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Enhancement of geraniol resistance of *Escherichia coli* by MarA overexpression

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Improvement of a microorganism's tolerance against organic solvents is required for a microbial factory producing terpenoid based biofuels. The bacterial genes, *marA*, *imp*, *cls* and *cti* have been found to increase organic solvent tolerance. Thus, the tolerance against the following terpenoids (isopentenol, geraniol, myrcene, and farnesol) was studied with overexpression of *marA*, *imp*, *cls* and *cti* genes in *Escherichia coli*. The *marA* overexpression significantly enhanced the tolerance of *E. coli* against geraniol, whereas there was no tolerance improvement against the terpenoids by overexpression of *cls* and *cti* genes. The *imp* overexpression even yielded sensitive phenotype to the tested solvents. The colony forming efficiency of the *marA* overexpressing *E. coli* was increased by 10^4-fold in plate overlay of geraniol compared to that of wild type *E. coli* and a two-fold decrease of intracellular geraniol accumulation was also observed in liquid culture of geraniol. Single knock-out mutations of *marA*, or one of the following genes (*acrA*, *acrB* and *tolC*) encoding AcrAB–TolC efflux pump made *E. coli* hypersensitive to geraniol. The geraniol tolerance conferred by *marA* overexpression was attributed to the AcrAB–TolC efflux pump that is activated by MarA.

[**Key words:** *Escherichia coli*; Efflux pump; Geraniol; MarA; Terpenoid tolerance]

Terpenoids (also called isoprenoids) are widespread in nature and range from essential cell components to unique secondary metabolites (1). Terpenoids comprise the largest and most structurally diverse group with over 50,000 compounds identified to date (2). Despite their tremendous structural diversity, terpenoids are derived from two common precursors, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (3) which are assembled and modified in various ways as defined by the enzymatic reactions in an organism. Terpenoids have been used in human nutrition, traditional herbal remedies and fragrance industry, and are also under investigation for antibacterial, anticancer, and other pharmaceutical functions (1). Recently, some hemi(C5)-, mono(C10)- and sesqui(C15)-terpenoids have been investigated as alternatives to fossil fuels (4–6). However, mass production of biofuel candidate terpenoids has been hindered due to their toxicity to host strains including *Escherichia coli* (7). Therefore, terpenoid tolerance of the host strain should be explored through genetic engineering. The toxicity of organic solvents to bacteria has been known to be related to the log P_{ow} of the solvent. Log P_{ow} is an index of solvent polarity and is defined as the common logarithm of the partition coefficient (P_{ow}) of a given organic solvent between *n*-octanol and water. An organic solvent with a low log P_{ow} value is generally considered toxic to microorganisms (8). Bacteria have various strategies to decrease the influx of solvents in cells to protect against their toxicity (9–15). Both short and long term adaptive changes were observed in the cell membrane of *Pseudomonas putida* after exposure to organic solvents, i.e., the *cis* to *trans* isomerization of unsaturated fatty acids mediated by *cis/trans*-isomerase (Cti) and the increase of cardiolipin content by cardiolipin synthase (Cls) (16–18). The Cls knock-out strain is very sensitive to toluene and several drugs as reported previously (17). Imp, which is also called OstA due to its relation to organic solvent tolerance, is involved in biogenesis of cell envelops and decrease of membrane permeability. An Imp mutant strain of *E. coli* showed increases of both membrane permeability and organic solvents sensitivity (12,19,20). Along with the decrease of solvent influx by the modified membrane barrier, an activated efflux system against the solvents that penetrate cells is essential to ensure a significant level of solvent tolerance. Thus, bacterial efflux pumps can provide an alternative direct mechanism for reducing solvent toxicity (14,15,21). AcrAB–TolC is a major multidrug efflux pump in *E. coli*, exporting a broad range of substrates such as detergents, dyes, antibiotics, and organic solvents. Expression of the AcrAB–TolC efflux pump is positively regulated by MarA (corresponding to Multiple Antibiotic Resistance) (22). MarA also regulates the expression of a set of genes, i.e., *sodA*, *nfo*, *micF*, *inaA*, *fumC*, *zef* and *fpr* genes belonging to *mar* and *soxRS* regulons, which are involved in tolerance of antibiotics, superoxide-generating reagents, heavy metals, and organic solvents (14,21,23). Therefore, we have investigated the effect of

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marA, *imp*, *cls* and *cti* genes on terpenoid tolerance in *E. coli* that has been used as a host strain for terpenoid production.

MATERIALS AND METHODS

Bacterial strains and culture conditions The bacterial strains used in this study are listed in Table 1. *E. coli* DH5α was used for all DNA manipulations and terpenoid tolerance study. The effect of *marA* gene deletion on terpenoid tolerance was investigated with *E. coli* BW25113 and its mutants, *E. coli* JW5249 (Δ*marA*), JW0452 (*ΔacrA*), JW0451 (*ΔacrB*) and JW5503 (*ΔtolC*) obtained from Keio collection (24). The terpenoid tolerance assay was carried out in a modified Luria–Bertani medium (LBGMg) which contains 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 0.1% (w/v) glucose and 10 mM $MgSO_4$. Antibiotics were added as required in concentrations of 100 μg/ml of ampicillin, 50 μg/ml of chloramphenicol and 50 μg/ml of kanamycin. IPTG at a concentration of 0.1 mM was used for overexpression of the tolerance genes *marA*, *imp*, *cls* and *cti*, whereas *acrA*, *acrB* and *tolC* were expressed under no IPTG condition. Cell growth in liquid culture was determined by measuring the optical density at 660 nm (OD_{660}).

DNA manipulation and plasmids construction The genomic DNA and plasmids preparation, restriction digestions, transformations and other molecular biological techniques were carried out as described in the literature (25). Plasmids and PCR primers used in this study are summarized in Table 1. The *marA*, *imp* and *cls* genes were amplified by *Pfu* DNA polymerase from *E. coli* genomic DNA and the *cti* gene, from *P. putida* P8 genomic DNA. The PCR products were inserted between corresponding restriction sites of pTrc99A and pSTV28 for the construction of pTmarA, pTimp, pTcls and pScti plasmids (Table 1).

Chemicals and reagents The restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA, USA). Gel extraction and plasmid preparation kits were purchased from Qiagen (Valencia, CA, USA). Isopentenol, geraniol, myrcene, farnesol and hexane were obtained from Sigma chemicals (St. Louis, MO). *Pfu* DNA polymerase and primers were purchased from SolGent and Bioneer (South Korea), respectively. Bacto-tryptone and yeast extract were obtained from Difco Laboratories (Sparks, MD, USA).

TABLE 1. Strains, plasmids and primers used in this work.

Names	Descriptions	References
Strains		
E. coli DH5α	F⁻ f80, D*lacZ*, DM15, D (*lacZ*YA-*arg*F), U169*deo*R, *rec*A1, *end*A1, *hsd*R17 (r_k^-, m_k^+), *pho*A, *sup*E44λ⁻ *thi*-1, *gyr*A96 *rel*A1	ATCC 53868
E. coli BW25113	*lacI*^{q}, Δ*lacZ*$_{WJ16}$, Δ*ara*BAD$_{AH33}$, Δ*rha*BAD$_{LD78}$, *hsd*R514, and r*rnB*$_{T14}$	(24)
E. coli JW5249	*E. coli* BW25113 (Δ*marA*)	(24)
E. coli JW5503	*E. coli* BW25113 (Δ*tolC*)	(24)
Pseudomonas putida P8	Wild type	ATCC 49451
Plasmids		
pBluescript	P_{lac}, ColE1 origin, lacZ, and Amp^r	Stratagene
pTrc99A	P_{trc}, pBR322 origin, lacI^q, and Amp^r	Amersham Bioscience
pSTV28	P_{lac}, pACYC184 origin, lacZ, and Cm^r	Takara Co., Ltd.
pTmarA	pTrc99A containing *marA* gene from *E. coli*	This study
pTcls	pTrc99A containing *cls* gene from *E. coli*	This study
pTimp	pTrc99A containing *imp* gene from *E. coli*	This study
pScti	pSTV28 containing *cti* gene from *P. putida* P8	This study
pTtolC	pTrc99A containing *tolC* gene from *E. coli*	This study
Primers[a]		
marA-F	G*GAGCTC*A**GGAGGTATGACGATG**TCCAGACGCA	This study
marA-R	C*TCTAGA***TGTCACGTTTTCAACTAG**CTGTTGTAAT	This study
cls-F	G*CCATGG*AGGAG**GTCTAATCTATGA**CAACCGTTT**ATACGTTG**	This study
cls-R	C*TCTAGA***CCTCTGTTGGCGACGTTTTACAG**	This study
imp-F	G*GGATCC*AGGAGG**ATCAACGTATGA**AAAAAACGT**ATCCCCAC**	This study
imp-R	C*TCTAGA****TTA***CAAAGTGTTTTGATACGGCAGAATG**	This study
cti-F	C*GAGCTC*AGGAGG**TTTCGCACATGGTGCATCG**	This study
cti-R	C*TCTAGA***GGTCAGCCTACCCGATCAGAG**	This study
acrA-F	G*GAGCTCAG***GAGGTTTACATATGAACAAAAACA****GAGG**	This study
acrA-R	C*TCTAGA***CCTGTTTAAGTTAAGACTTGGACTGTTC**	This study
acrB-F	C*TCTAGA***AGGAGCCGTTAAGACATGCCTA**	This study
acrB-R	G*AAGCTT***ACGTTGTATCAATGATGATCGACAG**	This study
tolC-F	C*GAGCTC***CACCACAAGGAATGCAAATGAAG**	This study
tolC-R	G*TCTAGA***GTCATCAGTTACGGAAAGGGTTAT**	This study

[a] Template binding regions are indicated by bold letters, start and stop codons are underlined, and restriction sites are italic underlined.

Organic solvent tolerance assay by plate overlay Terpenoid tolerance levels of *E. coli* overexpressing *marA*, *imp*, *cls* and *cti* genes were analyzed by measuring colony forming efficiency (CFE: colonies per number of cells plated) on LBGMg agar plate. Isopentenol (C5), geraniol (C10), myrcene (C10) and farnesol (C15) were used for the terpenoid tolerance study. The recombinant *E. coli* was cultured aerobically at 37°C in LBGMg medium. When the cell growth (OD_{660}) reached 1.5, serial ten-fold dilutions of the culture were prepared with saline solution. Each cell suspension of 5 μl was spotted onto LBGMg agar plate and then overlaid with different terpenoid solutions in a thickness of 3 mm. The plates were sealed and incubated at 30°C for 24 h (26). Hexane was used as a dilution solvent in preparation of the terpenoid solutions because it has been widely used as a diluent in biphasic solvent industry.

In order to decide the terpenoid concentration levels applied for the plate overlay, the cell growth of *E. coli* DH5α was tested in the LBGMg medium with the addition of up to 1.0% (v/v) of the selected terpenoids. Culture was performed in 50-ml screw cap tube containing 5 ml of LBGMg medium with an inoculation of overnight grown cells. Terpenoids were added in early exponential phase (OD_{600}, 0.1) and cultivated at 250 rpm and 30°C for 24 h.

Organic solvent tolerance assay by broth overlay Recombinant *E. coli* strains harboring pTmarA, pTimp, pTcls and pScti plasmids were inoculated in LBGMg liquid medium and incubated under shaking condition of 250 rpm at 37°C. When the cell growth (OD_{660}) reached 1.0, the culture was overlaid with terpenoid solutions in 10% volume of culture broth and cultivated at 250 rpm and 30°C in shaking incubator (27). The lower culture temperature was applied to reduce a volatile loss of the terpenoids solutions. Cell growth was measured every hour during the 4 h of cultivation. Viable cell count was performed as a measure to represent the number of viable cells in the culture.

Analysis of geraniol penetrated in *E. coli* by gas chromatography The broth overlay culture was performed for 4 h with 1.0% (v/v) geraniol solution. Cells were harvested from the culture broth by centrifugation at 13,000 rpm for 10 min and then washed with 1.0 ml saline solution containing 10 mM $MgSO_4$. After measurement of the wet cell weight (WCW), the cells were resuspended in 1.0 ml of the same washing solution. Intracellular geraniol was extracted by addition of an equal volume of chloroform to the cell suspension and then agitation at 200 rpm for 90 min at 25°C (27). The chloroform extract was analyzed by gas chromatography (Agilent technologies 7890A) to measure the amount of geraniol penetrated into the cells. The sample was injected at a split ratio of 1:10 and each analyte from the 1 μL samples was separated on a 19091J HP-5 capillary column (30 m length, 0.32 mm internal diameter, and 250 μm film thickness). The oven temperature was initially maintained at 80°C for 1 min, then raised with a gradient of 10°C/min to 250°C and was kept for 1 min. Nitrogen was used as a carrier gas with pressure of 39 *psi* inlet and the detector temperature was maintained at 260°C.

RESULTS

Effect of *marA*, *imp*, *cls* and *cti* genes on terpenoid tolerance in plate overlay culture In our previous study, *E. coli* DH5α was found to be the best host strain for the production of terpenoids including carotenoids (3). Therefore, terpenoid tolerance of *E. coli* DH5α overexpressing the genes of *marA*, *imp*, *cls*, and *cti* was investigated by cultivating the cells on LBGMg agar plates overlaid with different terpenoids. In our preliminary study, isopentenol (a hemiterpene alcohol, log $P_{ow} = 1.25$) and geraniol (a monoterpene alcohol, log $P_{ow} = 2.65$) were found to be significantly toxic to *E. coli* compared to myrcene (a monoterpene alkene, log $P_{ow} = 4.58$) and farnesol (a sesquiterpene alcohol, log $P_{ow} = 4.62$) (Fig. 1). Thus, isopentenol and geraniol were applied to the plate overlay culture in a lower concentration of 1% (v/v) than myrcene and farnesol that were used at a final concentration of 6% (v/v) (Fig. 2). In a preliminary study, myrcene and farnesol were also applied to the plate overlay culture at a concentration of 1% (v/v), which was gradually increased to 6% (v/v) (data not shown). However, no significant growth inhibition was observed up to this high concentration due to their low toxicity. It has been known that overexpression of membrane proteins to a high level is toxic to host cells (28). The Cti membrane protein severely inhibited cell growth, when it was expressed in pTrc99A, a high-copy number vector with a strong P_{trc} promoter (data not shown). Therefore, the mild expression vector pSTV28 was herein used for *cti* overexpression. The cell growth of the plate overlay culture after 24 h of incubation is shown in Fig. 2. Not surprisingly, the

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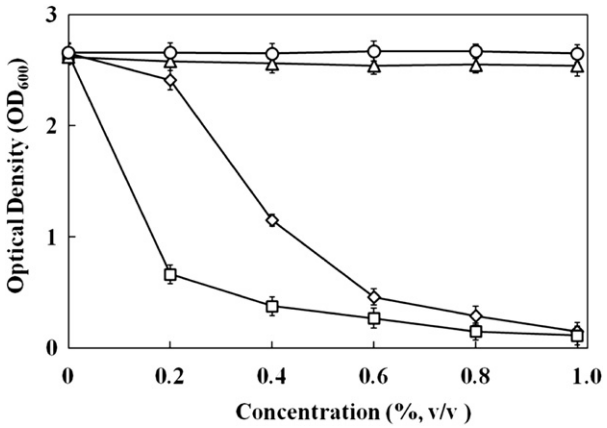

FIG. 1. Toxicity of terpenoids on *E. coli* DH5α. Cell growth of *E. coli* DH5α was measured by optical density at 600 nm after 24 h of cultivation at 30°C in LBGMg media containing isopentenol (diamonds), geraniol (squares), myrcene (circles), and farnesol (triangles) at different concentrations of 0–1.0% (v/v).

CFEs of the recombinant *E. coli* strains expressing *marA*, *imp*, *cls* and *cti* were similar to those of the controls harboring empty vectors (pTrc99A or pSTV28) under no solvent overlaying condition. The CFEs were somewhat lowered by hexane used for the dilution of terpenoid solutions, albeit the complete growth inhibition of the recombinant *E. coli* (pTimp). The overlay of 1% (v/v) geraniol or isopentenol in hexane resulted in much lower CFEs compared to the overlay of hexane solvent only, whereas there was no significant change in the CFEs of the 6% (v/v) myrcene or farnesol overlays. It indicates that isopentenol and geraniol with lower log P_{ow} values are more toxic to cells than myrcene and farnesol. The cellular toxicity of organic solvent is known to be inversely related to its log P_{ow} value in general (8). The overexpression of *imp*, *cls* and *cti* genes did not increase the CFEs against the terpenoids used in this study, unexpectedly. The solvent tolerance mechanisms of Imp, Cls, and Cti did not seem to work against the terpenoids. However, MarA increased the CFEs against geraniol, which was the most toxic among the studied terpenoids, by 10^4-fold compared to the control of pTrc99A. It suggests that the overexpression of *marA* gene could contribute to the geraniol tolerance of *E. coli*. Based on these results, further works were thus conducted with 1% (v/v) geraniol.

Effect of *marA*, *imp*, *cls* and *cti* genes on geraniol tolerance in broth overlay culture To confirm the effect of *marA*, *imp*, *cls* and *cti* genes on geraniol tolerance, the cell growth of *E. coli* DH5α harboring pTmarA, pTimp, pTcls and pScti was investigated in LBGMg liquid medium overlaid with 1% (v/v) geraniol. The optical density (OD660) of *E. coli* DH5α (pTcls, pScti, pTrc99A or pSTV28) did not change (Fig. 3A). The overexpression of *imp* gene resulted in a decrease of the optical density with visible clearing of turbidity, which indicates a lysis of cells. The OD660 of *E. coli* DH5α (pTmarA) increased in the first hour and then declined afterwards. Since the optical density measurement cannot discriminate between the viable and dead cells in the geraniol overlay broth culture, the viable cell numbers of the recombinant strains were counted every hour during the culture (Fig. 3B). The viable cells numbers of the *E. coli* (pTcls, pScti, pTrc99A, or pSTV28) sharply decreased and no viable cell was seen after 2 h of culture. Cell death was even more severe in *E. coli* (pTimp), where no viable cells were observed after only 1 h of culture. However, *marA* overexpression caused a mild decrease in the viable cell number, and there was a large amount of viable cells (4.2×10^5/ml) still present even after 4 h of culture. It is confirmed that the overexpression of *marA* gene contributes to the geraniol tolerance of *E. coli*.

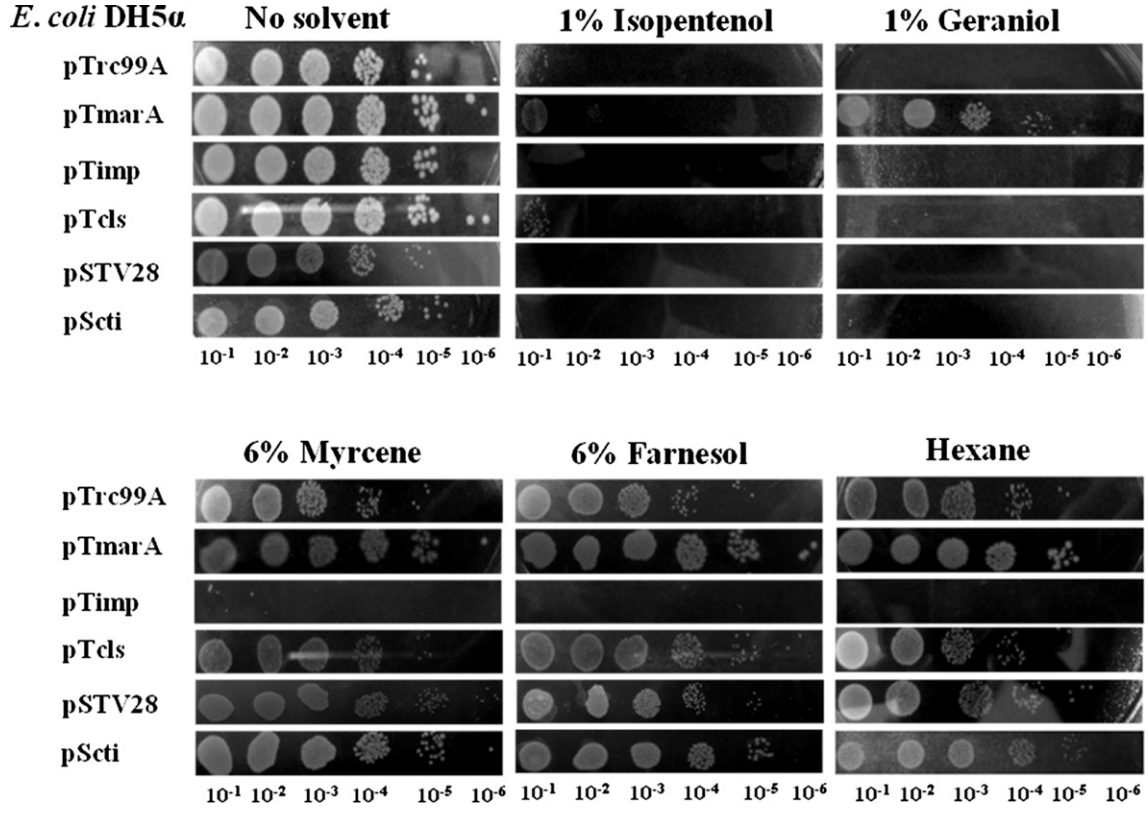

FIG. 2. Colony formation efficiencies of *E. coli* DH5α overexpressing *marA*, *imp*, *cls* and *cti* on terpenoid overlay plates. *E. coli* DH5α harboring pTrc99A, pTmarA, pTimp, pTcls, pSTV28 and pScti were spotted on LBGMg agar plates in serial ten-fold dilutions, overlaid with terpenoids solutions, and incubated at 30°C for 24 h.

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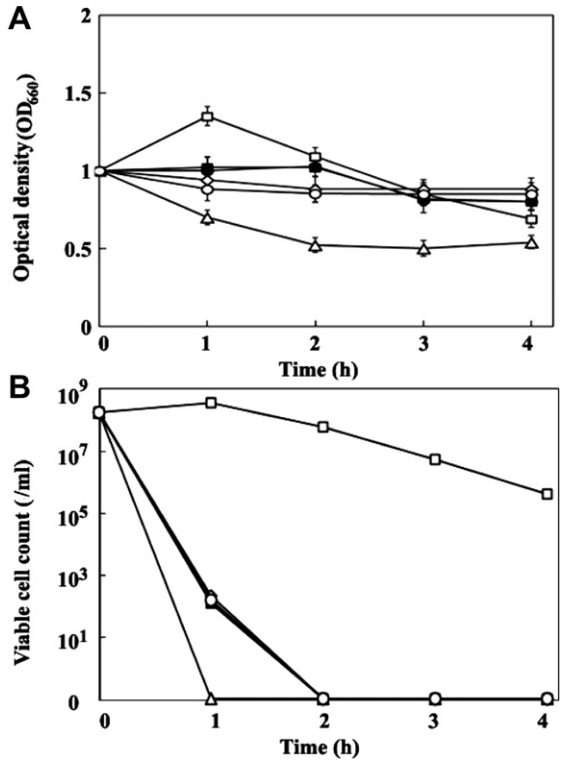

FIG. 3. Effect of overexpression of *marA*, *imp*, *cls* and *cti* genes on cell growth (A) and viability (B) in geraniol overlay broth culture. Optical density and viable cell number were measured in broth overlay culture of 1% (v/v) geraniol. *E. coli* DH5α harboring pTrc99A (closed squares), pTmarA (open squares), pTimp (open triangles), pTcls (open diamonds), pSTV28 (closed circles) and pScti (open circles) were used for the culture.

MarA regulates AcrAB–TolC solvent efflux pump MarA overexpression was shown to enhance geraniol tolerance. In order to investigate the relationship between MarA and geraniol tolerance, we conducted the plate overlay culture with a *marA* knock-out mutant of *E. coli* BW25113 (Fig. 4A). On the plate with no solvent overlay, the cell growth (CFEs) was not affected by the deletion of *marA*. However, the cell growth of the *E. coli* (Δ*marA*) harboring the empty vector pTrc99A was severely inhibited on the plate with overlay of 1% (v/v) geraniol. The growth inhibition of the *marA* knock-out mutant by geraniol was restored with the introduction of pTmarA. MarA has been known to activate the expression of AcrAB–TolC efflux pump that is responsible for an increase of organic solvent tolerance (29). To address if AcrAB–TolC efflux pump was involved in geraniol tolerance, the plate overlay culture was also performed with *acrA*, *acrB* and *tolC* knock-out mutants (Fig. 4B). The cell growth of the *acrA*, *acrB* and *tolC* knock-out mutants was completely inhibited by geraniol. The growth inhibition was complemented by the introduction of pTacrA, pTacrB and pTtolC in the *E. coli* (ΔacrA/pTacrA, ΔacrB/pTacrB and ΔtolC/pTtolC, respectively), whereas *marA* overexpression in *E. coli* (ΔacrA/pTmarA, ΔacrB/pTmarA and ΔtolC/pTmarA, respectively) failed to restore the cell growth. These results suggest that the geraniol tolerance of *E. coli* overexpressing *marA* is due to the AcrAB–TolC efflux pump mediated process. However, the *acrA*, *acrB* and *tolC* overexpression did not improve the geraniol tolerance of the *E. coli* (wild/pTtacrA, wild/pTtacrB and wild/pTtolC, respectively) (Fig. 4C). This is a natural consequence, because a single overexpressed component cannot constitute the AcrAB–TolC efflux pump without the co-overexpression of the others. In the plate culture of no solvent overlay, the cell growth (CFEs) was not affected by the deletion of *acrA*, *acrB* and *tolC*. A higher geraniol tolerance was observed in *E. coli* BW25113 compared to *E. coli* DH5α (Fig. 2 and Fig. S1). The organic solvent tolerance of *E. coli* has been known to differ significantly with different strains (14). An increased geraniol tolerance was also obtained by *marA* overexpression in the BW25113 strain, although the increase was less significant compared to the DH5α strain.

Decrease of intracellular geraniol concentration by overexpression of *marA* We assumed that the AcrAB–TolC efflux pump triggered by *marA* overexpression could export geraniol out of cells and increase the cellular tolerance against geraniol. This was addressed with the measurement of intracellular geraniol concentration in the broth overlay culture of geraniol (Table 2). The intracellular geraniol concentration of *E. coli* BW25113 (pTmarA) was observed at 6.3 μg/mg WCW, which was two-fold lower than 12.9 μg/mg WCW of *E. coli* BW25113 (pTrc99A). The deletions of *marA* and *tolC* genes caused significant increases of the intracellular geraniol concentration by 18.4 and 16.3 μg/mg WCW, respectively. The intracellular geraniol concentration was inversely related to the cell growth (CFEs) observed in Fig. 4. Activation of the AcrAB–TolC efflux pump by MarA is suspected to maintain the intracellular geraniol concentration of *E. coli* in a low level and to contribute to the geraniol tolerance of *E. coli*.

DISCUSSIONS

Improvement of microbial tolerance against organic solvents is required for the mass production of biofuel molecules. Organic solvent tolerance of *E. coli* has been improved by high level expression of stress responsive genes (13,14,26,27). The bacterial genes such as *marA*, *cls*, *imp* and *cti* have been found to increase organic solvent tolerance and their deletions in wild type strains resulted in an increase of sensitivity towards organic solvents. Therefore, we conducted overexpression of *marA*, *imp*, *cls* and *cti* genes to improve terpenoid tolerance of *E. coli*. Interestingly, we found that *marA* overexpression significantly improved geraniol tolerance. Overexpression of *marA* has been known to increase organic solvent tolerance in *E. coli* either by positive regulation of the stress responsive genes such as *sodA*, *nfo*, *micF*, *inaA*, *fumC*, *zef* and *fpr* or by activation of AcrAB–TolC efflux pump (14,21,23). In this study, we observed a decrease of intracellular geraniol concentration by *marA* overexpression, whereas geraniol concentration was increased by *marA* and *tolC* deletions. It was also found that the Δ*marA*, Δ*acrA*, Δ*acrB* and Δ*tolC* mutant strains were hypersensitive to geraniol. We hypothesized that geraniol tolerance was inversely related to the intracellular geraniol concentration and that the *marA* activated AcrAB–TolC efflux pump reduced the geraniol concentration in cells. It was confirmed by the *marA* overexpression in the Δ*acrA*, Δ*acrB* and Δ*tolC* mutant strains which failed to increase geraniol tolerance. However, an increase of geraniol tolerance was not observed in the *acrA*, *acrB* and *tolC* overexpression strains because of the limitation of their respective partner proteins. The overexpression of AcrAB–TolC efflux pump needs to be performed carefully because all of its constituents, AcrA, AcrB, and TolC, are membrane associated proteins. Overexpression of membrane proteins often causes a severe inhibition on cell growth (30). In this study, severe growth inhibition was also observed when AcrA, AcrB and TolC were highly overexpressed from pTacrA, pTacrB, and pTtolC (based on the strong expression vector pTrc99A, respectively) with 0.1 mM IPTG induction (data not shown here). Therefore, the recombinant *E. coli* harboring pTacrA, pTacrB and pTtolC were cultivated without IPTG induction and the genes were expressed in the LBGMg complex medium by leaky expression. All things taken into consideration, the augmentation of AcrAB–TolC efflux

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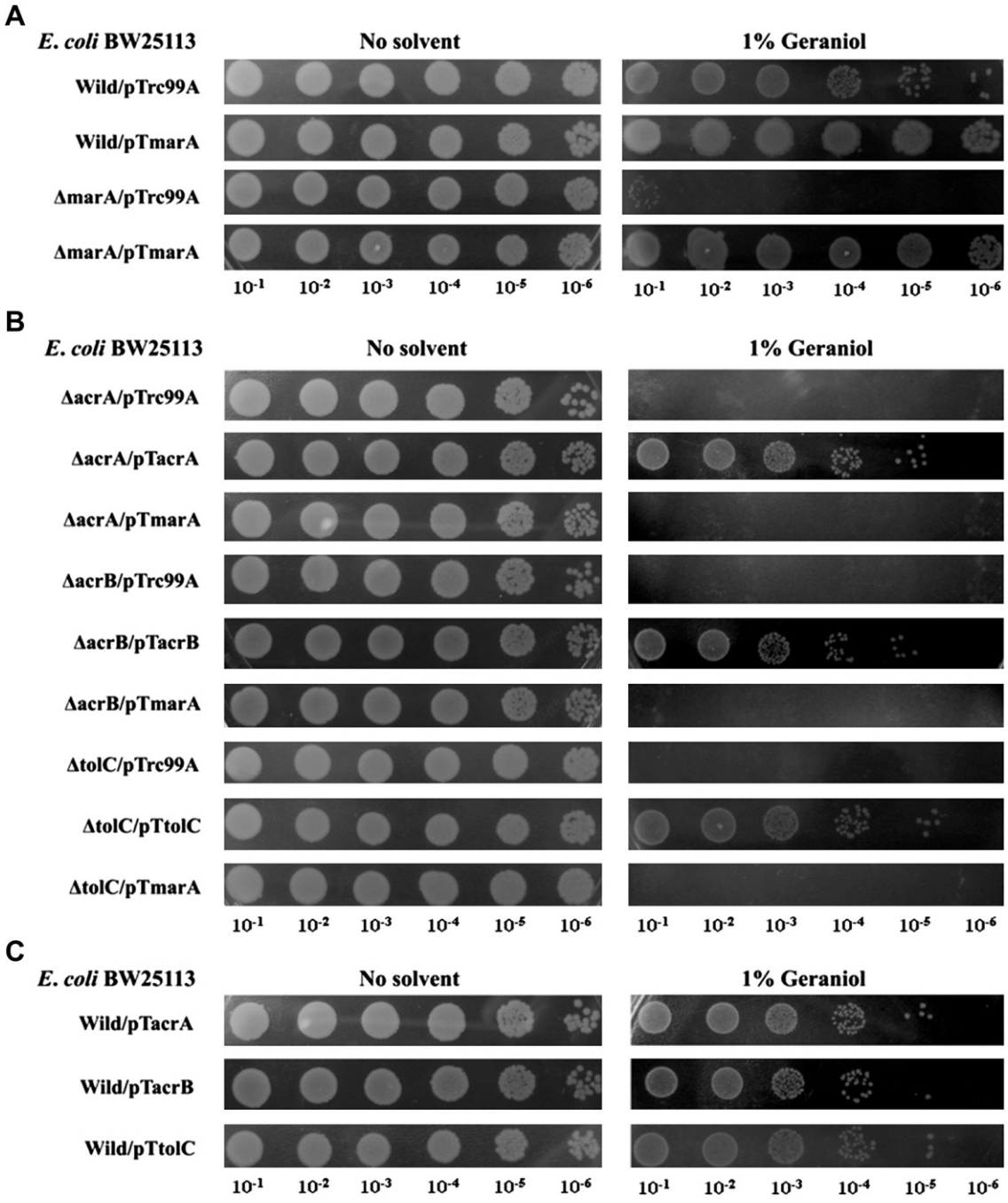

FIG. 4. Colony formation efficiencies depending on deletion and overexpression of *marA* (A), deletion of *acrA*, *acrB* and *tolC* (B), and overexpression of *acrA*, *acrB* and *tolC* (C). *E. coli* BW25113 and its *marA*, *acrA*, *acrB* and *tolC* knock-out mutants were transformed with pTrc99A, pTmarA, pTacrA, pTacrB and pTtolC. Each strain was spotted on LBGMg agar plates in serial ten-fold dilutions, overlaid with 1% (v/v) geraniol, and then incubated at 30°C for 24 h.

TABLE 2. Intracellular geraniol concentration of *E. coli* BW25113 and its mutant strains.

E. coli strains	Intracellular geraniol concentration (μg/mg WCW)[a]
BW25113 (pTrc99A)	12.9 ± 1.1
BW25113 (pTmarA)	6.3 ± 0.7
JW5249 (Δ*marA*)	18.4 ± 2.3
JW5503 (Δ*tolC*)	16.3 ± 1.9

[a] It indicates the amount of intracellular geraniol penetrated inside *E. coli* cells for 4 h in the broth overlay culture of geraniol.

pump by the overexpression of the MarA regulator alone can be a more simple and efficient way to increase geraniol tolerance. The MarA effect was more significant in the *E. coli* DH5α strain, which was found to be a good host for terpenoids production (3), rather than the *E. coli* BW25113 strain.

In conclusion, it was found that geraniol tolerance in *E. coli* was driven by the overexpression of *marA*, the transcriptional activator of AcrAB–TolC efflux pump. It seems that the AcrAB–TolC efflux pump is effective in pumping out geraniol from the cells. Therefore, a positive regulation of AcrAB–TolC efflux pump by MarA overexpression is beneficial to geraniol tolerance which results in high geraniol production.

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