HOKKAIDO UNIVERSITY

Title	Identification of cheese rancidity-related lipases in Aspergillus oryzae AHU 7139
Author(s)	Chintagavongse, Napaporn; Kumura, Haruto; Hayakawa, Toru et al.
Citation	Journal of bioscience and bioengineering, 137(5), 381-387 https://doi.org/10.1016/j.jbiosc.2024.01.016
Issue Date	2024-03-01
Doc URL	https://hdl.handle.net/2115/92618
Rights	© <2024>. This manuscript version is made available under the CC-BY-NC-ND 4.0 license http://creativecommons.org/licenses/by-nc-nd/4.0/
Rights(URL)	https://creativecommons.org/licenses/by-nc-nd/4.0/
Type	journal article
File Information	JBB R2-marked u.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Identification of cheese rancidity-related lipases in *Aspergillus oryzae* AHU 7139

A short title: Identification of cheese rancidity-related lipases

Napaporn Chintagavongse[1], Haruto Kumura[1,*], Toru Hayakawa[1], Jun-ichi Wakamatsu[1], Koichi Tamano[2]

[1]*Laboratory of Applied Food Science, Graduate School and Research Faculty of Agriculture, Hokkaido University, 060-8589, N9, W9, Sapporo, Japan;*[2]*Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), 2-17-2-1 Tsukisamu-Higashi, Toyohira-ku, Sapporo, Hokkaido 062-8517, Japan*

* Corresponding author: Haruto Kumura

Mailing address: Laboratory of Applied Food Science, Graduate School and Research Faculty of Agriculture, Hokkaido University, 060-8589, N9, W9, Sapporo, Japan

Tel: +81-11-706-3642, E-mail: kumura@agr.hokudai.ac.jp

ABSTRACT

The adjunct product with enzymatic activity from *Aspergillus oryzae* is beneficial for flavor enrichment in the ripened cheese. However, an excessive lipolytic reaction leads to the release of volatile free fatty acids. Accordingly, a strong off-flavor (i.e., rancidity) has been detected when *A. oryzae* AHU 7139 is used. To identify the rancidity-related lipase from this strain, we evaluated the substrate specificity and lipase distribution using five mutants cultured on a whey-based solid medium under different initial pH conditions. The results showed a higher diacylglycerol lipase activity than triacylglycerol lipase activity. Moreover, an initial pH of 6.5 for the culture resulted in higher lipolytic activity than a pH of 4.0, and most of the activity was found in the extracellular fraction. Based on the gene expression analysis by RT-PCR and location and substrate specificity, five genes (No. 1, No. 19, *mdlB*, *tglA*, and *cutL*) were selected among 25 annotated lipase genes to identify the respective knockout strains. Because Δ*tglA* and Δ*mdlB* showed an outstanding involvement in the release of free fatty acids, these strains were applied to *in vitro* cheese curd experiments. In conclusion, we posit that triacylglycerol lipase (TglA) plays a key role as the trigger of rancidity and the resulting diglycerides have to be exposed to diacylglycerol lipase (MdlB) to stimulate rancidity in cheese made with *A. oryzae* AHU 7139. This finding could help screen suitable *A.oryzae* strains as cheese adjuncts to prevent the generation of the rancid-off flavor.

KEYWORDS: *Aspergillus oryzae*, lipase, substrate, free fatty acids, rancidity, *in vitro* curds

INTRODUCTION

The filamentous fungus *Aspergillus oryzae* is used in traditional Japanese cuisine (washoku). *A. oryzae* is recognized as safe because it is missing the aflatoxin synthesis gene (1) and is assumed to encode as much as 135 protease genes based on its genomic analysis (2).

In contrast to washoku, *A. oryzae* is rarely used in Western foods. Kumura et al. (3) focused on the high proteolytic potential of *A. oryzae* and attempted to apply its culture products (CP) as adjuncts for cheese flavor enrichment. As solid-state fermentation is known to be superior to liquid fermentation for abundant enzyme production (4), Kumura et al. (3) prepared solid CP of *A. oryzae* AHU 7139 as adjuncts for making Gouda-type cheese and confirmed the enhancement of proteolysis during cheese ripening. Moreover, a remarkable content of free fatty acid (FFA) due to lipolysis was detected. They reported that the application of CPs whose fermentation had started with a pH of 6.5 resulted in a pronounced increase in FFA compared to the fermentation that started with a pH of 4.0, implying the initial pH of the culture may affect the profile of the lipases produced.

Although proper lipolysis provides the characteristic flavor and aroma to cheese, excess FFA accumulation during cheese ripening causes an undesirable off-flavor known as rancidity (5) by the release of volatile short-chain fatty acids over an organoleptic threshold. Furthermore, once milk triacylglycerols are degraded by lipases (the first step of lipolysis), the resulting diglycerides are exposed and become the subsequent substrates for diacylglycerol lipase (the second step of lipolysis). Thus, the adjunct materials of *A. oryzae* should be prepared with high proteolytic but limited lipase activity.

According to the genomic analysis of a wild-type strain, about 30 genes are annotated to encode lipase genes (FungiDB database, https://fungidb.org/fungidb/app/). Expression of some lipases is likely to be dependent on the culture conditions, and the identification of the rancid-inducible lipase molecules could provide valuable information to screen suitable *A. oryzae* strains for preventing flavor defects in dairy application.

Hence, we compared lipase gene expression under different pH conditions and selected some candidate lipase genes. Then, the influence of mutations on the lipase activity was evaluated. Finally, we identified the most influential lipase molecules on the rancidity, using *in vitro* cheese curd experiments.

MATERIALS AND METHODS

Strain and culture condition

The strain used in this study was *A. oryzae* AHU 7139 from the culture collection of Hokkaido University and was grown on potato dextrose agar (PDA) (Merck KGaA, Darmstadt, Germany) at 30°C for 7 days. Spore suspensions were prepared by adding 9.0 g L^{-1} sodium chloride (NaCl) solution into the grown culture on PDA and diluted to the concentration of 2.5×10^5 spores mL^{-1}, which was counted by using haemocytometer (NanoEntek, Seoul, Korea). The spore suspension (150 μL) was inoculated on whey solid medium whose pH was adjusted to 4.0 or 6.5, and cultivated at 20°C for 7 days (6).

RNA isolation and RT-PCR

The solid culture product (CP) was recovered and immediately freeze-dried and ground with a mortar and pestle to obtain freeze-dried powder (FDP). On average, 0.27 g FDP was obtained from 1 g CP. The FDP (0.5 mg) was transferred into 5 mL of neutralized phenol-saturated water containing 2 mol L^{-1} NaCl, mixed well, and incubated in a waterbath 55℃ for 10 min. The sample was centrifuged at 10,000 ×g, 4℃ for 15 min and the upper aqueous phase was transferred to a new tube followed by the addition of chloroform:isoamylalcohol (49:1; CIA). After centrifugation at 10,000 ×g, 4℃ for 10 min, the upper phase was transferred to a new tube followed by addition of water-saturated acidic phenol/CIA (1:1) and centrifuged again (this step was repeated two times). Then, the upper phase was poured into a new tube and CIA was added into the tube, followed by centrifugation for 5 min. After transferring the upper phase to a new tube, 0.5 volume of 7.5 mol L^{-1} LiCl (Kanto Chemical, Tokyo,

Japan) was added to the tube. The mixture was incubated at 4℃ overnight, followed by centrifugation at 12,000 ×g, 4℃ for 30 min. The supernatant was removed and the pellet was rinsed with 2.5 mol L^{-1} LiCl and centrifuged twice at 12,000 ×g , 4℃ for 5 min. The air-dried pellet was finally dissolved in formamide to obtain total RNA and stored at -80℃. The RNA concentration was estimated by measuring the absorbance at 260 nm with a NanoDrop spectrophotometer (Thermo scientific, US). The recovery of total RNA was evaluated by electrophoresis using a commercial kit (Dynamarker RNA High for Easy Electrophoresis, Biodynamics Laboratory, Tokyo, Japan) according to the manufacturer's instructions (Fig. S1).

The mRNA was isolated from the total RNA using a commercial kit (Pharmacia Biotech, Stockholm, Sweden) according to the manufacturer's instructions. The mixture containing 50 ng of mRNA sample, random primer (Promega, Wisconsin, US), and dNTP was heated at 65ºC for 5 min and immediately put on ice. Then, the mixture containing 5x RT buffer, RNase inhibitor (Toyobo, Osaka, Japan), and reverse transcriptase (M-MLV, Nippon Gene, Tokyo, Japan) was added and incubated at 37ºC for 60 min according to the manufacturer's instructions. The diluted cDNA sample (0.5 μL) was used as a template and mixed with the designed primers (1.5 μL), nuclease-free water (3 μL), and 5 μL of the PCR mixture solution (GoTaq® DNA polymerase and 5x green reaction buffer, Promega, Wisconsin, US). The PCR amplification involved denaturing at 95ºC for 2 min, followed by 40 cycles at 95ºC for 45 s, annealing at 55ºC for 30 s for all candidate lipase genes, and an extension at 73 ºC for 1 min. A final extension at 73ºC for 10 min was followed by cooling to 4ºC. All the primers used in this study are listed in Table S1. The reliability of all primer sets was checked by PCR using genomic DNA from *A. oryzae* AHU 7139 and RIB40 as the template (data not shown).

Disruption of individual genes in *A. oryzae* AHU 7139

Five candidate genes (AO090012000690, AO090005001319, AO090701000644, AO090003001507, and AO090005000029, named No.1, No. 19, *mdlB*, *tglA*, and *cutL*, respectively) were selected to detect the lipase activity after gene disruption. All the primers used throughout the gene disruption are shown in Table S2, based on the genomic sequence of *A. oryzae* wild-type strain RIB40 (2), used as a reference for designing primers.

Prior to the candidate gene disruption, the *pyrG* gene (AO090011000868) was knocked out in AHU 7139 by homologous recombination under selective pressure by 5-fluoroorotic acid (5-FOA). The *pyrG* knockout was aimed at conferring the uracil auxotrophy to AHU 7139 so as to make the *pyrG* available as a selectable marker. The DNA cassette for *pyrG* knockout was prepared by fusion PCR. Briefly, the promoter and terminator regions of *pyrG* were amplified using LUpyrG/LLpyrG and RUpyrG/RLpyrG primer pairs, respectively. The AHU 7139 chromosomal DNA was used as the template, and the KOD-PLUS DNA polymerase was used for DNA amplification (Toyobo Co. Ltd., Osaka, Japan). The amplified DNA fragments were purified by gel extraction using the Wizard SV Gel and PCR Clean-Up System (Promega Co., Madison, WI, USA) according to the manufacturer's instructions. Two types of purified DNA fragments were then mixed and joined by fusion PCR using a LUpyrG/RLpyrG primer pair and KOD-PLUS (Fig. S2). The resulting 2035 bp-long DNA fragment was purified by gel extraction and then applied to the transformation of AHU 7139. Protoplasts of AHU 7139 were prepared as reported previously (7) and used for the transformation. To generate transformants, Czapek–Dox (CD) minimal agar medium supplemented with 1.2 mol L^{-1} sorbitol, 1 mg mL^{-1} 5-FOA, 5 mmol L^{-1} uridine, and 10 mmol L^{-1} uracil was used. Because the non-homologous end-joining

(NHEJ) activity is originally high in filamentous fungi including *A. oryzae*, about 20 single colonies of transformants were screened. After single-spore isolation of transformants was performed three times using CD agar supplemented with 1 mg mL^{-1} 5-FOA, 5 mmol L^{-1} uridine, and 10 mmol L^{-1} uracil, the *pyrG* knockout was checked by PCR using a LUpyrG/cLpyrG primer pair. Only one positive homokaryon clone of the *pyrG* knockout mutant was acquired and named AHU 7139 [$pyrG^{-}$] (Fig. S3).

Then, five candidate genes were individually disrupted using AHU 7139 [$pyrG^{-}$] as a host. To increase the probability of the locus-specific homologous recombination, the *pyrG* marker-split method was applied to the candidate gene disruption (8). The former and latter parts of *pyrG* were amplified by PCR using PU/PLsplit and PUsplit/PL primer pairs, respectively. The 548 bp-long DNA region overlapped between them. Approximately 1 kb of promoter and terminator of the candidate genes were also amplified by PCR, using LU/LL and RU/RL primer pairs, respectively. After purification of these four types of DNA fragments by gel extraction, both pairs of promoter/*pyrG* (former part) and *pyrG* (latter part)/terminator were concatenated by fusion PCR, using LU/PLsplit and PUsplit/RL primer pairs, respectively. As a result, two types of fusion PCR DNA fragments were prepared as the gene disruption cassettes per candidate gene. The triple crossover between the chromosomal DNA and the two cassettes that occurred in the marker-split method is illustrated in Fig. S4A–E. Protoplasts of AHU 7139 [$pyrG^{-}$] were prepared as mentioned above. The protoplasts were applied to co-transformation with the two types of DNA fragments as gene disruption cassettes. In other words, two types of DNA fragments were simultaneously introduced into the protoplasts for each candidate gene disruption. Consequently, the transformants were generated on CD agar supplemented with 1.2 mol L^{-1} sorbitol, and five single colonies were selected per candidate gene. The selected clones were

178 subjected to single-spore isolation on CD agar at least three times, followed by
179 evaluating the candidate gene disruption by PCR, using cU/cL primer pairs (data not
180 shown).

181 The candidate gene disruption was further confirmed by Southern hybridization (Fig.
182 S5A–E). Aliquots (8 μg) of genomic DNA were digested with each restriction enzyme
183 (*Hinc*II, *Pst*I, *Sph*I, and *Pvu*II), fractionated on 0.6% agarose gel, and transferred onto
184 a Nytran SuPerCharge membrane (GE Healthcare Co., Piscataway, NJ, USA).
185 Hybridization and signal detection were performed using a digoxigenin (DIG) system
186 according to the manufacturer's instructions (GE Healthcare Co.). Briefly, DIG-
187 labeled probes (300–800 bases long) were prepared using a PCR DIG Probe Synthesis
188 Kit (Roche Applied Science AG, Mannheim, Germany) with SBU/SBL primer pairs,
189 followed by agarose gel extraction. The probes were hybridized to the DNA fragments
190 bound to the membrane, and the signals of the probes were detected via
191 chemiluminescence arising from the anti-DIG Fab fragment alkaline phosphatase
192 conjugate (Roche Applied Science AG), using the CDP-Star Detection Reagent (GE
193 Healthcare Co.). A WSE-6100H LuminoGraph I gel imager (ATTO Co. Ltd., Tokyo,
194 Japan) was used for the signal detection of Southern hybridization. The acquired
195 disruptants of the candidate genes (△1, △19, △*mdlB*, △*tglA*, and △*cutL*) and the
196 parent strain were used to measure lipase activities and FFA levels in the *in vitro*
197 cheese model.

198 **Biomass measurement by glucosamine assay**

199 The glucosamine content was determined to estimate the fungal biomass in the solid
200 medium, using the method of Fuji et al. (9) and Kasuga (10) with some modifications.
201 The CP (0.5 g) was washed with 50 mmol L^{-1} sodium phosphate buffer pH 7.0 and

shake vigorously by hand to obtain a homogeneous dispersion. Then, the samples were centrifuged at 1,070 ×g for 10 min at room temperature, and the supernatant was discarded. After washing three times, 15 mL of the same buffer was added to disperse the solid culture, followed by the addition of 20 mg of Yatalase (Takara Bio, Tokyo, Japan). Then, the tube was shaken at 60 rpm, 37°C for 1.5 h, followed by centrifugation. The supernatant (10 mL) was filtered through a 0.45 μm filter and collected in a 15 mL conical tube. The samples were kept at -20°C until use.

One hundred microliters of the sample or N-acetylglucosamine (GlcNAc) standard solution were transferred to a 1.5 mL microtube and then mixed with 40 μL of 0.4 mol L^{-1} potassium tetraborate tetrahydrate solution. The sample tubes were heated at 100°C for 3 min and were then cooled down at room temperature. Then, 600 μL of p-dimethylaminobenzaldehyde (DMAB) solution (0.1 g of DMAB powder dissolved in a mixture of 17.3 mol L^{-1} acetic acid and 0.37 mol L^{-1} HCl) was added to the tube and incubated at 37°C for 20 min. Finally, 150 μL of each sample solution was transferred in duplicate into a 96-well microplate to measure absorbance at 595 nm, using a microplate reader (Infinite F200 PRO, Tecan, Switzerland). One milligram of dried koji mycelium contains 0.628 μmol of GlcNAc according to Fujii et al. (9).

Preparation of the extracellular and intracellular enzymes

The CPs were mixed with an equal weight of deionized water and treated by a stomacher for 5 min. The materials were then transferred to a centrifugal tube and centrifuged at 21,130 ×g, 4°C, 10 min. The supernatant was recovered and the precipitate was re-extracted with deionized water another two times. The pooled supernatants were used as the extracellular enzyme. The precipitate was washed once more with deionized water and freeze-dried. The freeze-dried sample was ground into

226 a fine powder with a mortar and pestle and then dispersed in a simulated milk
227 ultrafiltrate (SMUF) at pH 5.5 (11), which was used as the intracellular enzyme.

228 **Measurement of fractionated lipase activity**

229 The extracellular enzyme (70 μL) was mixed with 230 μL of SMUF buffer pH 5.5,
230 while the suspension comprising 0.05 g of intracellular FDP dispersed in 300 μL of
231 SMUF pH 5.5 was used as the intracellular enzyme. The lipase activity was determined
232 as previously described, with some modifications (12). In brief, one gram of purified
233 triolein or diolein emulsified with 100 mL of 2% polyvinyl alcohol (degree of
234 polymerization 2000, Kishida Chemical, Osaka, Japan) was used as the substrate.
235 Purification of triolein was carried out by loading commercial triolein (Kanto
236 Chemical, Tokto, Japan) on the column packed with silica gel 60 N (Kanto Chemical,
237 Tokyo, Japan) pre-equilibrated with an organic solvent mixture of hexane and
238 chloroform (5:1). Triolein was recovered by the isocratic elution of the same solvent.
239 Purification of diolein was carried out by loading commercial diolein (Kanto Chemical,
240 Tokyo, Japan) on the same column pre-equilibrated with the organic solvent mixture
241 of hexane and chloroform (5:1). Then, diolein was recovered by the isocratic elution
242 of another solvent mixture of hexane and chloroform (1:1). The purity of these lipids
243 was confirmed by conventional thin layer chromatography (TLC) (Fig. S6).

244 The substrate was divided into 2 mL for each test tube and subjected to pre-incubation
245 at 30ºC for 5 min, followed by the addition of the enzyme to be tested (300 μL). The
246 reaction was carried out at 30ºC for 30 min. The reaction was terminated by adding
247 7.5 mL of extract solution (heptane : isopropanol : 0.5 mol L^{-1} sulfuric acid = 48:48:4).
248 FFA measurement was performed using a spectrophotometer at 570 nm, and the values
249 were corrected by subtracting the value obtained from the blank, which was subjected

to the same treatment except for the incorporation of the enzyme after the addition of the extract solution. Enzyme activity was expressed as μmol of released oleic acid from the substrate per 1 h at the defined temperature from one gram of the CPs (1 μmol oleic acid/h/g-CP = 1 lipase unit: LU). The specific lipase activity was calculated based on the biomass and expressed as the total lipase unit per mg of dried koji biomass (LU/mg koji).

Study of the FFA levels of mutants in a cheese curd model

The *in vitro* cheese curds used in this study were prepared as previously described (6) with the addition of 2% NaCl. After producing the curds, 0.2 g of FDP prepared using the culture with an initial pH of 4.0 or 6.5 was mixed with 200 g of curds, and 20 g was divided into nine bags, and then vacuum-sealed. The culture with the parent strain (P4, P6.5) or mutants, including Δ*mdlB* (M4, M6.5) and Δ*tglA* (T4, T6.5), were used. The control cheese (C) had no addition of any adjunct material. The curd packs were ripened at 12ºC and sampled at 4 weeks.

For FFA determination, the triplicate curd sample (0.2 g) was transferred to 2 mL of 7.7 mol L^{-1} HCl in a test tube and placed into boiling water to dissolve the sample. FFAs, extracted by the same extraction reagent as was used in the lipase activity measurement were determined by the phenol-red method (13) and its content was expressed as millimoles per 100 g of curds using oleic acid as the standard.

Statistical analysis

FFA content in the curds was analyzed using Tukey-Kramer's multiple comparison test. The data were analyzed by JMP software (Pro 17; SAS Institute, Inc., Tokyo, Japan). Differences were considered to be statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Qualitative gene expression analysis by RT-PCR to pre-screen of candidate lipase genes for mutation

The results of RT-PCR performed with twenty-five candidate primers revealed that initial cultures with a pH of either 4.0 or 6.5 resulted in the expression of eight and six genes respectively (Fig. 1). gDNA contamination in cDNA was unlikely because no amplification was found when the mRNA preparation was used as a template (Fig. S7). Annotated lipase genes Nos. 1, 20, 22, *mdlB*, *tglA*, and *cutL* were expressed in *A. oryzae* AHU 7139 with either an initial pH 4.0 or 6.5, whereas Nos. 19 and 21 showed limited expression in the culture with an initial pH of 4.0.

To narrow down the candidates for mutation, we evaluated the substrate specificity and distribution of the lipase in the culture products (CPs) of *A. oryzae* AHU 7139 to compare with the gene expression profile. Introducing purified substrates revealed that the parent strain of *A. oryzae* AHU 7139 produced DG lipolytic activity approximately twenty times higher than TG lipase activity at both initial pHs (Fig. 2). The activity was predominantly found in the extracellular fraction although the CPs prepared with an initial pH of 4.0 showed a higher ratio of intracellular DG lipase than those prepared at pH 6.5. It should be noted that the number of genes expressed at pH 4.0 was greater than at pH 6.5 (Fig. 1) despite the lower total lipase activity of the initial culture at pH 4.0 compared to pH 6.5 (Fig. 2). The gene No.1 (AO090012000690) annotated as a triglyceride lipase and the gene No. 19 (AO090005001319), annotated as a lipase and carboxyl ester hydrolase, were the focus of further study because of the limited information available regarding substrate specificity. On the other hand, Nos. 20 (AO090005001602), 21 (AO090023000717)

297 and 22 (AO090003000839) were screened out because they were reported to be
298 intracellular TG lipases (14) while we found limited intracellular lipase activity toward
299 triolein under either initial pH (Fig. 2). The expression of the *cutL* (15), *mdlB* (16),
300 and *tglA* (17) genes has been demonstrated in liquid culture and this study
301 demonstrated their expression in a solid-state medium as well. Taking the lipase
302 activity and the gene expression into account, two annotated lipase genes (Nos. 1 and
303 19), *mdlB*, *tglA*, and *cutL* were selected to obtain mutants.

304 **Lipase production and distribution in the mutant strains of *A. oryzae* AHU**
305 **7139**

306 We introduced a single-gene knockout of *A. oryzae* AHU 7139 to confirm the
307 lipase productivity. Considering the growth rate difference of the mutants, the specific
308 lipase activity that represent the activity based on the biomass was compared (Table
309 1). Reduced specific TG lipase activity was observed in Δ1 (694.1 LU/mg koji), Δ19
310 (430.3 LU/mg koji) and Δ*cutL* (1079.8 LU/mg koji) when cultivation was initiated at
311 pH 4.0, but the activity was elevated when cultivation was initiated at pH 6.5 (9165.5,
312 20209.0, and 12047.9 LU/mg koji, respectively) compared to the parent strain (1934.7
313 and 5570.3 LU/mg koji at pH 4.0 and 6.5, respectively). CutL from *A. oryzae* was
314 assumed to be one of the factors responsible for rancidity in dairy products since it
315 showed higher activity on esters of short-chain fatty acids (15). However, CutL as well
316 as Nos. 1 and 19, were unlikely to be related to rancidity because the rancidity was
317 more pronounced in cheese prepared using the adjuncts produced at an initial culture
318 pH of 6.5 rather than pH 4.0. In contrast, Δ*mdlB* showed the opposite response because
319 specific TG lipase activity at pH 6.5 was reduced to 61.9% (3450 LU/mg koji)
320 compared with that of the parent strain with slight increase of 2153.7 LU/mg koji at

pH 4.0. Although no substrate specificity of MdlB toward triacylglyceride has been reported (16). Lan et al. (18, 19) reported that the single point mutation of mono- and diacylglyceride lipase increased the lipolytic activity toward TG and DG making it function as a TG lipase. Thus, we cannot rule out that certain modifications of *mdlB* in *A. oryzae* AHU 7139 conferred hydrolytic capability toward triacylglycerides. On the other hand, an 83% reduction of TG lipase activity was detected in Δ*tglA* (968.1 LU/mg koji) when the cultivation was initiated at pH 6.5 whereas only a 25% reduction of TG lipase activity was found in Δ*tglA* when the cultivation was initiated at pH 4.0 (1451 LU/mg koji). Regarding the DG lipase, Nos. 1, 19, and CutL were proven to be active toward DG as well as TG since the specific lipase activity of the culture initiated at pH 4.0 was reduced by 59%, 77% and 34.6% in the Δ1 (19889.9 LU/mg koji), Δ19 (11003.0 LU/mg koji) and Δ*cutL* (31720.8 LU/mg koji) mutants, respectively. We initially speculated that the biological significance of these lipases under acidic circumstance was related to the transition into the sporulation phase of *A. oryzae* (14) because the spore-forming ability was recognized in the CP initiated at pH 4.0 but not in that initiated at pH 6.5 (Fig. S8). In fact, it has been suggested that the metabolism of storage lipids (20) and two intracellular lipases in *A. oryzae* BCC7051 (14) are involved in fungal spore production. However, Δ1, Δ19 and Δ*cutL* exhibited similar spore formation at the end of the cultivation (data not shown), which indicated that these three lipases are independent on spore formation. It is unclear that an increase in the specific DG and TG lipase activity in Δ19 in the culture initiated at pH 6.5, despite No. 19 was unexpressed under the initial culture condition at pH 6.5 (Fig.1). Δ*mdlB* showed the greatest lipase reduction (84%) compared to the parent strain under either pH condition (from 48519.8 LU/mg koji to 7844.1 LU/ mg koji at pH 4.0 and from 104114.6 LU/ mg koji to 16393.0 LU/ mg koji at pH 6.5). Thus, major DG lipase

346 activity could be ascribed to MdlB. Furthermore, major intracellular DG lipase found
347 in the culture at 4.0 was regarded as MdlB because Δ*mdlB* showed limited intracellular
348 lipase accumulation (Fig. 2). The MdlB found in the intracellular fraction was
349 probably due to the limited secretion period, followed by the rapid life cycle transition
350 into conidiation. As Toida et al. (17) reported the hydrolytic ability of TglA toward
351 DG, partial reduction of specific DG lipase activities of Δ*tglA* was confirmed in both
352 initial pHs. Overall, in the culture initiated at pH 4.0, the predominant TG lipase
353 activity was derived from Nos. 1, 19 and CutL, whereas that was due to TglA in the
354 culture initiated at pH 6.5 (Table 1). These results demonstrate that the production of
355 triglyceride lipase molecules depends on the initial culture pH. On the other hand,
356 MdlB was the major DG lipase irrespective of the initial culture pH. Considering the
357 higher level of total lipase activity in the culture initiated at pH 6.5, TglA and MdlB
358 were more likely to be related to rancidity. Therefore, Δ*tglA* and Δ*mdlB* were selected
359 for further study.

360 ***In vitro* studies of FFA profiles in the cheese curd model**

361 Finally, we conducted an *in vitro* cheese curd experiment to evaluate the effects
362 of triacylglycerol lipase (TglA) and mono-, diacylglycerol lipase (MdlB) on the free
363 fatty acid (FFA) levels in the curds. Table 2 shows the whole lipase activity of freeze-
364 dried powder (FDP), used for mixing with curds. The lipase activity was higher in all
365 CPs with an initial pH of 6.5 compared to those with an initial pH of 4.0, except for
366 the activity of mutant Δ*tglA* toward TG. The triacylglycerol lipase activity in all
367 mutants was lower than that in the parent strain for both initial culture pHs. Moreover,
368 lipase activity toward DG was not detected in Δ*mdlB* initially cultured at pH 4.0, and
369 low activity was observed with an initial pH of 6.5 (302.3 LU). After 4 weeks of

ripening, the addition of the CPs from the mutant Δ*tglA* resulted in a significantly lower amount of FFA than that from the parent strain ($p < 0.05$) and retained a comparable FFA level to that of the control sample (Fig. 3). Moreover, the addition of CPs from the mutant Δ*mdlB* initially cultured at pH 6.5 resulted in a significantly lower FFA level than that of the parent, although higher than that of the control cheese (Fig. 3).

FFA accumulation during cheese ripening is mainly attributed to the action of exogenous lipases because the endogenous milk lipase is heat-labile and can be inactivated by pasteurization (21). We found that the released FFA in the *in vitro* curds seemed to correlate with the TG lipase activity levels in the adjuncts (Fig.3, Table 2). Since the FFA accumulation in Δ*tglA* was significantly reduced in the ripening curds regardless of the initial pH, we suggested that TglA triggers hydrolysis that results in rancidity. As described above, lipases No. 1, No. 19, and CutL should still be active in either CPs from Δ*tglA* and Δ*mdlB*. However, the CPs from Δ*tglA* showed comparable FFA levels to the control, which demonstrated that those lipases were not as important as TglA under the cheese ripening circumstance. This is probably due to the reduced catalytic activity of other lipases at the temperature of cheese ripening (12℃) and/ or due to the degradation by concomitant rennet, which is supplied as the milk coagulation enzyme. The reduction of the FFA levels of Δ*mdlB* indicates that MdlB lipase should not be overlooked when considering rancidity. Okumura et al. (22) reported that mono- and diacylglycerol lipases, from *Penicillium cyclopium* M1, more easily hydrolyze acylglycerols than triacylglycerol lipase, and the synergistic action of mono- and diacylglycerol lipase with triacylglycerol lipase could accelerate lipolysis. Wong et al. (23) found elevated FFA productivity in *A. oryzae* RIB 40 when they

394 applied the overexpressed strain AO090701000644 (*mdlB*). Based on these results, we
395 postulated a rancidity process for ripened cheese caused by *Aspergillus* lipolysis (Fig.
396 4). Extracellular triacylglycerol lipase (TglA) is crucial for the degradation of
397 triglycerides in milk and the release of FFA and DG as the first step of rancidity.
398 Subsequently, the resulting DGs are provided as the substrate for MdlB lipase. The
399 dual effect of TglA, followed by MdlB, is likely to cause an excess amount of volatile
400 fatty acids and rancidity in cheese products made with *A. oryzae*.

401 It might be possible to distinguish MdlB from TglA only if highly purified
402 triolein is used as the substrate. Suzuki et al. (24) found a poor correlation between
403 lipase activity and butyric acid levels in cheese products made with *A. oryzae* when
404 they used a synthetic substrate of *p*-nitrophenol butyrate, which might represent whole
405 esterase activity. Even though butter oil was used as the substrate for lipase activity
406 measurement, FFA levels in the resulting cheese products were not correlated with the
407 lipase activities in the adjuncts prepared using *Aspergillus* CPs (6). As butter oil
408 included TG as the major component, with DG and MG as minor components, it could
409 be as the substrate not only for TG lipase but also for the abundant DG lipase. Thus,
410 when suitable strains of *A. oryzae* are screened with respect to lipase activity for the
411 preparation of adjuncts for cheesemaking, a strict evaluation of TG activity should be
412 performed.

413 In conclusion, *A. oryzae* is expected to generate adjuncts to enrich the flavor
414 of ripened cheese if the excessive accumulation of volatile fatty acids due to lipase
415 activity is reduced. In this study, we demonstrated that *tglA* is the most pivotal lipase
416 gene encoded in *A. oryzae*. Therefore, a TglA-inactive strain might be a candidate for
417 use as an adjunct for cheese ripening to prevent the production of rancid-off flavor.

We are currently investigating the reduction of TglA catalytic activity by substituting amino acid(s) and inserting or deleting nucleotides, leading to a frameshift of *tglA* transcripts. Furthermore, genome editing might be an alternative to obtain desirable strains.

ACKNOWLEDGEMENTS

This research was partially supported by grants from The Tojuro Iijima Foundation for Food Science and Technology and Sapporo Bioscience Foundation. The authors are very grateful to Yu Toba and Hiroki Matsumo, Field Science Center for Northern Biosphere, Hokkaido University, for their assistance with cheese curds preparation. We also thank Professor Satoshi Koike for the experimental support.

REFERENCES

1. Barbesgaard, P., Heldt-Hansen, H. P., and Diderichsen, B.: On the safety of *Aspergillus oryzae:* a review. Appl. Microbiol. Biotechnol., **36**, 569–572 (1992).

2. Machida, M., Asai, K., Sato, M., Tanaka, T., Kumagai, T., Terai, G., Kusumoto, K., Arima, T., Akita, O., Kashiwagi, Y., and other 53 others: Genome sequencing and analysis of *Aspergillus oryzae*, Nature, **438,** 1157–1161 (2005).

3. Kumura, H., Saito, C., Taniguchi, Y., Machiya, T., and Takahashi, Y.: Adjunctive application of solid-state culture products from *Aspergillus oryzae* for semi-hard cheese, J Adv Dairy Res, **5**(3), 1000188 (2017).

4. Viniegra-González, G., Favela-Torres, E., Aguilar, C. N., Rómero-Gomez, S. J., Díaz-Godínez, G., and Augur, C.: Advantages of fungal enzyme production in solid state over liquid fermentation systems, Biochem. Eng. J., **13**, 157–167 (2003).

5. Ray, P. R., Chatterjee, K., Chakraborty, C., and Ghatak, P. K.: Lipolysis of milk: A Review, Int. J. Agric. Vet. Sci., **1**, 58-74 (2013).

6. Chintagavongse, N., Takiguchi, H., Ming-Hsuan, C., Tamano, K., Hayakawa, T., Wakamatsu, J., Mitani, T., and Kumura, H.: A study of lipolysis induced by adjuncts from edible *Aspergillus* sp. solid culture products on ripened semi-hard cheese, J. Sci. Food Agric., **102**, 4355-4362 (2022).

7. Tamano, K., Satoh, Y., Ishii, T., Terabayashi, Y., Ohtaki, S., Sano, M., Takahashi, T., Koyama, Y., Mizutani, O., Abe, K., and Machida, M.: The beta-1,3-exoglucanase gene exgA (exg1) of *Aspergillus oryzae* is required to catabolize extracellular glucan, and is induced in growth on a solid surface, Biosci. Biotechnol. Biochem., **71**(4), 926-934 (2007).

8. Niu, J., Arentshorst, M., Seelinger, F., Ram, A.F., and Ouedraogo, J.P.: A set of isogenic auxotrophic strains for constructing multiple gene deletion mutants and parasexual crossings in *Aspergillus niger*, Arch. Microbiol., **198**(9), 861-868 (2016).

9. Fujii, F., Ozeki, K., Kanda, A., Hamachi, M., and Nunokawa, Y.: A simple method for the determination of grown mycelial content in ricekoji using commercial cell wall lytic enzyme, Yatalase. J. Brew. Soc. Jpn., **87**(10), 757-759 (1992) (in Japanese).

10. Kasuga, M.: A simple method for measuring of mycelial content in soy sauce koji culture, J. Brew. Soc. Jpn., **94**(2), 122-125 (1999) (in Japanese).

11. Jenness, R. and Koops, J.: Preparation and properties of a salt solution which simulates milk ultrafiltrate, Neth. Milk Dairy J., **16**, 153-164 (1962).

461 12. Kumura, H., Satoh, M., Machiya, T., Hosono, M., Hayakawa, T., and Wakamatsu,
462 J.: Lipase and protease production of dairy *Penicillium* sp. on milk-protein-based solid
463 substrates, Int. J. Dairy Technol., **72**, 403-408 (2019).

464 13. Saito, Z.: Application of the phenol-red method for investigations on the lipolysis
465 of raw milk, Jap J Zootech Sci, **50**, 710–715 (1979) (in Japanese).

466 14. Anantayanon, J., Jeennor, S., Panchanawaporn, S., Chutrakul, C., and Laoteng, K.:
467 Significance of two intracellular triacylglycerol lipases of *Aspergillus oryzae* in lipid
468 mobilization: A perspective in industrial implication for microbial lipid production,
469 Gene, **793**, 145745 (2021).

470 15. Ohnishi, K., Yoshida, Y., and Sekiguchi, J.: Lipase production of *Aspergillus*
471 *oryzae*, J. Biosci. Bioeng., **77**(5), 490-495 (1994).

472 16. Toida, J., Kondoh, K., Fukuzawa. M., Ohnishi, K., and Sekiguchi, J.: Purification
473 and characterization of a lipase from *Aspergillus oryzae*, Biosci. Biotechnol. Biochem.,
474 **59**(7), 1199-1203 (1995).

475 17. Toida, J., Arikawa, Y., Kondou, K., Fukuzawa, M., and Sekiguchi, J.: Purification
476 and characterization of triacylglycerol lipase from *Aspergillus oryzae*, Biosci.
477 Biotechnol. Biochem., **62**(4), 759-763 (1998).

478 18. Lan, D., Popowicz. G.M., Pavlidis, I.V., Zhou, P., Bornscheuer, U.T., and Wang,
479 Y.: Conversion of a mono- and diacylglycerol lipase into a triacylglycerol lipase by
480 protein engineering, ChemBioChem, **16**, 1431-1434 (2015).

481 19. Lan, D., Zhao, G., Holzmann, N., Yuan, S., Wang, J., and Wang, Y.: Structure-
482 guided rational design of a mono- and diacylglycerol lipase from *Aspergillus oryzae*:

483 a single residue mutant increases the hydrolysis ability, J. Agric. Food Chem., **69**(18),
484 5344-5352 (2021).

485 20. Engebrecht, J.A.: Cell signaling in yeast sporulation, Biochem. Biophys. Res.
486 Commun., **306**(2), 325-328 (2003).

487 21. Shipe, W. F. and Senyk, G. F.: Effects of processing conditions on lipolysis in milk,
488 J. Dairy Sci., **64**(11), 2146-2149 (1981).

489 22. Okumura, S., Iwai, M., and Tsujisaka, Y.: Purification and properties of partial
490 glyceride hydrolase of *Penicillium cyclopium* M1, J. Biochem., **87**(1), 205–211 (1980).

491 23. Wong, P.S., Tamano, K., and Aburatani, S.: Improvement of free fatty acid
492 secretory productivity in *Aspergillus oryzae* by comprehensive analysis on time-series
493 gene expression, Front. Microbiol., **12**, 605095 (2021).

494 24. Suzuki, S., Ohmori, H., Hayashida, S., Nomura, M., Kobayashi, M., Hagi, T.,
495 Narita, T., Tomita, S., Yamashita, H., Arakawa, Y., and other 3 authors: Lipase and
496 protease activities in Koji cheeses surface-ripened with *Aspergillus* strains, Food Sci.
497 Technol. Res., **27**(3), 543-549 (2021).

Figure Legends

Fig. 1. Agarose gel (1%) electrophoresis of RT-PCR products with cDNA from CP initial pH 4.0 (A) and pH 6.5 (B). M = DNA ladder marker, lane no.1-22 = annotated lipase no. 1-22, m = *mdlB*, t = *tglA*, and c = *cutL* gene. Red box shows the expected amplified band while others are non-specific bands.

Fig. 2. Lipase activities of *A. oryzae* AHU 7139 (parent) and five selected mutants (Δ*mdlB*, Δ*tglA*, Δ*cutL*, Δ1, Δ19) toward purified triolein (TG) and diolein (DG) from the solid culture products (CP) initiated with pH 4.0 and pH 6.5. Values are expressed in average value (n = 2).

Fig. 3. Free fatty acid (FFA) profiles in cheese model at 4 weeks.

C = curds only (control), P4 = parent AHU 7139 with initial culture pH 4, M4 = mutant AHU 7139 Δ*mdlB* with initial pH 4, T4 = mutant AHU 7139 Δ*tglA* with initial pH 4, P6.5 = parent AHU 7139 with initial pH 6.5, M6.5 = mutant AHU 7139 Δ*mdlB* with initial pH 6.5, T6.5 = mutant AHU 7139 Δ*tglA* with initial culture pH 6.5. Results of FFA level are expressed as the mean ± SE (n = 3). The different letters indicate a significant difference between the samples using Tukey-Kramer test ($p < 0.05$)

Fig. 4. The proposed rancidity process in the ripened cheese caused by *Aspergillus* lipolysis. As triacylglycerol (TG) is the major lipid in milk, triacylglycerol lipase (TglA) hydrolyzes TG at any fatty acid position ((A) sn-1, 3 or (B) sn-2) and generates diacylglycerol (DG) and free fatty acids (FAs) during ripening. Subsequently, diacylglycerol lipase (MdlB and, to some extent, TglA) hydrolyzes DG and produces FFA. The predominant lipases (TglA or MdlB) at each hydrolytic step are shown in bold, although other genes might be involved. The excess amounts of free volatile fatty acids released by fungal lipolysis could cause rancidity.

Supplementary Figure Legends

Fig. S1. RNA integrity check by non-denaturing agarose gel electrophoresis. Total RNA was isolated from *A. oryzae* AHU 7139 inoculated on whey solid culture adjusted to pH 4.0 and pH 6.5. +con = positive control of 28S and 18S RNA bands from bovine endometrial stromal cells.

Fig. S2. Construction of the DNA fragment for *pyrG* (AO090011000868) knockout in *A. oryzae* AHU 7139. The 2035 bp-long DNA fragment was constructed for the knockout. Primers used for the construction and clone check are shown as colored and black arrows, respectively.

Fig. S3. Clone check of the *pyrG* knockout mutant isolated after transformation. Transformant clone Nos. 1-3 are shown. Lane M: 1 kb DNA ladder marker [1-10 kb], lane R: RIB40 strain as a negative control, lane A: AHU 7139 strain as a negative control. Amplified DNA size: Positive clone, 1.0 kb; Negative clone, 2.1 kb. The clone No. 3 in red was considered a positive homokaryon, which was named AHU 7139 [pyrG^{-}].

Fig. S4A. Construction of the DNA fragment for disrupting AO090003001507 in *A. oryzae* AHU 7139 [pyrG^{-}]. The 2048 bp-long and 2375 bp-long DNA fragments were constructed for the disruption. Primers used for the construction and clone check are shown as colored and black arrows, respectively.

Fig. S4B. Construction of the DNA fragment for disrupting AO090701000644 in A. oryzae AHU 7139 [pyrG^{-}]. The 2021 bp-long and 2377 bp-long DNA fragments were constructed for the disruption. Primers used for the construction and clone check are shown as colored and black arrows, respectively.

Fig. S4C. Construction of the DNA fragment for disrupting AO090005000029 in *A. oryzae* AHU 7139 [pyrG^{-}]. The 2035 bp-long and 2392 bp-long DNA fragments were

547 constructed for the disruption. Primers used for the construction and clone check are
548 shown as colored and black arrows, respectively.
549 **Fig. S4D.** Construction of the DNA fragment for disrupting AO090012000690 in *A.*
550 *oryzae* AHU 7139 [pyrG$^-$]. The 2031 bp-long and 2403 bp-long DNA fragments were
551 constructed for the disruption. Primers used for the construction and clone check are
552 shown as colored and black arrows, respectively.
553 **Fig. S4E.** Construction of the DNA fragment for disrupting AO090005001319 in *A.*
554 *oryzae* AHU 7139 [pyrG$^-$]. The 2045 bp-long and 2408 bp-long DNA fragments were
555 constructed for the disruption. Primers used for the construction and clone check are
556 shown as colored and black arrows, respectively.
557 **Fig. S5A.** Southern hybridization analysis of AO090003001507 disruptant constructed
558 from AHU 7139. 1: AHU 7139; 2: AO090003001507 disruptant.
559 **Fig. S5B.** Southern hybridization analysis of AO090701000644 disruptant constructed
560 from AHU 7139. 1: AHU 7139; 2: AO090701000644 disruptant.
561 **Fig. S5C.** Southern hybridization analysis of AO090005000029 disruptant constructed
562 from AHU 7139. 1: AHU 7139; 2: AO090005000029 disruptant.
563 **Fig. S5D.** Southern hybridization analysis of AO090012000690 disruptant constructed
564 from AHU 7139. 1: AHU 7139; 2: AO090012000690 disruptant.
565 **Fig. S5E.** Southern hybridization analysis of AO090005001319 disruptant constructed
566 from AHU 7139. 1: AHU 7139; 2: AO090005001319 disruptant.
567 **Fig. S6.** Thin layer chromatography of the purified lipids. M = control monoglyceride,
568 D = control diglyceride, T = control triglyceride (tributyrin), PD = purified commercial
569 diolein using silica gel chromatography, PT = purified commercial triolein using silica
570 gel chromatography, CD = commercial diolein, CT = commercial triolein. Migration
571 of the sample on the silica gel plate was carried out using chloroform : methanol :

formic acid : DI water (45:20:2.5:1), stained with 0.05% primulin dissolved in acetone : water (4:1 v/v), and observed under UV light.

Fig. S7. Agarose gel (1%) electrophoresis of PCR using mRNA from CP with initial pH 4.0 (A) and pH 6.5 (B) as a template with all candidate lipase gene primers. M = DNA ladder marker, lane no.1-22 = annotated lipase no. 1-22, m = *mdlB*, t = *tglA*, and c = *cutL* gene. Confirming no gDNA in the mRNA sample.

Fig. S8. Growth and appearance of *A. oryzae* AHU 7139 on the WPC media incubated at 20°C for 7 days. The initial culture pH were of (A) pH 4.0 and (B) pH 6.

Table 1. Specific lipase activity of *A. oryzae* AHU 7139 and its mutants toward triacylglycerol (TG) and diacylglycerol (DG).

Sample	dried biomass (mg)	TG LU[(1)]/ mg koji	DG LU[(1)]/ mg koji
CP initial pH 4.0			
parent	0.008	1934.7	48519.8
Δ1	0.014	694.1	19889.9
Δ19	0.027	430.3	11003.0
Δ*mdlB*	0.004	2153.7	7844.1
Δ*tglA*	0.013	1451.0	32850.0
ΔcutL	0.015	1079.8	31720.8
CP initial pH 6.5			
parent	0.016	5570.3	104114.6
Δ1	0.018	9165.5	99601.9
Δ19	0.005	20209.0	330880.8
Δ*mdlB*	0.013	3450.7	16393.0
Δ*tglA*	0.017	968.1	88648.6
ΔcutL	0.017	12047.9	108810.5

Specific lipase activiy (LU/ mg koji) is calculated by total LU based on fungal biomass (n = 2).

[(1)] LU represents the sum of total extracellular and intracellular lipase activity.

Table 2. Whole lipase activity of freeze-dried powder (FDP) for *in vitro* cheese model

Sample	Whole lipase activity	
	TG	DG
P4	25.6	1632.4
M4	15.4	0.0
T4	8.9	808.9
P6.5	86.6	2433.3
M6.5	66.8	302.3
T6.5	4.8	2226.6

P = parent strain of *A. oryzae* AHU 7139, M = Δ*mdlB*, T = Δ*tglA*. FDP recovered from cultures initial pH 4.0 (4) and pH 6.5 (6.5). Lipase activity is shown as total LU per 100 g of curds (n = 2).

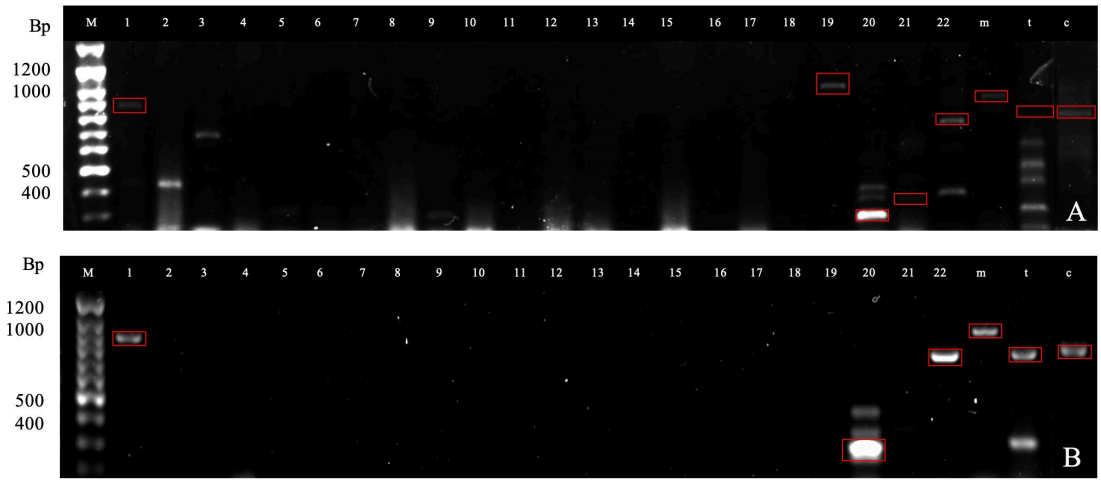

Fig. 1. Agarose gel (1%) electrophoresis of RT-PCR products with cDNA from CP initial pH 4.0 (A) and pH 6.5 (B). M = DNA ladder marker, lane no.1-22 = annotated lipase no. 1-22, m = *mdlB*, t = *tglA*, and c = *cutL* gene. Red box shows the expected amplified size band while others are non-specific bands.

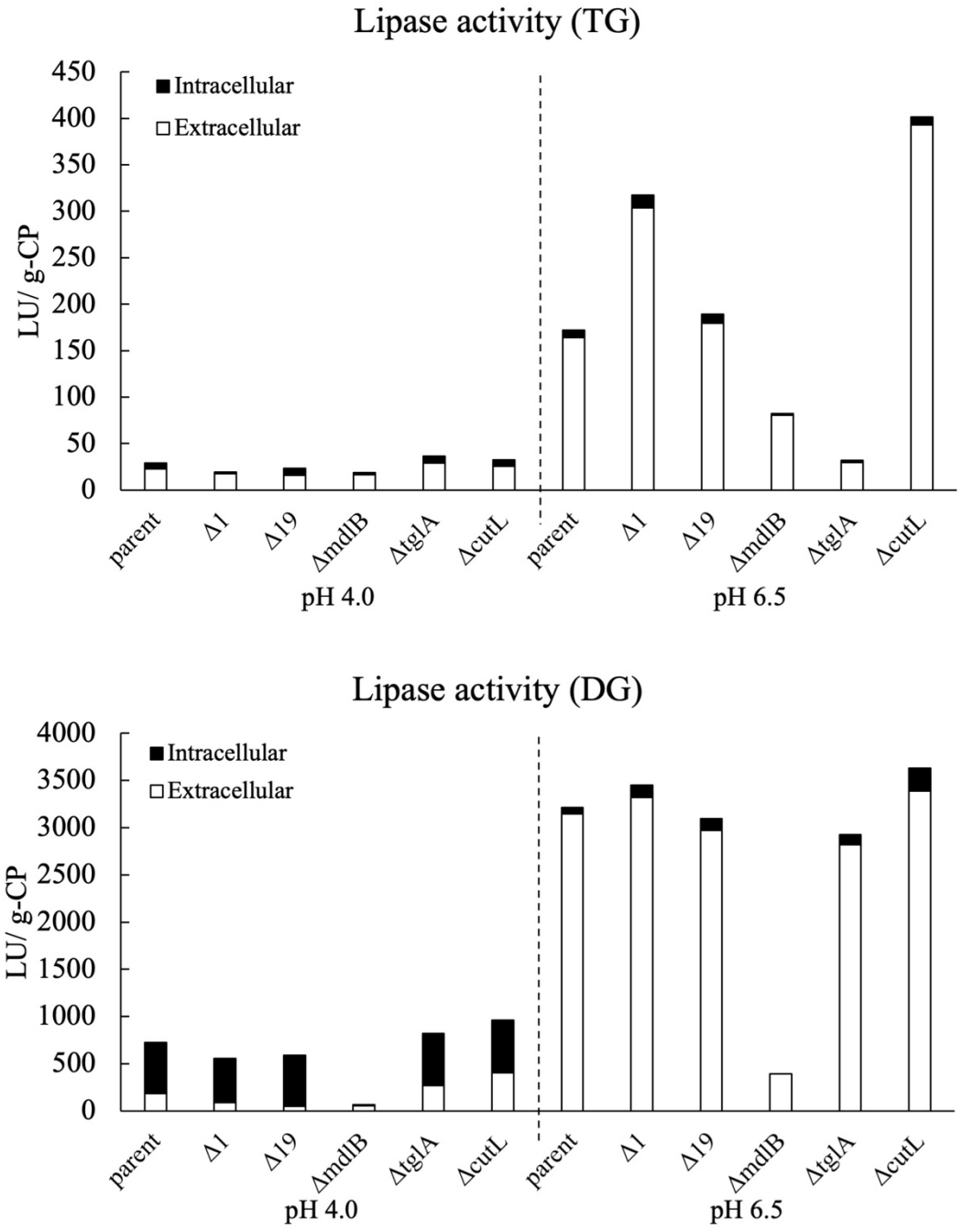

Fig. 2. Lipase activities of *A. oryzae* AHU 7139 (parent) and five selected mutants (Δ*mdlB*, Δ*tglA*, Δ*cutL*, Δ1, Δ19) toward purified triolein (TG) and diolein (DG) from the solid culture products (CP) initiated with pH 4.0 and pH 6.5. Values are expressed in average value (n = 2).

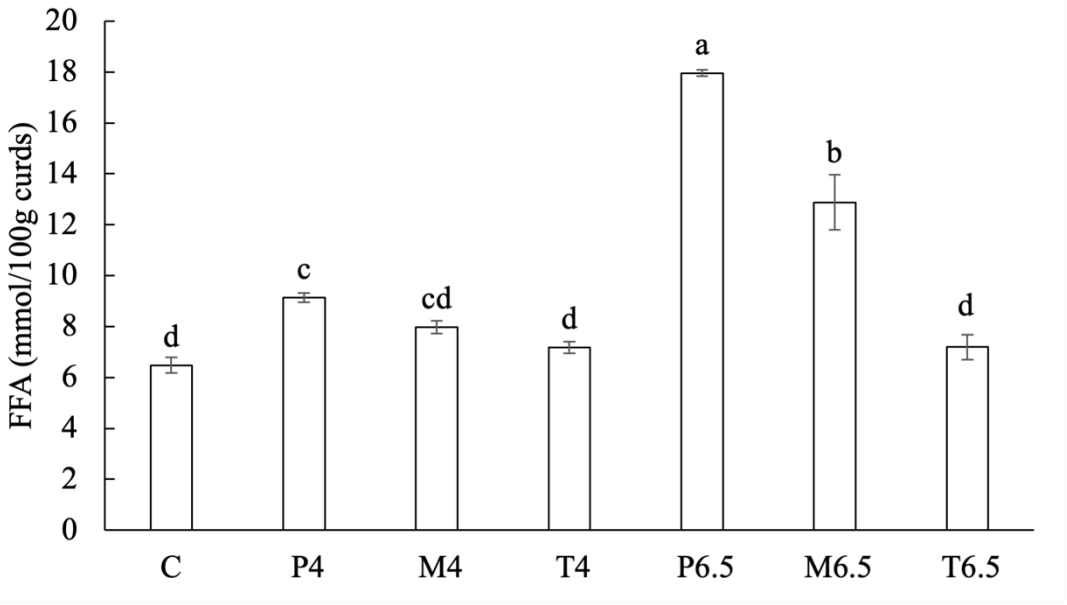

Fig. 3. Free fatty acid (FFA) profiles in cheese model at 4 weeks.

C = curds only (control), P4 = parent AHU 7139 with initial culture pH 4, M4 = mutant AHU 7139 Δ*mdlB* with initial pH 4, T4 = mutant AHU 7139 Δ*tglA* with initial pH 4, P6.5 = parent AHU 7139 with initial pH 6.5, M6.5 = mutant AHU 7139 Δ*mdlB* with initial pH 6.5, T6.5 = mutant AHU 7139 Δ*tglA* with initial culture pH 6.5. Results of FFA level are expressed as the mean ± SE (n = 3). The different letters indicate a significant difference between the samples using Tukey-Kramer test ($p < 0.05$)

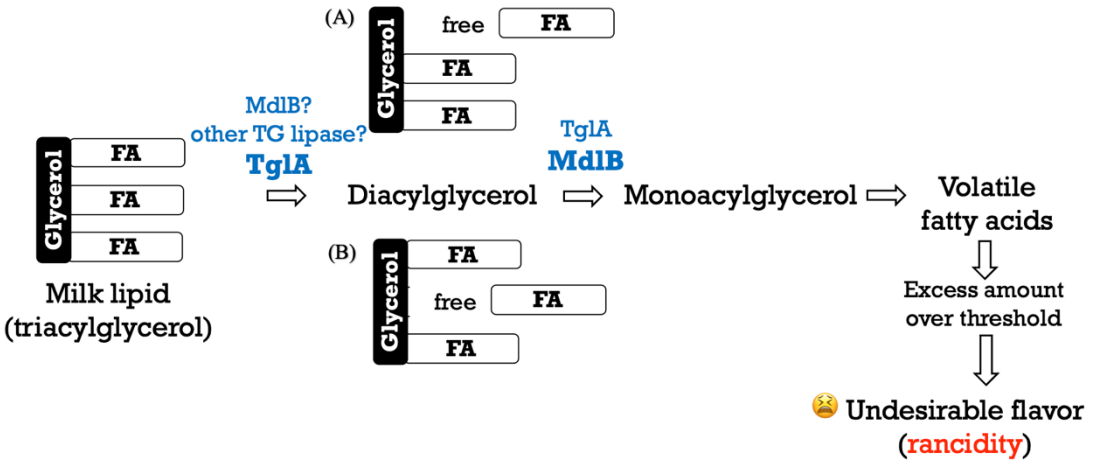

Fig. 4. The proposed rancidity process in the ripened cheese caused by *Aspergillus* lipolysis. As triacylglycerol (TG) is the major lipid in milk, triacylglycerol lipase (TglA) hydrolyzes TG at any fatty acid position ((A) sn-1, 3 or (B) sn-2) and generates diacylglycerol (DG) and free fatty acids (FAs) during ripening. Subsequently, diacylglycerol lipase (MdlB and, to some extent, TglA) hydrolyzes DG and produces FFA. The predominant lipases (TglA or MdlB) at each hydrolytic step are shown in bold, although other genes might be involved. The excess amounts of free volatile fatty acids released by fungal lipolysis could cause rancidity.

Table S1. Primers used for gene expression analysis.

Gene	Primer	Sequence (5' - 3')	Predicted amplified size (bp)	
			genomic DNA	cDNA
Triacylglycerol lipase (*tglA*) (AO090003001507)	Forward	CTTTCACGGAGCCCTTCCAC	957	765
	Reverse	CGTAAATTAGTTCGCAGCCGC		
Mono-, di-acylglycerol lipase (*mdlB*) (AO090701000644)R	Forward	TGAGTAGACCCTGCGAAGCAC	1024	921
	Reverse	CGAATTAGCGCAATGGCAATCCAG		
Cutinase 1 (*cutL*) (AO090005000029)	Forward	GCTTTGCCCCAGGAAGAAT	987	834
	Reverse	ACTGACCTCCATCAAATGGGAG		
AO090012000690 (1)	Forward	GAGATTTGATCGCACTGATGGC	1003	864
	Reverse	GCCAAGCTTTTGGCTTTCAC		
AO090701000692 (2)	Forward	CTACCTTAGCCCGCAAGTGC	1245	1038
	Reverse	CCACTAACAAACCATCCCTTCAAC		
AO090003001432 (3)	Forward	GCCGTATGTCCTCTGCCTCTG	1362	1362
	Reverse	CCACAATCAAGGAATTAGCCGACG		
AO090003001315 (4)	Forward	TGAGCTTTTCACCGTCTCCC	1269	1269
	Reverse	ACACATTCGTCCAAACATGGC		
AO090001000143 (5)	Forward	GCTGACTTCGCAAGGGATATTC	1056	888
	Reverse	TCAAGGCTGGCAGAAGTCTC		
AO090010000619 (6)	Forward	CGGCACTGAAGTGTCTGCATAATC	1505	1368
	Reverse	TAGATGGTGGGGTCTCTGGGAG		
AO090012000103 (7)	Forward	AGTGCGATCAATTCCCTACCC	1420	1353
	Reverse	CCCACATAACTCCCGTTTGAG		
AO090124000015 (8)	Forward	CCTTCTGTCCCTTGGACCTG	1102	894
	Reverse	CAGTACTGTAGGTCAGCTTTCC		

Table S1. (continue) Primers used for gene expression analysis.

Gene	Primer	Sequence (5' - 3')	Predicted amplified size (bp)	
			genomic DNA	cDNA
AO090103000172 (9)	Forward	CGGGCAGTTCCCATGTCATC	1344	1344
	Reverse	GGCACCACAAAGAAGCGAAAG		
AO090138000167 (10)	Forward	TCCCGACCTCACCAGGATAG	1009	894
	Reverse	CGATCTTTCTCAGCTATAATTCGGC		
AO090038000214 (11)	Forward	CTGTTGCTTTGTATAGTGTCACG	1153	1065
	Reverse	TCTGTCACCCAGAGCAGATG		
AO090020000171 (12)	Forward	AAGAGATACCCAACACCCAC	1131	957
	Reverse	CCCAGTGACCCGATGTATC		
AO090020000609 (13)	Forward	GCACGCCAAAGACGCTTAATG	1588	1356
	Reverse	CACAATCTGACCACATCATTCGG		
AO090003000022 (14)	Forward	ACTGCGCTTTCCCAACAAAC	1327	1263
	Reverse	TCTATCACCCCGTCATTGCG		
AO090023000125 (15)	Forward	AACCACGCCACACCCATATC	1781	1644
	Reverse	GGGAAAGTGTTGGTTTAGACTGG		
AO090138000014 (16)	Forward	CCCATATAACGCTCGAAACATC	1054	915
	Reverse	ACGGATAAGGGTTGTAACTTAGG		
AO090003000851 (17)	Forward	TCGACCAGCATTACTCCGC	1033	933
	Reverse	TAAACCCATGTGGTAGCCCC		
AO090005000930 (18)	Forward	TGACCCAAGTTCACATCCGC	1190	1125
	Reverse	CATCCAAGACATTGATCCCGC		
AO090005001319 (19)	Forward	CAGCCCACCCCCTTCAATAG	918	918
	Reverse	ACGGAACGATTCTTGACGAAAAC		
AO090005001602 (20)	Forward	GGTTGCTCGTGGTAGGAATG	425	284
	Reverse	CAGTCGGCCCAGAATGAGTC		
AO090023000717 (21)	Forward	GGTGCTGTATCTGAAAATGCG	449	361
	Reverse	ATCACAACGACCTTTCGCTG		
AO090003000839 (22)	Forward	GCGCACCGATATCCCTATTAG	837	780
	Reverse	CACTCGACCTTCTTGTCATCC		

No. 1-22 in the bracket represents the annotated lipase genes from the bioinformatics database of *A. oryzae* RIB 40.

Table S2. Primers used for candidate gene disruption.			
Use application		Name	Sequence (5' to 3')
Knockout of AO090011000868 (*pyrG*)	Front arm	LUpyrG	CGACTAAGCCACGATCTCGATCAT
		LLpyrG	aaaggagtacgtatccaccactacCAATTGCCGCGAAAAATTAA
	Rear arm	RUpyrG	GTAGTGGTGGATACGTACTC
		RLpyrG	CTCCGTTGCGGATCTTGCTGCTTG
	Clone check	cLpyrG	GAGTACGTATCCACCACTAC
*pyrG*_former part		PU	GTCCATATATCGAGGCAGGT
		PLsplit	ATTGACCTACAGCGCACGC
*pyrG*_latter part		PUsplit	CCGGTAGCCAAAGATCCCTT
		PL	TCCTCATTTACTCCCGAGAT
Disruption of AO090003001507	Front arm	LU1	CGACACGGAGAATTTCCCGATGTA
		LL1	agacacctgcctcgatatatggacAAGTGGAAGGGCTCCGTGAA
	Rear arm	RU1	gcagatctcgggagtaaatgaggaTTTACGATAAGGGCTCCATG
		RL1	ACGCTTTTGTAAGAGCCAGCCCAC
	Clone check	cU1	TCCGAAGTCAGTAGATCGAC
		cL1	AGCCACTTATTTCCGTGACC
Disruption of AO090701000644	Front arm	LU2	GAGGGAAATTCTGGACGATTCTCG
		LL2	agacacctgcctcgatatatggacCTTTGCCAGTGTGCTTCGCA
	Rear arm	RU2	gcagatctcgggagtaaatgaggaCTTACATGATTTGGACGGAC
		RL2	GCATCACTCGGCAATCCTACCTAA
	Clone check	cU2	CTCGACTACGTCAGAGGAGC
		cL2	CAACCACAGAATCTATCACC
Disruption of AO090005000029	Front arm	LU3	CTGTCTTCTTTTATTCCGCTCACCAC
		LL3	agacacctgcctcgatatatggacGAGGAAAGTGTTTTAGAAAGCG
	Rear arm	RU3	gcagatctcgggagtaaatgaggaTCTGCTGCCTTGCGTCGGAT
		RL3	TTGCTGAACCATGCCCTGCTATCG
	Clone check	cU3	CAAGCTACTATGGTGTGGAT
		cL3	GCAACCTCCACATCATCTCC

Table S2. (continued) Primers used for candidate gene disruption.			
Use application		Name	Sequence (5' to 3')
Disruption of AO090012000690	Front arm	LU4	CTCTCTCGTGGAAGTATGTAAGCG
		LL4	agacacctgcctcgatatatggacCAGTGCGATCAAATCTCAAAC
	Rear arm	RU4	gcagatctcgggagtaaatgaggaATTCTCATCGCCATCACTA
		RL4	CCAGGGCTCACTATTGAGGTATTG
	Clone check	cU4	GGGTTTAGCGAGATCTTATC
		cL4	TATCATCAGGGTCTGGTAGA
Disruption of AO090005001319	Front arm	LU5	TACCAAAGTGCCCGTCACCTCATT
		LL5	agacacctgcctcgatatatggacTGTGATATCGATCACGGTTTC
	Rear arm	RU5	gcagatctcgggagtaaatgaggaCAAATTCAAGTCAAGGCTATCG
		RL5	GTGCTGAACAGTAGCCTCAATCCA
	Clone check	cU5	GCATTCAACAATGGCGATGC
		cL5	TTAGCAGCACCATCTAGTCG
Southern hybridization	Probe 1	003-1507_SBU	CCAACATACTGTTGTCACAC
		003-1507_SBL	TCAACAGTACGGTTTCACTC
	Probe 2	701-0644_SBU	CTTACATGATTTGGACGGAC
		701-0644_SBL	GTGTAGTGTGCTTGGCCGAC
	Probe 3	005-0029_SBU	TCTCCTCTCTCTCTGTGTCT
		005-0029_SBL	CTCGCTTCGGATTGTATGAT
	Probe 4	012-0690_SBU	CGTGAGCGACATGACTAAGT
		012-0690_SBL	CCGTTGTTGAGCTGGAATGT
	Probe 5	005-1319_SBU	GTGGATCAAATCGGGATGAA
		005-1319_SBL	GAATCTTGGACAGGAATCGT
Tails of primers used for overlapping in fusion PCR are shown in lower case.			

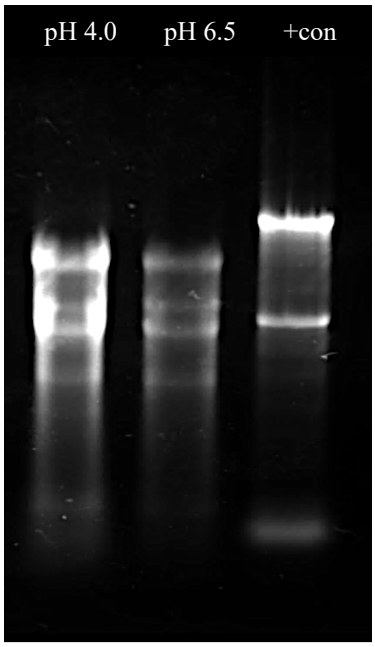

Fig. S1. RNA integrity check by non-denaturing agarose gel electrophoresis. Total RNA was isolated from *A. oryzae* AHU 7139 inoculated on whey solid culture adjusted to pH 4.0 and pH 6.5. +con = positive control of 28S and 18S RNA bands from bovine endometrial stromal cells.

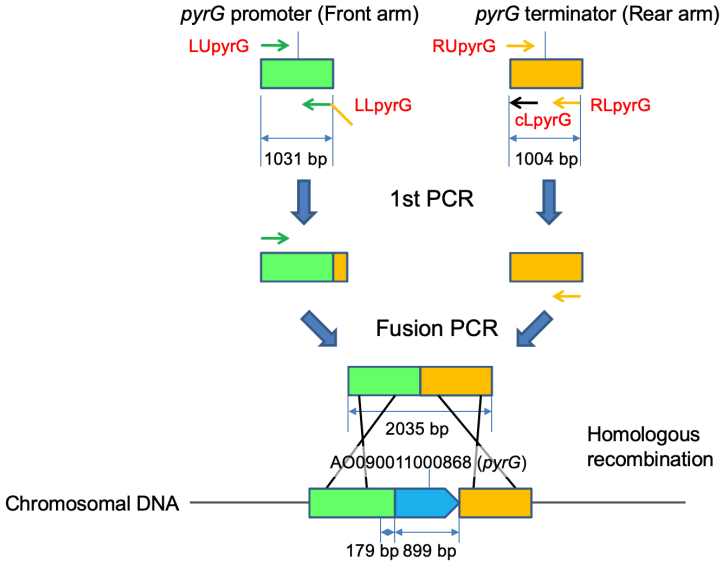

Fig. S2. Construction of the DNA fragment for *pyrG* (AO090011000868) knockout in *A. oryzae* AHU 7139. The 2035 bp-long DNA fragment was constructed for the knockout. Primers used for the construction and clone check are shown as colored and black arrows, respectively.

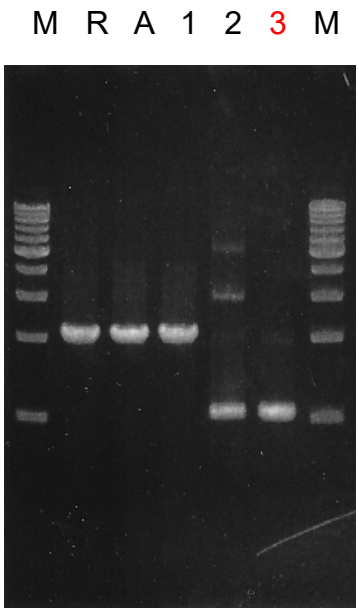

Fig. S3. Clone check of the *pyrG* knockout mutant isolated after transformation. Transformant clone Nos. 1-3 are shown. Lane M: 1 kb DNA ladder marker [1-10 kb], lane R: RIB40 strain as a negative control, lane A: AHU 7139 strain as a negative control. Amplified DNA size: Positive clone, 1.0 kb; Negative clone, 2.1 kb. The clone No. 3 in red was considered a positive homokaryon, which was named AHU 7139 [pyrG$^-$].

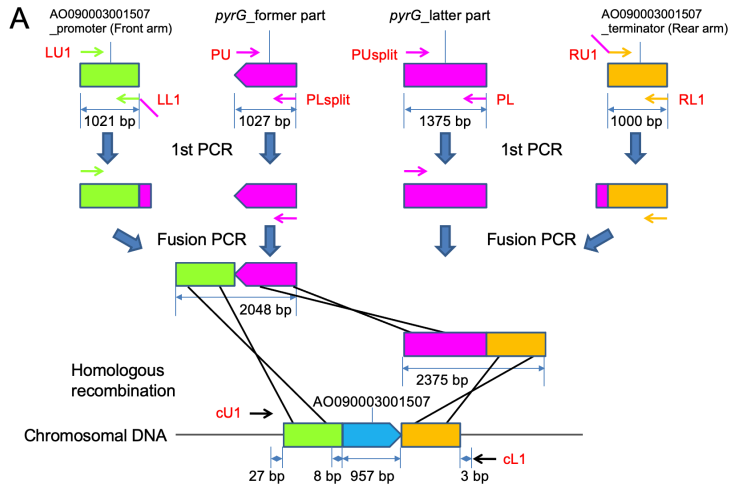

Fig. S4A. Construction of the DNA fragment for disrupting AO090003001507 in *A. oryzae* AHU 7139 [pyrG$^-$]. The 2048 bp-long and 2375 bp-long DNA fragments were constructed for the disruption. Primers used for the construction and clone check are shown as colored and black arrows, respectively.

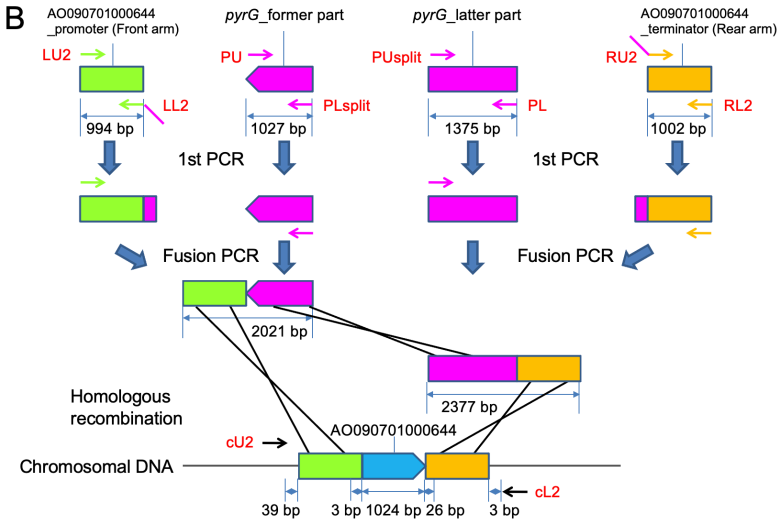

Fig. S4B. Construction of the DNA fragment for disrupting AO090701000644 in *A. oryzae* AHU 7139 [pyrG$^-$]. The 2021 bp-long and 2377 bp-long DNA fragments were constructed for the disruption. Primers used for the construction and clone check are shown as colored and black arrows, respectively.

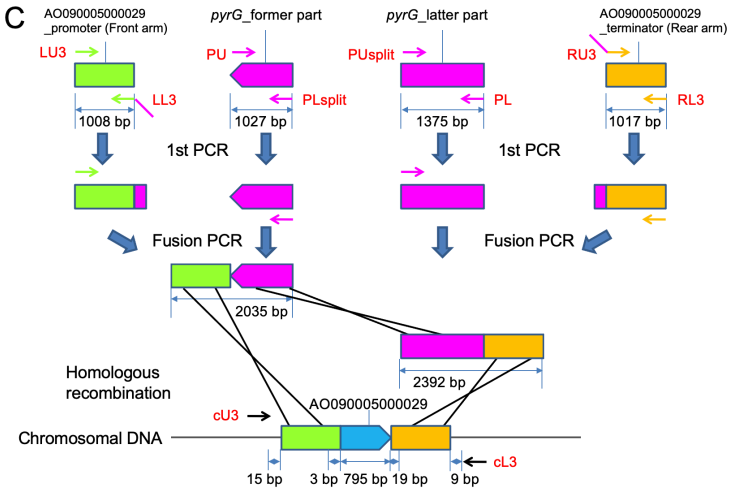

Fig. S4C. Construction of the DNA fragment for disrupting AO090005000029 in *A. oryzae* AHU 7139 [pyrG$^-$]. The 2035 bp-long and 2392 bp-long DNA fragments were constructed for the disruption. Primers used for the construction and clone check are shown as colored and black arrows, respectively.

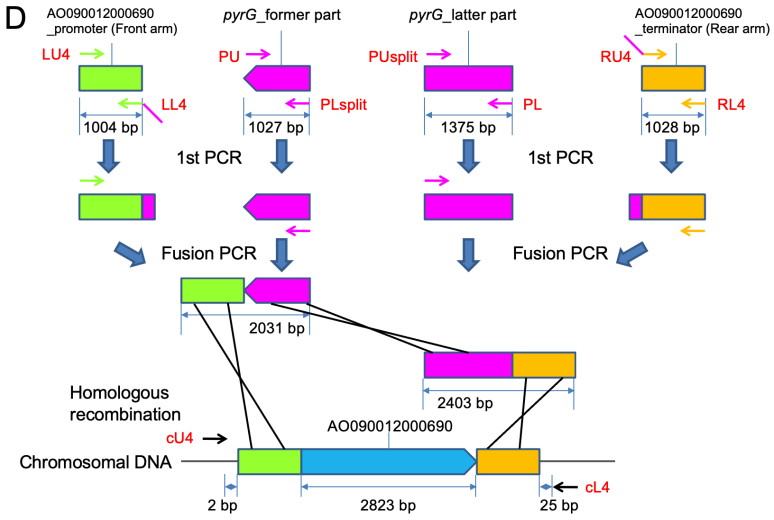

Fig. S4D. Construction of the DNA fragment for disrupting AO090012000690 in *A. oryzae* AHU 7139 [pyrG$^-$]. The 2031 bp-long and 2403 bp-long DNA fragments were constructed for the disruption. Primers used for the construction and clone check are shown as colored and black arrows, respectively.

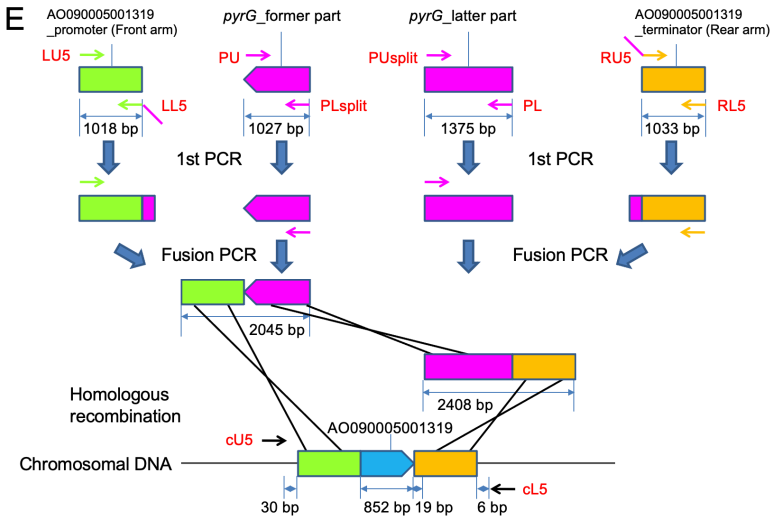

Fig. S4E. Construction of the DNA fragment for disrupting AO090005001319 in *A. oryzae* AHU 7139 [pyrG$^-$]. The 2045 bp-long and 2408 bp-long DNA fragments were constructed for the disruption. Primers used for the construction and clone check are shown as colored and black arrows, respectively.

A

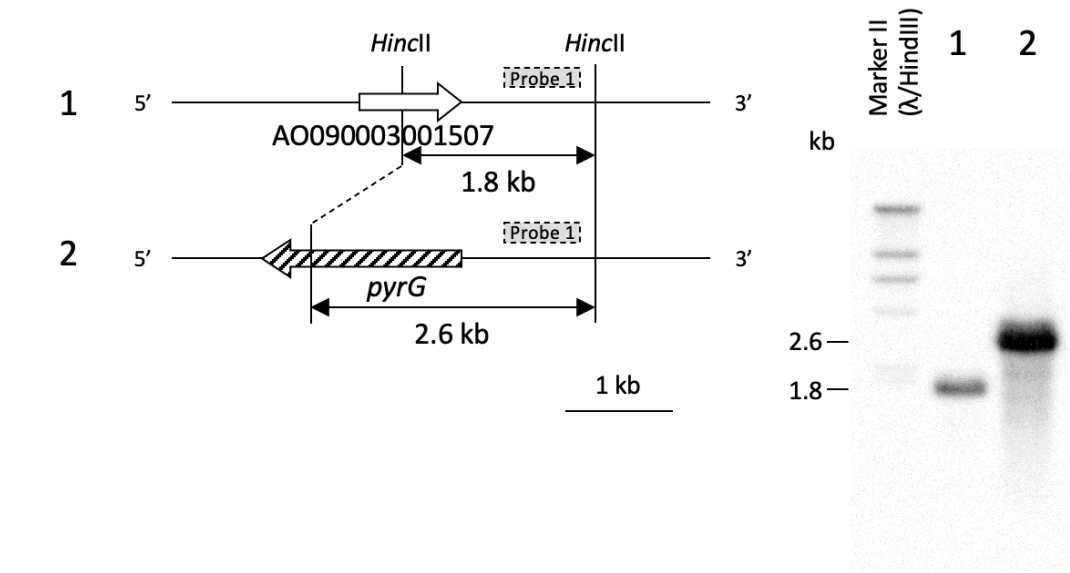

Fig. S5A. Southern hybridization analysis of AO090003001507 disruptant constructed from AHU 7139. 1: AHU 7139; 2: AO090003001507 disruptant.

B

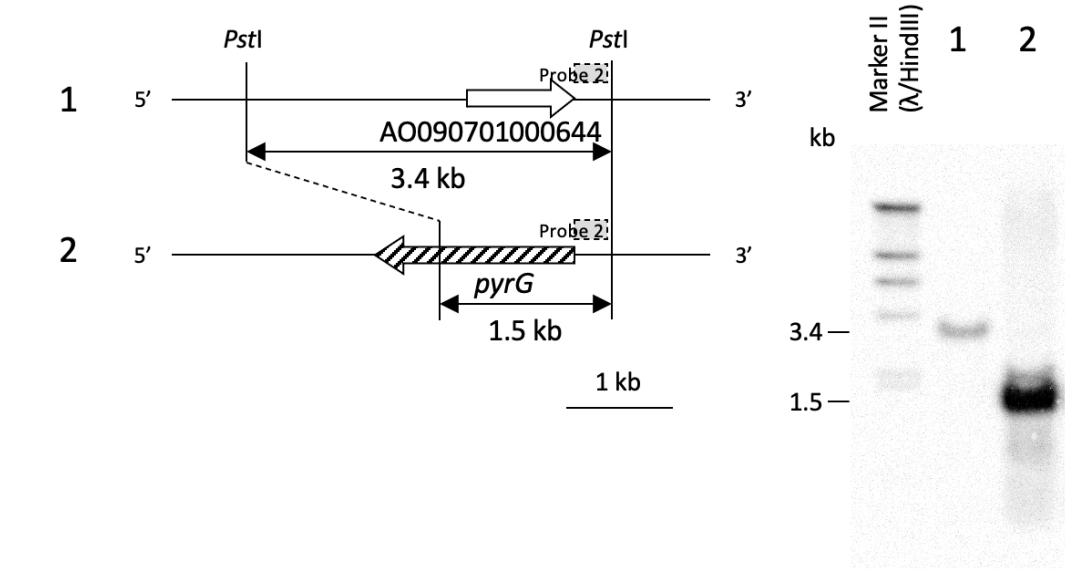

Fig. S5B. Southern hybridization analysis of AO090701000644 disruptant constructed from AHU 7139. 1: AHU 7139; 2: AO090701000644 disruptant.

C

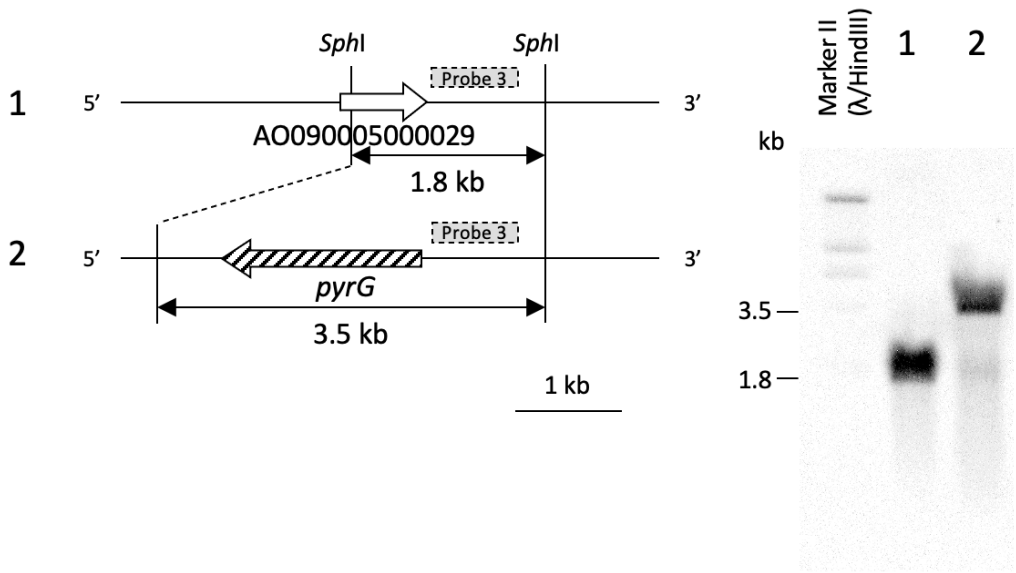

Fig. S5C. Southern hybridization analysis of AO090005000029 disruptant constructed from AHU 7139. 1: AHU 7139; 2: AO090005000029 disruptant.

D

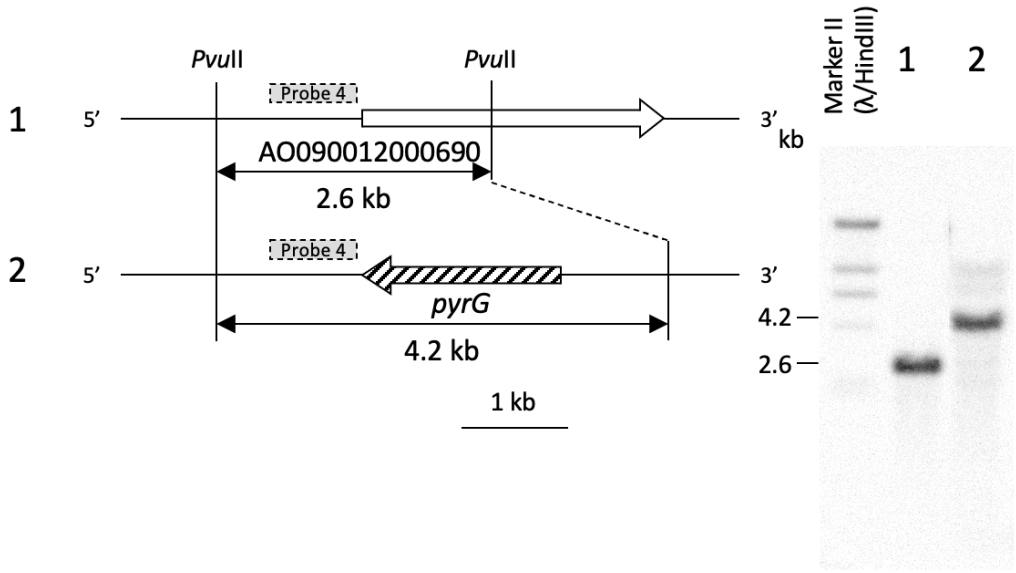

Fig. S5D. Southern hybridization analysis of AO090012000690 disruptant constructed from AHU 7139. 1: AHU 7139; 2: AO090012000690 disruptant.

E

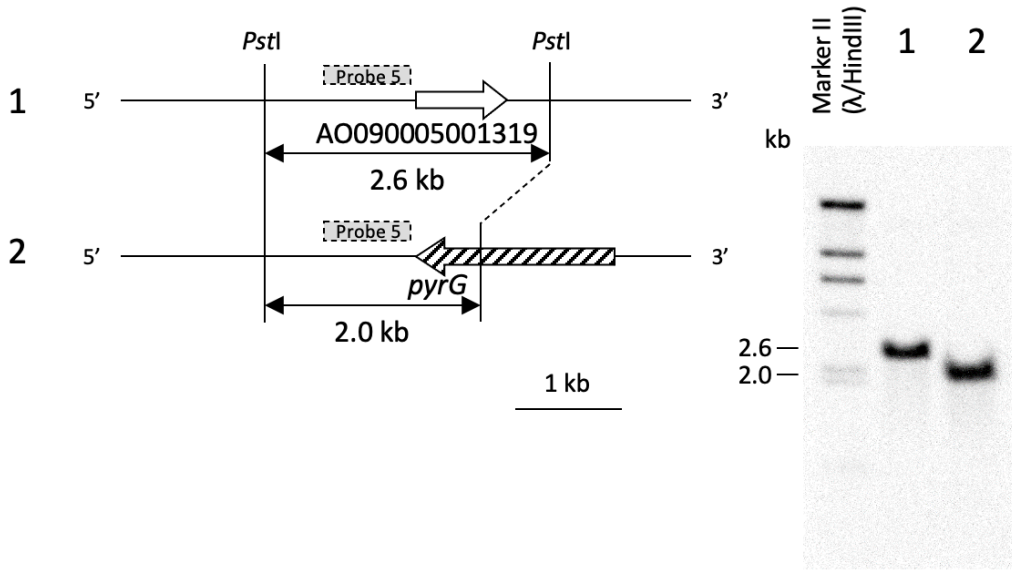

Fig. S5E. Southern hybridization analysis of AO090005001319 disruptant constructed from AHU 7139. 1: AHU 7139; 2: AO090005001319 disruptant.

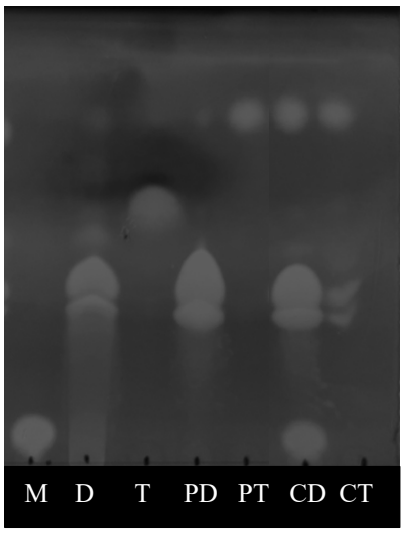

Fig. S6. Thin layer chromatography of the purified lipids. M = control monoglyceride, D = control diglyceride, T = control triglyceride (tributyrin), PD = purified commercial diolein using silica gel chromatography, PT = purified commercial triolein using silica gel chromatography, CD = commercial diolein, CT = commercial triolein. Migration of the sample on the silica gel plate was carried out using chloroform : methanol : formic acid : DI water (45:20:2.5:1), stained with 0.05% primulin dissolved in acetone : water (4:1 v/v), and observed under UV light.

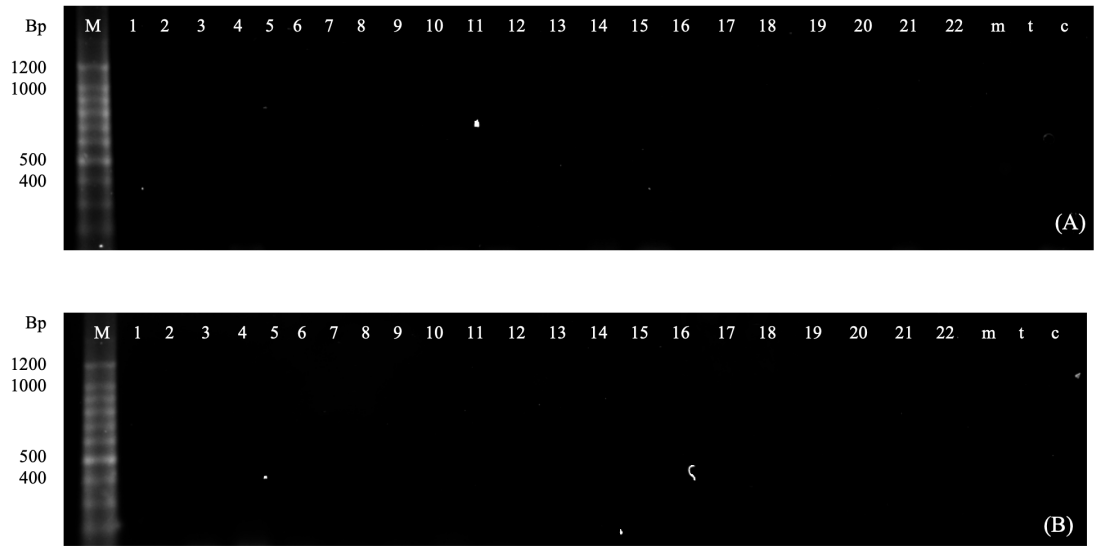

Fig. S7. Agarose gel (1%) electrophoresis of PCR using mRNA from CP with initial pH 4.0 (A) and pH 6.5 (B) as a template with all candidate lipase gene primers. M = DNA ladder marker, lane no.1-22 = annotated lipase no. 1-22, m = *mdlB*, t = *tglA*, and c = *cutL* gene. Confirming no gDNA in the mRNA sample.

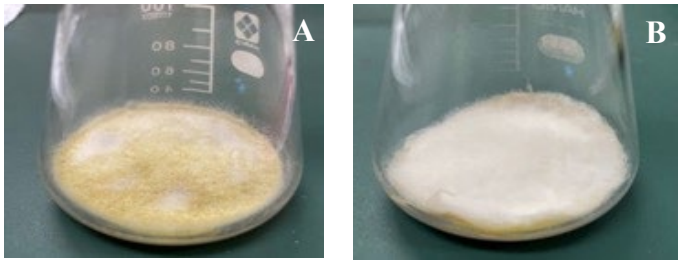

Fig. S8. Growth and appearance of *A. oryzae* AHU 7139 on the WPC media incubated at 20°C for 7 days. The initial culture pH were of (A) pH 4.0 and (B) pH 6.5.