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Metabolomics-based profiling of three terminal alkene-producing *Jeotgalicoccus* spp. during different growth phase

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Production of terminal alkenes by microbes has gained importance due to its role as a chemical feedstock in commercial industries. *Jeotgalicoccus* species has been widely unexplored despite being well-known as a natural producer of terminal alkene, catalyzing the one-step fatty acid decarboxylation reaction by OleT$_{JE}$ cytochrome P450. In this study, widely targeted ion-pair LC-MS/MS was used to monitor central carbon metabolism of *Jeotgalicoccus halotolerans* JCM 5429, *Jeotgalicoccus huakuii* JCM 8176, and *Jeotgalicoccus psychrophilus* JCM 5429 at logarithmic and stationary phases. Growth and production profile of terminal alkene, alcohols and organic acids were also measured. Among the three strains used in this study, *J. halotolerans* and *J. psychrophilus* showed higher terminal alkene production compared to *J. huakuii*. All strains achieved maximum terminal alkene production at logarithmic phase and therefore, detailed analysis of the metabolite profiles of the three strains were performed in logarithmic phase. PCA analysis showed that the strains were discriminated based on their ability to produce terminal alkene along PC1 and some of the important metabolites corresponding to this separation is the acetyl-CoA and 2-oxoglutarate. This study is the first report on metabolite profiling of three *Jeotgalicoccus* spp. in different growth phases. The results from this study can provide a better understanding of the changes that occur in the metabolome level during growth and production of terminal alkene in *Jeotgalicoccus* species.

[**Key words:** *Jeotgalicoccus*; Terminal alkene; Metabolomics; Liquid chromatography; Mass spectrometry]

Terminal alkenes (1-alkenes) are defined by the carbon-to-carbon double bond at the end of the carbon chain, and this bond is responsible for the characteristic properties of these compounds (1). Demand for terminal alkenes has raised due to their role in commercial industries (2,3). Terminal alkenes have been widely used as lubricating fluids, detergents, and other industrial products (4). Industrial terminal alkene is currently produced only by chemical synthesis such as by Hofmann elimination, ester pyrolysis, and Tebbe olefination (5–7). There is a demand for an alternative and environmentally-friendly production source for terminal alkenes that is more sustainable. Some microorganisms are capable of synthesizing alkenes naturally via various pathways (8,9). *Jeotgalicoccus* genus has been reported to be able to naturally produce terminal alkene by using a unique cytochrome P450 fatty acid decarboxylase enzyme named OleT$_{JE}$, which directly decarboxylates free fatty acids into terminal alkenes (2,10). *Jeotgalicoccus* spp. are non-pathogenic gram-positive, non-spore forming, and non-motile bacteria (11–13). To date, scientific reports on terminal alkene production only focus on the study of *Jeotgalicoccus halotolerans* JCM 5429 and *J. psychrophilus* JCM 5429 as model species for terminal alkene research. Previous reports on this genus were valuable to serve as the basis of our experimental design and therefore, we selected these two strains and one additional strain with close phylogenetic relationship *Jeotgalicoccus huakuii* JCM 8176 in our study. Current studies on terminal alkene-producing *Jeotgalicoccus* have been focused on OleT$_{JE}$ enzyme mechanism (14,15) and on use of this cytochrome P450 fatty acid decarboxylase for developing industrially relevant strains of *Escherichia coli* and *Saccharomyces cerevisiae* for production of terminal alkene (2,16). Metabolomics is an emerging omics field that can be used to obtain information on cellular metabolites derived from the central carbon metabolism, and can be directly used to describe biochemical networks reflecting cell physiology (17–20). Through metabolic profiling approach, changes in metabolites can be monitored during different growth phase and the obtained information can aid in the development of the strain.

In this study, we demonstrate the utility of a widely targeted metabolome analysis approach for profiling central carbon metabolism at logarithmic and stationary phase of three *Jeotgalicoccus* spp., namely *J. halotolerans*, *J. huakuii*, and *J. psychrophilus*, to reveal metabolites that have high correlation with terminal alkene production. This work is the first report of metabolomics approach for the study of terminal alkene-producing *Jeotgalicoccus* spp. at different growth phase.

MATERIALS AND METHODS

Bacterial strains and reagents *J. halotolerans* JCM 5429, *J. huakuii* JCM 8176, and *J. psychrophilus* JCM 5429 (RIKEN, Japan) were used for characterization of growth and terminal alkene production. Acetic acid, pyruvic acid, succinic acid,

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formic acid, lactate, ethanol, and chloroform were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Medium and growth conditions Marine broth (MB) (19.45 g NaCl, 5.9 g $MgCl_2$, 3.24 g $MgSO_4$, 1.8 g CaCl, 0.55 g KCl, 0.16 $NaHCO_3$, 0.1 g $C_6H_5FeO_7$, 0.08 g KBr, 34 mg $SrCl_2$, 22 mg H_3BO_3, 8 mg Na_2HPO_4, 4 mg Na_2SiO_3, 2.4 mg NaF per liter of ultrapure water) supplemented with 1 % glucose was used. Pre-culture was grown in MB with 1 % glucose at 30 °C, aerobically, overnight until mid-log phase. Pre-culture was transferred to 20 mL of new MB medium-1 % glucose with initial OD_{600} of 0.1 in 250 mL rubber cap flask at 30 °C and 200 rpm under aerobic conditions.

Fast filtration and extraction of intracellular metabolites For intracellular metabolome analysis, sampling by fast filtration method was conducted. Five OD_{600} units of cells were collected at logarithmic phase (18 h) and stationary phase (48 h) with a 0.45 μm pore size, 25 mm diameter nylon membrane filter (Millipore, MA, USA). The cells were quenched in liquid nitrogen to stop the metabolism immediately and stored at −80 °C until extraction. Extraction was performed using 3 mL of extraction solvent (methanol:ultra-pure water:chloroform; 5:2:2 v/v/v of ratio) containing 20 μg/L of (+)-10 camphorsulfonic acid as internal standard. Thawing incubation was performed at −80 °C for 1 h and then −30 °C for 30 min (three cycles). After thawing, 1 mL of supernatant was transferred to a new tube and mixed with 200 μL of ultra-pure water. Samples were centrifuged at 10,000 × *g* for 10 min at 4°C and 950 μL of the upper polar phase was collected after filtration (0.2 μm PTFE hydrophilic membrane, Millipore). The sample was concentrated using a spin dryer for 3 h and freeze-dried overnight. Lyophilized sample was dissolved in 50 μL ultra-pure water and centrifuged at 10,000 × *g* for 10 min at 4°C. For each sample, 40 μL was transferred to a glass vial for LC-MS/MS analysis.

Analysis of intracellular metabolites by LC-MS/MS Widely targeted analysis of intracellular metabolites was carried out using ion-pair liquid chromatography tandem mass spectrometry (LC-MS/MS). Nexera UHPLC system coupled with LCMS 8030 plus (Shimadzu, Kyoto, Japan) was used. The column from Chemicals Evaluation and Research Institute (CERI, Tokyo, Japan) L-column 2 ODS (150 mm × 2.1 mm, particle size 3 μm) was used. For LC, 10 mM of tributylamine with 15 mM acetate was used as mobile phase A and methanol was used as mobile phase B. Flow rate was 0.2 mL/min and the oven temperature was maintained at 45 °C. Concentration of mobile phase B was kept at 0 % for 1 min and then increased at a gradient of 10 %/min until 15 % and held for 3 min, 7 %/min until 50 %, 5 %/min until it reached 100%, then held for 1 min and decreased to 0 % in 30 s. The sample injection volume was 3 μL, position of probe was +1.5 nm with temperature of desolvation line (DL) 250 °C, heat block was 40 °C, drying gas flow rate was 15 L/min, and nebulizer gas flow rate at 2 L/min. Intracellular metabolites were ionized with negative ion mode (19,31). Abf file converter (Reifycs Inc., Tokyo, Japan) was used to convert raw data from LC-MS/MS to abf file and analyzed by MRMPROBS (21).

Multivariate analysis SIMCA-P+ version 13 (Umetrics, Umeå, Sweden) was used for principal component analysis (PCA). The metabolome data was normalized by internal standard. The data was mean centered and scaled to unit variance.

Quantification of extracellular metabolites by gas chromatography-flame ionization detector and ultra fast liquid chromatography-photo diode array The supernatant of the culture medium was collected at 18 h and 48 h. The collected supernatant was centrifuged at 16,000 × *g* for 5 min at 4°C and filtered using 0.2 μm pore size filter (Millipore). The alcohols were quantified using a GC-2010 system (Shimadzu), with a GL Science (Tokyo, Japan) InertCap Pure-WAX capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness), coupled with a flame ionization detector and an AOC-20i/s auto-injector (Shimadzu). The injection volume was 0.5 μL in 1:15 ratio of split mode. The temperature of injector was maintained at 225 °C. The initial temperature of the column was 50 °C for 1 min and then raised with gradient of 7 °C/min until 80 °C and was held for 1 min. Afterwards it was raised with a gradient of 20 °C/min until 120 °C, held for 2 min and then finally raised with a gradient of 50 °C/min until 250 °C and held for 5 min. Nitrogen gas was used as carrier gas with the flow rate 2.09 mL/min. Organic acids were measured using a Prominence ultra fast liquid chromatography-photo diode array (UFLC) system (Shimadzu) with a photo diode array detector SPD-M20A (Shimadzu) and Aminex HPX-87H column (300 mm × 7.8 mm, particle size 9 μm) from Bio-Rad (Hercules, CA, USA). The column temperature was maintained at 50 °C and 10 mM H_2SO_4 used as the mobile phase with 0.3 mL/min of flow rate. For quantification the 210 nm wavelength was used. Concentration of alcohols and organic acids in the samples were determined by extrapolation from standard curves.

Quantification of terminal alkene Cell pellets (11 Log CFU/mL) were collected after centrifuging at 16,000 × *g* for 5 min at 4°C. Terminal alkenes were extracted by suspending cell pellets with 1 mL of methanol (Kanto chemical, Japan) and sonicated for 30 min. After sonication, 4 mL of hexane (Wako, Japan) was added, the suspension was vortexed and centrifuged at 16,000 × *g* for 5 min at 4°C. Then, 1 mL of upper phase was transferred to a new tube and the sample was concentrated using a spin dryer for 30 min. The terminal alkenes extracted were resuspended in 0.1 mL of hexane. The terminal alkenes were quantified using Shimadzu QP-2010 gas chromatography-mass spectrometry (GC-MS). Column InertCap 5 MS/NP (30 m by 0.25 mm i.d., 0.25 μm film thickness, GL Sciences, USA) was used. Injection volume was 2 μL and analysis was carried out under splitless injection condition, with inlet temperature set to 250 °C. The oven temperature was held at 80 °C for 2 min. Then, the temperature was increased to 330 °C at a rate of 15 °C/min and held for 6 min at 330 °C. The mass spectrometer detector was set to 250 °C and scanned at 30–500 m/*z* in the scan mode. To help peak identification, authentic standard references C10-C19 (Tokyo Chemical Industry, Japan) were used and their retention time (rt) and fragmentation arrangement were used to compare with the extracted alkenes. Concentration of each terminal alkene in the samples were determined by extrapolation from standard curves.

Quantification of free fatty acid Five OD_{600} units of cells were collected at 18 h and 48 h by fast filtration with a 0.45 μm pore size, 25 mm diameter nylon membrane filter (Millipore) and quenched immediately in liquid nitrogen. Extraction solvent (methanol:chloroform; 2:1 v/v of ratio) containing 10 μmol/L of decanoic acid (C10:0) as internal standard. Thawing was performed at room temperature for 15 min. In the samples, 100 μL of ultra-pure water was added and incubated for 15 min. Samples were centrifuged at 10,000 × *g* for 10 min at room temperature and 500 μL of the supernatant was dried up by nitrogen gas. The samples were reconstructed with 100 μL of methanol. Relative quantification of free fatty acids was conducted using liquid chromatography tandem mass spectrometry (LC-MS/MS). Nexera UHPLC system coupled with LCMS 8050 (Shimadzu) was performed. The column from GL Sciences InertSustain C18 (50 mm × 2.1 mm, 3) was used. In liquid chromatography, mobile phase A (ultra-pure water:acetonitrile:ammonium acetate; 40:60:0.1 v/v/w) and mobile phase B (methanol:2-propanol:ammonium acetate; 10:90:0.1 v/v/w) were used. Flow rate was 0.4 mL/min and the oven temperature was managed at 50 °C. Gradually, concentration of mobile phase B was kept at 0 % and then increased at a gradient of 10 %/min until it reached 100%, then held for 1 min and decreased to 0 % in 30 s. The sample injection volume was 2 μL and position of probe was +1.5 nm with temperature of DL 250 °C, heat block was 40 °C, drying gas flow rate was 10 L/min, and nebulizer gas flow rate at 3 L/min.

Glucose measurement The remaining glucose in the culture medium was measured using GL Science GL-7400 high performance liquid chromatography system coupled with a column oven GL-7432 and refractive index detector GL-7454 (HPLC-RI). The column Shim-pack SPR-Pb (250 mm length and i.d., 7.8 mm (Shimadzu) was used. The supernatant of the culture medium was centrifuged at 10,000 × *g* for 5 min at 4°C. Three hundred microliter sample was transferred to Whatman syringeless LC vial (Merck). Sample injection volume was 10 μL and the oven temperature was set at 80 °C. Ultrapure water was used as mobile phase with 0.6 mL/min flow rate and 189 psi of back pressure.

RESULTS AND DISCUSSION

Growth profile of *Jeotgalicoccus* spp. on marine broth medium We tested the growth of three *Jeotgalicoccus* spp. in marine broth (MB) medium with supplementation of 1 % glucose as the carbon source and, with 2 % NaCl for optimum growth. *J. halotolerans*, *J. huakuii* and *J. psychrophilus* are halotolerant and moderately halophilic. The three species are able to grow under salinity as high as 20 %, with optimum growth at 2–5 % salinity (11–13). In order to monitor the metabolome of a particular microbe, it is necessary to determine appropriate sampling time to reflect the physiology of microbial cells in a given time for growth phases (17,22,23). In this study, measurement of growth curve of *Jeotgalicoccus* spp. was performed. Based on the growth curve, two time points corresponding to logarithmic (18 h) and stationary phase (48 h), were chosen for analysis of extracellular and intracellular metabolites. Fig. 1A shows glucose consumption and growth profile for the three *Jeotgalicoccus* species. Glucose consumption curve showed that glucose was consumed in lag and logarithmic phase of growth and stopped during the stationary phase. At the end of the cultivation, there was about 5 g/L of glucose remaining in the culture medium. Fig. 1B shows the pH changes during cultivation in the MB medium, indicating production of organic acid(s) by *Jeotgalicoccus* spp. during cultivation. Fig. 1C shows the terminal alkene titer of all *Jeotgalicoccus* spp. and shows highest titer at 18 h during cultivation. In addition, terminal alkene production reached a plateau at 48 h for all species. From this point forward, we selected these two sampling points for comparison of intracellular and extracellular data.

Terminal alkene production in *Jeotgalicoccus* spp. The *Jeotgalicoccus* genus has been reported as the native terminal alkene producer (10). Biosynthesis of terminal alkene is carried out by decarboxylation of free fatty acids (14,24,25). Enzyme $OleT_{JE}$ is a

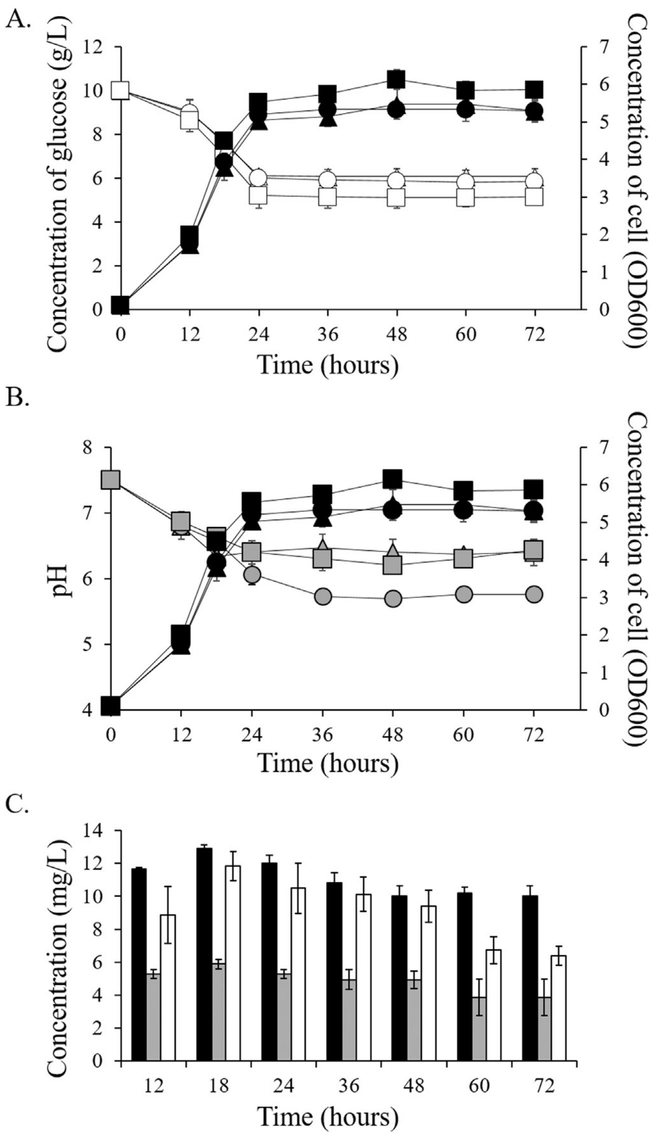

FIG. 1. (A) Cell growth profile of *Jeotgalicoccus* spp. (closed symbols) and glucose consumption profile (open symbols). (B) pH changes during cultivation (shaded symbols) and cell growth (closed symbols) (0, 12, 18, 24, 48, 60, 72 h) of *J. halotolerans* (triangles), *J. huakuii* (circles), and *J. psychrophilus* (squares). (C) Terminal alkene titer of *J. halotolerans* (closed bars), *J. huakuii* (shaded bars), and *J. psychrophilus* (open bars). Results are the average of three biological replicates with error bars showing the standard deviation from the mean value.

P450 cytochrome belonging to the cyp152 family of peroxygenases. OleT_{JE} catalyzes the one-step fatty acid decarboxylation reaction for terminal alkene production (Fig. 2A). Terminal alkene titer of *J. halotolerans*, *J. huakuii* and *J. psychrophilus* cultivated in MB medium was between 6 mg/L to 13 mg/L depending on the species and growth phase. Quantification of intracellular terminal alkene was performed by GC/MS analysis and the results showed that all three species produced three terminal alkenes namely 1-nonadecene (C_{19}), 1-heptadecene (C_{17}) and 1-pentadecene (C_{15}) (Fig. 2B, Table 1). *J. huakuii* produced less terminal alkene than the other two strains, suggesting difference in production ability of different *Jeotgalicoccus* spp. Total terminal alkene titer at logarithmic phase (18 h) was 12.90, 5.87, and 11.82 mg/L for *J. halotolerans*, *J. huakuii*, and *J. psychrophilus*, respectively. At stationary phase (48 h), 10.02, 5.53, and 9.39 mg/L of terminal alkene titer was produced by *J. halotolerans*, *J. huakuii*, and *J. psychrophilus*, respectively. 1-Nonadecene (C_{19}) was the major terminal alkene produced by all three species. Comparison of terminal alkene titer showed that there was a decrease in terminal alkene concentration in stationary phase compared to logarithmic phase, suggesting that the cells achieved maximum terminal alkene titer during logarithmic phase.

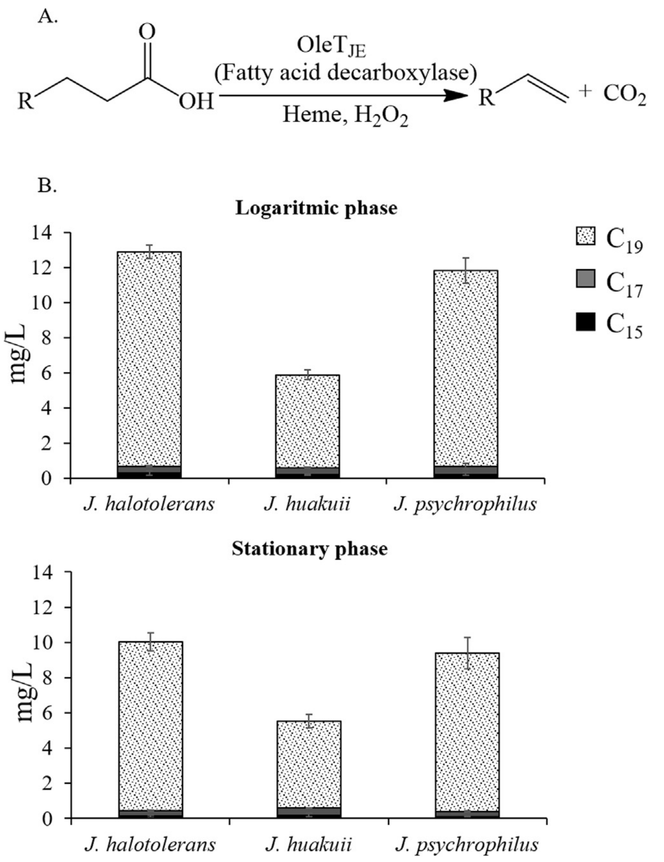

FIG. 2. (A) Synthesis of terminal alkene by OleT_{JE} through fatty acid decarboxylation. (B) Distributions of different chain length alkenes produced by *Jeotgalicoccus* spp. at logarithmic phase (18 h) and stationary phase (48 h). Alkenes with different chain lengths (C_{15}, C_{17}, and C_{19}) are represented with different colors. Results are the average of three biological replicates with error bars showing the standard deviation from the mean value.

Intracellular metabolic profiling of *Jeotgalicoccus* spp. during different growth phases Metabolic profiling during the logarithmic and stationary phase were carried out with widely-targeted ion-pair LC-MS/MS. Eighty five metabolites belonging to amino acids, sugar phosphates, sugars, nucleosides, nucleoside phosphates, organic acids, co-factors, and CoA-compounds were annotated in *J. halotolerans*, *J. huakuii* and *J. psychrophilus*. Relative intensity of each metabolite was obtained by normalization with an internal standard, (+)-10 camphorsulfonic acid. (+)-10 camphorsulfonic acid was used as an internal standard as it is not present in any biological samples and is stable in the extraction solvent (26,27). Principal component analysis (PCA) of the LC-MS/MS dataset showed that two principle component (PC) described 78.9 % of the total variance at logarithmic (Fig. 3) and 78.2 % of the total variance at stationary phase (Fig. S1). The PCA score plot showed the same trend in both logarithmic and stationary phase.

Considering that growth, glucose consumption as well as terminal alkene production are most active during the logarithmic phase, monitoring the changes in metabolite concentration during the logarithmic phase may best reflect the physiological state of the microbial cells during growth and production of terminal alkene. As such, detailed analysis of PCA was conducted for logarithmic phase.

TABLE 1. Terminal alkene titer (mg/L).

Terminal alkene	*J. halotolerans*		*J. huakuii*		*J. psychrophilus*	
	Phase		Phase		Phase	
	Logarithmic	Stationary	Logarithmic	Stationary	Logarithmic	Stationary
Pentadecene (C_{15})	0.29 ± 0.12	0.13 ± 0.06	0.20 ± 0.04	0.20 ± 0.10	0.21 ± 0.07	0.10 ± 0.03
Heptadecene (C_{17})	0.38 ± 0.02	0.30 ± 0.05	0.39 ± 0.03	0.39 ± 0.03	0.45 ± 0.18	0.28 ± 0.03
Nonadecene (C_{19})	12.21 ± 0.37	9.57 ± 0.50	5.27 ± 0.26	4.93 ± 0.39	11.15 ± 0.71	8.99 ± 0.89

Data represent mean ± S.D. of *Jeotgalicoccus* spp. of three separate biological samples.

The results showed that *J. huakuii* with the other 2 species were separated along PC 1, accounting for 44.2% of the total variance (Fig. 3). This results might be related to terminal alkene production phenotype of the three strains. *J. halotolerans* and *J. psychrophilus* have similar production of terminal alkene which is notably different with *J. huakuii*. Acetyl-CoA and pyruvate contributed negatively towards the separation by PC1. On the other hand, 2-oxoglutarate (2OG) and malonyl-CoA contributed positively towards the separation by PC1 (Fig. 3). This result might suggest that acetyl-CoA and malonyl-CoA are some of the important precursors as they are the starting metabolites for production of free fatty acid, which is converted to terminal alkene. Acetyl-CoA accumulated more in *J. huakuii* as compared to other strains. However, malonyl-CoA and free fatty acid relative intensity was comparable to that in *J. halotolerans* and *J. Psychrophilus* (Fig. 4). Low terminal alkene production in *J. huakuii* could be due to slower conversion from fatty acids to terminal alkene in this species. Furthermore, it is possible that excess acetyl-CoA was converted to citrate or pyruvate preferably than to malonyl-CoA. The results of PC2 accounting for 34.7 % of total variance separated *J. psychrophilus* with other two species. The separation in PC2 may be correlated with growth as *J. psychrophilus* showed higher specific growth rate (Table S2) compared to the other two species. In the loading plot UTP and CTP were revealed as positively correlating metabolites, whereas GMP and UMP were negatively correlating metabolites (Fig. 3). This result might indicate that pyrimidine biosynthesis was regulated by the intracellular concentration of UTP, CTP, GMP and UMP (28–30). In several gram-positive bacteria, UTP is produced from UMP in the pathway of pyrimidine biosynthesis and is then further used for biomass production. Based on these pyrimidine biosynthesis metabolites, it can be suggested that separation by PC2 might be correlated with growth profiles of the three species, specifically higher specific growth rate of *J. psychrophilus* (Fig. 1, Table S2).

Monitoring central metabolism of *Jeotgalicoccus* spp. The intracellular level of central metabolites of *Jeotgalicoccus* spp. were measured at different growth phases of the cultivation. Fig. 1C shows that quantification of terminal alkene production at 18 h achieved highest production for all *Jeotgalicoccus* species. At this time point (logarithmic phase), the cell actively produced terminal alkene compared to at 48 h (stationary phase). We wanted to observe the metabolome during the highest production of terminal alkene (18 h) and during the time when the terminal alkene production reached a plateau (48 h). Such insight may be useful to consider from metabolome perspective on what kind of metabolite changes can be observed when production of terminal alkene reached a halt, to better provide understanding on what pathways may be affected during the time when cells actively produce terminal alkene and when cells reached stationary phase where growth, glucose uptake and terminal alkene production reached a plateau. Quenching was performed to rapidly arrest metabolism by using fast filtration followed by rapid freezing in liquid nitrogen (31). One of the most important parameter to indicate that the quenching process was adequate to rapidly stop the microbial metabolism is to observe the concentration of glucose 6-phosphate (G6P) and fructose 6-phosphate (F6P) (Fig. 4). Based on two glycolytic metabolites (G6P and F6P) suggested that the quenching method for gram positive *Jeotgalicoccus* spp. used in this study was suitable for our purpose (32,33). The result also suggested glucose 6-phosphate (G6P) relative intensity was similar among the three species at both growth phases, however, a steady increase was observed at stationary phase. The decreasing concentration of fructose 1,6-phosphate (F1,6P) in all species from logarithmic to stationary phase indicates that the glucose was converted to F1,6P via the glycolysis pathway (33) (Fig. 4).

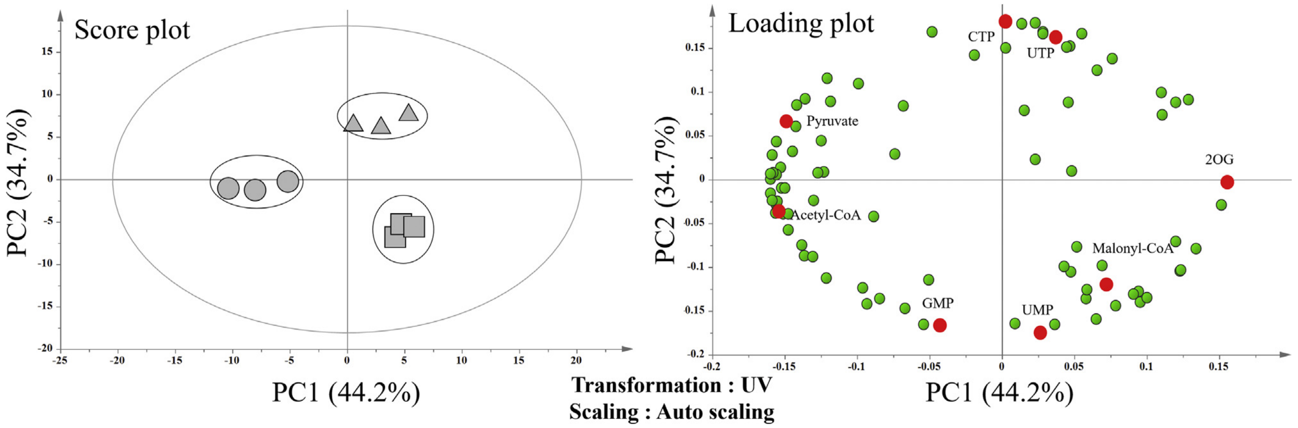

FIG. 3. PCA score plot and loading plot for metabolic profiling of *J. halotolerans* (triangles), *J. huakuii* (circles), and *J. psychrophilus* (squares) at logarithmic phase (18 h). The ellipse demonstrate the 95% confidence border on Hotelling's T^2.

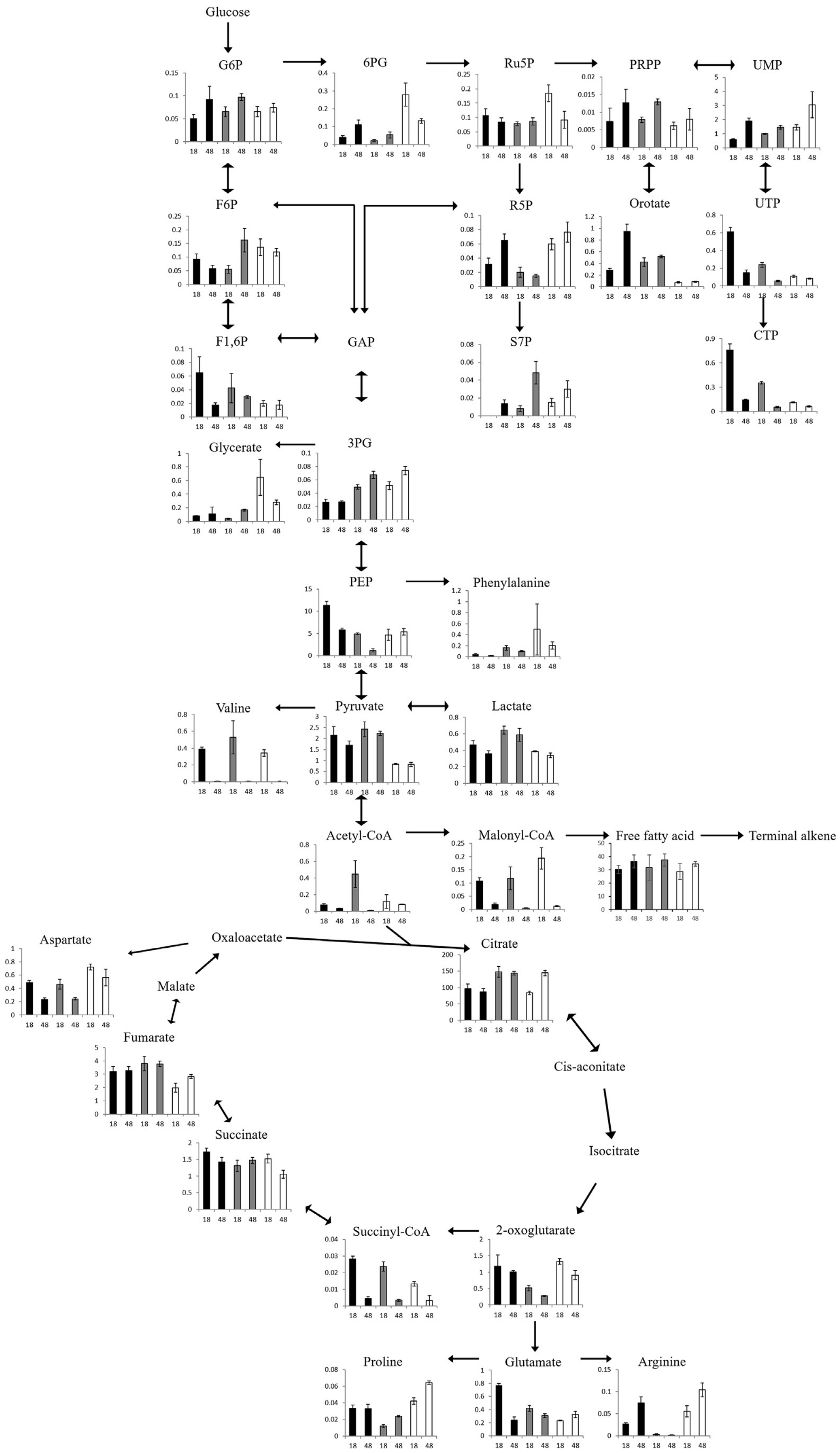

FIG. 4. Relative intensity of carbon central metabolism (glycolysis, EMP, and tricarboxylic acid pathway metabolites). Data obtained from the samples taken at logarithmic phase (18 h) and stationary phase (48 h). Closed bars, *J. halotolerans*; shaded bars, *J. huakuii*; and open bars, *J. psychrophilus*. Results are the average of three biological replicates with error bars showing the standard deviation from the mean value.

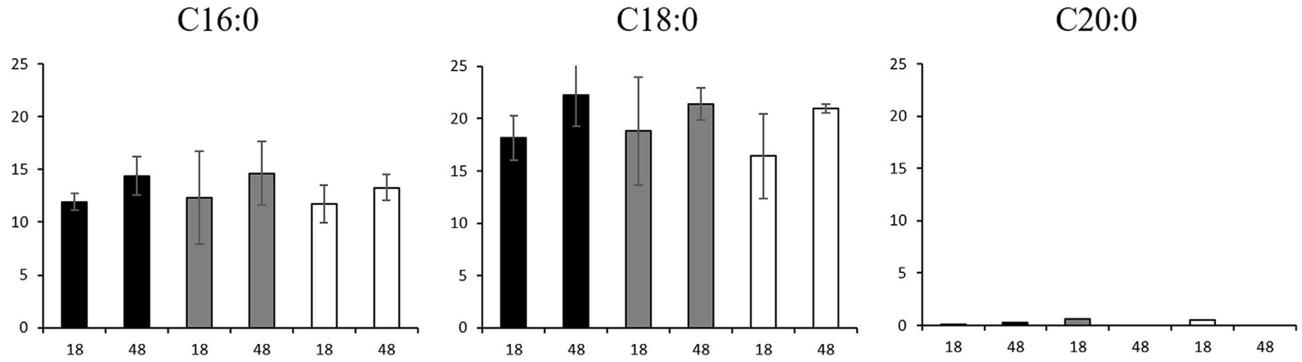

FIG. 5. Relative intensity distributions of different chain length free fatty acids produced by *Jeotgalicoccus* spp. at logarithmic phase (18 h) and stationary phase (48 h). Fatty acids with different chain lengths (C16:0, C18:0, and C20:0) are represented with different colors. Closed bars, *J. halotolerans*; shaded bars, *J. huakuii*; and open bars, *J. psychrophilus*. Results are the average of three biological replicates with error bars showing the standard deviation from the mean value.

Relative intensity of ribulose 5-phosphate (Ru5P), ribose 5-phosphate (R5P), and sedoheptulose 7-phosphate (S7P) in 3 *Jeotgalicoccus* spp. is shown in Fig. 4. Presence of these metabolites indicates glucose is converted through the pentose phosphate pathway (PPP) linked via glycolysis at F6P and GAP. The metabolite 6-phosphogluconate (6PG) was detected at both time points in all 3 *Jeotgalicoccus* spp., indicating that glucose also entered PPP through Ru5P via G6P of the glycolysis pathway.

The three pivotal intermediates of TCA cycle, i.e., citrate, succinate, and fumarate, were found at higher concentration in the logarithmic and stationary phase (Fig. 4). Concentration of 2-oxoglutarate (2OG) decreased at stationary phase for all *Jeotgalicoccus* spp., possibly due to the direct conversion of 2OG to succinate and glutamate. Glutamate is possibly converted to proline and arginine (amino acids), both of which show general increasing trend from logarithmic to stationary phase. Increasing concentration of these two amino acids may explain growth in *Jeotgalicoccus* spp. as they are channeled into pyrimidine metabolism resulting in biomass production. Specifically, *J. psychrophilus* shows higher intensity of proline and arginine which might have contributed to higher growth, both at logarithmic and stationary phases as compared to other two species (28).

Intracellular relative quantification of free fatty acids (C20:0, C18:0, and C16:0) was performed and the results showed that eicosanoic acid (C20:0) intensity is low compared with two other fatty acids (Fig. 5). Lower intensity of eicosanoic acid may explain terminal alkene titer of 1-nonadecene was the major terminal alkene produced by all three species (Fig. 2B). OleT_{JE} catalyzes the free fatty acid decarboxylation reaction for terminal alkene production that eicosanoic acid was mainly converted to 1-nonadecene (10).

Extracellular metabolite production in *Jeotgalicoccus* spp. To provide a comprehensive overview of the metabolism of *Jeotgalicoccus* spp., measurement of extracellular metabolites was also performed to reveal the correlation between intracellular metabolomics data and its by-products (organic acid and alcohol productions). The annotated intracellular metabolites showed that butanoyl-CoA was present in high relative intensity in *J. huakuii* and *J. psychrophilus* but not in *J. halotolerans* (Table S1). Extracellular 1-butanol and butanoate titer were measured by gas chromatography-flame ionization detector (GC-FID) and liquid chromatography-photo diode array (LC-PDA), respectively. *J. psychrophilus* produced 21.13 and 22.07 mg/L of 1-butanol at logarithmic and stationary phases, respectively (Table 2). This result may suggest that *J. psychrophilus* is one of the bacteria that is capable of producing 1-butanol. To gain insight into the pyruvate distribution, within the cell, to various metabolites such as lactate and acetate, organic acids that are involved in the central carbon metabolism were measured using LC-PDA. Lactate in the extracellular medium increased during stationary phase in *Jeotgalicoccus* spp. (Table 2). The accumulation of lactate during growth suggests that pyruvate was channeled into lactate (19). High acetate accumulation in the extracellular sample was also observed (Table 2). Considering that both acetate and terminal alkene use acetyl-CoA as an important precursor, acetate synthesis may be a competing reaction for terminal alkene production. Identification of major by-products in *Jeotgalicoccus* spp. may be useful for further engineering of this species for higher terminal alkene production.

Finally, we suggest plausibility of 1-nonadecene (C_{19}) as the major terminal alkene produced by all three species. Our results show that cells achieved maximum terminal alkene production during logarithmic phase. We monitored metabolic changes between logarithmic and stationary phase in an attempt to reveal the central metabolites of terminal alkene-producing *Jeotgalicoccus* spp. that correlate with different growth phase by using widely-targeted metabolome analysis. We suggested new insights on important metabolite changes for terminal alkene production

TABLE 2. Extracellular metabolites (mg/L).

Metabolite	*J. halotolerans*		*J. huakuii*		*J. psychrophilus*	
	Phase		Phase		Phase	
	Logarithmic	Stationary	Logarithmic	Stationary	Logarithmic	Stationary
Succinate	353.02 ± 55.41	377.26 ± 64.07	438.35 ± 18.19	446.13 ± 20.93	201.44 ± 87.19	334.97 ± 82.05
Lactate	157.67 ± 23.79	172.65 ± 29.95	203.67 ± 13.79	210.44 ± 6.68	143.91 ± 40.45	146.56 ± 28.34
Acetate	995.11 ± 159.32	1011.66 ± 173.63	1018.25 ± 50.46	963.47 ± 29.35	569.65 ± 188.46	636.76 ± 95.51
Butanoate	N/D	N/D	193.72 ± 23.64	239.51 ± 14.18	211.29 ± 18.65	289.95 ± 10.29
Ethanol	N/D	N/D	N/D	N/D	26.71 ± 0.50	28.62 ± 0.69
1-Butanol	N/D	N/D	N/D	N/D	21.13 ± 0.39	22.07 ± 0.98

Data represent mean ± S.D. of *Jeotgalicoccus* spp. of three separate biological samples. N/D, not detected.

during growth phase. These results can provide a better understanding of terminal alkene production by *Jeotgalicoccus* spp. at the metabolome level, allowing for the further strain development.

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