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Effective Trapping of Fruit Flies with Cultures of Metabolically Modified Acetic Acid Bacteria

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Acetoin in vinegar is an attractant to fruit flies when combined with acetic acid. To make vinegar more effective in attracting fruit flies with increased acetoin production, *Komagataeibacter europaeus* KGMA0119 was modified by specific gene disruption of the acetohydroxyacid isomeroreductase gene (*ilvC*). A previously constructed mutant lacking the putative ligand-sensing region in the leucine-responsive regulatory protein (*Ke*Lrp, encoded by *Kelrp*) was also used. The *ilvC* and *Kelrp* disruptants (KGMA5511 and KGMA7203, respectively) produced greater amounts of acetoin (KGMA5511, 0.11%; KGMA7203, 0.13%) than the wild-type strain KGMA0119 (0.069%). KGMA7203 produced a trace amount of isobutyric acid (0.007%), but the other strains did not. These strains produced approximately equal amounts of acetic acid (0.7%). The efficiency of fruit fly attraction was investigated with cultured *Drosophila melanogaster*. *D. melanogaster* flies (approximately 1,500) were released inside a cage (2.5 m by 2.5 m by 1.5 m) and were trapped with a device containing vinegar and a sticky sheet. The flies trapped on the sticky sheet were counted. The cell-free supernatant from KGMA7203 culture captured significantly more flies (19.36 to 36.96% of released flies) than did KGMA0119 (3.25 to 11.40%) and KGMA5511 (6.87 to 21.50%) cultures. Contrastingly, a 0.7% acetic acid solution containing acetoin (0.13%) and isobutyric acid (0.007%), which mimicked the KGMA7203 supernatant, captured significantly fewer flies (0.88 to 4.57%). Furthermore, the KGMA0119 supernatant with additional acetoin (0.13%) and isobutyric acid (0.007%) captured slightly more flies than the original KGMA0119 supernatant but fewer than the KGMA7203 supernatant, suggesting that the synergistic effects of acetic acid, acetoin, isobutyric acid, and unidentified metabolites achieved the efficient fly trapping of the KGMA7203 supernatant.

Bacterial cultures can function as attractants for insects (1). Fruit flies, such as *Drosophila melanogaster*, are recognized as an index for unsanitary conditions in urban areas because the flies infest damaged and overripe fruits and rotten foods. One of the important challenges is to keep the numbers of flies low, particularly in the food industries. A closely related species, the spotted-wing drosophila (SWD) (*Drosophila suzukii*), which is native to southeastern Asia, is a newly emerging invasive pest for soft-skinned fruits such as blueberries, strawberries, and peaches in North America and Europe (2–4). Because the SWD breaks the skin of maturing healthy fruits using a serrated ovipositor to oviposit, unlike other *Drosophila* species, the fly causes significant damage to soft-skinned fruits (2). The damage caused by the SWD promotes microbial decay in the fruits, which results in a secondary infestation of other *Drosophila* species. The damaged fruits are likely to be rejected at the processing plant or export terminal (5). Although fruits are protected with sprays of chemical insecticides once the SWD is detected, growers risk the rejection of harvested fruits when the levels of residual insecticides exceed the maximum limits (5). Serious economic losses are estimated to be caused by the SWD, including increased management costs and rejection of crops, in the fruit-growing industries of the United States and Europe (6, 7). Therefore, effective flytraps are important to establish a systematic pest management program to prevent economic damage by protecting fruits from the attack of the SWD and other *Drosophila* species. Such traps could also help growers reduce pesticide applications, which is preferable for the safety of the consumer.

Recently, flytraps that used fermented foods instead of chemicals were developed for general household use in response to increasing consumer demands. Vinegar and wine strongly attract fruit flies (1, 8). Several physiologically active compounds in vinegar and wine were identified by gas chromatography coupled with electroantennographic detection (GC-EAD) (7, 9, 10). Acetic acid is a primary volatile compound in vinegar and plays a key role in fruit fly attraction (7, 8, 11). Combined with acetic acid, acetoin, which is a fermentation compound primarily found in dairy foods (12) that has a heavy cheese-like odor, exerts a strong synergistic effect on fruit fly attraction (7, 11). These volatile compounds are important signals for fruit flies to find feeding and oviposition sites (10). Therefore, vinegar with increased amounts of acetoin is expected to be a powerful attractant for effective trapping of flies.

Acetic acid bacteria (AAB) are Gram-negative obligately aerobic microorganisms. Recently, it has been reported that some

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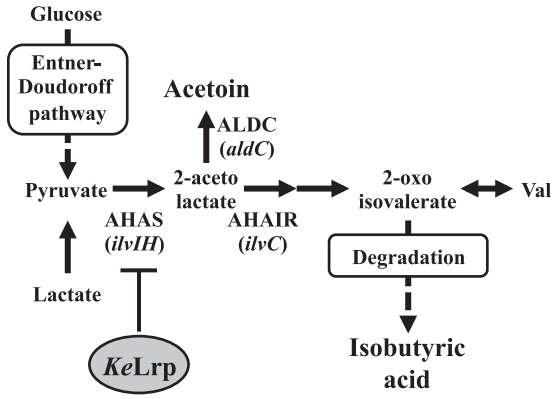

FIG 1 Biosynthetic pathway for the production of valine, acetoin, and isobutyric acid. AHAS, acetohydroxyacid synthase; AHAIR, acetohydroxyacid isomeroreductase; ALDC, α-acetolactate decarboxylase; *Ke*Lrp, leucine-responsive regulatory protein in *K. europaeus*.

unique AAB are applicable not only for vinegar production but also for other beneficial purposes, such as cellulose production for transplantation therapy (13), production of shikimate (precursor of oseltamivir) (14), and nitrogen fixation in agriculture (15). *Komagataeibacter europaeus* (previously classified as *Gluconacetobacter europaeus*) (16) is widely used in the industrial production of vinegar because of its ability to oxidize ethanol and high tolerance to acetic acid (17). In our previous study, a targeted gene disruption system using the endogenous *pyrE* gene as a selectable marker was developed for use in *K. europaeus*, and the pathway for acetoin biosynthesis in *K. europaeus* was determined (18). The pathway shares intermediary metabolites (pyruvate and 2-acetolactate) with the pathway for branched-chain amino acid (BCAA; valine, leucine, or isoleucine) biosynthesis (Fig. 1). The disruption of *ilvC*, which is one of the genes for BCAA biosynthesis and encodes acetohydroxyacid isomeroreductase, was expected to promote acetoin production by shifting the carbon flux from valine to acetoin (Fig. 1). Our previous study also revealed that the *K. europaeus* leucine-responsive regulatory protein (*Ke*Lrp [previously designated *Ge*Lrp]; encoded by *Kelrp*) repressed the transcription of *ilvIH*, encoding a rate-limiting enzyme in BCAA biosynthesis (acetohydroxyacid synthase). Increased expression of *ilvIH* occurred when the repression was released by the truncation of the C-terminal ligand-sensing region in *Ke*Lrp (Fig. 1) (19). Thus, the *Kelrp* disruptant (designated KGMA7203) was predicted to accumulate increased amounts of acetoin as a by-product (Fig. 1).

To enhance attractiveness to fruit flies, changes in raw materials and their blending ratios are effective techniques. Currently, a variety of flytraps that use fermented foods (e.g., apple cider vinegar, rice vinegar, and balsamic vinegar) as attractants are available on the market. In this study, we first constructed an *ilvC* disruptant (designated KGMA5511). Fly-trapping experiments were then conducted with traps baited with cell-free supernatants from cultures of the *ilvC* disruptant KGMA5511 and the previously constructed *Kelrp* disruptant KGMA7203 (19), and the attractiveness of the supernatants to fruit flies was investigated. Fruit fly trapping was effectively enhanced by the culture supernatants of these metabolically modified AAB.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used in the study are presented in Table 1. Strain KGMA7203 [Δ*pyrE Kelrp*Δ(397-511)::*pyrE* {where "Δ(397-511)" indicates deletion of nucleotides 397 to 511}] was a valine- and leucine-overproducing strain with a specific deletion at the C-terminal ligand-binding domain in *Ke*Lrp (19). Strain KGMA0119 (wild type, isolated from rice vinegar) (18) and its derivatives were cultured in yeast peptone dextrose (YPD) broth (18) at 30°C with reciprocal shaking at 150 rpm. To isolate the *ilvC* disruptant

TABLE 1 Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant characteristics or sequence (5′ to 3′)[a]	Source or purpose
Strains		
E. coli DH5α	F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ$^-$ *thi-1 gyrA96 relA1*	20
K. europaeus		
KGMA0119	Wild type isolated from rice vinegar	18
KGMA0704	KGMA0119 derivative; Δ*pyrE*	18
KGMA5511	KGMA0704 derivative; Δ*pyrE* Δ*ilvC*::*pyrE*	This work
KGMA7203	KGMA0704 derivative; Δ*pyrE Kelrp*Δ(397-511)::*pyrE*	19
Plasmids		
pBR322	Amp^r	21
pBR322-Δ*ilvC*::*pyrE*	pBR322 derivative; Δ*ilvC*::*pyrE* cassette composed of 5′ upstream and 3′ downstream regions of *ilvC* and a *pyrE* cassette (3,374 bp)	This work
Primers		
ilvC-F	AAGAATTCTCACGGTATTCCGAAAGCTACAGT	*ilvC* disruption
ilvC-R	AAGGATCCGTATTCAGGCGGTATAGGTGACGTT	*ilvC* disruption
ilvC-i1	ATGATGTCATGGATCGGCAAGAACAAGCTG	*ilvC* disruption
ilvC-i2	ATCGCGATCGTAATAGACGCGCATGGTCTT	*ilvC* disruption
EP-F2	CTGCCATATCCCGTGTTCGT	*ilvC* disruption
E-R4	TCGCCATAGGGAAAGACTGC	*ilvC* disruption
ilvC-F2	GTGGATACAATATCGAAAGCCTGAC	Genotyping
ilvC-R2	CTGGCTTGGAAAAGAAGAAAGGTTC	Genotyping

[a] Restriction enzyme sites are underlined. Amp, ampicillin.

strain KGMA5511 (see below), a minimal medium (18) containing 12 mM BCAAs (12 mM valine, 12 mM leucine, and 12 mM isoleucine) was used. As well, 12 mM BCAAs were added to YPD broth when the strain KGMA5511 was cultured. Unless otherwise indicated, 0.4% (wt/vol) ethanol and 0.5% (wt/vol) acetic acid were added to both media. For plate cultures, 0.9% (wt/vol) agar was added to both media.

Escherichia coli DH5α (20) (TaKaRa Bio, Ohtsu, Shiga, Japan) and pBR322 (21) (TaKaRa Bio) were used to construct the *ilvC* disruption vector (see Fig. S1A in the supplemental material). *E. coli* DH5α was cultured in lysogeny broth (22) containing 50 μg/ml of ampicillin at 37°C.

DNA manipulation and sequencing. General DNA manipulations were performed as described by Green and Sambrook (22). PCR was conducted using KOD plus (Toyobo, Osaka, Japan) as a DNA polymerase. Restriction and modifying enzymes were purchased from TaKaRa Bio or Nippon Gene (Tokyo, Japan). PCR products and restriction fragments were separated by agarose gel electrophoresis, and the DNA fragments were recovered using the NucleoSpin Gel and PCR Cleanup kit (TaKaRa Bio). DNA sequencing was performed with a BigDye Terminator Cycle Sequencing kit, ver. 3.1, and model 3130 capillary sequencer (Applied Biosystems, Foster City, CA, USA).

Construction of *ilvC* disruption vector and *ilvC* disruptant. The theoretical background for the specific gene disruption was described previously (18, 23–25). The *pyrE* gene, encoding orotate phosphoribosyltransferase, and strain KGMA0704, lacking *pyrE*, were used as a selectable marker and a host strain, respectively. The primers used for the construction of the *ilvC* disruption vector are listed in Table 1 and are shown in Fig. S1A in the supplemental material. A 1.5-kb *pyrE* marker cassette containing its promoter region was amplified from KGMA0119 chromosomal DNA using 5′-phosphorylated primers EP-F2 and E-R4. A 2.8-kb fragment containing the *ilvC* open reading frame (ORF) together with its 5′ (1.0 kb)- and 3′ (0.9 kb)-flanking regions was amplified using primers ilvC-F and ilvC-R (see Fig. S1A in the supplemental material). The amplified fragment was subcloned into pBR322 between the EcoRI and BamHI sites. The 5′- and 3′-flanking regions of *ilvC* and the plasmid backbone, excluding the 945 bp of the *ilvC* ORF, were amplified from the intermediary plasmid using primers ilvC-i1 and ilvC-i2 (see Fig. S1A in the supplemental material). This PCR fragment was then ligated with a 5′-phosphorylated *pyrE* cassette, and the resultant plasmid was designated pBR322-Δ*ilvC*::*pyrE* (see Fig. S1A in the supplemental material). The pBR322-Δ*ilvC*::*pyrE* plasmid was introduced into KGMA0704 competent cells by electroporation, and the cells were allowed to recover in 1 ml of YPD broth for 3 h at 30°C. The cells were then harvested by centrifugation, washed with saline (0.85% [wt/vol] NaCl), and transferred to 5 ml of the minimal medium lacking uracil and containing 12 mM BCAAs. The cells were cultured for at least 48 h to concentrate uracil prototrophic and BCAA auxotrophic cells. An aliquot of the culture was diluted with saline, spread onto the minimal agar plate lacking uracil and containing BCAAs, and incubated at 30°C for 4 to 5 days. The genotypes of positive clones were investigated with PCR using primers ilvC-F2 and ilvC-R2 (see Fig. S1A in the supplemental material) and by sequencing the obtained PCR fragments. The resultant *ilvC* disruptant was designated KGMA5511 (Δ*pyrE* Δ*ilvC*::*pyrE*) (see Fig. S1 in the supplemental material).

Measurements of volatile compounds and organic acids and preparation of culture supernatants for fly trapping. The strains KGMA0119, KGMA5511, and KGMA7203 (19) were cultured in 30 ml of YPD broth supplemented with 1.0% (wt/vol) sodium L-lactate (and 12 mM BCAAs for KGMA5511). Aliquots of cultures were sampled sequentially, and cell-free supernatants were prepared by centrifugation (4°C, 10,000 × *g*, 5 min) and filtration (0.45-μm-pore-size Millex LH filter; Millipore, Bedford, MA, USA). The concentrations of ethanol, acetic acid, and acetoin in the supernatants were measured with a GC-2014 gas chromatograph (GC; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a packed column (PEG20M 10%, Shincarbon A 60/80, 2.1 m by 3.2 mm; Shinwa Chemical, Kyoto, Japan). The column oven temperature was initially held at 60°C for 3 min, which was followed by an increase of 10°C per min up to 200°C.

The level of isobutyric acid in the supernatants (filtered with a 0.45-μm filter) was quantified by high-performance liquid chromatography (HPLC; Organic Acid Analysis System Prominence; Shimadzu) on an ion-exclusion column (Shim-pack SCR-102H; Shimadzu). The column was equilibrated with a mobile phase (5 mM *p*-toluenesulfonate; Wako Pure Chemicals, Osaka, Japan) at a flow rate of 0.8 ml/min at 40°C. The eluted organic acids were automatically mixed with a reaction buffer composed of 5 mM *p*-toluenesulfonate, 100 μM EDTA (Dojindo Molecular Technologies, Kumamoto, Japan), and 20 mM Bis-Tris [bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; Dojindo Molecular Technologies] at a flow rate of 0.8 ml/min at 40°C and were monitored with an electrical conductivity detector (CDD-10AVP; Shimadzu).

After cultivation for 34 h, cell-free supernatants of the three strains were prepared as described above using the entire cultures, which were then used for the fly-trapping experiments (see below).

To evaluate concentrations of acetic acid and acetoin in supernatants and the final cell yields (optical density at 600 nm [OD_{660}]) of the three strains after 34 h of cultivation, mean values ($n = 3$) were statistically analyzed by Tukey's test at P values of 0.05 following an analysis of variance (ANOVA).

Breeding of fruit flies. *D. melanogaster* was kindly provided by the Department of Biological Sciences, Tokyo Metropolitan University, Japan. A banana medium (10 g of pasted banana supplemented with 0.5 g of dry yeast [Oriental Yeast, Tokyo, Japan] and 3 ml of beer [Kirin, Tokyo, Japan]) was used routinely as a feed for the fruit flies. The flies were transferred to a 30-ml glass vial containing 6 ml of the banana medium and capped with a cotton plug, and the flies were reared at room temperature (22 to 25°C) for 10 to 14 days. To maintain the fly population, newly eclosed flies were transferred to glass vials containing fresh medium, and this was repeated every 10 days. For the trapping experiments, the adult flies, larvae, and pupae in the glass vials were transferred to a 5-liter glass bottle baited with fresh banana medium and further reared for 5 to 10 days until the population reached approximately 1,500 individuals. The cultured flies were used in the subsequent trapping experiments.

Fly-trapping experiments. As a flytrap device, a plastic bottle of the Victor Fly Magnet system (Woodstream Co., Lititz, PA, USA) was used with modifications as follows: a sticky sheet (11 cm by 21 cm; Sankyo-Shodoku Co., Tokyo, Japan) was formed into a cylinder and attached to a clear plastic bottle (Fig. 2A). Thirty milliliters of the cell-free supernatant was added to the bottle as an attractant, and the bottle was capped by a lid with entry holes for the attracted flies and a shade that promoted the spread of attractant (Fig. 2A). Synthetic vinegar composed of 0.7% (wt/vol) acetic acid, 0.13% (wt/vol) acetoin, and 0.007% (wt/vol) isobutyric acid was used as a reference solution. The traps were hung in the corners of a cage (2.5 m by 2.5 m by 1.5 m) placed outside and fixed with a mosquito net. The height at which the traps were hung was 1.0 m from the ground (Fig. 2B). To evaluate the attractiveness of the supernatants, the cultured flies (approximately 1,500) were released inside the cage, and the flies captured on the sticky sheets were monitored every 15 min for 45 min. At the conclusion of the trapping experiments, the flies not captured in the traps were killed with an insecticide and enumerated. The number of flies killed was added to that of captured flies to determine the total number of released flies. The attractiveness of supernatants was indexed as the ratio of the number of flies captured in traps to the fly population released (number of captured flies/total number of released flies [%]). The trapping experiments were conducted in Sanda City, Hyogo, Japan. The first trapping experiments were conducted in triplicate ($n = 3$) on 1 and 9 July 2014 (see Fig. 6). The first, second, and third trials were performed from 13:30 to 14:15 on July 1 under sunny conditions (30°C) (see Fig. 6A), from 11:00 to 11:45 on July 9 under cloudy conditions (28°C) (see Fig. 6B), and from 14:00 to 14:45 on July 9 under cloudy conditions (28°C) (see Fig. 6C), respectively. The second trapping experiments to examine an effect of additional acetoin and isobutyric acid on fly-trapping effi-

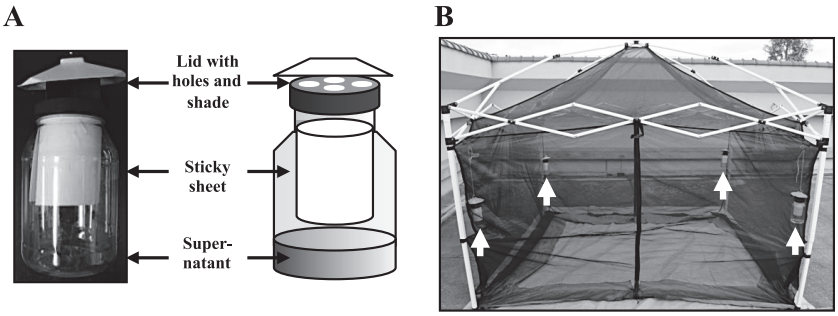

FIG 2 Design of fly-trapping experiment. (A) Trap design. Victor Fly Magnet (Woodstream Co.) with sticky sheet. The cell-free supernatants of cultures from strains KGMA0119 (wild type), KGMA5511 (Δ*pyrE* Δ*ilvC*::*pyrE*), and KGMA7203 [Δ*pyrE Kelrp*Δ(397-511)::*pyrE*] were used as attractants. (B) Cage with a mosquito net used for trapping experiments. Traps were hung in the corners of the cage (white arrows). The cultured flies were released inside the cage, and the flies captured in traps were monitored (Fig. 6 and 7).

ciency were also conducted in triplicate ($n = 3$) on 9 and 20 October 2014 (see Fig. 7). The first, second, and third trials were performed from 13:40 to 14:25 on 9 October under cloudy conditions (26°C) (see Fig. 7A), from 15:05 to 15:50 on 9 October under cloudy conditions (25°C) (see Fig. 7B), and from 13:40 to 14:25 on 20 October under cloudy conditions (25°C) (see Fig. 7C), respectively.

Nucleotide sequence accession numbers. The nucleotide sequences of *ilvC* and *Kelrp* were deposited in the DDBJ, EMBL, and GenBank databases under accession numbers AB899162 and AB899159, respectively.

RESULTS

Construction of the *ilvC* disruptant. The biosynthesis pathway for acetoin shares intermediary metabolites with the pathway for BCAAs in *K. europaeus* (Fig. 1). The disruption of the *ilvC* gene was expected to change the carbon flux toward acetoin and result in increased production of acetoin. The *ilvC* disruption vector pBR322-Δ*ilvC*::*pyrE* was introduced into the *pyrE*-lacking strain KGMA0704 (see Fig. S1A in the supplemental material). The resultant *ilvC* disruptant was designated KGMA5511 (Δ*pyrE* Δ*ilvC*::*pyrE*) (see Fig. S1A in the supplemental material). The PCR analysis confirmed that the *ilvC* locus of KGMA5511 was longer than that of each of the two parental strains, KGMA0704 and the wild-type strain KGMA0119 (see Fig. S1B in the supplemental material). Sequence analysis of the targeted region also confirmed that the *ilvC* locus in KGMA5511 was replaced with the *pyrE* marker as expected. The KGMA5511 strain strictly required three types of BCAA (valine, leucine, and isoleucine) for its growth (Fig. 3A) and showed impaired growth when it was cultured in a nutrient-rich YPD broth in the absence of BCAAs (Fig. 3B). To restore this growth defect, 12 mM BCAAs (12 mM valine, 12 mM leucine, and 12 mM isoleucine) was needed. The impaired growth of KGMA5511 was not fully restored by the addition of lower concentrations (0.5 or 4 mM each) of BCAAs to the medium (Fig. 3B).

Volatile compounds and organic acid profiles of gene disruptants. Our previous study revealed that the genes for BCAAs biosynthesis (*ilvI* and *ilvH*) were highly expressed in the *Kelrp* disruptant KGMA7203 (19). Thus, it was expected that the strain accumulated increased amounts of acetoin as a by-product (Fig. 1). To estimate acetoin productivity, the strains KGMA0119, KGMA5511, and KGMA7203 were cultured in YPD broth supplemented with 1.0% sodium L-lactate (and 12 mM BCAAs for KGMA5511), and the concentrations of acetoin, acetic acid, and ethanol in the cultures were measured at various growth phases by GC (Fig. 4). The growth profiles were also monitored. The final cell yields of KGMA5511 and KGMA7203 were slightly lower than those of KGMA0119 (Fig. 4A); however, there were no statistical differences among the cell yields of the three strains (Tukey's test following ANOVA, $F = 5.14$, $df = 8$, $P = 0.017$) (Table 2). Whereas all the strains produced acetoin from the late logarithmic

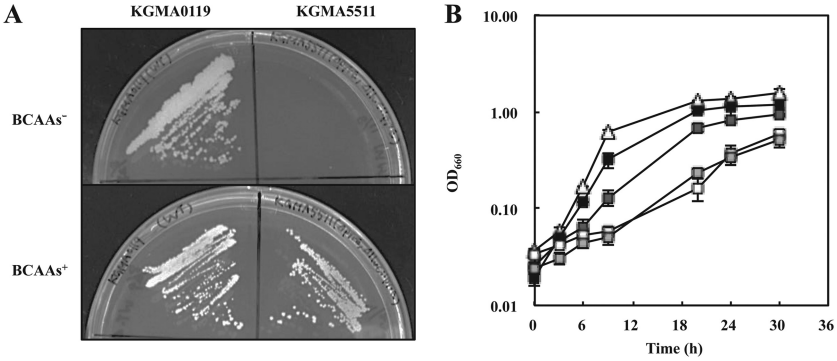

FIG 3 The BCAA auxotrophy of KGMA5511 (Δ*pyrE* Δ*ilvC*::*pyrE*). (A) BCAA auxotrophy of strain KGMA5511. Strains KGMA0119 (wild type) and KGMA5511 were streaked onto a minimal agar plate lacking (upper panel) or containing (lower panel) 12 mM BCAAs. (B) Growth profiles of KGMA0119 and KGMA5511 strains. KGMA0119 was cultured in YPD broth lacking BCAAs, and KGMA5511 was cultured in YPD broth lacking or containing various concentrations of BCAAs. White triangles, KGMA0119 without BCAAs; white squares, KGMA5511 without BCAAs; light gray squares, KGMA5511 with 0.5 mM BCAAs; dark gray squares, KGMA5511 with 4 mM BCAAs; black squares, KGMA5511 with 12 mM BCAAs. Experiments were conducted in triplicate, and error bars indicate standard deviations.

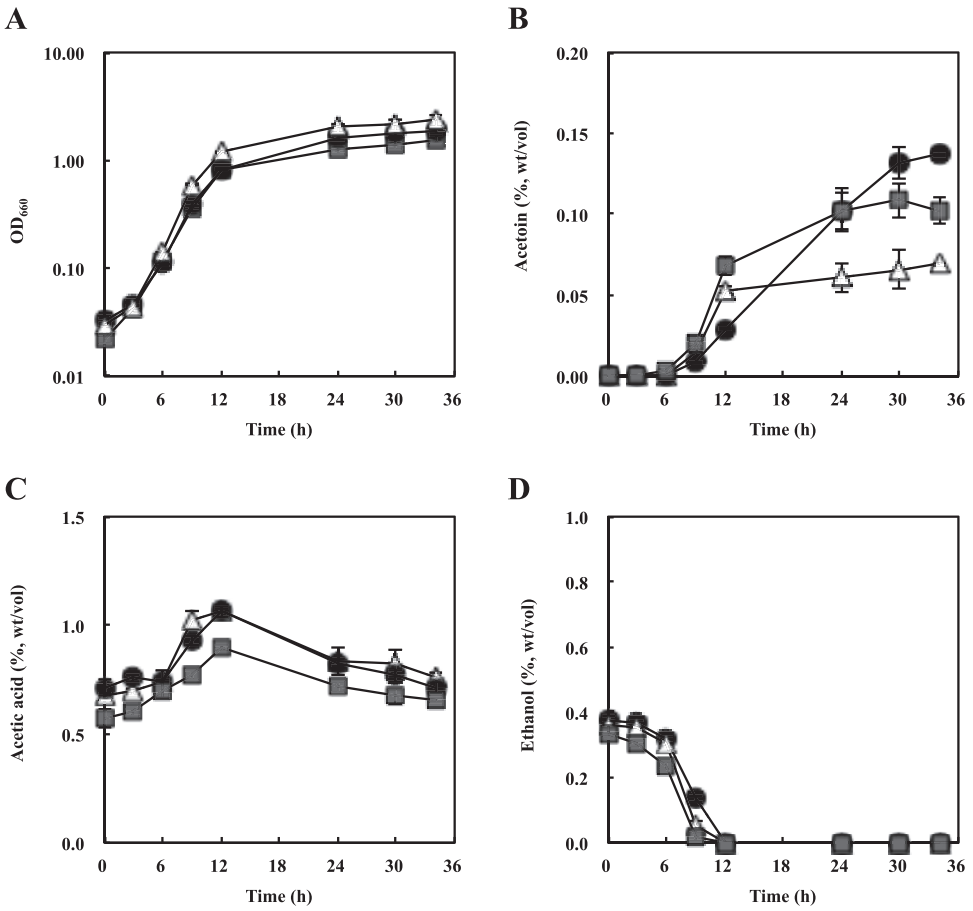

FIG 4 Growth and volatile compound profiles of strains KGMA0119 (wild type), KGMA5511 (Δ*pyrE* Δ*ilvC*::*pyrE*), and KGMA7203 [Δ*pyrE Kelrp*Δ(397-511)::*pyrE*]. The three strains were cultured in YPD broth supplemented with 1.0% sodium L-lactate (and 12 mM BCAAs for KGMA5511). The amounts of acetoin, acetic acid, and ethanol in cultures were measured by GC. Shown are profiles of growth (OD_{660}) (A), acetoin (% [wt/vol]) (B), acetic acid (% [wt/vol]) (C), and ethanol (% [wt/vol]) (D). White triangles, KGMA0119; dark gray squares, KGMA5511; black circles, KGMA7203. Experiments were conducted in triplicate, and error bars indicate standard deviations.

phase of growth, strains KGMA5511 (0.11%) and KGMA7203 (0.13%) accumulated significantly greater amounts of acetoin than the wild-type KGMA0119 (0.069%) (Tukey's test following ANOVA, $F = 5.14$, $df = 8$, $P = 0.00011$) (Fig. 4B and Table 2). The profiles of ethanol oxidization and acetic acid production were not significantly different in the three strains (Fig. 4C and D), and the concentrations of acetic acid after 34 h of culture were approximately 0.7% (Tukey's test following ANOVA, $F = 5.14$, $df = 8$, $P = 0.15$) (Fig. 4C and Table 2). Ethanol was not detected in cultures of the three strains after 34 h of growth (Fig. 4D).

To investigate the composition in more detail, the organic acids that accumulated in the cultures after 34 h growth were analyzed with HPLC. Strain KGMA7203 accumulated a trace amount of isobutyric acid (0.007%), whereas the other two strains did not (Fig. 5). Cell-free supernatants were then prepared and used for the fly-trapping experiments.

Fly-trapping experiments. The attractiveness to fruit flies was evaluated using traps baited with the supernatants of the three strains and a synthetic vinegar with concentrations of acetic acid (0.7%), acetoin (0.13%), and isobutyric acid (0.007%) that were adjusted to those of the KGMA7203 supernatant obtained after 34 h of growth. The synthetic vinegar captured fewer than 5% of released flies (0.88 to 4.57%) (Fig. 6), which indicated that it was not attractive to flies. In contrast, the culture supernatants of the

TABLE 2 Volatile compounds in cultures and cell yields of KGMA0119 (wild type) and its derivatives after 34 h of cultivation[a]

Strain (relevant characteristics)	Mean amt ± SD of volatile compound (%, wt/vol)		Final cell yield (OD_{660} at 34 h)
	Acetic acid	Acetoin	
KGMA0119 (wild type)	0.76 ± 0.022 A	0.069 ± 0.00082 A	2.33 ± 0.28 A
KGMA5511 (Δ*pyrE* Δ*ilvC*::*pyrE*)	0.69 ± 0.046 A	0.11 ± 0.0059 B	1.56 ± 0.12 A
KGMA7203 [Δ*pyrE Kelrp*Δ(397-511)::*pyrE*]	0.71 ± 0.025 A	0.13 ± 0.0081 B	1.87 ± 0.11 A

[a] Values shown are consistent with the date of 34 h of cultivation in Fig. 4. Values followed by different capital letters (A or B) are significantly different by Tukey's test at *P* values of 0.05. For acetic acid, ANOVA $F = 5.14$, $df = 8$, $P = 0.15$. For acetoin, ANOVA $F = 5.14$, $df = 8$, $P = 0.00011$. For cell yield, ANOVA $F = 5.14$, $df = 8$, $P = 0.017$.

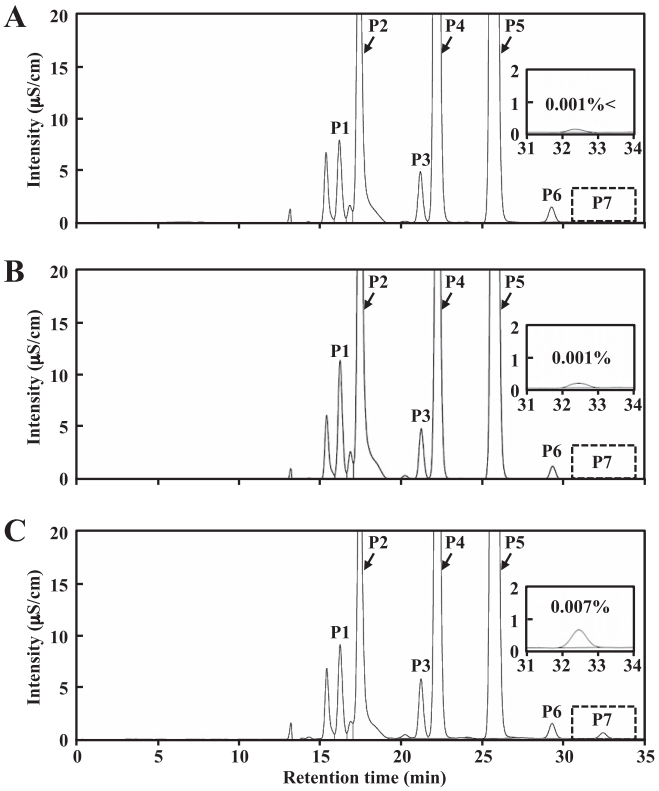

FIG 5 HPLC profiles of organic acids that accumulated in cultures after 34 h of growth. The chromatograms are consistent with the data of 34 h of cultivation in Fig. 4. The data of chromatograms (31 to 34 min) denoted by dotted-line boxes are highlighted. Peaks: P1, citric acid (16.3 min); P2, gluconic acid (17.5 min); P3, succinic acid (21.2 min); P4, lactic acid (22.2 min); P5, acetic acid (25.8 min); P6, propionic acid (29.3 min); P7, isobutyric acid (32.5 min). (A) KGMA0119 (wild type); (B) KGMA5511 (Δ*pyrE* Δ*ilvC*::*pyrE*); (C) KGMA7203 [Δ*pyrE Kelrp*Δ(397-511)::*pyrE*].

two gene disruptants were more attractive to flies than that of the KGMA0119 strain (Fig. 6). The supernatant of KGMA7203 had the highest attractiveness to flies and captured significantly more flies (19.36 to 36.96%) than those of KGMA0119 (3.25 to 11.40%) and KGMA5511 (6.87 to 21.50%) during a 45-min period (Fig. 6).

Effects of additional acetoin and isobutyric acid on the fly-trapping efficiency of KGMA0119 supernatant. The data described above indicated that acetic acid, acetoin, and isobutyric acid were important to attract fruit flies. Therefore, acetoin and isobutyric acid were added to the KGMA0119 supernatant for final concentrations of 0.13% and 0.007%, respectively, and then used in a fruit fly-trapping experiment. The KGMA0119 supernatant that contained additional acetoin (0.13%) and isobutyric acid (0.007%) captured slightly more flies (4.11 to 6.85%) than the original KGMA0119 supernatant (0.34 to 3.08%) (Fig. 7A and B), although the opposite tendency was observed in the third trial (KGMA0119, 5.92%; KGMA0119 with additional acetoin and isobutyric acid, 3.95%) (Fig. 7C). The KGMA7203 supernatant showed the highest attractiveness to fruit flies in all the trials conducted (5.82 to 13.36%) (Fig. 7).

DISCUSSION

To enhance the attractiveness of vinegar to fruit flies, we focused on acetoin and constructed two gene disruptants that were expected to produce increased amounts of acetoin (Fig. 4B and Table 2). The KGMA7203 supernatant attracted significantly more flies than the wild-type supernatant or synthetic vinegar (Fig. 6). Although acetoin levels in the cultures of the KGMA5511 and KGMA7203 strains were approximately equal (Fig. 4B and Table 2), the supernatant of KGMA7203 was more attractive to flies than that of KGMA5511, which suggested that KGMA7203 produced other attractants in addition to acetoin. The HPLC analyses revealed that KGMA7203 accumulated a trace amount of isobutyric

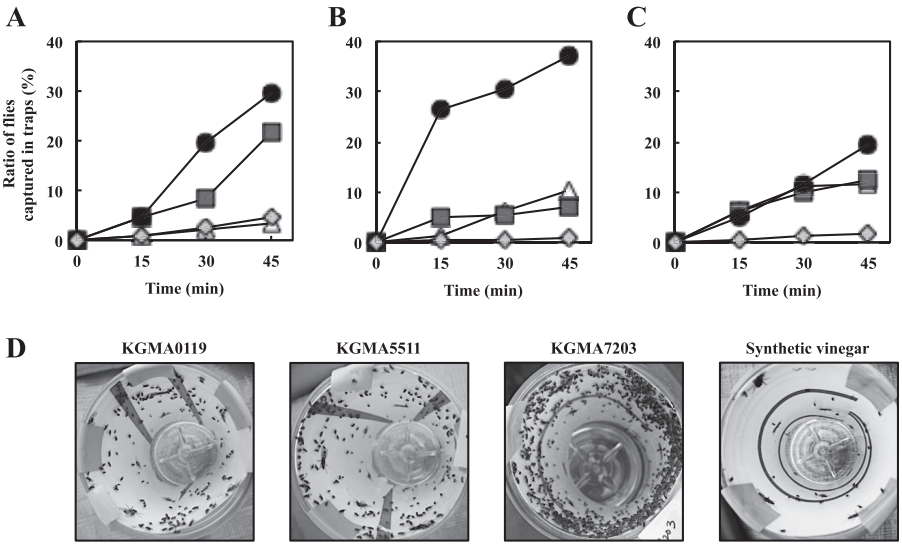

FIG 6 The fly-trapping experiments with culture supernatants from various strains. Strains KGMA0119 (wild type), KGMA5511 (Δ*pyrE* Δ*ilvC*::*pyrE*), and KGMA7203 [Δ*pyrE Kelrp*Δ(397-511)::*pyrE*] were cultured in YPD broth supplemented with 1.0% sodium L-lactate (and 12 mM BCAAs for KGMA5511). After culture for 34 h, cell-free supernatants were prepared and used for fly-trapping experiments. (A to C) Attractiveness of the supernatants to fruit flies. The attractiveness of the supernatants was indicated as a ratio of flies captured in traps to the total fly population released (%). The synthetic vinegar was composed of 0.7% acetic acid, 0.13% acetoin, and 0.007% isobutyric acid. The trapping experiments were conducted in triplicate. The first (A), second (B), and third (C) trials were conducted in the sunny afternoon (30°C), in the cloudy morning (28°C), and in the cloudy afternoon (28°C), respectively. See Materials and Methods for more-detailed conditions during the experiments (date and time). White triangles, KGMA0119; dark gray squares, KGMA5511; black circles, KGMA7203; light gray diamonds, synthetic vinegar. (D) Flies captured in traps. The results are consistent with the data of 45 min in panel B.

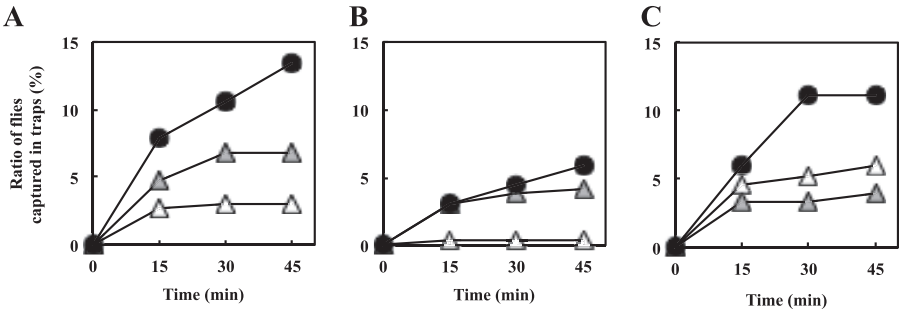

FIG 7 Effects of additional acetoin and isobutyric acid on the fly-trapping efficiency of KGMA0119 supernatant. Strains KGMA0119 (wild type) and KGMA7203 [Δ*pyrE Kelrp*Δ(397-511)::*pyrE*] were cultured in YPD broth supplemented with 1.0% sodium L-lactate. After 34 h of cultivation, cell-free supernatants were prepared and used for fly-trapping experiments. To investigate the effects of increased amounts of attractive compounds, the KGMA0119 supernatant, whose final concentrations of acetoin (0.13%) and isobutyric acid (0.007%) were adjusted to those in KGMA7203 supernatant, was used. The attractiveness of the supernatants was indicated as the ratio of the number of flies captured in traps to the total fly population released (%). The trapping experiments were conducted in triplicate. The first (A), second (B), and third (C) trials were conducted in the cloudy afternoon (26°C), in the cloudy late afternoon (25°C), and in the cloudy afternoon (25°C), respectively. White triangles, KGMA0119; light gray triangles, KGMA0119 with additional acetoin (0.13%) and isobutyric acid (0.007%); black circles, KGMA7203. See Materials and Methods for more-detailed conditions during the experiments (date and time).

acid in the culture, whereas the other strains did not (Fig. 5). Our previous study showed that the genes for BCAAs biosynthesis were highly expressed in KGMA7203 and resulted in the overproduction of valine (19). The genome analysis predicted that *K. europaeus* possessed the degradation pathways of BCAAs and their corresponding precursors, which were similar to those in other bacteria (26, 27). The isobutyric acid in the KGMA7203 supernatant would be derived from the degradation of the overproduced valine and its precursor (2-oxoisovalerate) (Fig. 1). Kleiber et al. reported that some short-chain acids, such as formic acid and valeric acid, attracted fruit flies in greenhouse assays (28). The high attractiveness of the KGMA7203 supernatant might be attributed to a synergistic effect of acetic acid, acetoin, and isobutyric acid. In contrast, synthetic vinegar (0.7% acetic acid, 0.13% acetoin, and 0.007% isobutyric acid), which mimicked the KGMA7203 supernatant, captured significantly fewer flies (Fig. 6). Furthermore, the KGMA0119 supernatant that contained additional acetoin (0.13%) and isobutyric acid (0.007%) captured slightly more flies than the original KGMA0119 supernatant but fewer than the KGMA7203 supernatant (Fig. 7). These results suggested that the efficient fly trapping of the KGMA7203 culture was achieved by the synergistic effect of acetic acid, acetoin, isobutyric acid, and unidentified metabolites.

The disruption of *ilvC* was effective in increasing the production of acetoin (Fig. 4B and Table 2). However, the *ilvC* disruptant KGMA5511 required abnormally large amounts of BCAAs (12 mM) for normal growth (Fig. 3B), and the impaired growth of KGMA5511 was not fully restored by the addition of lower concentrations (0.5 or 4 mM) of BCAAs to the growth medium (Fig. 3B). *E. coli* possesses effective BCAA transport systems encoded by the *livKHMGF* gene cluster and *livJ* gene (29). The systems are composed of periplasmic BCAA-binding proteins (LivJ and LivK), hydrophobic transmembrane proteins (LivH and LivM), and cytoplasmic ATP-binding components (LivG and LivF). The active transport of BCAAs is driven by the energy from hydrolysis of ATP (29). Genome analysis predicted that *K. europaeus* lacked some orthologs (LivJ, LivK, LivH, and LivM) of the proteins for BCAA transport reported in *E. coli* (26). Abnormal BCAA auxotrophy of the strain KGMA5511 might be attributed to the lack of essential components for BCAA transport systems. Such a characteristic of this strain would be a disadvantage for the production of attractants, particularly at a larger scale. Instead of *ilvC* disruption, however, the enhanced expression of the *aldC* gene (which encodes α-acetolactate decarboxylase) (Fig. 1) would be more practical for the efficient production of acetoin. Shuttle plasmid vectors between *E. coli* and *K. europaeus* were developed recently (30), and trials to overexpress *aldC* are in progress.

Lrp is a global transcriptional regulator that controls the expression of approximately 10% of all genes in *E. coli*, and the Lrp regulon coordinates various cellular processes such as the metabolism and transport of amino acids (31, 32). The binding of leucine to its C-terminal region modulates the activity of Lrp on target genes (31, 32). *Ke*Lrp is an Lrp ortholog in *K. europaeus* and was predicted to regulate the expression of numerous genes and control BCAA biosynthesis and export (19). Strain KGMA7203 expressed a mutated *Ke*Lrp with the C-terminal ligand-binding region deleted (19). Thus, it was anticipated that the *Ke*Lrp variant would be insensitive to a signal molecule and that a number of downstream metabolisms would be significantly affected in KGMA7203. As a result, strain KGMA7203 might accumulate unidentified metabolites attractive to fruit flies as well as acetoin. Cha et al. reported that methionol increased fly response to a mixture of acetic acid and ethanol (7). Further analyses are needed to identify the other attractants in the KGMA7203 culture.

The numbers of captured flies in the experiments conducted in the afternoon (Fig. 6A and C) appeared to be lower than those in the experiment conducted in the morning (Fig. 6B). It has been well known that endogenous circadian clocks control rhythmic phenomena, such as locomotor activity, physiology, and metabolisms in *D. melanogaster* (33). The clocks can synchronize these phenomena to the environmental cycles through *Zeitgebers* ("time giver") such as light intensity and temperature (34). *D. melanogaster* generally shows peaks of locomotor activity at dawn and dusk, and the activity falls down to its minimum at several hours before dusk (35). The fluctuation and decrease in captured flies in the afternoon experiments might be due to the lower locomotor activity of flies caused by the circadian clocks. Similarly, the numbers of captured flies in the second trapping experiments (Fig. 7) were smaller than those in the first trapping experiments (Fig. 6). Since *D. melanogaster* is an ectotherm, its locomotor activity

would be significantly affected by the slight change in temperature (as little as 3°C) (34, 36, 37). The lower efficiency in fly attraction in the second experiments could be attributed to the temperature being lower (25 to 26°C) (Fig. 7) than that of the first experiments (28 to 30°C) (Fig. 6). Temperature also influences the release rate of attractants. Overall, however, the supernatant of KGMA7203 exerted the highest attractiveness to fruit flies in all the experiments that we conducted, emphasizing the advantage of strain KGMA7203 in fruit fly attraction.

For effective fly trapping, several factors such as the concentration, blending ratio, release rate of attractants, and trap device shape (5, 38) should be optimized. Additionally, the raw materials and bacterial strains used for vinegar fermentation are important to enhance attractiveness. From our observations, the odor of vinegar varies significantly depending on the raw materials and bacterial strains used in its production. In the United States, apple cider vinegar has been recommended as a desirable attractant to fruit flies (1). According to a report of Landolt et al. (8), rice vinegar is also preferable for the effective trapping of flies. Our results indicate that KGMA7203 is a suitable strain for developing effective flytraps with boosted attractiveness, and we hypothesize that the vinegars fermented by the strain are expected to be useful to attract fruit flies, including the SWD. We are now investigating the trapping efficiency of the KGMA7203 supernatant to various fruit flies besides *D. melanogaster*.

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