Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Antimicrobial properties and mechanism of volatile isoamyl acetate, a main flavour component of Japanese sake (Ginjo-shu)

H. Ando[1], A. Kurata[2] and N. Kishimoto[2]

1 Health Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Ikeda City, Osaka, Japan
2 Department of Applied Biological Chemistry, Faculty of Agriculture, Kinki University, Nara City, Nara, Japan

Keywords
antimicrobial activity, antimicrobial mechanism, isoamyl acetate, proteome, volatile organic compounds.

Correspondence
Noriaki Kishimoto, Department of Applied Biological Chemistry, Faculty of Agriculture, Kinki University, 3327-204 Nakamachi, Nara City, Nara 631-8505, Japan.
E-mail: kisimoto@nara.kindai.ac.jp

2014/2003: received 26 September 2014, revised 21 December 2014 and accepted 20 January 2015

doi:10.1111/jam.12764

Abstract

Aims: To evaluate the antimicrobial properties of the main Ginjo-flavour components of sake, volatile isoamyl acetate and isoamyl alcohol.
Methods and Results: Volatile isoamyl acetate and isoamyl alcohol both inhibited growth of the five yeast and 10 bacterial test strains. The minimum inhibitory dose and minimum bactericidal (fungicidal) dose of isoamyl acetate were higher than those of isoamyl alcohol. *Escherichia coli* and *Acetobacter aceti* were markedly sensitive to isoamyl acetate and isoamyl alcohol. In *E. coli* exposed to isoamyl acetate for 5 h, changes in expression were noted in proteins involved in sugar metabolism (MalE, MglB, TalB and PtsI), tricarboxylic acid cycle (AceA, Pfl and AcnB) and protein synthesis (EF-Tu, EF-G, and GlyS). Expression of acid and alcohol stress-response proteins was altered in *E. coli* exposed to isoamyl acetate. Esterase activity was detected in *E. coli*, suggesting that isoamyl acetate was hydrolyzed to acetic acid and isoamyl alcohol. Acetic acid and isoamyl alcohol damaged *E. coli* cell membranes and inactivated membrane proteins, impairing respiration.
Conclusions: Volatile isoamyl acetate and isoamyl alcohol were effective in inactivating various micro-organisms, and antimicrobial mechanism of volatile isoamyl acetate against *E. coli* was clarified based on proteome analysis.
Significance and Impact of the Study: To the best of our knowledge, this is the first report to examine the antimicrobial mechanism of volatile organic compound using proteome analysis combining two-dimensional difference gel electrophoresis with peptide mass fingerprinting.

Introduction

Some micro-organisms produce volatile organic compounds (VOCs) with antimicrobial activity (Kai *et al.* 2009). VOCs produced by filamentous fungi, *Muscodor* sp. and *Trichoderma* sp. (Strobel *et al.* 2001; Humphris *et al.* 2002), yeast, *Saccharomyces* sp. and *Pichia* sp. (Masoud *et al.* 2005; Fialho *et al.* 2010) and bacteria, *Bacillus* sp. and *Pseudomonas* sp. (Fernando *et al.* 2005; Yuan *et al.* 2012) have been shown to inhibit growth of phytopathogenic filamentous fungi.

Many of these VOCs are alcohols, aldehydes or esters. Identification, antimicrobial activity levels and antimicrobial spectra of VOCs produced by micro-organisms have been reported, but the growth inhibition mechanism has not been fully elucidated. The main component of VOCs produced by *Saccharomyces cerevisiae* strain CR-1 is ethanol. And the VOCs inhibit activities of the hyphal extension enzyme laccase and tyrosinase produced by phytopathogenic filamentous fungi (Fialho *et al.* 2010, 2011). VOCs containing heptanal and amyl alcohol produced by *Trichoderma* spp. inhibit protein synthesis of phytopathogenic filamentous fungi, but the relationship between the type of inhibited proteins and growth inhibition has not been clarified (Humphris *et al.* 2002).

We have isolated *Candida maltosa* NP9 from fermented food, which produces VOCs that inhibit spore germination of filamentous fungi, and we identified that isoamyl acetate and isoamyl alcohol were responsible for the inhibition of spore germination (Ando *et al.* 2012). Isoamyl acetate and isoamyl alcohol are well known as Ginjo-flavour components produced by sake yeast in unrefined sake mash. The level of isoamyl acetate in Japanese sake is strongly correlated with sake quality (Yoshizawa 1999). Therefore, the biosynthesis pathway and control system of isoamyl acetate and the establishment of a highly productive strain have been extensively investigated (Fukuda *et al.* 1998; Arikawa *et al.* 2000; Kitagaki and Kitamoto 2013). However, there has been no report on the antimicrobial mechanism of isoamyl acetate.

In this study, we first investigated the antimicrobial spectra of isoamyl acetate and isoamyl alcohol, and observed high antimicrobial activity against *Escherichia coli*. We then clarified that the cell membrane was damaged, and protein expression was changed in proteome analysis in *E. coli* exposed to isoamyl acetate. Based on these findings, the antimicrobial mechanism of isoamyl acetate against *E. coli* was investigated.

Materials and methods

Chemicals, micro-organisms and culture conditions

Isoamyl acetate and isoamyl alcohol were purchased from Nacalai Tesuque Inc. (Kyoto, Japan). All of the other chemicals were of analytical grade.

The following strains were used in this study: yeast: *S. cerevisiae* NBRC 10217^T, *Candida albicans* NBRC 12204, *Pichia anomala* NBRC 12210, *Zygosaccharomyces bailii* NBRC 1098^T, and *Kluyveromyces marxianus* NBRC 10005^T; Gram-positive bacteria: *Bacillus subtilis* IAM 1213, *Listeria denitrificans* JCM 11481, *Staphylococcus aureus* IFO 3060, *Lactobacillus plantarum* IAM 1041, *Lactobacillus brevis* IAM 1082, *Pediococcus pentosaceus* IAM 12296, and *Streptococcus salivarius* ATCC 9758; and Gram-negative bacteria: *Pseudomonas aeruginosa* IAM 1514^T, *E. coli* NBRC 3301, and *Acetobacter aceti* IAM 1802. The yeast strains were cultured on yeast malt (YM) agar (3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g dextrose and 15 g agar per litre, pH 6·2) at 28°C for 24 h. The bacterial strains were cultured on nutrient agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) at 37°C for 24 h. After incubation, yeast and bacterial colonies were suspended in 0·8% sterile saline and adjusted to $3{\cdot}2 \times 10^4$ CFU ml^{-1} with sterile saline. The cell concentration was determined using the 10-fold serial dilution method.

Antimicrobial activities of isoamyl acetate and isoamyl alcohol

Antimicrobial activities of isoamyl acetate and isoamyl alcohol were determined by the vapour–agar contact method as described previously (Ando *et al.* 2012). Briefly, the test strain was inoculated at 10 μl suspensions to agar medium in a small glass vessel, and the vessel was placed into a Petri dish. Another small glass vessel with the volatile compound was placed into the Petri dish at the same time as the inoculated vessel. Then, the Petri dish containing the two glass vessels was sealed and placed into a plastic container, and the container was incubated at 28°C for 48 h. After 48 h incubation, the colony of the test strain exposed to volatile compound was compared with that of the unexposed control. The minimum inhibitory dose (MID) was defined as the lowest addition (μl dish^{-1}) that completely inhibited colony formation of the test strain. The minimum bactericidal (fungicidal) dose (MBD) was defined as minimum concentration (μl dish^{-1}) which bactericidally (fungicidally) inhibited the growth of the test strain determined as follows: the strain which completely inhibited colony formation, despite the treatment with the test compound for 48 h, was aseptically removed and transferred into a new agar plate to examine survival at 24 h. Each experiment was repeated three times.

Effect of isoamyl acetate on growth of *Escherichia coli*

To obtain *E. coli* cells exposed to isoamyl acetate, the vapour–agar contact method was slightly modified. Ten microliters of *E. coli* suspensions were inoculated into nutrient agar medium in a small vessel, and the vessel was placed into a Petri dish to be cultured at 28°C. After 24-h culture, the bacterial colony was exposed to isoamyl acetate (160 μl dish^{-1}), which was in another small vessel placed in the Petri dish, at 28°C. The exposed colony was scraped with a sterile cotton swab and spread on new nutrient agar medium to examine the effect on the growth of *E. coli* at 24 h. Each experiment was repeated three times.

Protein extraction

Thirty milligrams of *E. coli* cells exposed to isoamyl acetate by the method described above were suspended in 500 μl Tris–HCl buffer (50 mmol l^{-1}, pH 7·4). The cell suspension was sonicated for 30 s and cooled for 60 s on ice, repeated five times. The sonicated suspension was centrifuged at 6000 ***g*** for 20 min at 4°C. Protein in the supernatant was used as a soluble protein fraction. The membrane protein fraction was extracted using the

ReadyPrep Protein Extraction Kit (Membrane I) (Bio-Rad Laboratories, Hercules, CA), which is based on the use of Triton X-114, according to manufacturer's instructions. The fraction was further purified using the ReadyPrep 2-D Cleanup Kit (Bio-Rad Laboratories) to remove salt, nucleic acid, lipid and some other small ions. Finally, membrane protein pellets were dissolved in lysis buffer (pH 8·0–9·0) containing 7 mol l^{-1} urea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and 30 mmol l^{-1} Tris.

Protein concentrations in soluble and membrane protein fractions were determined by the Bradford method (Bradford 1976) using Protein Assay CBB Solution (Nacalai Tesuque Inc.), and the bicinchoninic acid method (Smith *et al.* 1985) using the Protein Assay Bicinchoninate kit (Nacalai Tesuque Inc.). Bovine serum albumin was used as a standard for protein determinations.

Two-dimensional difference gel electrophoresis analysis

The extracted proteins from unexposed control and exposed cells were labelled with IC3-OSu and IC5-OSu (Dojindo Laboratories, Kumamoto, Japan), respectively. One hundred micrograms of protein was reacted with 2·0 μl IC3-OSu or IC5-OSu (400 pmol μl^{-1}) for 15 min at room temperature in the dark. The reaction was stopped by the addition of 2·0 μl lysine (10 mmol l^{-1}; Nacalai Tesuque Inc.) that was treated for 15 min at room temperature in the dark. Equal volumes (100 μg) of each of the labelled proteins were mixed and made up to a volume of 250 μl with DeStreak Rehydration solution (GE Healthcare, Piscataway, NJ) and 0·5% IPG buffer 3-11 NL (GE Healthcare). Isoelectric focusing was performed on an Immobiline DryStrip pH 3-11 NL, 13 cm (GE Healthcare) using an Ettan IPGphor 3 IEF System (GE Healthcare), with the following conditions: temperature 20°C, 500 V (step and hold) for 1 h, 1000 V (gradient) for 1 h, 8000 V (gradient) for 2·5 h, and 8000 V (step and hold) for 25 min. The focused strips were equilibrated in equilibration buffer (6 mol l^{-1} urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS) and 50 mmol l^{-1} Tris) with 65 mmol l^{-1} dithiothreitol for 15 min, and the same buffer with 135 mmol l^{-1} iodoacetamide for 15 min. Second-dimension electrophoresis was performed on 10% SDS polyacrylamide gel electrophoresis using a Hoefer SE 600 Ruby (GE Healthcare). The gels were run at 20 mA per gel, for 5 h. Labelled proteins were visualized using a Typhoon 9200 image analyzer (GE Healthcare). The IC3-OSu images were scanned using a 532-nm excitation laser and a 580-nm emission filter, whereas the IC5-OSu images were scanned using a 633-nm excitation laser and a 670-nm emission filter. The labelled proteins were visualized as green (IC3-OSu), red (IC5-OSu) and yellow (overlaying IC3-OSu and IC5-OSu). To identify the interesting proteins, unlabeled protein fractions were separated by 2D GE under the same conditions, and the gels were stained with Coomassie Brilliant Blue (CBB).

In-gel digestion and identification of proteins

For protein identification by peptide mass fingerprinting (PMF), protein spots in the CBB-stained gels were excised, digested with trypsin (Promega Corporation, Madison, WI), mixed with saturated solution of α cyano-4-hydroxycinnamic acid (Sigma–Aldrich, St Louis, MO) in 50% acetonitrile/0·1% trifluoroacetic acid, and subjected to matrix-assisted laser desorption/ionization-time of flight analysis (Microflex LRF 20; Bruker Daltonics, Bremen, Germany) as described by Fernandez *et al.* (1998). Spectra were collected from 300 shots per spectrum over an *m/z* range of 600–3000 and calibrated by 2-point internal calibration using Trypsin autodigestion peaks (*m/z* 842·5099 and 2211·1046). Peak list was generated using Flex Analysis 3·0. The threshold used for peak-picking was as follows: 500 for minimum resolution of monoisotopic mass, 5 for S/N. The search program MASCOT, developed by Matrixscience (http://www.matrixscience.com), was used for protein identification by PMF. The following parameters were used for the database search: trypsin as the cleaving enzyme, a maximum of one missed cleavage, iodoacetamide (Cys) as a complete modification, oxidation (Met) as a partial modification, monoisotopic masses, and a mass tolerance of ±0·1 Da. PMF acceptance criteria is probability scoring.

Esterase activity

To determine the esterase activity of whole cells, *E. coli* was grown on nutrient agar at 37°C for 24 h. The cells were harvested and washed twice in Tris–HCl buffer (50 mmol l^{-1}, pH 7·4), and resuspended in 5 ml of the same buffer to a final OD_{660} of 10·0 ($1·2 \times 10^9$ CFU ml^{-1}). Cell suspension (920 μl) was mixed with 50 mmol l^{-1} *p*-nitrophenyl acetate (30 μl; Nacalai Tesuque Inc.) as the substrate. The amount of released *p*-nitrophenol after 10 min at 28°C was measured by determining the increase in absorbance at 400 nm with a spectrophotometer (UV-1200; Shimadzu Corporation, Kyoto, Japan). One unit of esterase activity was defined as the amount of enzyme releasing 1 pmol *p*-nitrophenol min^{-1} under the assay conditions. Each experiment was repeated three times.

Effects of isoamyl alcohol and acetic acid on the growth of *Escherichia coli*

Antimicrobial activity of acetic acid (Nacalai Tesuque Inc.), and the effects of isoamyl alcohol (160 μl dish^{-1}) and acetic acid (160 μl dish^{-1}) on the growth of *E. coli* were assayed by the methods described above. Each experiment was repeated three times.

Florescence microscopy observations

Membrane injury and respiratory activity in *E. coli* cells exposed to antimicrobial VOCs were determined by propidium iodide (PI) and 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC) staining methods, respectively. One hundred microliters of *E. coli* suspension (10^9 CFU ml^{-1}) was counter-stained with 1 μl 4', 6-diamidino-2-phenylindole (DAPI), a nucleus-staining dye (1 μg μl^{-1}; Dojindo Laboratories). After 5 min staining, the cell suspension was also stained with 1 μl PI solution (1 μg μl^{-1}; Dojindo Laboratories) for 5 min. Another cell suspension was stained with 10 μl CTC solution (50 mmol l^{-1}) and 2·5 μl Enhancing Reagent B from a Bacstain CTC Rapid Staining Kit (Dojindo Laboratories) for 30 min, and counter-stained with DAPI. All staining reactions were carried out at room temperature in the dark. Morphological changes in the exposed cells were assessed visually under an Olympus BX51 fluorescence microscope (Olympus Corporation, Tokyo, Japan), equipped with WU, WIG and WIB excitation filters for DAPI, PI and CTC staining, respectively.

Table 1 Antimicrobial spectrum of volatile isoamyl acetate and isoamyl alcohol against yeasts and bacteria

		Isoamyl acetate		Isoamyl alcohol	
		MID	MBD	MID	MBD
Yeasts					
Saccharomyces cerevisiae	NBRC 10217^T	40	80	20	40
Candida albicans	NBRC 12204	160	>160	20	80
Pichia anomala	NBRC 12210	40	80	20	40
Zygosaccharomyces bailii	NBRC 1098^T	80	>160	20	>160
Kluyveromyces marxianus	NBRC 10005^T	80	>160	20	80
Gram-positive bacteria					
Bacillus subtilis	IAM 1213	80	>160	20	>160
Listeria denitrificans	JCM 11481	80	>160	20	40
Staphylococcus aureus	IFO 3060	80	>160	40	80
Lactobacillus plantarum	IAM 1041	160	>160	20	160
Lactobacillus brevis	IAM 1082	80	>160	40	160
Pediococcus pentosaceus	IAM 12296	160	>160	40	80
Streptococcus salivarius	ATCC 9758	160	>160	20	80
Gram-negative bacteria					
Pseudomonas aeruginosa	IAM 1514^T	80	>160	20	>160
Escherichia coli	NBRC 3301	20	20	20	40
Acetobacter aceti	IAM 1802	20	20	20	20

MID was defined as the lowest concentration (μl dish^{-1}) with antimicrobial activity. MBD was defined as the minimum concentration (μl dish^{-1}) that bactericidally (fungicidally) inhibited growth. All assays were repeated three times.

Results

Antifungal and antibacterial activities of volatile isoamyl acetate and isoamyl alcohol

Volatile isoamyl acetate and isoamyl alcohol both inhibited growth of five yeast and 10 bacterial test strains. The MID and MBD of isoamyl acetate were higher than those of isoamyl alcohol (Table 1). Isoamyl acetate inhibited growth of all yeast species at 160 μl dish^{-1}, and the inhibition was fungicidal at 80 μl dish^{-1} for *S. cerevisiae* and *P. nomala* excluding *C. albicans*, *Z. bailii* and *K. marxianus*. Isoamyl alcohol inhibited growth of all yeast species at 20 μl dish^{-1}, and the inhibition was fungicidal at 80 μl dish^{-1} for all five species excluding *Z. bailii*.

Isoamyl acetate (160 μl dish^{-1}) bacteriostatically inhibited growth of Gram-positive bacteria but could not kill them, whereas isoamyl alcohol (160 μl dish^{-1}) bactericidally inhibited *L. denitrificans, Staph. aureus, Lact. plantarum, Lact. brevis, Ped. pentosaceus and Strep. salivarius* and but not *B. subtilis*. Isoamyl acetate killed Gram-negative *E. coli* and *Acet. aceti* at 20 μl dish^{-1}, and isoamyl alcohol showed bactericidal inhibition at 20–40 μl dish^{-1}.

Escherichia coli and *Acetobacter aceti* were markedly sensitive to isoamyl acetate and isoamyl alcohol.

Volatile isoamyl acetate exposure time required to kill *Escherichia coli*

When bacteria were exposed to isoamyl acetate at 160 μl dish^{-1} for 3 h using the vapour–agar contact method, *E. coli* growth was inhibited. Growth was strongly inhibited by 4-h exposure, and no colony was formed after 5-h exposure. The same findings were observed when the isoamyl acetate concentration was decreased to 20 μl dish^{-1}.

Proteome analysis of *Escherichia coli* exposed to volatile isoamyl acetate

Soluble protein fractions were prepared from *E. coli* with and without exposure to isoamyl acetate (160 μl dish^{-1}) for 5 h and subjected to differential expression analysis using two-dimensional difference gel electrophoresis (2D-DIGE). The expression level was reduced by exposure at four protein spots (1–4, Fig. 1a). Membrane proteins were similarly analyzed, and the expression level was increased by exposure at 11 spots (5–15, Fig. 1b).

These 15 proteins with changes in expression level were identified by the PMF method. The identified proteins included many involved in sugar metabolism (MalE, MglB, TalB and PtsI), tricarboxylic acid (TCA) cycle (AceA, Pfl and AcnB) and protein synthesis (EF-Tu, EF-G and GlyS) (Table 2).

Escherichia coli esterase activity

The esterase activity level in *E. coli* cells (10^5 CFU) was measured using *p*-nitrophenyl acetate as a substrate, and the activity level was 45·7 mU.

Influence of hydrolysis products of isoamyl acetate, isoamyl alcohol and acetic acid on growth of *Escherichia coli*

The MID and MBD of isoamyl alcohol against *E. coli* determined using the vapour–agar contact method were 20 and 40 μl dish^{-1}, respectively (Table 1), and those of

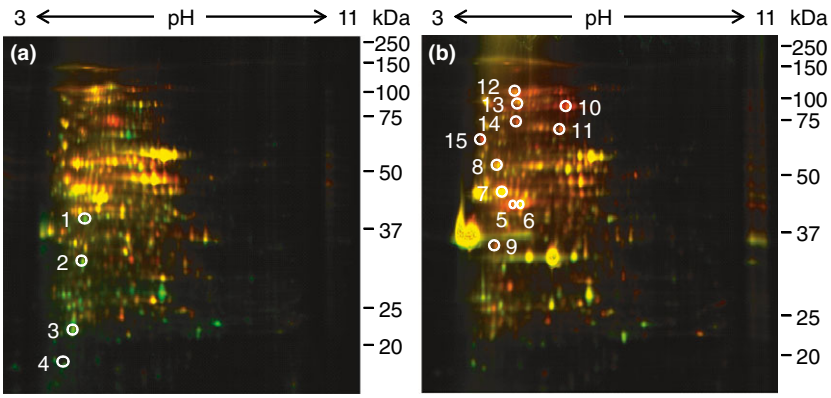

Figure 1 Two-dimensional difference gel electrophoresis (2D-DIGE) images of soluble protein fraction (a) and membrane protein fraction (b) of *Escherichia coli* exposed to volatile isoamyl acetate. Green: proteins down–regulated by isoamyl acetate exposure; red: proteins up–regulated by isoamyl acetate exposure; yellow: same amount of expressed proteins. The numbered spots indicate the identified proteins listed in Table 2.

Table 2 Identification of up- or down-regulated proteins in *Escherichia coli* exposed to volatile isoamyl acetate using peptide mass fingerprinting

Spot no.	Protein name	Description	NCBI gi no.	MASCOT score	Peptides matched	Sequence cov. (%)	pI/MW
1	MalE	Chain A, engineered high-affinity maltose-binding protein	gi|34809631	148	11/18	34	5·21/40031
2	MglB	D-galactose-binding periplasmic protein	gi|323184227	107	9/20	35	5·36/34217
3	AhpC	Alkyl hydroperoxide reductase submit C	gi|15800320	160	14/33	60	5·03/20862
4	Crr	Glucose-specific component IIA of the PTS system	gi|1314675	102	6/11	41	4·79/17459
5	EF-Tu	Translation elongation factor Tu	gi|300946929	168	18/27	46	5·20/44525
6	EF-Tu	Translation elongation factor Tu	gi|118137902	107	13/36	51	4·98/37020
7	AceA	Isocitrate lyase	gi|556177	254	23/44	65	5·44/47485
8	AldA	Aldehyde dehydrogenase A	gi|15801728	144	16/36	48	5·07/52377
9	TalB	Transaldolase B	gi|1941982	330	29/52	80	5·10/35237
10	Pfl	Pyruvate formate lyase I	gi|16128870	271	32/56	49	5·69/85588
11	PrkA	Serine kinase family protein	gi|194428508	264	29/52	47	5·63/74758
12	AcnB	Bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase	gi|16128111	333	35/59	52	5·24/94009
13	EF-G	Translation elongation factor G	gi|300926981	336	38/57	60	5·24/77677
14	GlyS	Glycine tRNA synthetase, β subunit	gi|16131430	372	34/47	52	5·29/76936
15	PtsI	PEP-protein phosphotransferase of PTS system (enzyme I)	gi|16130342	112	18/55	37	4·78/63750

acetic acid were 5 and 20 μl dish^{-1}, respectively. The duration of exposure to acetic acid (160 μl dish^{-1}) and isoamyl alcohol (160 μl dish^{-1}) required to kill *E. coli* was 8 and 17 h, respectively.

Florescence microscopic observation of *Escherichia coli* exposed to isoamyl acetate

Escherichia coli exposed to isoamyl acetate (160 μl dish^{-1}) for 5 h developed red fluorescence on PI staining, but unexposed *E. coli* developed no fluorescence (Fig. 2a–d). Unexposed *E. coli* developed red fluorescence on CTC staining, but exposed *E. coli* developed no fluorescence (Fig. 2e–h). *Escherichia coli* exposed to acetic acid (160 μl dish^{-1}) for 8 h and isoamyl alcohol (160 μl dish^{-1}) for 17 h developed red fluorescence on PI staining and developed no fluorescence on CTC staining.

Discussion

We clarified the antimicrobial activities of the main Ginjo-flavour components of sake, volatile isoamyl acetate and isoamyl alcohol, and the antimicrobial mechanism was analyzed using the proteome technique.

Volatile isoamyl acetate showed broad antimicrobial activity against filamentous fungi (Ando *et al.* 2012), bacteria, and yeast (Table 1). *Muscodor* sp. have also been reported to produce volatile esters, and isoamyl acetate shows the highest antimicrobial activity level against filamentous fungi and bacteria (Strobel *et al.* 2001). In addition to isoamyl acetate, esters, such as pentyl acetate, ethyl hexanoate, and isoethyl hexanoate, are produced by micro-organisms and inhibit filamentous fungal spore germination (Huang *et al.* 2011).

Among the VOCs produced by micro-organisms, several antimicrobial mechanisms of volatile alcohols and aldehydes have been reported, but no mechanism of volatile esters including isoamyl acetate has been reported. In *E. coli* exposed to isoamyl acetate for 5 h, changes in expression were noted in proteins involved in sugar metabolism (MalE, MglB, TalB, and PtsI), TCA cycle (AceA, Pfl, and AcnB), and protein synthesis (EF-Tu, EF-G, and GlyS) (Fig. 1, Table 2). Proteome analyses of *E. coli* under various culture and stress conditions have been reported (Han and Lee 2006). In *E. coli* exposed to acid stress, the expression levels of AceA and EF-Tu increase, and that of MalE decreases (Blankenhorn *et al.* 1999; Zhang *et al.* 2011). These changes in expression are consistent with those noted in proteins induced by isoamyl acetate in *E. coli*. In *E. coli* exposed to alcohol stress, changes in expression have been noted in genes involved in the TCA cycle, such as *aceA*, *aceE*, *acnA*, *sdhABCD*, and *sucA* (Brynildsen and Liao 2009; Horinouchi *et al.* 2010; Rutherford *et al.* 2010; Segura *et al.* 2012; Chong *et al.* 2013). Expression of alcohol-stress-response proteins (AceA, Pfl and AcnB) was also altered in isoamyl acetate-exposed *E. coli* (Fig. 1, Table 2). Expression of acid- and alcohol-stress-response proteins was altered in *E. coli* exposed to isoamyl acetate, and esterase activity was detected in *E. coli*, inferring that isoamyl acetate was hydrolyzed to acetic acid and isoamyl alcohol. Pacaud (1982) also detected esterase activity in the inner and outer membranes of *E. coli*. However, although the

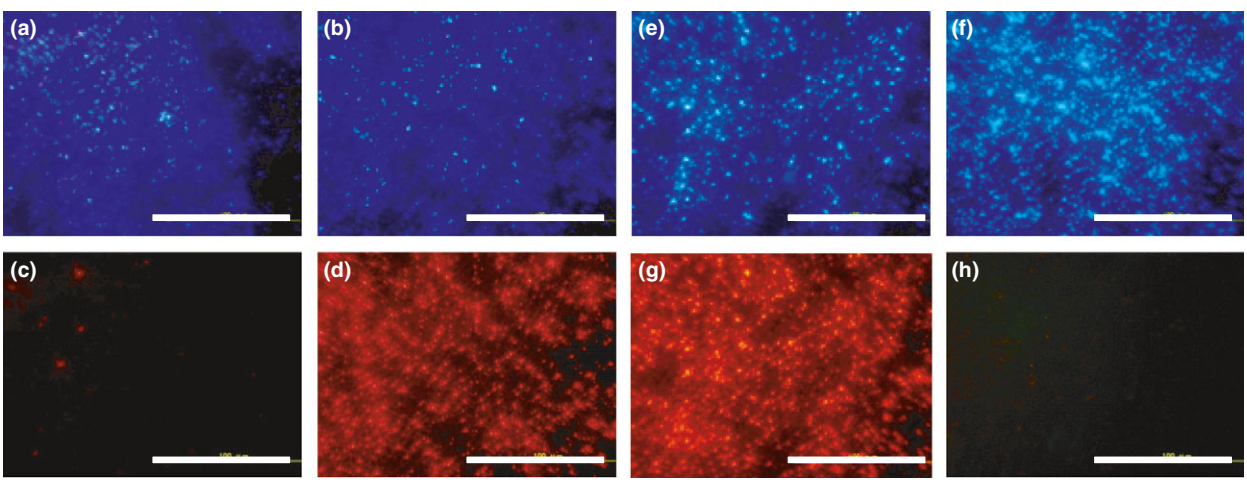

Figure 2 Fluorescence microscope images of *Escherichia coli* cells. Membrane damage of *E. coli* cells caused by exposure to volatile isoamyl acetate (a–d). a and c: unexposed cells after 5 h; b and d: cells after exposure for 5 h. a and b: stained with DAPI (blue); c and d: stained with propidium iodide (PI) (red). Loss of respiratory activity of *E. coli* cells caused by exposure to volatile isoamyl acetate (e–h). e and g: unexposed cells after 5 h; f and h: cells after exposure for 5 h. e and f: stained with DAPI (blue); g and h: stained with 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC) (red). Bars represent 100 μm.

mechanism is still unclear, further consideration will be needed to yield any findings about the mechanism.

The MBDs of acetic acid and isoamyl alcohol, produced by hydrolysis of isoamyl acetate against *E. coli*, were 20 and 40 μl dish^{-1}, respectively, and these showed antimicrobial activities similar to that of isoamyl acetate. *Escherichia coli* cell membrane was damaged by exposure to acetic acid and isoamyl alcohol; similar to damage by isoamyl acetate, and respiration activity was absent. Based on these findings, hydrolysis products of isoamyl acetate damaged the cell membrane of *E. coli* and arrested respiration, similar to isoamyl acetate, showing high antimicrobial activity.

Alcohol including ethanol, hexanol and phenethyl alcohol is amphipathic, and the hydrophobic region is dissolved in the cell membrane and increases membrane fluidity, damaging the cell membrane (Ingram and Buttke 1984; Seward *et al.* 1996; Fialho *et al.* 2010). Long-chain alcohols are more hydrophobic than ethanol, such as isoamyl alcohol and *n*-butanol, and therefore, it has a high affinity for the cell membrane and abolishes respiration in *E. coli* by damaging the cell membrane (Heipieper *et al.* 1994; Brynildsen and Liao 2009; Rutherford *et al.* 2010). Acetic acid has also been reported to damage *E. coli* cell membrane, although it is weaker than alcohol (Zaldivar and Ingram 1999). Acetic acid and isoamyl alcohol damage *E. coli* cell membrane and inactivate membrane proteins, such as succinate dehydrogenase (an enzyme involved in both the TCA cycle and electron transport system), impairing respiration. To resist this, *E. coli* increases expression of enzymes (AceA, Pfl and AcnB) of the TCA cycle involved in succinic acid biosynthesis. In addition, the expression levels of proteins (EF-Tu, EF-G and GlyS) involved in synthesis of these proteins increases. In this way, bacteria may cope with inactivation of membrane proteins by acetic acid and isoamyl alcohol. It is also possible that *E. coli* avoids acid stress by enhancing TCA cycle activity and consuming acetic acid.

Based on these findings, we propose the following mechanism for inhibition of *E. coli* growth by volatile isoamyl acetate. First, volatile isoamyl acetate adheres to the superficial layer of *E. coli* and enters the periplasm. Isoamyl acetate is hydrolyzed to acetic acid and isoamyl alcohol by esterase produced by *E. coli*. The resulting acetic acid and isoamyl alcohol damage the cell membrane of *E. coli* and impair the electron transport system, leading to loss of respiration. *E. coli* may compensate for this by increasing the expression of proteins involved in the TCA cycle and protein synthesis to maintain respiration, resisting the acid and alcohol stress of acetic acid and isoamyl alcohol.

Among the VOCs, aldehydes show the highest antimicrobial activity, followed by alcohol, and the activity of esters is generally low. This may be due to the absence of free functional groups in esters, in contrast to aldehydes and alcohol that contain highly reactive free functional groups (carbonyl and hydroxyl groups). However, even though antimicrobial activity of volatile esters is low, when they are adsorbed to the superficial layer of cells and hydrolyzed by cellular esterase to highly antimicrobial acid and alcohol, they subsequently exhibit strong activity. Consideration of antimicrobial activity of hydrolysis products may be important with regard to antimicrobial activity of volatile esters.

Conflict of Interest

No conflict of interest declared.

References

Ando, H., Hatanaka, K., Ohata, I., Yamashita-Kitaguchi, Y., Kurata, A. and Kishimoto, N. (2012) Antifungal activities of volatile substances generated by yeast isolated from Iranian commercial cheese. *Food Control* **26**, 472–478.

Arikawa, Y., Yamada, M., Shimosaka, M., Okazaki, M. and Fukuzawa, M. (2000) Isolation of sake yeast mutants producing a high level of ethyl caproate and/or isoamyl acetate. *J Biosci Bioeng* **90**, 675–677.

Blankenhorn, D., Phillips, J. and Slonczewski, J.L. (1999) Acid- and base-induced proteins during aerobic and anaerobic growth of *Escherichia coli* revealed by two-dimensional gel electrophoresis. *J Bacteriol* **181**, 2209–2216.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.

Brynildsen, M.P. and Liao, J.C. (2009) An integrated network approach identifies the isobutanol response network of *Escherichia coli*. *Mol Syst Biol* **5**, 1–13.

Chong, H., Huang, L., Yeow, J., Wang, I., Zhang, H., Song, H. and Jiang, R. (2013) Improving ethanol tolerance of *Escherichia coli* by rewiring its global regulator cAMP receptor protein (CRP). *PLoS One* **8**, e57628.

Fernandez, J., Gharahdaghi, F. and Mische, S.M. (1998) Routine identification of proteins from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels or polyvinyl difluoride membranes using matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS). *Electrophoresis* **19**, 1036–1045.

Fernando, W.G.D., Ramarathnam, R., Krishnamoorthy, A.S. and Savchuk, S.C. (2005) Identification and use of potential bacterial organic antifungal volatiles in biocontrol. *Soil Biol Biochem* **37**, 955–964.

Fialho, M.B., Toffano, L., Pedroso, M.P., Augusto, F. and Pascholati, S.F. (2010) Volatile organic compounds produced by *Saccharomyces cerevisiae* inhibit the *in vitro* development of *Guignardia citricarpa*, the causal agent of citrus black spot. *World J Microbiol Biotechnol* **26**, 925–932.

Fialho, M.B., Ferreira, L.F.R., Monteiro, R.T.R. and Pascholati, S.F. (2011) Antimicrobial volatile organic compounds affect morphogenesis-related enzymes in *Guignardia citricarpa*, causal agent of citrus black spot. *Biocontrol Sci Technol* **21**, 797–807.

Fukuda, K., Yamamoto, N., Kiyokawa, Y., Yanagiuchi, T., Wakai, Y., Kitamoto, K., Inoue, Y. and Kimura, A. (1998) Balance of activities of alcohol acetyltransferase and esterase in *Saccharomyces cerevisiae* is important for production of isoamyl acetate. *Appl Environ Microbiol* **64**, 4076–4078.

Han, M.J. and Lee, S.Y. (2006) The *Escherichia coli* proteome: past, present, and future prospects. *Microbiol Mol Biol Rev* **70**, 362–439.

Heipieper, H.J., Weber, F.J., Sikkema, J., Keweloh, H. and de Bont, J.A. (1994) Mechanisms of resistance of whole cells to toxic organic solvents. *Trends Biotechnol* **12**, 409–415.

Horinouchi, T., Tamaoka, K., Furusawa, C., Ono, N., Suzuki, S., Hirasawa, T., Yomo, T. and Shimizu, H. (2010) Transcriptome analysis of parallel-evolved *Escherichia coli* strains under ethanol stress. *BMC Genomics* **11**, 579.

Huang, R., Li, G.Q., Zhang, J., Yang, L., Che, H.J., Jiang, D.H. and Huang, H.C. (2011) Control of postharvest Botrytis fruit rot of strawberry by volatile organic compounds of *Candida intermedia*. *Phytopathology* **101**, 859–869.

Humphris, S.N., Bruce, A., Buultjens, E. and Wheatley, R.E. (2002) The effects of volatile microbial secondary metabolites on protein synthesis in *Serpula lacrymans*. *FEMS Microbiol Lett* **210**, 215–219.

Ingram, L.O. and Buttke, T.M. (1984) Effects of alcohols on micro-organisms. *Adv Microb Physiol* **25**, 253–300.

Kai, M., Haustein, M., Molina, F., Petri, A., Scholz, B. and Piechulla, B. (2009) Bacterial volatiles and their action potential. *Appl Microbiol Biotechnol* **81**, 1001–1012.

Kitagaki, H. and Kitamoto, K. (2013) Breeding research on sake yeasts in Japan: history, recent technological advances, and future perspectives. *Annu Rev Food Sci Technol* **4**, 215–235.

Masoud, W., Poll, L. and Jakobsen, M. (2005) Influence of volatile compounds produced by yeasts predominant during processing of *Coffea arabica* in East Africa on growth and ochratoxin A (OTA) production by *Aspergillus ochraceus*. *Yeast* **22**, 1133–1142.

Pacaud, M. (1982) Identification and localization of two membrane-bound esterases from *Escherichia coli*. *J Bacteriol* **149**, 6–14.

Rutherford, B.J., Dahl, R.H., Price, R.E., Szmidt, H.L., Benke, P.I., Mukhopadhyay, A. and Keasling, J.D. (2010) Functional genomic study of exogenous *n*-butanol stress in *Escherichia coli*. *Appl Environ Microbiol* **76**, 1935–1945.

Segura, A., Molina, L., Fillet, S., Krell, T., Bernal, P., Muñoz-Rojas, J. and Ramos, J.L. (2012) Solvent tolerance in gram-negative bacteria. *Curr Opin Biotechnol* **23**, 415–421.

Seward, R., Willettes, J.C., Dinsdale, M.G. and Lloyd, D. (1996) The effects of ethanol, hexan-1-ol, and 2-phenylethanol on cider yeast growth, viability, and energy status; synergistic inhibition. *J Inst Brew* **102**, 439–443.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M. *et al.* (1985) Measurement of protein using bicinchoninic acid. *Anal Biochem* **150**, 76–85.

Strobel, G.A., Dirkse, E., Sears, J. and Markworth, C. (2001) Volatile antimicrobials from *Muscodor albus*, a novel endophytic fungus. *Microbiology* **147**, 2943–2950.

Yoshizawa, K. (1999) Sake: production and flavor. *Food Rev Int* **15**, 83–107.

Yuan, J., Raza, W., Shen, Q. and Huang, Q. (2012) Antifungal activity of *Bacillus amyloliquefaciens* NJN-6 volatile compounds against *Fusarium oxysporum* f. sp. *cubense*. *Appl Environ Microbiol* **78**, 5942–5944.

Zaldivar, J. and Ingram, L.O. (1999) Effect of organic acids on the growth and fermentation of ethanologenic *Escherichia coli* LY01. *Biotechnol Bioeng* **66**, 203–210.

Zhang, D.F., Li, H., Lin, X.M., Wang, S.Y. and Peng, X.X. (2011) Characterization of outer membrane proteins of *Escherichia coli* in response to phenol stress. *Curr Microbiol* **62**, 777–783.