculated and presented. Positions of supercoiled (SC) and open circular (OC) plasmids are indicated.

Table 3. Properties of pYqhD and pUC19

Properties[a]	pUC19	pYqhD
Plasmid abundance[b] (plasmid/gDNA)	1.5	2.1
Relative growth rate[c] ($\Delta OD_{600}/\Delta t$)	1	0.925

[a] Determined from the experiments conducted five times.
[b] Plasmid abundance was estimated by the ratio of plasmid DNA to genomic DNA (gDNA), which was determined by electrophoretic separation of total DNA on 1.2% agarose gel. The density was estimated using the Image J program (http://rsb.info.nih.gov/ij/).
[c] Changes in OD_{600} during 7 h were compared between DH10β/pYqhD and DH10β/pUC19.

ments based on propanal/butanal selection and compared pUC19 and pYqhD as cloning vectors. The DNA inserts were prepared by amplifying the *sodB* region, located 1,733,402 to 1,733,983 base pairs of genomic DNA, and by attaching a *Bam*HI adaptor to both ends (Table 1). The insert and vector DNAs were completely digested with *Bam*HI, and the DNA inserts were ligated to *Bam*HI site of MCS in the vectors of pUC19 and pYqhD ('Materials and Methods'). The recombinant plasmids were used to transform DH10β for selection in the presence or absence of propanal/butanal. Concentrations of propanal/butanal were varied from 30 to 90 mM, from which best result was obtained at 90 mM (data not shown). The positive clones were identified by colony PCR using primers recognizing the insert and the vector (Table 1, 'Materials and Methods'). The recombinant plasmids were also confirmed by enzyme digestion (data not shown). As a result, more than 90% of colonies were positive when selected with 90 mM propanal/butanal (Table 2). However, fewer than 15% of colonies were positive in both pUC19 and pYqhD without a propanal/butanal selection. When the suicidal selection was applied to *nemA* gene, encoding N-ethylmaleimide reductase, inactivation of *yqhD* gene was concomitantly observed with the loss of butanal resistance (data not shown), indicating that the system does not discriminate types of genes used as inserts.

Copy number of pYqhD plasmid

The pYqhD transformants were compared with those of parent pUC19 in terms of their effects on growth by measuring optical density at 600 nm (Fig. 3A). Growth of DH10β/pYqhD was slightly lower than that of DH10β/pUC19. The relative growth rates are shown in Table 3. Copy number of pYqhD was measured by the ratio of plasmid DNA to genomic DNA (Table 3 and Fig. 3B). The amount of pYqhD was estimated to be 1.4 times greater than that of pUC19. Since the size of pYqhD (3,689 bp) is 1.4 times larger than pUC19 (2,686 bp), the copy number of pYqhD is roughly equal to that of pUC19. Despite its growth-retarding effect, pYqhD is stably maintained similarly as in its parent plasmid.

Table 4. Stability of pYqhD and pUC19

Culturing time (h)		pUC19	pYqhD
25	Ap^r/total number of colonies[a]	132/249	171/192
	Percentage (%)	53	89
50	Ap^r/total number of colonies	31/158	124/127
	Percentage (%)	19	97

[a] The total number of cells cultured 25/50 h in LB broth was determined by counting cells, which were diluted and spreaded onto LB plates. The number of ampicillin-resistant (Ap^r) cells was estimated by replica plating on ampicillin containing plates.

Expression of YqhD provides a selective advantage to the plasmid-bearing cells

YqhD was reported to detoxify carbonyl compounds, such as glyoxal, glycolaldehyde, and methylglyoxal, through enzymatic reduction (Lee *et al.*, 2010). The carbonyl compounds are formed spontaneously *in vivo* by oxidative degradation of glucose and by lipid peroxidation (Wells-Knecht *et al.*, 1995; Mlakar *et al.*, 1996). Because of its reactive carbonyl group, accumulation of carbonyl compounds causes various damages in macromolecules. For example, glycations of nucleotide and protein cause mutagenesis and malfunction of protein, respectively (Kasper *et al.*, 2000; Thornalley, 2002). Since YqhD converts carbonyl compounds to non-toxic alcohols, an expression of YqhD from plasmid could be advantageous over carbonyl stress.

We investigated the selective advantage of pYqhD plasmid in DH10β strain (Table 4). Cells containing the plasmid were grown in LB broth without ampicillin (100 μg/ml) for 25 and 50 h. After spreading onto LB plates free of antibiotics, total numbers of cells were determined by counting colonies. At the same time, the plasmid-containing cells were estimated by counting ampicillin-resistant colonies after replica plating of the colonies onto LB plates containing antibiotics. Interestingly, the pYqhD plasmid was stably maintained, more than 90%, after more than 25 h and up to 50 h of incubations. In contrast, the majority of pUC19 was cured and thus found only about 50 and 20% left after 25 and 50 h, respectively. Therefore, it is likely that although pYqhD affects growth of the cell, it has a selective advantage, presumably by relieving cellular stress of carbonyl compounds.

Discussion

We introduced a suicidal selection system based on butanal/propanal susceptibility due to an over-expression of YqhD protein in *E. coli*. Problems associated with the previous cloning systems, such as the requirement of indicators, inefficiency in the suicidal mechanism, and mutational background, are no longer a matter of concern. Furthermore, the substrates for pYqhD, propanal, and butanal, are easily available and inexpensive, especially for large-scale and high throughput screening. For convenience in cloning, the MCS sequence from pUC19 vector is now embedded in the pYqhD plasmid. As a suicidal selection system, the *sacB* gene is widely used. However, some bacterial species actively metabolize sucrose, making it difficult to use the *sacB* suicidal vector. In addition, toxicity of *sacB* is not fully manifested in Gram-positive bacteria. Since the toxic mechanism of alcohol is universal, the pYqhD system can be ideally used as a cloning system.

The positive selection of pYqhD transformants based on butanal/propanal resistance has been demonstrated successfully, in which more than 90% of transformants contained desirable clones. Conventionally, a cloning experiment achieves less than 15% efficiency of screening positive clones

without any selection pressure. Other merit of using the pYqhD system is its stable maintenance in host cells, which is presumably due to the function of YqhD protein relieving the stress of intracelluar aldehydes. Therefore, the pYqhD suicidal selection system allows us to select positive recombinants with high efficiency, serving as a reliable vector-cloning system.

As a future work, the exact mechanism of butanal/propanal susceptibility in using the pYqhD system has to be elucidated. It is thought that the toxicity is due to either the metabolic products of YqhD itself, i.e. butanol and propanol, or a redox imbalance as a result of NADPH depletion, a cofactor of YqhD. Various substrates of YqhD were identified in the previous studies, espcially for aldehyde compounds (Jarboe, 2011). When we tested acetaldehyde, a susceptibility was not clearly observed (data not shown), presumably due to a lack of enzymatic specificity. It was reported that YqhD exhibits preference for substrate containing more than three carbons (Sulzenbacher *et al*., 2004). This might be the reason why YqhD converts propanal and butanal efficiently to corresponding alcohols. As a matter of fact, the toxic mechanism of alcohols produced by YqhD is still uncertain. At any rate, propanal and butanal are the most efficient substrates for pYqhD selection system tested so far. On the other hand, different types of replicon might be used as *yqhD*-carrying vehicles, and so does the host for transformation, e.g. *E. coli* with a *yqhD* deletion.

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