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# Gas-phase bioproduction of a high-value-added monoterpenoid (*E*)-geranic acid by metabolically engineered *Acinetobacter* sp. Tol 5

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**Abstract**

Gas-phase bioproduction, in which immobilized biocatalysts are employed and chemical reactions are performed in a gas phase, has attracted researchers' attention as a green process. However, there is difficulty in the employment of whole cell catalysts for gas-phase bioproduction due to the lack of a suitable cell immobilization method. *Acinetobacter* sp. Tol 5 is a unique bacterium, which is remarkably sticky and can be easily immobilized onto various material surfaces through the adhesive bacterionanofiber protein AtaA. In this study, we demonstrate the gas-phase bioproduction of (*E*)-geranic acid (GA), a high-value-added monoterpenoid, from geraniol using immobilized Tol 5 transformant cells, into which a gene involved in a (*E*)-GA synthetic pathway was introduced. Time course analysis of the liquid-phase bioproduction of (*E*)-GA revealed the inherent metabolism of Tol 5 involved in the degradation of (*E*)-GA. By disrupting the *fadD4*-ortholog gene, which encodes a key enzyme of the (*E*)-GA degradation, we successfully generated a (*E*)-GA-accumulating strain, Tol 5 Δ*fadD4* (pGeoA). The immobilized cells of this mutant strain on a polyurethane support enabled the production of (*E*)-GA with a passive supply of gaseous geraniol in a batch gas-phase reaction. A major fraction of the (*E*)-GA, which was produced, was adsorbed onto the polyurethane support but easily extracted into ethanol, a safe solvent without environmental impact. This is the first example of gas-phase bioproduction of a complex and high-value-added compound. Tol 5 is a highly promising platform for gas-phase bioproduction.

## Introduction

Bioproduction employing biocatalysts is a green process which can be used to realize the production of chemicals that are inaccessible by organic synthesis, under ordinary temperature and pressure conditions. Biocatalysts include whole cell catalysts and isolated enzymes, but the former is more cost-efficient than the latter because it does not require a purification process. A cascade reaction with multiple steps is also expected to proceed intercellularly via multiple enzymes[1, 2]. Whole cell catalysts, microbial cells in most cases, are usually used in suspension in an aqueous medium, that is, liquid-phase bioproduction. Many substrates (feedstocks) used in bioproduction are water-soluble materials including carbohydrates, ionic compounds, alcohols, amines, and nitriles. However, hydrophobic substrates with low water-solubility are not suitable for conventional liquid-phase bioproduction because this process requires vigorous agitation with high energy consumption to dissolve the substrates in an aqueous solution[3, 4]. Alternatively, the two-liquid-phase partitioning bioprocess, in which an organic solvent is used for dissolving a hydrophobic substrate, has been developed for not only bioproduction but also biodegradation of hazardous chemicals[5-8]. In this system, the organic solvent works as a substrate sink but vigorous agitation is also required to increase the interface between two immiscible liquids.

For hydrophobic gaseous substrates, a unique system has been proposed, that is, a gas-phase bioprocess, in which there is no aqueous solution to act as a suspension medium for biocatalysts[9-11]. In this process, biocatalysts are used in an immobilized state on solid supports and the biocatalytic reaction proceeds in a gas phase. This gas-phase bioproduction has the following three advantages over liquid-phase bioproduction: (1) efficient mass transfer due to high molecular diffusion rates in a gas phase, (2) availability of a passive supply of gaseous substrates with low energy

consumption, and (3) low water content reducing the microbial contamination risk. However, target products of gas-phase bioproduction have been limited to simple low molecular weight compounds with low boiling points. Another difficulty with gas-phase bioproduction is the lack of cell immobilization methods suitable for gas-phase reactions. A gel entrapment method, which is mostly used for whole cell catalysts, cannot offer actual gas-phase reaction because gels have a high water content. The Gram-negative bacterium *Acinetobacter* sp. Tol 5 exhibits nonspecific, high adhesiveness[12], and its cells can be easily immobilized onto various material surfaces[13-16] through the adhesive bacterionanofiber protein AtaA localized on the cell surface[17, 18]. Tol 5 can versatilely metabolize various chemicals including aromatic hydrocarbons such as toluene and benzene, organic solvents such as ethanol, and oils such as triacylglycerol[12, 19]. In the previous study, immobilized Tol 5 cells were used for the degradation of toluene in a gas phase[20].

Whole cell biocatalysts allow for the production of high-value-added derivatives oxygenated from inexpensive monoterpenoids through regio- and stereo-selective one-pot reactions that are difficult for chemical synthesis[21-25]. Most monoterpenoids are volatile, and therefore can become targets for gas-phase bioproduction. In the previous study, we generated Tol 5 transformant cells harboring the *geoA* gene from *Castellaniella defragrans*[26], which encodes geraniol dehydrogenase. This transformant produced small amounts of (*E*)-GA and (1*R*,3*R*,4*R*)-1-methyl-4-(1-methylethenyl)-1,3-cyclohexanediol [(1*R*,3*R*,4*R*)-MMC] from geraniol, a volatile monoterpenoid, in liquid-phase bioproduction[27]. (*E*)-GA is a high-value-added monoterpenoid with great potential for industrial applications such as a perfuming agent[28] and a building block for the production of natural flavor esters[29]. Additionally, it shows strong antifungal activities against corn phytopathogens[30] and tyrosinase inhibitory activity[31, 32]. We

hypothesized that the gas-phase bioproduction of (*E*)-GA was feasible using immobilized cells of a Tol 5 derivative. This study demonstrates, for the first time, the gas-phase bioproduction of a high-value-added monoterpenoid using whole cell biocatalysts.

## Experimental

### Chemicals, bacterial strains, and growth conditions

Geraniol and GA (mixture of isomers) were purchased from FUJIFILM Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Bacterial strains used in this study are listed in Table 1. *Acinetobacter* sp. Tol 5 and its derivative strains were grown in a basal salt (BS) medium[17] supplemented with toluene, lactate, geraniol, or GA ($3.3\times10^{-2}$ mol-carbon/L) or Luria-Bertani (LB) medium at 28 °C, with shaking. *Escherichia coli* strains were grown in LB medium containing the appropriate antibiotics at 37 °C. Antibiotics were used at the following concentrations, when required: ampicillin (500 μg/mL) and gentamicin (10 μg/mL) for Tol 5 derivative strains, ampicillin (100 μg/mL) and gentamicin (10 μg/mL) for *E. coli*. Arabinose was added to a final concentration of 0.2% (wt/vol) for the induction of *geoA* under the control of an arabinose-inducible promoter.

### Construction of a *fadD4*-ortholog deletion mutant in *Acinetobacter* sp. Tol 5

Plasmids and the nucleotide sequences of primers used in this study are listed in Tables 1 and 2, respectively. To construct a Δ*fadD4* mutant of Tol 5, we used a general gene replacement strategy, as described previously[33, 34]. Briefly, DNA fragments containing the 1.5-kb upstream and 1.5-kb downstream regions of *fadD4* were amplified by PCRs using primer sets pJQfadD4Fw/fadD4Re and fadD4Fw/pJQfadD4Re,

respectively. These fragments were cloned into the *BamH*I site of pJQ200sk by using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, Ipswich, MA), generating pJQfadD4; this plasmid was used as a suicide plasmid for the gene replacement of *fadD4*. The single crossover mutant was selected on a BS agar plate containing 100 μg/mL gentamycin supplemented with toluene, and then the second crossover mutant was selected on a BS agar plate containing 5% sucrose. The chromosomal integration of the plasmid in the single crossover mutant and the deletion of *fadD4* in the second crossover mutant were confirmed by PCRs using primer sets fadD4 dcofw2/scofadD4-Re and scofadD4-Fw/scofadD4-Re, respectively. Transformation of the Tol 5 Δ*fadD4* mutant with pGeoA was performed, as described previously[27].

**Immobilization of bacterial cells onto a polyurethane foam support**

Cells were immobilized onto a polyurethane (PU) foam support, as described previously[20]. Briefly, the overnight culture of cells was inoculated into 20 mL LB medium and incubated with shaking at 28 °C for 12 h. The cells were harvested by centrifugation, washed twice with deionized water ($dH_2O$), and resuspended in 20 mL BS medium at an optical density at 660 nm ($OD_{660}$) of 1.0 in a 100 mL Erlenmeyer flask. Four pieces of 1 $cm^3$ PU support with a specific surface area of 6.68 $cm^2/cm^3$ (CFH-13; Inoac Corporation, Nagoya, Japan) were placed into the cell suspension and shaken at 115 rpm at 28 °C for 1 h.

The PU support with or without immobilized cells was picked up from the cell suspension with tweezers, gently squeezed on Kimtowel (Nippon Paper Crecia, Tokyo, Japan) to remove most of the water, and weighed to determine its wet weight. Then, the PU support with or without immobilized cells was frozen at −80 °C for 2 h, lyophilized

to completely remove moisture, and weighed to determine its dry weight. The amount of water adsorption in the PU support with or without immobilized cells was calculated from the difference of the weights before and after the lyophilization.

**Reaction**

For liquid-phase reaction, precultured cells were grown in 20 mL LB medium for 12 h, harvested by centrifugation, washed with $dH_2O$, and resuspended in 20 mL BS medium at an $OD_{660}$ of 1.0 in a 100 mL Erlenmeyer flask. Dimethyl sulfoxide (DMSO), which dissolves geraniol, was added to the cell suspension for a final DMSO concentration of 20 mM, to give a final geraniol concentration of 5 to 80 mM. During the incubation at 28 °C for 10 days with shaking at 115 rpm, 200 μL of culture was harvested using a syringe (MS-N500; ITO Corporation, Fuji, Japan) every other day. The culture sample was saturated with NaCl and extracted with 200 μL of ethyl acetate (EtOAc) containing 0.5 mM thymol as an internal standard. For gas chromatography-mass spectrometry (GC-MS), 1 μL of the EtOAc extract was sampled using a syringe (MS-GFN10; ITO Corporation, Fuji, Japan).

For the gas-phase reaction, immobilized cells on a PU support were prepared as described above. Four pieces of the PU support with immobilized cells were suspended from the top of a 125 mL cylindrical vial using a stainless steel wire that was penetrated through a butyl rubber plug. A drop of geraniol was placed on the bottom of the vial using a syringe so as to supply more than 10 μmol/vial (1.7 μL-liquid/vial) of geraniol. To supply geraniol at a concentration of ≤1.0 μmol/vial, a drop of DMSO containing geraniol was placed in the vial. After incubation at 28 °C without agitation, gaseous (*E*)-GA was analyzed by direct headspace GC-MS or headspace solid phase-microextraction (HS-SPME)-GC-MS. For the analysis of adsorbed (*E*)-GA on the PU

158 support, immobilized cells on the PU support were removed beforehand by washing
159 with $dH_2O$. For this purpose, a piece of PU support was put into a 50 mL conical tube
160 with 200 μL of $dH_2O$, vortexed for 20 s, and transferred into a new tube. This washing
161 process was repeated twice. Thereafter, four pieces of the washed PU support were
162 treated with 400 μL ethanol containing 0.5 mM thymol as an internal standard to extract
163 (*E*)-GA adsorbed on the PU support. To analyze the (*E*)-GA which had accumulated
164 intracellularly, the detached cells were lysed completely with 400 μL chloroform. The
165 obtained extracts were centrifuged at 12,000 *g* for 5 min, dried over anhydrous sodium
166 sulfate, and then filtered through a 0.20 μm PTFE syringe driven filter unit (Millex-LG,
167 Millipore, Tokyo, Japan). One microliter of the organic phase was analyzed by GC-MS
168 using a syringe (MS-GFN10) for sampling.
169 To reuse immobilized cells for the gas-phase reaction, adsorbed (*E*)-GA was
170 extracted by immersing the PU support with the immobilized cells in 5 mL BS medium
171 containing 10% DMSO for 10 s after the 5 days reaction with 200 μmol/vial liquid
172 geraniol. One milliliter of the extract was re-extracted with 1 mL of EtOAc containing
173 0.5 mM thymol as an internal standard. This was then analyzed by GC-MS. The PU
174 support with the immobilized cells was gently squeezed on to a Kimtowel to remove
175 most of the water. Four pieces of the PU support with the immobilized cells were
176 subjected to the gas-phase reaction again.
177
178 **Examination of chemical inhibition and toxicity**
179 The inhibitory effect of geraniol and GA on Tol 5 cell growth was examined in LB
180 medium, as previously described[27]. The maximum growth rate at a given concentration
181 of geraniol or GA ($\mu_{max}$) was determined during the exponential growth phase 1 h after
182 the addition of geraniol or GA. The maximum growth rate without geraniol or GA

($\mu_{max0}$) was also determined during the exponential growth phase. Growth inhibition was evaluated using the ratio of $\mu_{max}$ and $\mu_{max0}$ ($\mu_{max}/\mu_{max0}$).

To examine the toxicity of gaseous geraniol and GA, immobilized cells were exposed to the chemical vapors for 12 h in a vial set as described above in the gas-phase reaction. After a 12 h incubation at 28 °C for the exposure, immobilized cells on the PU support were detached by washing with 20 mL $dH_2O$. The cell suspension was spread on LB agar after serial dilution and incubated at 28 °C overnight. Colonies were counted to determine the colony forming units (CFU).

**Gas chromatography-mass spectrometry**

GC-MS analysis was performed in the selected ion monitoring (SIM) mode on a model 7820A gas chromatograph (Agilent Technologies, Wilmington, DE) equipped with a model MSD 5977E mass spectrometer (Agilent Technologies). A fused-silica capillary Rtx-200 column (30 m length, 0.32 mm i.d.; Restex, Bellefonte, PA) was used. Helium was used as the carrier gas.

For the analysis of liquid samples, the oven temperature was programmed from 60 to 250 °C, increased at a rate of 4 °C/min. The injection port temperature was maintained at 245 °C. The flow rate of the carrier gas was set at 10 mL/min. The GC column effluent was introduced directly into the ion source via a transfer line at 280 °C. The ion source temperature was set at 230 °C. The split ratio for the injection and the voltage for electron ionization were set at 50:1 and 70 eV, respectively.

Gaseous geraniol and (*E*)-GA were measured by direct headspace GC-MS or HS-SPME-GC-MS using the method by Sanekata et al[35] with slight modifications. In the direct headspace GC-MS, 50 μL of the head space gas was taken using a gas tight

207 syringe (MS-GFN100; ITO Corporation, Fuji, Japan) and injected into the GC-MS
208 system. The same operating program was used with liquid samples.
209 In the HS-SPME GC-MS, gaseous chemicals were adsorbed to a SPME fiber made
210 from polydimethylsiloxane/divinylbenzene (65 μm film in thickness, Supelco,
211 Bellefonte, PA). The SPME fiber was conditioned beforehand, following the
212 manufacturer's instructions, and then placed into the head space of a vial. When
213 analyzed, the fiber was inserted into a GC injector at 250 °C for 3 min in the splitless
214 mode. The flow rate of carrier gas was set at 1.5 mL/min. The oven temperature
215 program was 60 °C for 3 min, raised to 250 °C, and increased at a rate of 5 °C/min,
216 followed by a 3 min isotherm. An external calibration curve method was used for
217 quantification. For the preparation of calibration curves, a drop of DMSO containing
218 0.01 to 5% (V/V) geraniol or GA was enclosed into the vial, vaporized completely at
219 28 °C for 24 h under atmospheric pressure, and analyzed by HS-SPME-GC-MS. The
220 calibration parameters are listed in Table S1. The maximum adsorption amounts of
221 geraniol and GA on the SPME fiber were 100 nmol and 400 nmol, respectively.
222 Favorable linearity ($R^2$ value of >0.99) for both chemicals was observed in the range of
223 1 nmol to the maximum adsorption amounts above per vial. The limits of detection
224 (LOD) and limits of quantitation (LOQ) were determined, following the method by
225 Chen et al[36].
226
227 **Results**
228 **Effects of geraniol and (*E*)-geranic acid concentrations on *Acinetobacter* sp. Tol 5**
229 Substrate/product inhibition is referred to as a main bottleneck hampering the
230 microbial production of monoterpenoids[37, 38]. To examine the tolerance of the Tol 5
231 wild type (WT) to the substrate geraniol and the product GA, a growth inhibition assay

was performed. The cell growth of Tol 5 WT was inhibited by geraniol in a dose-dependent manner, strongly affected at 50 mM, and completely ceased at 80 mM (Fig. 1A). Although Tol 5 WT showed higher tolerance to GA than to geraniol, its cell growth was also inhibited by GA in a dose-dependent manner, strongly affected at higher than 80 mM, and completely ceased at 120 mM (Fig 1B). Because no threshold concentration of the substrate/product inhibition was found, the effect of geraniol concentration on (*E*)-GA production by Tol 5 (pGeoA) was examined. Geraniol in the concertation range of 5 to 80 mM was added to a liquid culture of Tol 5 (pGeoA) and incubated at 28 °C with shaking for 10 days. Figure 2 shows the time course of (*E*)-GA production for each geraniol concentration. When supplemented with 10 mM geraniol, the largest amount of (*E*)-GA was produced. At all concentrations of geraniol, the amounts of produced (*E*)-GA decreased from the eighth day. (1*R*,3*R*,4*R*)-MMC, which was determined as the major product by Tol 5 (pGeoA) cells in the previous study[27], was detected only when supplemented with 80 mM geraniol; it was below the detection limit when supplemented with less than 60 mM geraniol.

Based on the decrease in the amount of the produced (*E*)-GA from the eighth day, we assumed that Tol 5 can intrinsically metabolize (*E*)-GA. To confirm this, Tol 5 WT cells were inoculated into the medium containing either GA or lactate as the sole carbon source. The cell growth of WT was observed in both media although the maximum values of $OD_{660}$ were different (Fig. 3). This indicated that Tol 5 is able to metabolize GA as the sole carbon and energy source.

**Metabolic engineering of *Acinetobacter* sp. Tol 5 for improving (*E*)-geranic acid production**

To repress the degradation of (*E*)-GA, we tried to disrupt a gene involved in the metabolism of (*E*)-GA in Tol 5. According to the metabolic pathway of (*E*)-GA reported in *Pseudomonas aeruginosa*[39], (*E*)-GA is converted to geranyl CoA by FadD4, which is one of six fatty acyl-CoA synthetases, and then degraded through the β-oxidation pathway followed by the TCA cycle (Fig. 4); thus, FadD4 is a key enzyme for degrading (*E*)-GA. To identify a FadD4-ortholog from the Tol 5 genome (unpublished data), a sequence similarity search was performed using BLASTP at an E-value cutoff of $10^{-30}$ using FadD4 from *P. aeruginosa* (GenBank accession number: WP_003114249) as a query. As a result, a FadD4-ortholog that showed 46.3% identity and 63.4% similarity was found. A Tol 5 mutant deficient in the *fadD4*-ortholog gene, Tol 5 Δ*fadD4*, was constructed using a general gene-replacement method. The disruption of the target gene was confirmed by PCR using primers annealing to the flanking region of the *fadD4*-ortholog gene that were used as homologous sites for recombination. The length of the amplicon (3,516 bp) from Tol 5 Δ*fadD4* was shorter than that from Tol 5 WT (5,110 bp) (Fig. S1), indicating the successful excision of the *fadD4*-ortholog gene. To confirm the repression of (*E*)-GA metabolism in Tol 5 Δ*fadD4*, the mutant strain was inoculated into a medium supplemented with either lactate or GA as the sole carbon source. The Tol 5 Δ*fadD4* mutant grew as well as the Tol 5 WT strain on lactate, but barely grew on GA (Fig. 3), implying that the Tol 5 Δ*fadD4* mutant was unable to metabolize (*E*)-GA.

The Tol 5 Δ*fadD4* mutant was transformed with the pGeoA plasmid, generating Tol 5 Δ*fadD4* (pGeoA), whose metabolic pathway of geraniol is shown in Fig. 4. Tol 5 Δ*fadD4* (pGeoA) cells were examined for (*E*)-GA production from 10 mM geraniol in the liquid-phase reaction for 10 days. Unlike when Tol 5 (pGeoA) cells were used, the amount of (*E*)-GA produced did not decrease from the eighth day when Tol 5 Δ*fadD4*

(pGeoA) cells were used (Fig. 5). After the sixth day, the amount of (*E*)-GA produced plateaued as the substrate geraniol was almost completely consumed (data not shown). The maximum amount of (*E*)-GA produced by Tol 5 Δ*fadD4* (pGeoA) cells was 5.7 ± 0.7 mM, which was about twice of that of the Tol 5 (pGeoA) cells (2.9 ± 0.2 mM). However, the mass balance between the substrate and the product was mismatched. A fraction (about 30%) of (*E*)-GA was vaporized and was not included in the product quantitated by GC-MS (data not shown). The other fraction of geraniol was assumed to be metabolized via the intermediate metabolite geranial in Tol 5 Δ*fadD4* (pGeoA) cells. To confirm this, Tol 5 WT, Tol 5 (pGeoA), and Tol 5 Δ*fadD4* (pGeoA) were inoculated into a medium containing geraniol as the sole carbon source; the two Tol 5 transformants can produce geranial from geraniol through the GeoA expressed in the cells while Tol 5 WT cannot. The cell growth of the Tol 5 transformants was observed and the maximum values of $OD_{660}$ were achieved at 11 h: the $OD_{660}$ of Tol 5 (pGeoA), which is able to metabolize (*E*)-GA, was approximately 0.35, whereas that of Tol 5 Δ*fadD4* (pGeoA), which is unable to metabolize (*E*)-GA, was about 0.15 (Fig. 6). This indicated that Tol 5 is able to intrinsically metabolize geranial to a small degree, using it as the sole carbon and energy source via an unknown intermediate as well as via (*E*)-GA (Fig. 4).

**Gas-phase bioproduction of (*E*)-geranic acid from gaseous geraniol**

To reveal the substrate concentration that can be supplied as a gas, we determined the saturated concentration of gaseous geraniol. For this purpose, 100 μL (576 μmol) of liquid geraniol was placed on the bottom of a 125 mL vial. The liquid geraniol gradually vaporized under the experimental conditions at 28 °C and atmospheric pressure (Fig. S2A). The concentration of gaseous geraniol was saturated at 1.5

μmol/mL-gas, which corresponded to 32.5 μL-liquid/vial, at 8 h, and liquid geraniol remained visible. The concentration dependence of geraniol vaporization was also examined under the same conditions. When 34.7 μL (200 μmol) of liquid geraniol was placed in a vial, the vaporization curve coincided with that of 100 μL-liquid/vial liquid geraniol and a saturated chemical vapor state (1.5 μmol/mL-gas) was reached. When less than 17.4 μL-liquid geraniol was placed, it was almost fully vaporized, with 95% and 98% vaporization of 17.4 μL (100 μmol) and 1.7 μL (10 μmol) of liquid geraniol, respectively (Fig.S2A). When 0.17 (1 μmol) or 0.017 μL (0.1 μmol) of liquid geraniol dissolved in 1.7 μL DMSO solution was placed, the droplet of DMSO remained at the bottom after 12 h incubation but geraniol was almost fully (99%) vaporized (Fig. S2B). We analyzed the geraniol contained in the DMSO droplet, but no geraniol was detected. Thus, geraniol could be supplied up to 1.5 μmol/mL as a gas to the microorganism.

To construct a batch system of the gas-phase bioproduction of (*E*)-GA, Tol 5 Δ*fadD4* (pGeoA) cells were immobilized onto a PU support using the adhesive properties of AtaA[17]. As a negative control, Tol 5 Δ*ataA* cells were also subjected to the same immobilization procedure. Tol 5 Δ*fadD4* (pGeoA) cells were able to be immobilized as efficiently as Tol 5 WT cells (Fig. S3). The amount of water adsorption on the PU support with or without the immobilized cells was 0.05 mL/cm$^3$ and 0.03 mL/cm$^3$, respectively. Therefore, water content was lower than 5% and there was no bulk water around the cells. The PU supports with the immobilized cells were suspended from the top of a vial. Liquid geraniol less than 34.7 μL/vial was supplied to give the cells up to the saturated gas concentration determined above (1.5 μmol/mL-gas). A drop of geraniol or DMSO solution containing geraniol was placed on the bottom of the vial so as not to make direct contact with the PU support pieces (Fig. 7A).

Prior to experiments for gas-phase bioproduction, the toxicity of gaseous geraniol and GA to Tol 5 was examined. In the concentration ranges of 0.8 to 1500 nmol/mL-gas of geraniol and GA, there were no significant differences in viable cell counts [ANOVA, $P = 0.94 > 0.05$ (geraniol), $P = 0.89 > 0.05$ (GA)] (Fig. S4), indicating that gaseous geraniol and GA were not toxic to Tol 5 in this batch system. We also confirmed that DMSO did not affect Tol 5 cell growth or (*E*)-GA production (data not shown).

Gaseous (*E*)-GA produced by the gas-phase reaction was taken from the headspace of the vial or adsorbed on the SPME fiber and quantified by GC-MS. We confirmed that geraniol is not autoxidized into (*E*)-GA and that PU does not work as a catalyst in the absence of the immobilized cells (Fig. S5). Tol 5 Δ*fadD4* (pGeoA) cells in a gas-phase produced gaseous (*E*)-GA from the gaseous substrate geraniol and (*E*)-GA detected in the gas-phase gradually increased (Fig. 7B). Although the concentration of gaseous (*E*)-GA increased concomitantly with geraniol concentration, the conversion of geraniol into gaseous (*E*)-GA was quite low (≤ 8.3 ± 2.1%) (Table 3). We assumed that most (*E*)-GA molecules produced by the gas-phase reaction were immediately adsorbed on the PU support. To confirm this, we attempted to extract adsorbed (*E*)-GA molecules from the PU surface into ethanol. Beforehand, the immobilized cells were detached from the PU support by washing with $dH_2O$, according to our previously established method utilizing the reversibility of AtaA adhesion[20]. Thereafter, the adsorbed (*E*)-GA was extracted with ethanol from the PU support and the detached cells, followed by quantification using GC-MS. (*E*)-GA was not detected from the ethanol extract of the detached cells (data not shown). Even when the detached cells were completely lysed with chloroform, (*E*)-GA was not detected (data not shown), indicating neither (*E*)-GA adsorption on the cell surface nor (*E*)-GA accumulation inside the cells. However, (*E*)-

GA was extracted from the PU support into ethanol. Figure 7C shows the time course of the adsorbed (*E*)-GA on the PU support. The amount of adsorbed (*E*)-GA increased with the amount of geraniol supplied and reached 114 μmol/vial at 120 h from 200 μmol/vial of geraniol. The yields and conversions of gaseous and adsorbed (*E*)-GA are summarized in Table 3. When less than 100 μmol/vial geraniol was supplied, the conversion almost coincided with the yield and their values were similar (around 65%). When 200 μmol/vial of geraniol was supplied, the conversion increased to 84% but the yield decreased to 58% due to the increase in the remaining substrate. (*E*)-GA was detected as the sole product from each concentration of geraniol given; that is, the selectivity was 100%. The maximum production rate of (*E*)-GA was 1.4 μmol/(mL-reaction volume·h), which was calculated from the (*E*)-GA amount produced from the 200 μmol/vial of geraniol between 8 to 12 h (Fig. S6). The reaction volume was considered to be the apparent volume of the PU support, 4 mL. This production rate was about 20 times higher than that of the liquid-phase reaction [70 ± 35 nmol/(mL-liquid·h)], which was calculated from the amount of (*E*)-GA produced from 4 to 6 days shown in Fig. 5.

Furthermore, the reusability of immobilized Tol 5 Δ*fadD4* (pGeoA) cells for the batch gas-phase reaction was examined. After the reaction, in which 200 μmol/vial liquid geraniol was supplied, (*E*)-GA adsorbed on the PU support with immobilized cells was extracted into BS medium containing 10% DMSO. During this extraction process, the cells were not detached from the PU support. Next, the cells that remained immobilized on the PU were subjected to the same gas-phase reaction. This production and extraction process was repeated 5 times and cells were subjected to the same gas-phase reaction 6 times. As shown in Fig. 8, the productivity of (*E*)-GA was maintained at the same level throughout the 6 reactions.

## Discussion

The bioproduction of monoterpenoids using whole cell biocatalysts has thus far been performed in liquid-phase reactions[27, 40-42]. Because it requires active aeration and mixing with high energy consumption, it is not a true green technology even when using biocatalysts. The gas-phase bioproduction of monoterpenoids overcomes this drawback of the liquid-phase bioproduction because the reaction proceeds with the passive supply of gaseous substrates. However, application of gas-phase bioproduction has been limited to simple compounds[43]. In particular, for gas-phase production using whole cell biocatalysts, only epoxidation reactions from simple $C_2$ and $C_3$ alkenes have been reported[44, 45]. These substrates and products can be easily handled as gases due to their low boiling points (b.p. = –103.7 to 34 °C). In this study, we succeeded in the first gas-phase production of a high-value-added monoterpenoid using a whole cell biocatalyst. While geraniol, an alcohol with volatility, is a suitable substrate for use in a gaseous state, (*E*)-GA, an acid with low volatility, is considered to be an unsuitable product for separating it in a gaseous state. In this study, high purity (*E*)-GA produced in the gas-phase was directly adsorbed onto the PU support, thereby enabling the simple separation of (*E*)-GA by extraction with a non-toxic organic solvent like ethanol. This led to the development of a true complete green bioproduction process spanning from reaction to product separation. Our results also suggest that the gas-phase bioproduction is applicable to slightly volatile compounds with a relatively high boiling point. Furthermore, it should be noted that the regio- and stereo-selective production of (*E*)-GA from geraniol is a cascade reaction from geraniol, via geranial, and to (*E*)-GA. This cascade reaction is catalyzed by intracellular enzymes in Tol 5 Δ*fadD4* (pGeoA). However, (*E*)-GA was not detected from the inside or surface of the cells. Therefore, we

consider that the produced (*E*)-GA was promptly secreted out of the cells, vaporized, and simultaneously or subsequently adsorbed onto the PU support. Although the cascade reaction has been recognized as one of the challenges in gas-phase bioproduction[46], this has never been achieved. Therefore, this study contributes to expanding the use of gas-phase bioproduction to a wide range of complex compounds.

In the previous study, we found that Tol 5 (pGeoA) cells produced small amounts of two terpenoids, (1*R*,3*R*,4*R*)-MMC as the major product and (*E*)-GA as the minor product, from 80 mM geraniol in a liquid-phase reaction [27]. In this study, we examined the toxicity of the substrate and the product, and (1*R*,3*R*,4*R*)-MMC was produced only at the geraniol concentration of 80 mM, at which cell growth was completely inhibited. If a large amount of (*E*)-GA is produced from the high concentration of geraniol, it should also show severe product toxicity (Fig. 1). Hence, the production of (1*R*,3*R*,4*R*)-MMC might be related to a cellular function of escaping from the toxic product.

The liquid-phase bioproduction of (*E*)-GA caused us to notice the presence of the metabolic pathway involved in the degradation of (*E*)-GA catalyzed by FadD4. By disrupting the gene of the FadD4-ortholog in Tol 5, we succeeded in improving the productivity of (*E*)-GA in a gas-phase. On the other hand, as shown in Table 3, the total conversion of geraniol into (*E*)-GA was approximately 65% to 85%, but not 100% in the gas-phase reaction. This may have been because the intermediate geranial was used as the sole carbon and energy source for cell growth and maintenance of this mutant (Fig. 4). When supplemented with ≤ 100 μmol/vial liquid geraniol, approximately 65% of the geraniol was converted into (*E*)-GA (Table 3) and the other 35% was considered to be used for cell maintenance via geranial derivatives other than (*E*)-GA. When supplemented with 200 μmol/vial liquid geraniol, the conversion increased, suggesting that the flux into (*E*)-GA production increased from the excess carbon source. If we

identify and delete the gene that is responsible for the inherent conversion of geranial, the conversion of geraniol into (*E*)-GA may be improved further. On the contrary, the metabolic flow of a small fraction of the substrate into cell maintenance and/or growth may become effective for the continuous production of (*E*)-GA for a long period while maintaining cellular activity.

Cell immobilization is essential for the construction of a gas-phase bioproduction system. To date, entrapment into aqueous gels, covalent cross-linking to the surface of support, and physical attachment to material surfaces have been employed as cell immobilization methods[43]. Generally, these methods are inefficient in gas-phase reactions due to mass transfer limitations, deactivation of cells, and the insufficient mass of immobilized biocatalysts. Unlike these conventional methods, our immobilization method utilizing AtaA overcomes all these drawbacks and can conveniently immobilize a sufficient amount of cells for gas-phase reactions[13]. In addition, cells immobilized by our method show enhanced tolerance to toxic compounds[15], and can be reversibly detached from the supports by washing with $dH_2O$ or by adding casein hydrolysates[20, 47]. This study demonstrates that the immobilized cells on the PU support were tolerant to the saturated concentration of gaseous geraniol and that the immobilized cells can be reused for batch gas-phase reactions after simple separation of the product by liquid extraction.

*Acinetobacter* sp. Tol 5, which inherently possesses AtaA, is a tractable strain that grows as early as *E. coli*, is versatile for substrate utilization, shows relatively high tolerance to various hazardous chemicals, and can be engineered using genetic manipulation tools developed by ourselves[15, 17, 34]. Therefore, we propose that this strain is a suitable platform to develop whole cell biocatalysts for gas-phase bioproduction.

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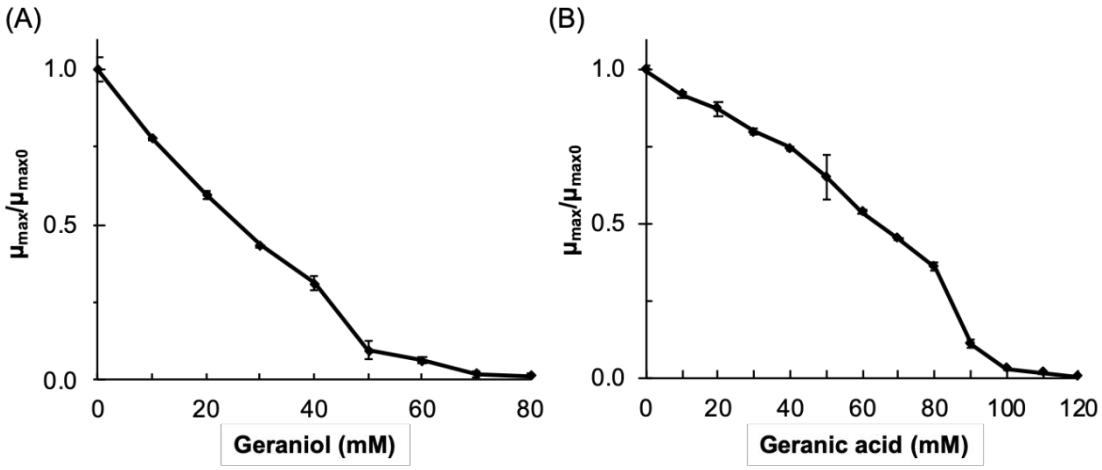


547

548 **Figure 1.** Growth inhibition of *Acinetobacter* sp. Tol 5 by geraniol (A) and geranic acid
549 (B). $\mu_{max}$, maximum growth rate at a given geraniol or geranic acid concentration; and
550 $\mu_{max0}$, maximum growth rate without geraniol or geranic acid. Data are expressed as the
551 mean ± standard deviation (SD) from three independent cultivations.

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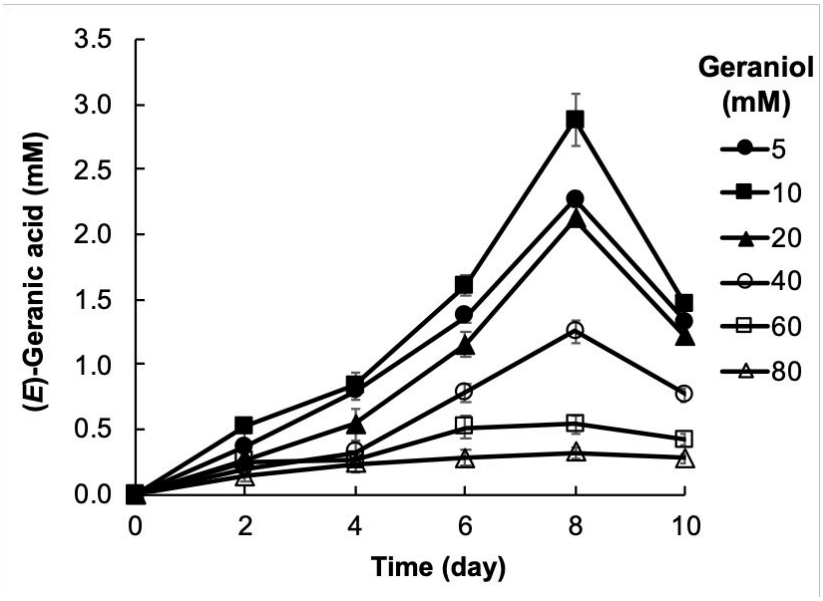


554

555 **Figure 2.** Effect of geraniol concentration on the production of (*E*)-geranic acid by

556 *Acinetobacter* sp. Tol 5 expressing the *geoA* gene in an aqueous liquid phase. Time

557 course of (*E*)-geranic acid production was examined at different geraniol concentrations

558 (5, 10, 20, 40, 60, and 80 mM). Data are expressed as the mean ± SD from three

559 independent reactions.

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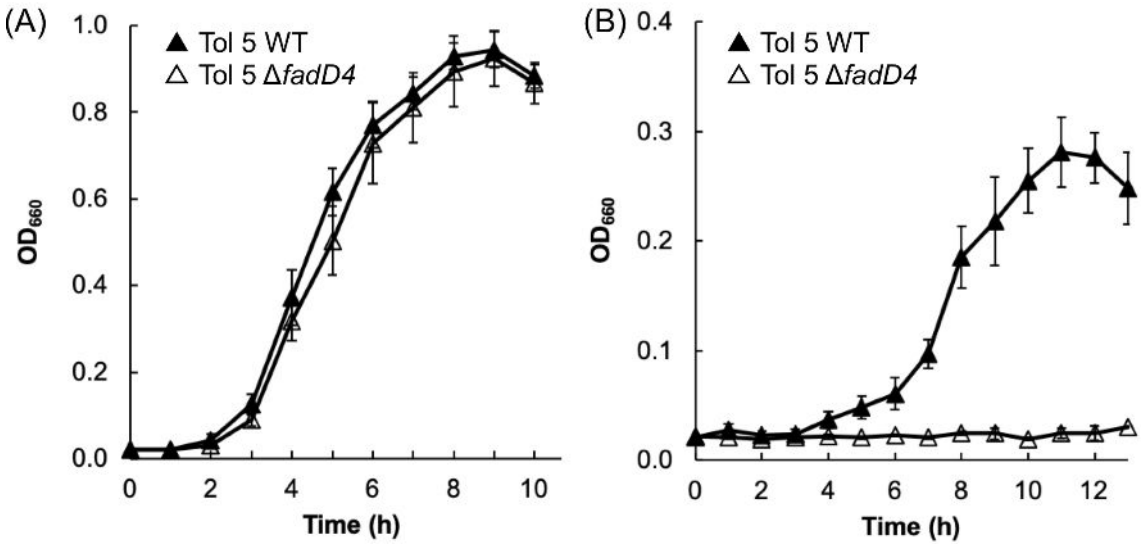



**Figure 3**. Growth curves of *Acinetobacter* sp. Tol 5 (Tol 5 WT) and its mutant lacking the *fadD4*-ortholog gene (Tol 5 Δ*fadD4*). Tol 5 WT or Tol 5 Δ*fadD4* was grown on lactate (A) and geranic acid (B). Data are expressed as the mean ± SD from three independent cultivations.

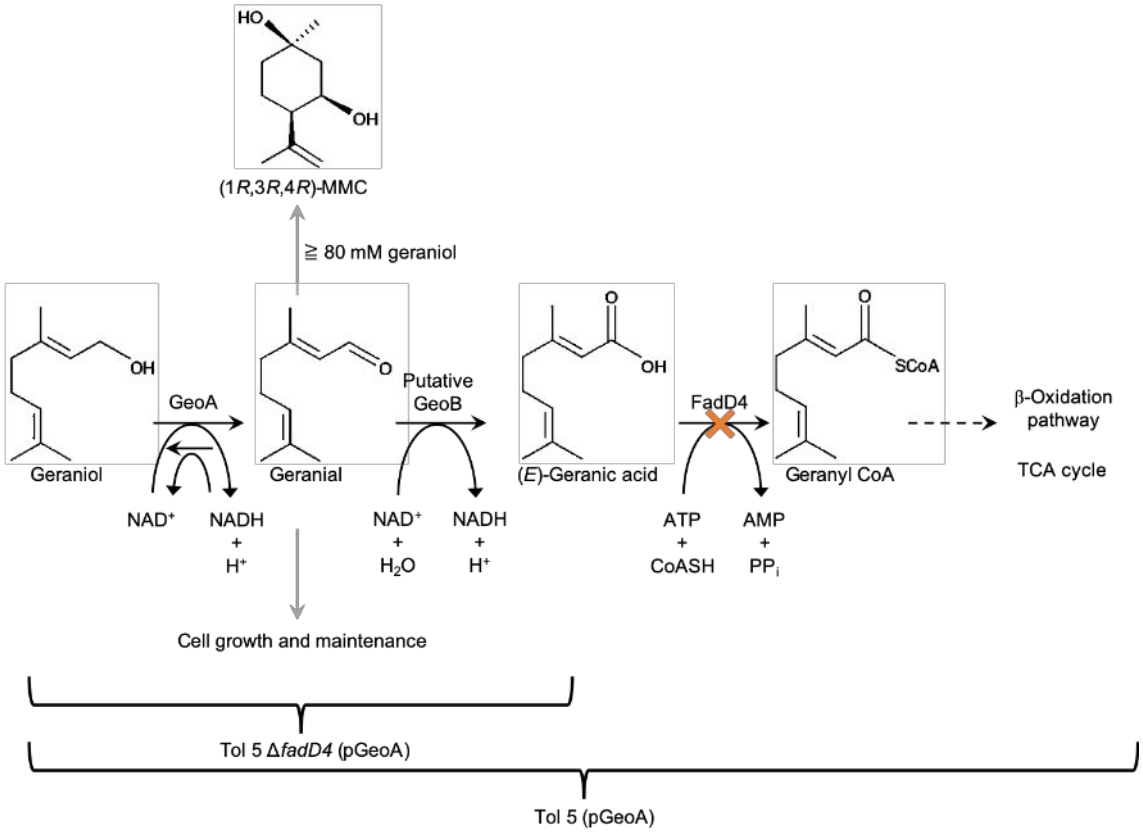
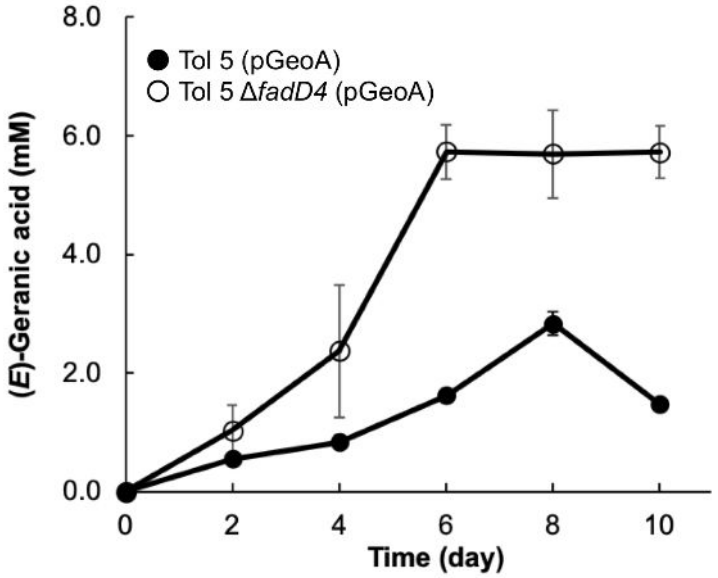


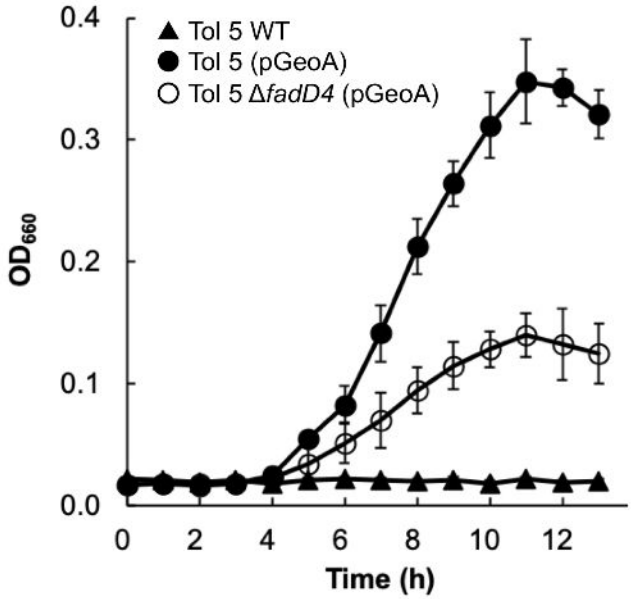

**Figure 4.** The possible pathway of the production and metabolism of (*E*)-geranic acid in *Acinetobacter* sp. Tol 5 derivative strains. Abbreviations: GeoA, geraniol dehydrogenase; GeoB, geranial dehydrogenase; FadD4, fatty acyl-CoA synthetase; and (1*R*,3*R*,4*R*)-MMC, (1*R*,3*R*,4*R*)-1-methyl-4-(1-methylethenyl)-1,3-cyclohexanediol. The gray arrows indicate minor pathways.




574

575 **Figure 5.** Production of (*E*)-geranic acid from 10 mM geraniol by metabolically
576 engineered strains of *Acinetobacter* sp. Tol 5, in an aqueous liquid-phase. Data are
577 expressed as the mean ± SD from three independent reactions.

578




579

580 **Figure 6**. Growth curves of *Acinetobacter* sp. Tol 5 WT and its metabolically

581 engineered strains grown in a medium containing geraniol as the sole carbon source.

582 Data are expressed as the mean ± SD from three independent cultivations.

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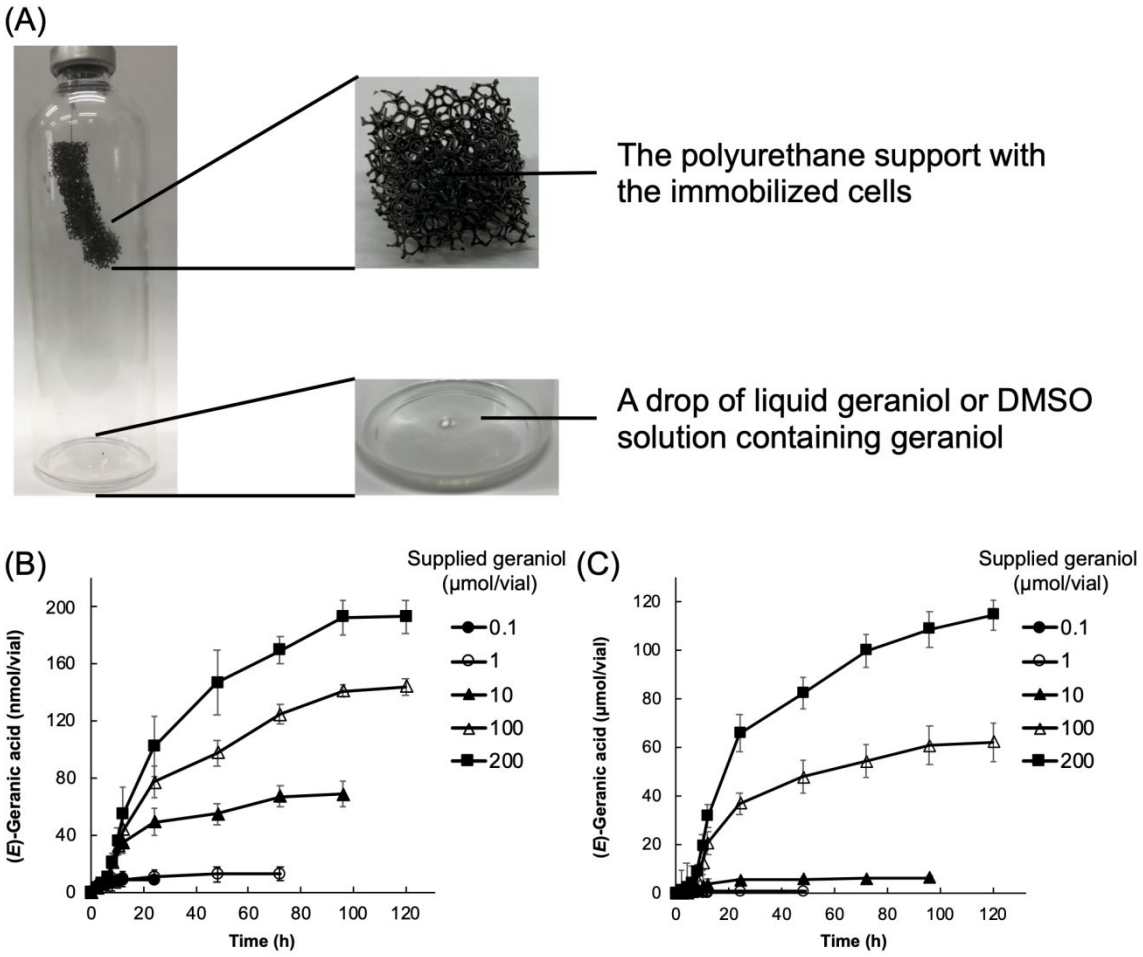
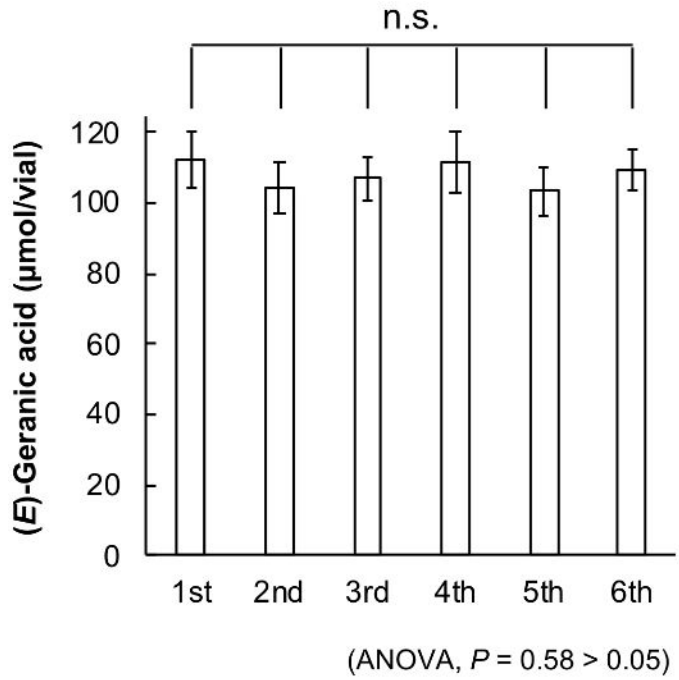



**Figure 7.** Production of (*E*)-geranic acid from gaseous geraniol by the metabolically engineered strains of *Acinetobacter* sp. Tol 5. (A) A photograph of the batch gas-phase bioproduction of (*E*)-geranic acid [(*E*)-GA]. Four pieces of polyurethane foam (PU) support with the immobilized Tol 5 Δ*fadD4* (pGeoA) cells were suspended from the top of a 125 mL cylindrical vial using a stainless steel wire. A drop of liquid geraniol or DMSO solution containing geraniol was placed on the bottom of the vial. Liquid geraniol gradually vaporized and gaseous geraniol was passively supplied to the cells on the PU support. (B) Time courses of (*E*)-GA produced in a gaseous state. (C) Time courses of (*E*)-GA produced and adsorbed on the PU support. Data are expressed as mean ± SD from three independent reactions.




595

596 **Figure 8.** Reusing immobilized *Acinetobacter* sp. Tol 5 Δ*fadD4* (pGeoA) cells for the
597 batch gas-phase bioproduction of (*E*)-geranic acid. After the reaction, in which 200
598 μmol/vial liquid geraniol was supplied, adsorbed (*E*)-GA was extracted into BS medium
599 containing 10% DMSO. Subsequently, the immobilized cells on the PU support were
600 subjected to the same gas-phase reaction after removing most of the water from the
601 support. This process was repeated 5 times. (*E*)-GA extracted into BS medium
602 containing DMSO was re-extracted into ethyl acetate, and quantified by GC-MS. Data
603 are expressed as mean ± SD from three independent reaction cycles.

604

**Table 1.** Bacterial strains and plasmids used in this study

| Strain or plasmid | Description | Reference |
|---|---|---|
| *Acinetobacter* sp. | | |
| Tol 5 | Wild-type strain | [12] |
| Tol 5 Δ*ataA* | Δ*ataA* mutant of Tol 5 strain | [34] |
| Tol 5 (pGeoA) | The transformant of Tol 5 strain harboring the pGeoA plasmid | [27] |
| Tol 5 Δ*fadD4* | Δ*fadD4* mutant of Tol 5 strain | This study |
| Tol 5 Δ*fadD4* (pGeoA) | The transformant of Tol 5 Δ*fadD4* strain harboring the pGeoA plasmid | This study |
| *Escherichia coli* | | |
| XL10-Gold | Host for routine cloning | Agilent |
| S17-1 | Donor strain for conjugation | [48] |
| Plasmid | | |
| pGeoA | *E. coli–Acinetobacter* shuttle expression harboring *geoA* | [27] |
| pJQ200sk | Suicide plasmid; $Gm^r$, SacB | [49] |
| pJQfadD4 | DNA fragment containing upstream and downstream regions of *fadD4* ligated into the *BamH*I site of pJQ200sk | This study |

605

**Table 2.** Primers used in this study

| Primer | Sequence (5' to 3') |
|---|---|
| fadD4Fw | TGCATTATTTCCATTTCTGGATAATTGGG |
| fadD4Re | CCAGAAATGGAAATAATGCACATGGGTATC |
| pJQfadD4Fw | CGAATTCCTGCAGCCCGGGGGATCGTGGCGCAGGAGATTG |
| pJQfadD4Re | CGGCCGCTCTAGAACTAGTGGATATCGTGGGTGAACACAGCAC |
| fadD4 dco fw2 | GCTGAAAGCCTCTACCAGAG |
| scofadD4-Fe | CAACAGCGCAAACGCTGCAAG |
| scofadD4-Re | GTAAGGCAGACATCTCTCTCTG |

606

607

**Table 3.** Gas-phase bioproduction of (*E*)-geranic acid from geraniol

| Substrate | | Product | | | Yield[b] (%) | | | Conversion[c] (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Supplied liquid geraniol (µmol/vial) | Consumed geraniol (µmol/vial) | Gaseous (*E*)-GA[a] (nmol/vial) | Adsorbed (*E*)-GA (µmol/vial) | Total (*E*)-GA (µmol/vial) | Gaseous | Adsorbed | Total | Gaseous | Adsorbed | Total |
| 0.1 | 0.1 | 0.01 ± 0.01 | 0.06 ± 0.01 | 0.07 ± 0.01 | 8.3 ± 2.1 | 57 ± 7 | 65 ± 10 | 8.3 ± 2.1 | 57 ± 7 | 65 ± 9 |
| 1 | 1 | 0.01 ± 0.01 | 0.7 ± 0.1 | 0.7 ± 0.1 | 1.3 ± 0.2 | 64 ± 1 | 65 ± 1 | 1.3 ± 0.2 | 64 ± 1 | 65 ± 1 |
| 10 | 9.7 ± 0.2 | 0.1 ± 0.1 | 6.3 ± 0.1 | 6.3 ± 0.1 | 0.7 ± 0.1 | 62 ± 3 | 63 ± 3 | 0.7 ± 0.1 | 64 ± 2 | 65 ± 2 |
| 100 | 96 ± 1 | 0.1 ± 0.1 | 62 ± 1 | 63 ± 1 | 0.2 ± 0.1 | 62 ± 1 | 63 ± 1 | 0.2 ± 0.1 | 65 ± 1 | 65 ± 1 |
| 200 | 138 ± 15 | 0.2 ± 0.1 | 114 ± 9 | 114 ± 9 | 0.1 ± 0.1 | 58 ± 1 | 58 ± 1 | 0.1 ± 0.1 | 83 ± 3 | 84 ± 6 |

[a] (*E*)-Geranic acid

[b] The yield was calculated from the concentration of (*E*)-GA at the end of the reaction and the supplied liquid geraniol concentration.

[c] The conversion was calculated from the concentration of (*E*)-GA at the end of the reaction and the consumed geraniol concentration.