Gas-Phase Bioproduction of a High-Value-Added Monoterpenoid (E)-Geranic Acid by Metabolically Engineered *Acinetobacter* Sp. Tol 5

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1 **Gas-phase bioproduction of a high-value-added monoterpene (E)-**
2 **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 monoterpene, 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 $\Delta fadD4$ (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 chemicals⁵⁻⁸. 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 biocatalysts⁹⁻¹¹. 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 adhesiveness¹², and its cells can be easily immobilized onto various material surfaces¹³⁻¹⁶ through the adhesive bacterionanofiber protein AtaA localized on the cell surface^{17, 18}. Tol 5 can versatily 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 phase²⁰.

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 synthesis²¹⁻²⁵. 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 monoterpene, in liquid-phase bioproduction²⁷. (*E*)-GA is a high-value-added monoterpene with great potential for industrial applications such as a perfuming agent²⁸ and a building block for the production of natural flavor esters²⁹. Additionally, it shows strong antifungal activities against corn phytopathogens³⁰ 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 monoterpene 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) medium¹⁷ supplemented with toluene, lactate, geraniol, or GA (3.3×10^{-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. The inhibitory effect of geraniol and GA on Tol 5 cell growth was examined, as previously described²⁷.

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 $\Delta fadD4$ 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 *Bam*HI 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 previously²⁷.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference
<i>Acinetobacter</i> sp.		
Tol 5	Wild-type strain	12
4140	Δ <i>ataA</i> mutant of Tol 5 strain	34
Tol 5 (pGeoA)	The transformant of Tol 5 strain harboring the pGeoA plasmid	27
Tol 5 Δ <i>fadD4</i>	Δ <i>fadD4</i> mutant of Tol 5 strain	This study
Tol 5 Δ <i>fadD4</i> (pGeoA)	The transformant of Tol 5 Δ <i>fadD4</i> strain harboring the pGeoA plasmid	This study
<i>Escherichia coli</i>		
XL10-Gold	Host for routine cloning	Agilent 48
S17-1	Donor strain for conjugation	
Plasmid		
pGeoA	<i>E. coli</i> – <i>Acinetobacter</i> shuttle expression harboring <i>geoA</i>	27
pJQ200sk	Suicide plasmid; Gm ^r , SacB	49
pJQfadD4	DNA fragment containing upstream and downstream regions of <i>fadD4</i> ligated into the <i>Bam</i> HI site of pJQ200sk	This study

Table 2. Primers used in this study

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

Immobilization of bacterial cells onto a polyurethane foam support

Cells were immobilized onto a polyurethane (PU) foam support, as described previously²⁰. 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 (dH₂O), and resuspended in 20 mL BS medium at an optical density at 660 nm (OD₆₆₀) of 1.0 in a 100 mL Erlenmeyer flask. Four pieces of 1 cm³ PU support with a specific surface area of 6.68 cm²/cm³ (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 dH₂O, and resuspended in 20 mL BS

medium at an OD₆₆₀ 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 every other day, saturated with NaCl, and extracted with ethyl acetate (EtOAc) containing 0.5 mM thymol as an internal standard. The EtOAc phases were analyzed by gas chromatography-mass spectrometry (GC-MS).

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 (1.7 µL) of 1% (V/V) geraniol solution in DMSO was placed on the bottom of the vial using a syringe so as to supply 100 nmol/vial of geraniol. After incubation at 28 °C without agitation, gaseous (*E*)-GA was analyzed by headspace-solid phase-microextraction (HS-SPME)-GC-MS. Prior to the analysis of adsorbed (*E*)-GA on the PU support, immobilized cells on the PU support were removed by washing with dH₂O. For this purpose, a piece of PU support was put into a 50 mL conical tube with 200 µL of dH₂O, vortexed for 20 s, and transferred into a new tube. This washing process was repeated twice. Four pieces of the washed PU support were treated with 400 µL EtOAc or ethanol containing 0.5 mM thymol as an internal standard to extract (*E*)-GA adsorbed on the PU support. The obtained extracts were centrifuged at 12,000 *g* for 5 min. When ethanol was used, the extract was dried over anhydrous sodium sulfate, and then filtered through a 0.20 µm PTFE syringe driven filter unit (Millex-LG, Millipore, Tokyo, Japan). The organic phase was analyzed by GC-MS.

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 HS-SPME-GC-MS using the method by Sanekata et al.³⁵ with slight modifications. SPME fiber made from polydimethylsiloxane/divinylbenzene (65 µm film in thickness, Supelco, Bellefonte, PA) was conditioned following the manufacturer's instructions. For the adsorption of gaseous geraniol and (*E*)-GA, the conditioned fiber was placed into the head space of a vial. When analyzed, the fiber was inserted into a GC injector at 250 °C for 3 min in the splitless mode. The flow rate of carrier gas was set at 1.5 mL/min. The oven temperature program was 60 °C for 3 min, raised to 250 °C, and increased at a rate of 5 °C/min, followed by a 3 min isotherm. An external calibration curve method was used for quantification. For the preparation of calibration curves, a drop of DMSO containing 0.01 to 5% (V/V) geraniol or GA was enclosed into the vial, vaporized completely at 28 °C for 24 h under atmospheric pressure, and analyzed by HS-SPME-GC-MS. The calibration parameters are listed in Table S1. The maximum adsorption amounts of

geraniol and GA on the SPME fiber were 100 nmol and 400 nmol, respectively. Favorable linearity (R^2 value of >0.99) for both chemicals was observed in the range of 1 nmol to the maximum adsorption amounts above per vial. The limits of detection (LOD) and limits of quantitation (LOQ) were determined, following the method by Chen et al³⁶.

Results

Effects of geraniol and (*E*)-geranic acid concentrations on *Acinetobacter* sp. Tol 5

Substrate/product inhibition is referred to as a main bottleneck hampering the microbial production of monoterpenoids^{37, 38}. To examine the tolerance of the Tol 5 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 concentration 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 study²⁷,

was detected only when supplemented with 80 mM geraniol; it was below the detection limit when supplemented with less than 60 mM geraniol.

Figure 1. Growth inhibition of *Acinetobacter* sp. Tol 5 by geraniol (A) and geranic acid (B). $\mu_{\max}$, maximum growth rate at a given geraniol concentration; and $\mu_{\max 0}$, maximum growth rate without geraniol. Data are expressed as the mean $\pm$ standard deviation (SD) from three independent cultivations.

Figure 2. Effect of geraniol concentration on the production of (*E*)-geranic acid by *Acinetobacter* sp. Tol 5 expressing the *geoA* gene in an aqueous liquid phase. Time course of (*E*)-geranic acid production was examined at different geraniol concentrations (5, 10, 20, 40, 60, and 80 mM). Data are expressed as the mean $\pm$ SD from three independent cultivations.

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 OD₆₆₀ were different (Fig. 3). This indicated that Tol 5 is able to metabolize GA as the sole carbon and energy source.

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

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

254 degrading (*E*)-GA. To identify a FadD4-ortholog from the Tol 5 genome (unpublished
255 data), a sequence similarity search was performed using BLASTP at an E-value cutoff
256 of 10^{-30} using FadD4 from *P. aeruginosa* (GenBank accession number:
257 WP_003114249) as a query. As a result, a FadD4-ortholog that showed 46.3% identity
258 and 63.4% similarity was found. A Tol 5 mutant deficient in the *fadD4*-ortholog gene,
259 Tol 5 $\Delta fadD4$, was constructed using a general gene-replacement method. The
260 disruption of the target gene was confirmed by PCR using primers annealing to the
261 flanking region of the *fadD4*-ortholog gene that were used as homologous sites for
262 recombination. The length of the amplicon (3,516 bp) from Tol 5 $\Delta fadD4$ was shorter
263 than that from Tol 5 WT (5,110 bp) (Fig. S1), indicating the successful excision of the
264 *fadD4*-ortholog gene. To confirm the repression of (*E*)-GA metabolism in Tol 5
265 $\Delta fadD4$, the mutant strain was inoculated into a medium supplemented with either
266 lactate or GA as the sole carbon source. The Tol 5 $\Delta fadD4$ mutant grew as well as the
267 Tol 5 WT strain on lactate, but barely grew on GA (Fig. 3), implying that the Tol 5
268 $\Delta fadD4$ mutant was unable to metabolize (*E*)-GA.

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.

The Tol 5 ΔfadD4 mutant was transformed with the pGeoA plasmid, generating Tol 5 ΔfadD4 (pGeoA), whose metabolic pathway of geraniol is shown in Fig. 4. Tol 5 ΔfadD4 (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 ΔfadD4 (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 ΔfadD4 (pGeoA) cells was $5.7 \pm$

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 $\Delta fadD4$ (pGeoA) cells. To confirm this, Tol 5 WT, Tol 5 (pGeoA), and Tol 5 $\Delta fadD4$ (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 OD₆₆₀ were achieved at 11 h: the OD₆₆₀ of Tol 5 (pGeoA), which is able to metabolize (*E*)-GA, was approximately 0.4, whereas that of Tol 5 $\Delta fadD4$ (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).

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

Figure 6. Growth curves of *Acinetobacter* sp. Tol 5 WT and its metabolically engineered strains grown in a medium containing geraniol as the sole carbon source. Data are expressed as the mean $\pm$ SD from three independent cultivations.

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

To construct a batch system of the gas-phase bioproduction of (*E*)-GA, Tol 5 (pGeoA) and Tol 5 Δ *fadD4* (pGeoA) cells were immobilized onto a PU support using the adhesive properties of AtaA¹⁷. As a negative control, Tol 5 Δ *ataA* cells were also subjected to the same immobilization procedure. Tol 5 (pGeoA) and Tol 5 Δ *fadD4* (pGeoA) cells were able to be immobilized as efficiently as Tol 5 WT cells (Fig. S2). The PU supports with the immobilized cells were suspended from the top of a vial and a drop of the DMSO solution containing 100 nmol geraniol was placed on the bottom of the vial so as to not make direct contact with the PU support pieces (Fig. 7A). The liquid geraniol contained in the DMSO solution gradually vaporized under the experimental conditions at 28 °C and atmospheric pressure, and completely changed to the gaseous state after 8 h (Fig. S3). Thus, the substrate geraniol was supplied as gas during the reaction. The amount of water adsorption on the PU support with or without

the immobilized cells was 0.05 mL/cm³ and 0.03 mL/cm³, respectively. Therefore, water content was lower than 5% and there was no bulk water around the cells.

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 (pGeoA) or 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 the DMSO solution containing 100 nmol 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 $\pm$ SD from three independent cultivations.

Gaseous (*E*)-GA produced by the gas-phase reaction was adsorbed on the SPME fiber and quantified by GC-MS. While gaseous (*E*)-GA was not detected in the vial containing immobilized Tol 5 (pGeoA) cells, immobilized 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). However, the reaction yield was quite low ($8.3 \pm 2.1\%$). We assumed that most (*E*)-GA molecules produced by the gas-phase reaction were immediately adsorbed on the PU support. To confirm this, we tried to extract adsorbed (*E*)-GA molecules from the PU surface into organic solvents. Beforehand, the immobilized cells were detached from the PU support by washing with dH₂O, according to our previously established method utilizing the reversibility of AtaA adhesion²⁰. Thereafter, the adsorbed (*E*)-GA was extracted with EtOAc from the PU support and the detached cells, followed by quantification using GC-MS. (*E*)-GA was not detected from the EtOAc extract of the detached cells (data not shown). However, (*E*)-GA was extracted from the PU support into EtOAc. Figure 7C shows the time course of the adsorbed (*E*)-GA on the PU support. With immobilized Tol 5 (pGeoA) cells, the amount of adsorbed (*E*)-GA increased gradually to 20.7 ± 3.9 nmol/vial for 10 h, but thereafter decreased to 1.3 ± 0.8 nmol/vial at 24 h due to its degradation by the FadD4-ortholog of Tol 5. On the other hand, with immobilized Tol 5 Δ *fadD4* (pGeoA) cells in the gas phase, the amount of adsorbed (*E*)-GA increased rapidly for 10 h and thereafter slowed, finally reaching 72.6 ± 4.3 nmol/vial at 24 h. (*E*)-GA adsorbed on the PU support can also be extracted with ethanol, a less harmful organic solvent than EtOAc, to a similar level: the extracted amount in ethanol was 65.4 ± 9.4 nmol/vial, which was not significantly different from that of EtOAc (Welch's *t* test, $P = 0.31 > 0.05$). The yields of gaseous and adsorbed (*E*)-GA are summarized in Table 3.

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

Strain	Yield (%) ^a		
	Gaseous	Adsorbed	Total
Tol 5 (pGeoA)	Not Detection	1.3 ± 0.8	1.3 ± 0.8
Tol 5 Δ <i>fadD4</i> (pGeoA)	8.3 ± 2.1	73 ± 4	81 ± 6

^aThe yield was calculated from the concentration of (*E*)-GA for 24 h-incubation and the initial geraniol concentration.

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 compounds⁴³. In particular, for gas-phase production using whole cell biocatalysts, only epoxidation reactions from simple C₂ and C₃ 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. Although the cascade reaction has been recognized as one of the challenges in gas-phase bioproduction⁴⁶, 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 reaction²⁷. 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. As shown in Table 3, the total (*E*)-GA yield from the substrate by Tol 5 Δ *fadD4* (pGeoA) cells was 81%, which was 62 times higher than that of Tol 5 (pGeoA) cells (1.3%), but it was not 100%. This might have been because the intermediate geranial was used as the sole carbon and energy source for cell growth and maintenance of this mutant (Fig. 6). If we identify and delete the gene that is responsible for the inherent conversion of geranial, the yield of (*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 methods⁴³. 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 reactions¹³. In addition, cells immobilized by our method show enhanced tolerance to toxic compounds¹⁵, and can be reversibly detached from the supports by washing with dH₂O or by adding casein hydrolysates^{20, 47}.

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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1 **Supplementary Figures**

2
3
4 **Figure S1.** PCR confirmation of *fadD4* disruption in *Acinetobacter* sp. Tol 5. PCR was
5 performed using the primers scofadD4-Fw and scofadD4-Re. The nucleotide sequences
6 of these primers are shown in Table 2. From the genome sequence information for Tol 5,
7 the lengths of PCR amplicons from the wild type (WT) and the Δ *fadD4* mutant are
8 estimated to be 5,110 bp, and 3,516 bp, respectively.

Figure S2. The immobilization ratios of *Acinetobacter* sp. Tol 5 and its derivative cells onto a polyurethane foam support. The cells were immobilized onto four pieces of polyurethane foam support in 20 mL BS medium in a flask with shaking and the immobilization ratio was calculated from a decrease in the OD₆₆₀ of the cell suspension. Data are expressed as the mean $\pm$ SD (n = 3).

Figure S3. Vaporization curve of 100 nmol geraniol in a 125 mL cylindrical vial. A drop of the DMSO solution containing 100 nmol geraniol was placed on the bottom of the vial. The vial was incubated at 28 °C. Gaseous geraniol in the head space of the vial was quantified by HS-SPME-GC-MS. Data are expressed as the mean $\pm$ SD (n = 3).

Table S1. Gas chromatography-mass spectrometry parameters for quantitation of geraniol and (*E*)-geranic acid by headspace-solid phase microextraction^a

Compound	quantifier ion (<i>m/z</i>)	qualifier ion (<i>m/z</i>)	<i>R</i> ²	slope	linear range (nmol/vial)	LOD (nmol/vial)	LOQ (nmol/vial)
geraniol	136	121	0.995	2.8×10^5	1–100	0.3	1.0
(<i>E</i>)-geranic acid	123	168, 100	0.999	4.0×10^4	1–400	0.4	1.1

^aLOD, limit of detection; LOQ, limit of quantification. LOD and LOQ were estimated as three and ten times the standard error of the intercept divided by the slope of the calibration equation³⁶

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