Accelerated Alcoholic Fermentation of Intact Grapes by Saccharomyces Cerevisiae in Symbiosis with Microbial Community Inhabiting Grape-skin

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Accelerated alcoholic fermentation of intact grapes by *Saccharomyces cerevisiae* in symbiosis with microbial community inhabiting grape-skin

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Abstract

Saccharomyces cerevisiae, an essential player in alcoholic fermentation during winemaking, is rarely found in intact grapes. Here, we addressed symbiotic interactions between *S. cerevisiae* and grape-skin residents upon spontaneous wine fermentation. When glucose was used as a carbon source, the yeast-like fungus *Aureobasidium pullulans*, a major grape-skin resident, had no effect on alcoholic fermentation by *S. cerevisiae*. In contrast, when intact grape berries as a sole carbon source, coculture of *S. cerevisiae* and *A. pullulans* accelerated alcoholic fermentation. Thus, grape-inhabiting microorganisms may increase carbon availability by degrading and/or incorporating grape-skin materials, such as cell wall and cuticles. *A. pullulans* exhibited broad spectrum assimilation of plant-derived carbon sources, including ω-hydroxy fatty acids, arising from degradation of cutin. In fact, yeast-type cutinase was produced from *A. pullulans* EXF-150 strain. The degradation and utilization of grape-skin materials by fungal microbiota may account for their colonization on grape-skin and symbiotic interactions with *S. cerevisiae*.

Introduction

The origin of wine yeasts, taxonomically categorized into *Saccharomyces cerevisiae* or its closely related species, has been an issue of great controversy. Historically, wine yeasts were first found on grape surfaces[1], whereas *Saccharomyces* species are absent or rare in fresh grape berries[2–4]. Resident microbiota in intact grapes predominantly consists of the yeast-like ascomycetous fungi *Aureobasidium pullulans* and basidiomycetous yeasts, such as *Cryptococcus*, *Rhodosporidium*, *Rhodotorula*, and *Sporobolomyces* (a.k.a. *Sporidiobolus* as teleomorphic forms) species, which are irrelevant to winemaking due to their lack of alcoholic fermentation ability[3–5]. Assuming that *Saccharomyces* yeast species are vectored by insects or migratory birds[6,7], the first colonist wine yeasts must endure and adapt in vineyard environments, nutritionally undesirable for survival and proliferation.

The grape-skin, the natural habitat for non-*Saccharomyces* oligotrophic microorganisms, covers and protects the pulp for nutrient storage[8,9]. The grape-skin occupies approximately 10% of the dry weight of grape berries and acts as a barrier against dehydration, physical damage, and microbial penetration. The plant primary cell wall components, cellulose, hemicellulose, and pectin, contribute to the structural integrity. The grape-skin is particularly abundant in pectinic acid, the methyl-esterified form of polygalacturonic acid[10,11]. As previously reported, many grape-inhabiting microorganisms secrete cellulase, pectinase, and relevant degrading enzymes to use the cell wall decomposition products as nutrients[12–15]. The microbial targeting ability to degrade and assimilate the plant cell wall compounds is likely a prerequisite for colonization and adaptation to vineyard environments. Wine yeasts of the *Saccharomyces* genus may need the aid of grape-skin residents to survive on the surface of grapes due to

the absence of degrading or metabolizing enzymes for plant cell wall and decomposition products[16,17].

The outermost layer of grape-skin is the cuticle, mainly composed of the lipid polyester cutin (i.e., ester-linked ω-hydroxy C_{16} and C_{18} fatty acids) and the chemically highly resistant biopolymer cutan[18,19]. On all aerial surfaces of land plants, thick and hydrophobic cuticle layers prevent desiccation, UV damage, and pathogen infection. At the frontline, the plant cuticle also serves multifunctional roles in triggering immunity during plant-pathogen interactions[20]. Thus, cuticle-degrading virulent pathogens can cause severe damage to terrestrial plants. The cutin-hydrolyzing enzyme cutinase has been extensively studied in typical plant pathogens of *Fusarium* species and in a limited number of molds, yeasts, and bacteria[21–23]. However, the degradation and assimilation of the plant cuticle compounds by non-pathogenic, grape-skin microorganisms are yet to be described.

This study proposed a novel tripartite relationship between grape berries, grape-skin microbiota, and *S. cerevisiae* during alcoholic fermentation. Similar plant-microbial interactions may typically occur in traditional production processes of fermented foods and beverages, where humans have optimized the growth of characteristic microbial communities over thousands of years[24]. Thus, this study provides an important clue to understand the dynamics and mechanism of plant-microbial ecosystems by analyzing the origin of wine fermentation as an experimentally tractable model.

Results

Isolation of yeasts and related microorganisms inhabiting grape-skin. Among chloramphenicol-tolerant, yeast-like colonies isolated from grape juice, surface-washed

suspensions, or enrichment cultures, 76 clones were identified to species level by sequencing the internal transcribed spacer (ITS) region of the nuclear rRNA gene (Supplementary Table S1). Isolated microorganisms were classified as yeast-like fungi, basidiomycetous yeasts, or ascomycetous yeasts. The yeast-like fungi included a single ascomycetous species *A. pullulans*[25–27], also known as "black yeast", frequently isolated from grape and wine environments in previous reports[3–5]. On nutrient-rich yeast extract-peptone-dextrose (YPD) medium, *A. pullulans* earlier produced smooth pale-pink colonies, which developed protrusions after long-term cultivation (Fig. 1a). Due to melanin-like pigment accumulation, *A. pullulans* cells were easily observed on the skin of spoiled grape berries (Fig. 1b). The isolated basidiomycetous yeasts mainly consisted of *Sporobolomyces* and *Papiliotrema* (formerly categorized as *Cryptococcus*) species[3–5]. Additionally, *Rhodotorula mucilaginosa* is a common ecological basidiomycete found in soil, air, water, and foods[26,28]. *Hanseniaspora uvarum*[26,29] was most frequently isolated from the wine grape variety, Pinot noir, among the ascomycetous yeast species. Several other ascomycetes were assigned to *Candida* (as anamorphic status) or *Pichia* genus. In most cases, every grape sample tested in this study contained a few yeasts or associated fungal species, excluding *Saccharomyces cerevisiae*. Thus, grape environments may be greatly suitable for the colonization and development of non-*Saccharomyces* oligotrophic fungal microbiota. Yeast-like fungus *A. pullulans*, three basidiomycetous yeast species (*Papiliotrema laurentii*, *Sporidiobolus pararoseus*, and *R. mucilaginosa*), and five ascomycetous yeast species (*H. uvarum*, *Torulaspora delbrueckii*, *Meyerozyma caribbica*, *Debaryomyces hansenii*, and *Pichia terricola*) were used as representative grape-skin microorganisms for further comparative analysis (Fig. 2).

Alcoholic fermentation of glucose by grape-skin fungi and *S. cerevisiae*. To assess alcoholic fermentation performance of the grape-skin fungi and *S. cerevisiae*, each microorganism was statically incubated in a 5-mL liquid yeast nitrogen base (YNB) medium, synthetic minimum medium for laboratory yeast strains, supplemented with 10% (w/v) glucose as a sole carbon source. As all species grew well in this medium (Supplementary Fig. S1; optical density of 600 nm (OD_{600}) > 1), carbon dioxide emission rate, glucose consumption, and ethanol production were quantified during the 6-d fermentation test (Fig. 3). *S. cerevisiae* exhibited a robust peak of fermentation rate and full glucose consumption to yield approximately 5% (v/v) ethanol. In contrast, yeast-like fungus *A. pullulans*, basidiomycetous yeasts, *P. laurentii*, *S. pararoseus*, *R. mucilaginosa*, and ascomycetous yeast *D. hansenii* showed a constant basal level of carbon dioxide emission, no detectable ethanol production, and little glucose consumption, and was categorized as non-fermenting species. The other ascomycetous yeasts, *H. uvarum*, *T. delbrueckii*, *M. caribbica*, and *P. terricola*, with intermediate phenotypes, were categorized as weak-fermenting species.

In the coculture experiment in 50-mL YNB medium containing 10% (w/v) glucose as a sole carbon source, the non-fermenting species *A. pullulans* displayed no significant interaction with *S. cerevisiae* in alcoholic fermentation (Fig. 4a). Notably, the coculture of *A. pullulans* and *S. cerevisiae* had no synergistic effect on the growth of both species (Supplementary Fig. S2).

Alcoholic fermentation of intact grapes by nonfermentative, grape-skin fungi and *S. cerevisiae*. In 50-mL YNB minimum medium plus 50-g intact grape berries as a sole carbon source (Fig. 4b), *A. pullulans* generated almost no carbon dioxide, whereas *S.*

cerevisiae slowly progressed alcoholic fermentation, reaching the maximum carbon emission rate 4 d after inoculation. Since robust peaks of fermentation rates were typically observed within 1−2 d after inoculation in the presence of glucose (Figs. 3a and 4a), grape-skin may function as a physical barrier against *S. cerevisiae* cells to protect fermentable sugars, such as glucose, fructose, and sucrose, inside grape berries.

When *A. pullulans* and *S. cerevisiae* were co-cultured, the initial carbon emission rate increased, and a maximum fermentation rate was observed at 3 d (Fig. 4b). Considering the minor contribution of *A. pullulans* to alcoholic fermentation and *S. cerevisiae* growth, *A. pullulans* may specifically contribute to an increase in sugar availability for *S. cerevisiae*, leading to accelerated alcoholic fermentation. Coculture of *S. cerevisiae* with the nonfermentative basidiomycetous yeast, *P. laurentii*, *S. pararoseus*, or *R. mucilaginosa*, gave similar results (Supplementary Fig. S3). These data suggest that *S. cerevisiae* cells make use of the ability of grape-skin microbiota to access fermentable sugars in grape berries upon triggering spontaneous wine fermentation.

Carbon assimilation profiles of grape-skin fungi and *S. cerevisiae*. To characterize carbon assimilation profile, *A. pullulans* and *S. cerevisiae* cells were cultivated in a YNB minimum medium with various possible carbon sources in grape berries (Fig. 5). Both *A. pullulans* and *S. cerevisiae* vigorously grew in the presence of fermentable sugars, such as glucose and sucrose (Fig. 5a, b). A major part of grape-skin consists of plant cell wall polymers, cellulose and pectin[10]. Although carboxymethyl cellulose (CMC), a water-soluble cellulose derivative, was used by neither *A. pullulans* nor *S. cerevisiae*, the main cellulose degradation product cellobiose was assimilated by *A. pullulans* (Fig. 5c, d). As previously reported[30], the activity of carboxymethyl cellulase (i.e., CMCase) is deficient

among some *A. pullulans* strains. The *A. pullulans* cells identified in this study may be unable to degrade cellulose by themselves but can grow by using cellobiose produced through cellulose degradation by other grape-skin fungi. The major pectic polysaccharide polygalacturonic acid and its building block galacturonic acid were assimilated by *A. pullulans*, but not by *S. cerevisiae* (Fig. 5e, f). Thus, *A. pullulans* likely degrades and assimilates a broad spectrum of plant cell wall-relevant materials, unmetabolized by *S. cerevisiae*, consistent with previous reports[12–15,26,27].

The plant cuticle, the outermost hydrophobic layer, is another major component of grape-skin[10]. Cutin in the plant cuticle is a polyester of ω-hydroxy C_{16} and C_{18} fatty acids and their derivatives. We discovered that *A. pullulans* exhibited weak but reproducible growth using ω-hydroxypalmitic acid as a sole carbon source (Fig. 5g). Since neither palmitic acid or ω-carboxypalmitic acid was assimilated (Fig. 5h, i), *A. pullulans* may possess a utilization system specific for ω-hydroxy fatty acids. Besides *A. pullulans*, three basidiomycetous yeasts, *P. laurentii*, *S. pararoseus*, and *R. mucilaginosa*, and two ascomycetous yeasts, *M. caribbica* and *D. hansenii*, showed significant growth in the presence of ω-hydroxy palmitic acid (Fig. 6). Notably, most species described above were nonfermentative microorganisms.

Cutinase-like esterase activity in *A. pullulans*. The secretion of cutin-degrading enzymes in *A. pullulans*, basidiomycetous yeasts, and *S. cerevisiae* was tested using a model polyester polycaprolactone (PCL)-plate (Fig. 7a). Known cutinases from the other species represent PCL degradation activity in previous studies[31–33]. The supernatant of the fully grown *A. pullulans* culture in YNB medium plus 2% glucose formed a clear halo, although no halo was observed using the other supernatant samples. Additionally, the

supernatant of the *A. pullulans* culture exhibited higher esterase activity toward *p*-nitrophenyl butyrate (*p*NPB) and *p*-nitrophenyl palmitate (*p*NPP) used as substrates than the supernatant of the *S. cerevisiae* culture (Fig. 7b). These data suggest that the isolated *A. pullulans* strain from grapes secretes cutinase to assist penetration into plant cuticles.

Previous whole-genome analysis of the *A. pullulans* EXF-150 strain[34] revealed nine candidate genes encoding cutinase-like enzymes, designated as ApCut1 to ApCut9 (Supplementary Fig. S4). These gene products contained a classical α/β-hydrolase catalytic triad Ser-His-Asp and a Gly-Tyr-Ser-Gln-Gly (GYSQG) motif conserved among cutinase catalytic sites[22,27]. Additionally, two pairs of cysteine residues forming disulfide bonds, important for spatial conformation[22,35], were also conserved in all cutinase candidates except for ApCut4, in which the amino terminus is truncated. Phylogenetic analysis indicated that ApCut1 to ApCut3 form a subgroup with yeast cutinases from *A. adeninivorans* and *Cryptococcus* sp. S-2[31,33], while ApCut5 to ApCut9 form a subgroup with mold cutinases from *F. solani*, *A. oryzae*, and *B. cinerea* (Fig. 8a)[36–38]. Whole-cell lysate of *E. coli* expressing recombinant ApCut1 indicated cutinase-like activity, based on the PCL-plate clearing assay and the *p*NPB hydrolysis assay (Fig. 8b−d). Also, the extracts of ApCut2- or ApCut3-expressing *E. coli* cells weakly degraded PCL (Supplementary Fig. S5). Since the expression of ApCut2 and ApCut3 was almost undetectable in the coomassie brilliant blue (CBB)-stained gel, more attention should be paid to the protease sensitivity, expression conditions, and synonymous codon usage bias. These results revealed the activity of the yeast-type cutinase isoenzymes in *A. pullulans*.

Discussion

Based on the experimental data, we propose that the grape-skin-resident microorganisms, including nonfermentative, yeast-like fungus *A. pullulans*, increase the accessibility to fermentable sugars in intact grape berries by degrading and assimilating the plant cell wall, cuticle compounds, or both. Grape-skin microbiota's high and versatile abilities to degrade and assimilate plant cell wall and/or cuticle is likely to be essential for their adaptation and proliferation on grape-skin. In contrast, *S. cerevisiae* cells without such abilities need the aid of grape-skin microbiota to survive in grape environments. Studies of wild populations of *S. cerevisiae* and its closest relative *Saccharomyces paradoxus* suggest that woodlands or primeval forests are natural habitats for these yeast species[39–41]. In this study, no *S. cerevisiae* clone was isolated even from fermented juice or enriched cultures in 5% sucrose. Moreover, *S. cerevisiae* was unable to assimilate plant cell wall, cuticle, and their components. Altogether, nutrient-poor, intact grape surfaces may be inappropriate for wine yeasts as their stable and permanent habitats. Although *Saccharomyces* species might have been accidentally brought to the vineyard by yeast-carrier animals[6,7], the primary cause of wine yeasts still needs to be experimentally explored. Our results provide an important clue to address how *S. cerevisiae* cells met and conquered grapes upon the origin of spontaneous wine fermentation.

What are the key grape-skin compounds that protect fermentable sugars in intact grape berries? Based on the assimilation tests of CMC and cellobiose (Fig. 5c, d), our isolated *A. pullulans* strain lacks cellulase. This is consistent with a previous report about intraspecific variations of cell wall-degrading enzymes in *A. pullulans*[30]. Thus, cellulose degradation may be nonessential for accelerated alcoholic fermentation of intact grapes. Among pectin-degrading enzymes, polygalacturonase is genetically encoded and

expressed by grape-skin resident species and by *S. cerevisiae*[42,43]. Recently, we revealed the importance of pectin as an initial target for the saprophytic bacterium *Bacillus subtilis* to recognize the surface of dead soybeans[44], whereas it is unlikely that pectin degradation by non-*Saccharomyces* microorganisms specifically accelerated alcoholic fermentation of intact grapes. As shown in Fig. 7a, the PCL-degrading activity was detected in *A. pullulans*, although not in the examined basidiomycetous yeast strains. Based on these data, the responsible enzymatic activity specific and common to all grape-skin residents is still unidentified. The degradation of the other plant cell wall or cuticle components should be focused on in future research. Alternatively, cooperative or synergistic degradation of plant epidermis by whole grape-skin microbiota needs to be investigated. Such complicated, highly ordered microbial interactions at the chemical, metabolic, genetic, and genomic levels will be the central issue in applied microbiology and microbial ecology.

This is the first report on ω-hydroxypalmitic acid assimilation as a carbon source by *A. pullulans* and several yeast species. Although ω-hydroxylation of fatty acids also occurs in mammals and insects, ω-hydroxy fatty acids play a broad and vital biological role in higher plants as major components of cutin and suberin[18–20,45–47]. Thus, the microbial ability to assimilate ω-hydroxy fatty acids may mainly contribute to symbiotic interactions with terrestrial plants. In the ω-oxidation process of animals and plants, known as a minor, fatty acid catabolic pathway, a hydroxy group is introduced onto the ω carbon of the medium to long-chain fatty acids[46,47]. The resultant ω-hydroxy fatty acids are oxidized to ω-carboxy fatty acids (i.e., dicarboxylic fatty acids), further degraded through the β-oxidation pathway. Grape-skin residents may probably metabolize ω-hydroxy fatty acids similarly. Based on our data, *A. pullulans* cells are suggested to have

an ω-hydroxy fatty acid-specific transporter because they can grow using ω-hydroxypalmitic acid as a sole carbon source not palmitic acid or ω-carboxypalmitic acid. Furthermore, this study revealed that *A. pullulans* may secrete cutinase to hydrolyze cutin into ω-hydroxy fatty acids and other minor components, such as glycerol. The combination of cutin degradation and ω-hydroxy fatty acid assimilation may characterize *A. pullulans* as the most abundant and persistent resident among grape-skin microbiota. Notably, the *A. pullulans* EXF-150 strain was first identified as a microorganism that acquired both yeast- and mold-type cutinase genes in the genome, which may be associated with the yeast-to-hyphal dimorphic transition of this species[25]. Enzymatic analysis of yeast- and mold-type cutinases in *A. pullulans* will reveal the significance of differences between both cutinase types.

Conclusively, this study focused on the symbiotic relationship between grape-skin microbiota and *S. cerevisiae* from the perspective of winemaking origin. Oligotrophic microorganisms, such as *A. pullulans*, have developed versatile abilities to use plant cell wall polysaccharides and plant cuticular lipids as nutrient sources to establish their ecological niche. Especially, degradation and assimilation of the plant cuticle, the outermost layer interacting with the environment, may be a prerequisite for oligotrophic resident microorganisms to trigger colonization and adaptation. In contrast, eutrophic yeasts, including *S. cerevisiae*, yield energy through the alcoholic fermentation of sugars in grape berries with the aid of oligotrophic microorganisms. Such tripartite interaction between grape berries, oligotrophic residents, and eutrophic yeasts determines the grape-skin microbiome dynamics and spontaneous wine fermentation. Thus, studying the origins and microbiota ecological succession in fermented foods will help elucidate the key principles governing plant-microbial ecosystems' emergence and development.

Materials

Materials and strains. To isolate yeasts or yeast-like fungi, (i) juice, (ii) surface-washed suspensions, or (iii) enrichment cultures were obtained from commercially available wine or table grape varieties belonging to *Vitis vinifera* species or *Vitis* interspecific hybrids (see Supplementary Table S1). Grape juice was obtained from freshly pressed grape berries using a food-grade juicer (Panasonic, Japan) and was used either immediately or after being incubated at 30°C for 3 d. Surface-washed suspensions were obtained by vigorously shaking the flask containing approximately 50-g grape berries and 25-mL sterilized water at 30°C for 15 min. Enrichment cultures were obtained by statically incubating the flask containing approximately 10-g grape berries and 40-mL sterilized water or 5% (w/v) sucrose at 30°C for 3 d. Each sample was spread on a nutrient-rich,YPD (1% yeast extract, 2% peptone, and 2% glucose) medium plate with 0.1% chloramphenicol to inhibit bacterial growth, and was incubated at 30°C. Single colonies representing yeast-like colony morphology were isolated by repeatedly streaking on YPD medium plates with 0.1% chloramphenicol. The isolated clones were identified by DNA sequencing of the rRNA gene ITS region, using the ITS_1F (5'-GTAACAAGGTYTCCGT-3') and ITS_1R (5'-CGTTCTTCATCGATG-3') primer pair and genomic DNA as PCR templates. The *S. cerevisiae* X2180 strain, obtained from the American Type Culture Collection (USA), was used for alcoholic fermentation tests or other control experiments.

Fermentation test. Cells were aerobically precultured at 30°C for 2 d in 0.67% YNB minimum medium containing 2% (w/v) glucose as a carbon source and harvested. For the alcoholic fermentation of glucose, cells were inoculated into a YNB medium containing

10% (w/v) glucose at a final OD_{600} of 0.1 and were then further incubated at 30°C without shaking. For the alcoholic fermentation of intact grapes, cells were inoculated into 50-mL YNB medium at a final OD_{600} of 0.1, mixed with approximately 50-g commercially available intact grape berries of Green Seedless, and were then further incubated at 30°C without shaking. Fermentation was continuously monitored by measuring the weight loss of evolved carbon dioxide for 5-mL test tube-scale tests or using a Fermograph II apparatus (Atto, Japan) for 50-mL-scale coculture tests. Glucose and ethanol concentrations were determined using the LabAssay glucose kit (Fujifilm Wako Pure Chemical, Japan) and the ethanol assay F-kit (Roche, Switzerland), respectively.

Carbon assimilation test. Cells were aerobically precultured at 30°C for 2 d in a YNB minimum medium containing 2% (w/v) glucose as a carbon source, harvested, washed by sterilized water, and inoculated into a YNB medium containing 0.5% (w/v) of carbon sources as below: glucose, sucrose, CMC, cellobiose, polygalacturonic acid, galacturonic acid, palmitic acid, 16-hydroxyhexadecanoic acid (i.e., ω-hydroxypalmitic acid), or heptadecanedioic acid (i.e., ω-carboxypalmitic acid). Upon inoculation, initial OD_{600} was adjusted to 0.1. For assimilation tests of palmitic acid, ω-hydroxypalmitic acid, or ω-carboxypalmitic acid, 0.05% (w/v) Tween 40 was added to the medium. In advance of measurement of OD_{600}, insoluble fatty acids were removed by washing pellets with hexane three times, and cells were dissolved in sterilized water[48].

Phylogenetic analysis of yeast and mold cutinases. Amino acid sequences of nine putative cutinase gene products in the *A. pullulans* EXF-150 strain and representative yeast and mold cutinases [Cut2 from *Arxula adeninivorans* (AaCut2), Cle1 from

Cryptococcus sp. strain S-2 (CS2Cle1), Cut1 from *Fusarium solani* (FsCut1), CutL from *Aspergillus oryzae* (AoCutL), and CutA from *Botrytis cinerea* (BcCutA)] were obtained from UniPlotKB. Multiple sequence alignment was conducted using Clustal Omega program, and the phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis v.10.2.2. The putative cutinase genes of *A. pullulans* were designated as below: M438DRAFT_340638 as *ApCUT1*, M438DRAFT_351543 as *ApCUT2*, M438DRAFT_352218 as *ApCUT3*, M438DRAFT_388226 as *ApCUT4*, M438DRAFT_264999 as *ApCUT5*, M438DRAFT_267580 as *ApCUT6*, M438DRAFT_341517 as *ApCUT7*, M438DRAFT_347465 as *ApCUT8*, and M438DRAFT_368700 as *ApCUT9*.

Expression of recombinant *A. pullulans* cutinase. The chemically synthesized *ApCUT1* gene (Eurofins, Luxembourg) was cloned into the BamHI-EcoRI site of the pET-21b(+) vector (Merck Millipore, USA) to express recombinant ApCut1p tagged with the T7 epitope at the amino terminus and the 6 × His epitope at the carboxy terminus. The resultant pET-21b(+)-*ApCUT1* plasmid was introduced into *Escherichia coli* BL21(DE3)pLysS cells (Novagen, Germany). A transformant was inoculated into 100-mL Luria-Bertani medium (0.5% yeast extract, 1% tryptone, and 1% sodium chloride) with 100-μg/mL ampicillin and 34-μg/mL chloramphenicol, and was cultured at 37°C to an OD_{600} of 0.6. Isopropyl thio-β-D-galactoside (IPTG) was added to the culture to a final concentration of 1 mM, and cells were further cultured at 37°C for 4 h. Whole-cell extracts were prepared from cell pellets suspended in the xTractor buffer (TakaraBio, Japan), followed by the addition of DNase I. After protein separation by SDS-PAGE, the proteins were detected by CBB staining or using His-Detect In-Gel Stain (Nacalai Tesque,

Japan).

Enzymatic assay. To assay the potential cutinase activity from the culture supernatants of fully grown *A. pullulans* or yeasts, PCL was used as a model polyester substrate. Turbid agar plates containing 0.05% PCL (Fujifilm Wako Pure Chemical, Japan) were prepared as previously described[32,33]. The assay for determining esterase activity was conducted according to previous reports[33,49], using *p*NPB and *p*NPP (Sigma-Aldrich, USA) as substrates.

References

1. Pasteur L. Nouvelles expériences pour démontrer que le germe de la levure qui fait le vin provient de l'extérieur des grains de raisin. *C R Acad Sci* 1872; **75**: 781–793.
2. Mortimer R, Polsinelli M. On the origins of wine yeast. *Res Microbiol* 1999; **150**: 199–204.
3. Barata A, Malfeito-Ferreira M, Loureiro V. The microbial ecology of wine grape berries. *Int J Food Microbiol* 2012; **153**: 243–259.
4. Stefanini I, Cavalieri D. Metagenomic approaches to investigate the contribution of the vineyard environment to the quality of wine fermentation: potentials and difficulties. *Front Microbiol* 2018; **9**: 991.
5. Loureiro V, Ferreira MM, Monteiro S, Ferreira RB. The microbial community of grape berry. In: Gerós H, Chaves MM, Delrot S (eds). *The Biochemistry of the Grape Berry.* Bentham Science: 2012, pp 241–268.
6. Stefanini I, Dapporto L, Legras J-L, Calabretta A, Di Paola M, De Filippo C *et al.* Role of social wasps in *Saccharomyces cerevisiae* ecology and evolution. *Proc Natl Acad Sci U S A* 2012; **109**: 13398–13403.
7. Francesca N, Carvalho C, Sannino C, Guerreiro MA, Almeida PM, Settanni L *et al.* Yeasts vectored by migratory birds collected in the Mediterranean island of Ustica and description of *Phaffomyces usticensis* f.a. sp. nov., a new species related to the cactus ecoclade. *FEMS Yeast Res* 2014; **14**: 910–921.
8. Gao Y, Zietsman AJJ, Vivier MA, Moore JP. Deconstructing wine grape cell walls with enzymes during winemaking: new insights from glycan microarray technology. *Molecules* 2019; **24**: 165.
9. Martínez-Lapuente L, Guadalupe Z, Ayestarán B. Properties of wine polysaccharides.

In: Masuelli M (ed). *Pectins - Extraction, Purification, Characterization and Applications.* IntechOpen: 2019, DOI: 10.5772/intechopen.85629.

10. Lecas M, Brillouet J-M. Cell wall composition of grape berry skins. *Phytochemistry* 1994; **35**: 1241–1243.
11. González-Centeno MR, Rosselló C, Simala S, Garau MC, López F, Femenia A. Physico-chemical properties of cell wall materials obtained from ten grape varieties and their byproducts: grape pomaces and stems. *LWT Food Sci Technol* 2010; **43**: 1580–1586.
12. Biely P, Heinrichová K, Kružiková M. Induction and inducers of the pectolytic system in *Aureobasidium pullulans*. *Curr Microbiol* 1996; **33**: 6–10.
13. Strauss ML, Jolly NP, Lambrechts MG, van Rensburg P. Screening for the production of extracellular hydrolytic enzymes by non-*Saccharomyces* wine yeasts. *J Appl Microbiol* 2001; **91**: 182–190.
14. Merín MG, Mendoza LM, Farías ME, Morata de Ambrosini VI. Isolation and selection of yeasts from wine grape ecosystem secreting cold-active pectinolytic activity. *Int J Food Microbiol* 2011; **147**: 144–148.
15. Úbeda J, Maldonado Gil M, Chiva R, Guillamón JM, Briones A. Biodiversity of non-*Saccharomyces* yeasts in distilleries of the La Mancha region (Spain). *FEMS Yeast Res* 2014; **14**: 663–673.
16. Huisjes EH, Luttik MAH, Almering MJH, Bisschops MMM, Dang DHN, Kleerebezem M *et al.* Toward pectin fermentation by *Saccharomyces cerevisiae*: expression of the first two steps of a bacterial pathway for D-galacturonate metabolism. *J Biotechnol* 2012; **162**: 303–331.
17. Casa-Villegas M, Polaina J, Marín-Navarro, J. Cellobiose fermentation by

Saccharomyces cerevisiae: comparative analysis of intra versus extracellular sugar hydrolysis. *Process Biochem* 2018; **75**: 59–67.

18. Domínguez E, Heredia-Guerrero JA, Heredia A. The biophysical design of plant cuticles: an overview. *New Phytol* 2011; **189**: 938–949.
19. Martin LBB, Rose JKC. There's more than one way to skin a fruit: formation and functions of fruit cuticles. *J Exp Bot* 2014; **65**: 4639–4651.
20. Ziv C, Zhao Z, Gao YG, Xia Y. Multifunctional roles of plant cuticle during plant-pathogen interactions. *Front Plant Sci* 2018; **9**: 1088.
21. Egmond MR, de Vlieg J. *Fusarium solani pisi* cutinase. *Biochimie* 2000; **82**: 1015–1021.
22. Chen S, Su L, Chen J, Wu J. Cutinase: characteristics, preparation, and application. *Biotechnol Adv* 2013; **31**: 1754–1767.
23. Nyyssölä A. Which properties of cutinases are important for applications? *Appl Microbiol Biotechnol* 2015; **99**: 4931–4942.
24. Wolfe BE, Dutton RJ. Fermented foods as experimentally tractable microbial ecosystems. *Cell* 2015; **161**: 49–55.
25. Slepecky RA, Starmer WT. Phenotypic plasticity in fungi: a review with observations on *Aureobasidium pullulans*. *Mycologia* 2009; **101**: 823–832.
26. Varela C, Borneman AR. Yeasts found in vineyards and wineries. *Yeast* 2017; **34**: 111–128.
27. Bozoudi D, Tsaltas D. The multiple and versatile roles of *Aureobasidium pullulans* in the vitivinicultural sector. *Fermentation* 2018; **4**: 85.
28. Wirth F, Goldani LZ. Epidemiology of *Rhodotorula*: an emerging pathogen. *Interdiscip Perspect Infect Dis* 2012; **2012**: 465717.

29. Martin V, Valera MJ, Medina K, Boido E, Carrau F. Oenological impact of the *Hanseniaspora/Kloeckera* yeast genus on wines--a review. *Fermentation* 2018; **4**: 76.
30. Sun P-F, Chien I-A, Xiao H-S, Fang W-T, Hsu C-H, Chou J-Y *et al*. Intraspecific variation in plant growth-promoting traits of *Aureobasidium pullulans*. *Chiang Mai J. Sci.* 2019; **46**: 15–31.
31. Masaki K, Kamini NR, Ikeda H, Iefuji H. Cutinase-like enzyme from the yeast *Cryptococcus* sp. strain S-2 hydrolyzes polylactic acid and other biodegradable plastics. *Appl Environ Microbiol* 2005; **71**: 7548–7550.
32. Murphy CA, Cameron JA, Huang SJ Vinopal RT. *Fusarium* polycaprolactone depolymerase is cutinase. *Appl Environ Microbiol* 1996; **62**: 456–460.
33. Bischoff F, Litwińska K, Cordes A. Three new cutinases from the yeast *Arxula adeninivorans* that are suitable for biotechnological applications. *Appl Environ Microbiol* 2015; **81**: 5497–5510.
34. Gostinčar C, Ohm RA, Kogej T, Sonjak S, Turk M, Zajc J *et al.* Genome sequencing of four *Aureobasidium pullulans* varieties: biotechnological potential, stress tolerance, and description of new species. *BMC Genomics* 2014; **15**: 549.
35. Kodama Y, Masaki K, Kondo H, Suzuki M, Tsuda S, Nagura T *et al.* Crystal structure and enhanced activity of a cutinase-like enzyme from *Cryptococcus* sp. strain S-2. *Proteins* 2009; **77**: 710-717.
36. Soliday CL, Dickman MB, Kolattukudy PE. Structure of the cutinase gene and detection of promoter activity in the 5'-flanking region by fungal transformation. *J Bacteriol* 1989; **171**: 1942–1951.
37. Ohnishi K, Toida J, Nakazawa H, Sekiguchi J. Genome structure and nucleotide sequence of a lipolytic enzyme gene of *Aspergillus oryzae*. *FEMS Microbiol Lett*

1995; **126**: 145–150.

38. van der Vlugt-Bergmans CJ, Wagemakers CA, van Kan JA. Cloning and expression of the cutinase A gene of *Botrytis cinerea*. *Mol Plant Microb Interact* 1997; **10**: 21–29.
39. Sniegowski PD, Dombrowski PG, Fingerman E. *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* coexist in a natural woodland site in North America and display different levels of reproductive isolation from European conspecifics. *FEMS Yeast Res* 2002; **1**: 299–306.
40. Fay JC, Benavides JA. Evidence for domesticated and wild populations of *Saccharomyces cerevisiae*. *PLoS Genet* 2005; **1**: 66–71.
41. Duan S-F, Han P-J, Wang Q-M, Liu W-Q, Shi J-Y, Li K *et al.* The origin and adaptive evolution of domesticated populations of yeast from Far East Asia. *Nat Commun* 2018; **9**: 2690.
42. Gognies S, Gainvors A, Aigle M, Belarbi A. Cloning, sequence analysis and overexpression of a *Saccharomyces cerevisiae* endopolygalacturonase-encoding gene (*PGL1*). *Yeast* 1999; **15**: 11–22.
43. Radoi F, Kishida M, Kawasaki H. Endo-polygalacturonase in *Saccharomyces* wine yeasts: effect of carbon source on enzyme production. *FEMS Yeast Res* 2005; **5**: 663–668.
44. Sugiura H, Nagase A, Oiki S, Mikami B, Watanabe D, Hashimoto W. Bacterial inducible expression of plant cell wall-binding protein YesO through conflict between *Glycine max* and saprophytic *Bacillus subtilis*. *Sci Rep* 2020; **10**: 18691.
45. Kolattukudy PE. Biopolyester membranes of plants: cutin and suberin. *Science* 1980; 208: 990–1000.

46. Miura Y. The biological significance of ω-oxidation of fatty acids. *Proc Jpn Acad Ser B Phys Biol Sci* 2013; **89**: 370–382.
47. Wertz PW. Naturally occurring ω-hydroxyacids. *Int J Cosmet Sci* 2018; **40**: 31–33.
48. Nakagawa T, Imanaka T, Morita M, Ishiguro K, Yurimoto H, Yamashita A *et al*. Peroxisomal membrane protein Pmp47 is essential in the metabolism of middle-chain fatty acid in yeast peroxisomes and is associated with peroxisome proliferation. *J Biol Chem* 2000; **275**: 3455–3461.
49. Chen S, Tong X, Woodard RW, Du G, Wu J, Chen J. Identification and characterization of bacterial cutinase. *J Biol Chem* 2008; **283**: 25854–25862.

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Author contributions

W.H. designed the study. D.W. performed the experiments. D.W. and W.H. analyzed the data. D.W. and W.H. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Figure legends

Fig. 1 *A. pullulans* on grape-skin. a Colony morphology of an *A. pullulans* strain isolated from Delaware grape berries in this study. Cells were streaked on YPD plate medium with 0.1% chloramphenicol and were incubated at 37°C for 3 d. Bar, 5 mm. **b** *A. pullulans* cells were observed on the surface of a spoiled Pinot noir grape berry. Arrows indicate typical *A. pullulans* cells characterized by accumulation of melanin pigment. Bar = 10 μm.

Fig. 2 Representative grape-skin residents isolated in this study. Microscopic images of the nine species of grape-skin residents and *S. cerevisiae* (*Sc*). Grape-skin residents include one yeast-like fungus *A. pullulans* (*Ap*), three basidiomycetous yeasts, *P. laurentii* (*Pl*), *S. pararoseus* (*Sp*), and *R. mucilaginosa* (*Rm*), and five ascomycetous yeasts, *H. uvarum* (*Hu*), *T. delbrueckii* (*Td*), *M. caribbica* (*Mc*), *D. hansenii* (*Dh*), and *P. terricola* (*Pt*). Bars = 10 μm.

Fig. 3 Alcoholic fermentation of glucose by grape-skin residents and *S. cerevisiae*. a Carbon dioxide emission rate during 6-d alcoholic fermentation in YNB medium containing 10% (w/v) glucose. **b** Glucose consumption (orange) and ethanol production (yellow) during 6-d alcoholic fermentation in YNB medium containing 10% (w/v) glucose. Data represent mean values and standard deviations from three independent experiments. *Ap*, *A. pullulans*; *Pl, P. laurentii*; *Sp*, *S. pararoseus*; *Rm*, *R. mucilaginosa*; *Hu*, *H. uvarum*; *Td*, *T. delbrueckii*; *Mc*, *M. caribbica*; *Dh*, *D. hansenii*; *Pt*, *P. terricola*; *Sc*, *S. cerevisiae*.

Fig. 4 Alcoholic fermentation of glucose or intact grapes in coculture of *A. pullulans* and *S. cerevisiae*. Carbon dioxide emission rate (upper) and total carbon dioxide emission (lower) during 6-d alcoholic fermentation in YNB medium containing 10% (w/v) glucose (**a**) or in a mixture of an equal weight of YNB medium and intact grape berries (**b**). Data represent mean values and standard deviations from three independent experiments. *Ap*, inoculated with *A. pullulans* (red); *Sc*, inoculated with *S. cerevisiae* (pink); *Ap* + *Sc*, coinoculated with *A. pullulans* and *S. cerevisiae* (violet). Asterisks indicate statistically significant increases in carbon dioxide emission compared with *Sc* (*t*-test, $p < 0.05$).

Fig. 5 Assimilation of possible carbon sources in grape berries by *A. pullulans* and *S. cerevisiae*. Graphs indicate growth curves of *A. pullulans* (*Ap*, red) and *S. cerevisiae* (*Sc*, pink) in YNB medium containing 0.5% glucose (**a**), sucrose (**b**), CMC (**c**), cellobiose (Cel, **d**), polygalacturonic acid (polyGalUA, **e**), galacturonic acid (GalUA, **f**), ω-hydroxypalmitic acid (C16:0-ωOH, **g**), palmitic acid (C16:0, **h**), ω-carboxypalmitic acid (C16:0-ωCOOH, **i**), or no carbon source (**j**). Data represent mean values and standard deviations from three independent experiments. Asterisks indicate statistically significant increases in carbon dioxide emission compared with *Sc* (*t*-test, $p < 0.05$).

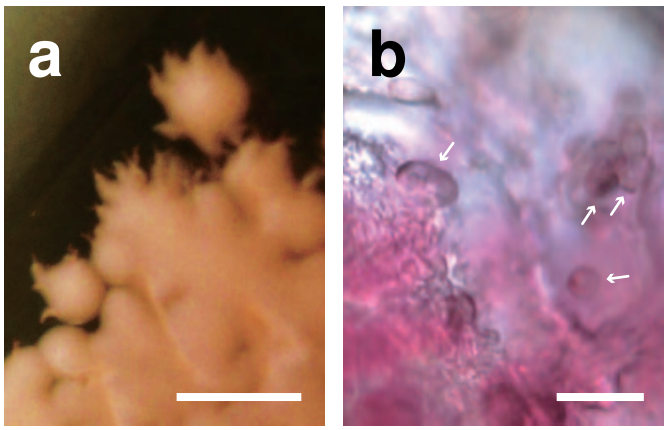
Fig. 6 Assimilation of ω-hydroxypalmitic acid by grape-skin residents and *S. cerevisiae*. The graph indicates OD_{600} values after 24-h incubation in YNB medium containing 0.5% ω-hydroxypalmitic acid. A red dashed line shows the initial OD_{600} value (OD_{600} = 0.1). Data represent mean values and standard deviations from three independent experiments. *Ap*, *A. pullulans*; *Pl, P. laurentii*; *Sp*, *S. pararoseus*; *Rm*, *R. mucilaginosa*; *Hu*, *H. uvarum*; *Td*, *T. delbrueckii*; *Mc*, *M. caribbica*; *Dh*, *D. hansenii*; *Pt*,

P. terricola; *Sc*, *S. cerevisiae*.

Fig. 7 Cutinase-like activity of the culture supernatants of grape-skin residents and *S. cerevisiae*. **a** PCL-plate clearing assay. **b** *p*NPB and *p*NPP hydrolysis assay. The supernatants were obtained from fully grown 5-d cultures in a YNB medium containing 2% (w/v) glucose. Data represent mean values and standard deviations from three independent experiments. *Ap*, *A. pullulans*; *Pl, P. laurentii*; *Sp*, *S. pararoseus*; *Rm*, *R. mucilaginosa*; *Sc*, *S. cerevisiae*. Asterisks indicate statistically higher specific activity than *Sc* (*t*-test, $p < 0.05$).

Fig. 8 Cutinase-like activity of recombinant ApCut1. **a** A phylogenetic tree of *A. pullulans* cutinase-like gene products. The number corresponds to each cutinase-like gene product in *A. pullulans*. ApCut1–ApCut3 (magenta) belongs to the yeast cutinase family, while ApCut5 to ApCut9 (cyan) belong to the mold cutinase family. Bar, 0.2 substitutions per nucleotide position. **b** Expression of recombinant ApCut1 in *E. coli*. Left and right panels indicate CBB-stained gel and His-detect-stained gel for specific detection of His-tagged proteins, respectively. **c** PCL-plate clearing assay. Bar, 1 cm. **d** *p*NPB hydrolysis assay. Data represent mean values and standard deviations from three independent experiments. An asterisk indicates a statistically higher specific activity than the negative control (IPTG (-), *t*-test, $p < 0.05$). EV, empty vector.

Fig. 1



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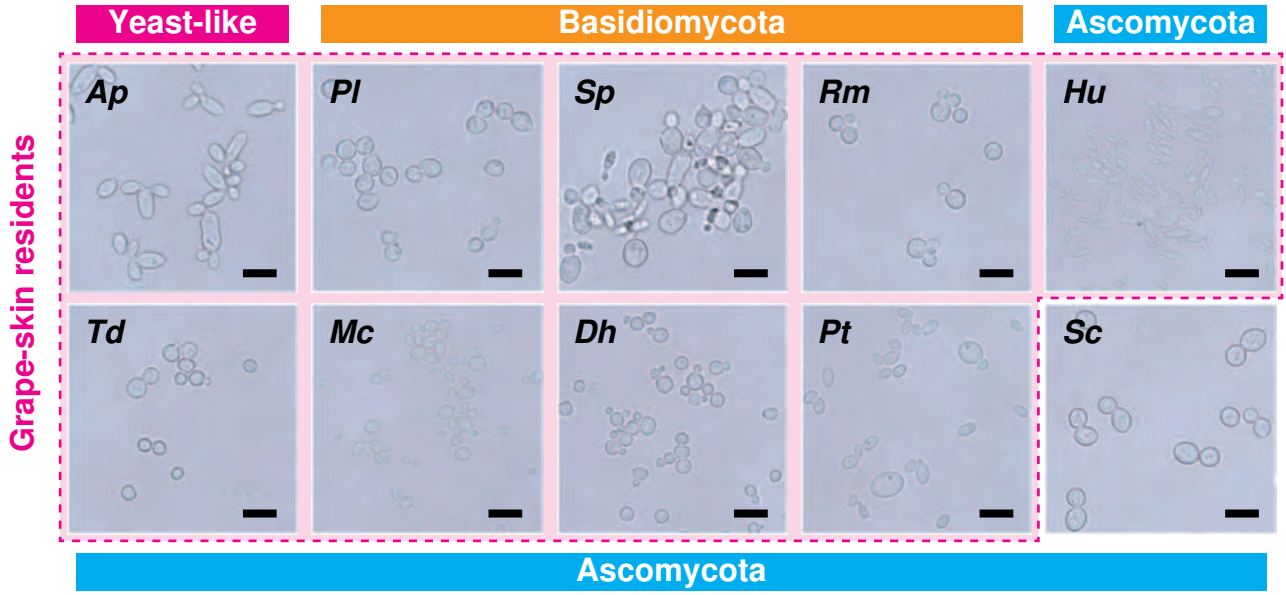
Yeast-like
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Ascomycota
Grape-skin residents
Ap
Pl
Sp
Rm
Hu
Td
Mc
Dh
Pt
Sc
Ascomycota

Fig. 3

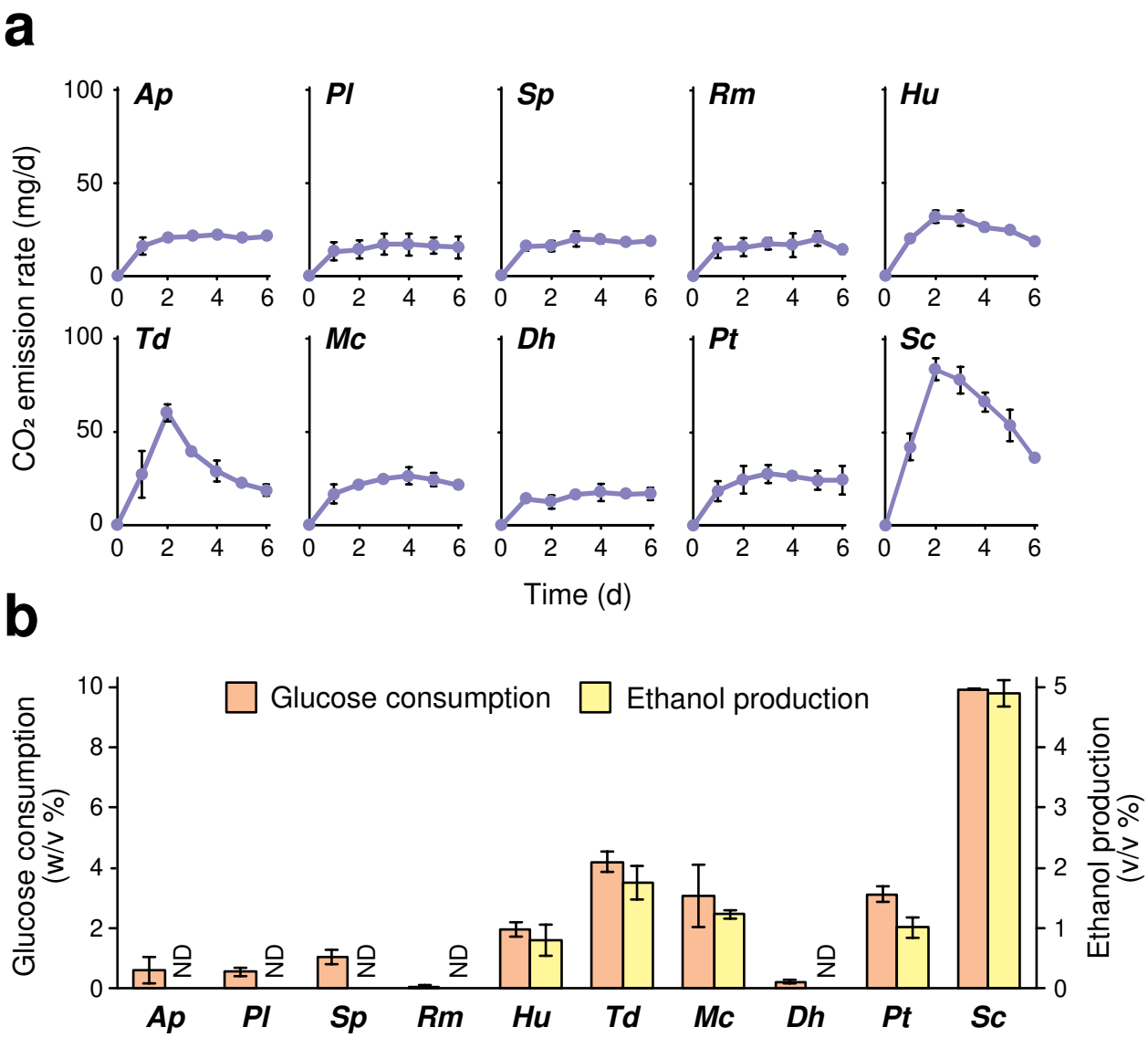

a
Ap
Pl
Sp
Rm
Hu
Td
Mc
Dh
Pt
Sc
CO₂ emission rate (mg/d)
Time (d)
b
Glucose consumption
Ethanol production
Glucose consumption (w/v %)
Ethanol production (v/v %)
ND

Fig. 4

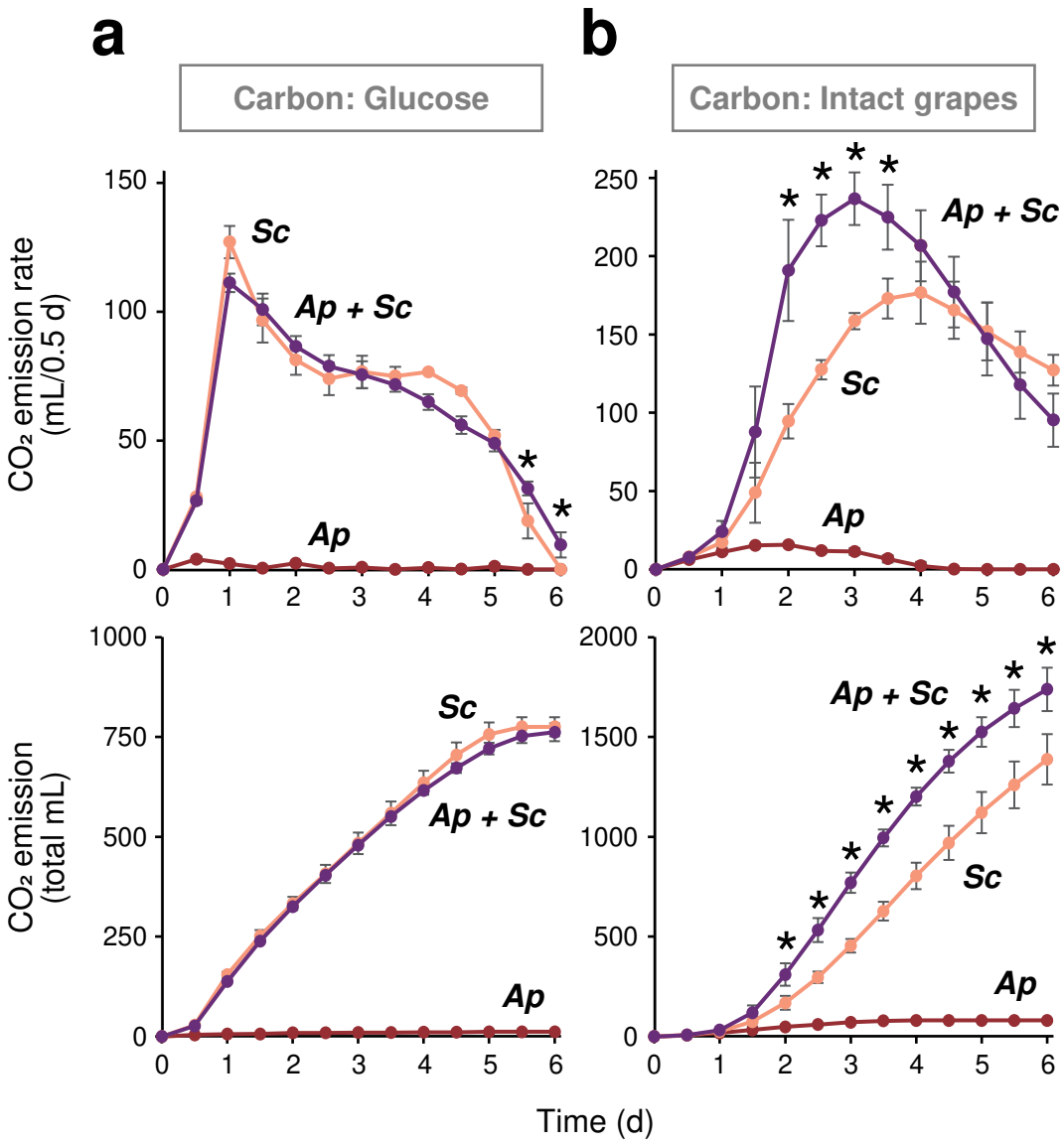
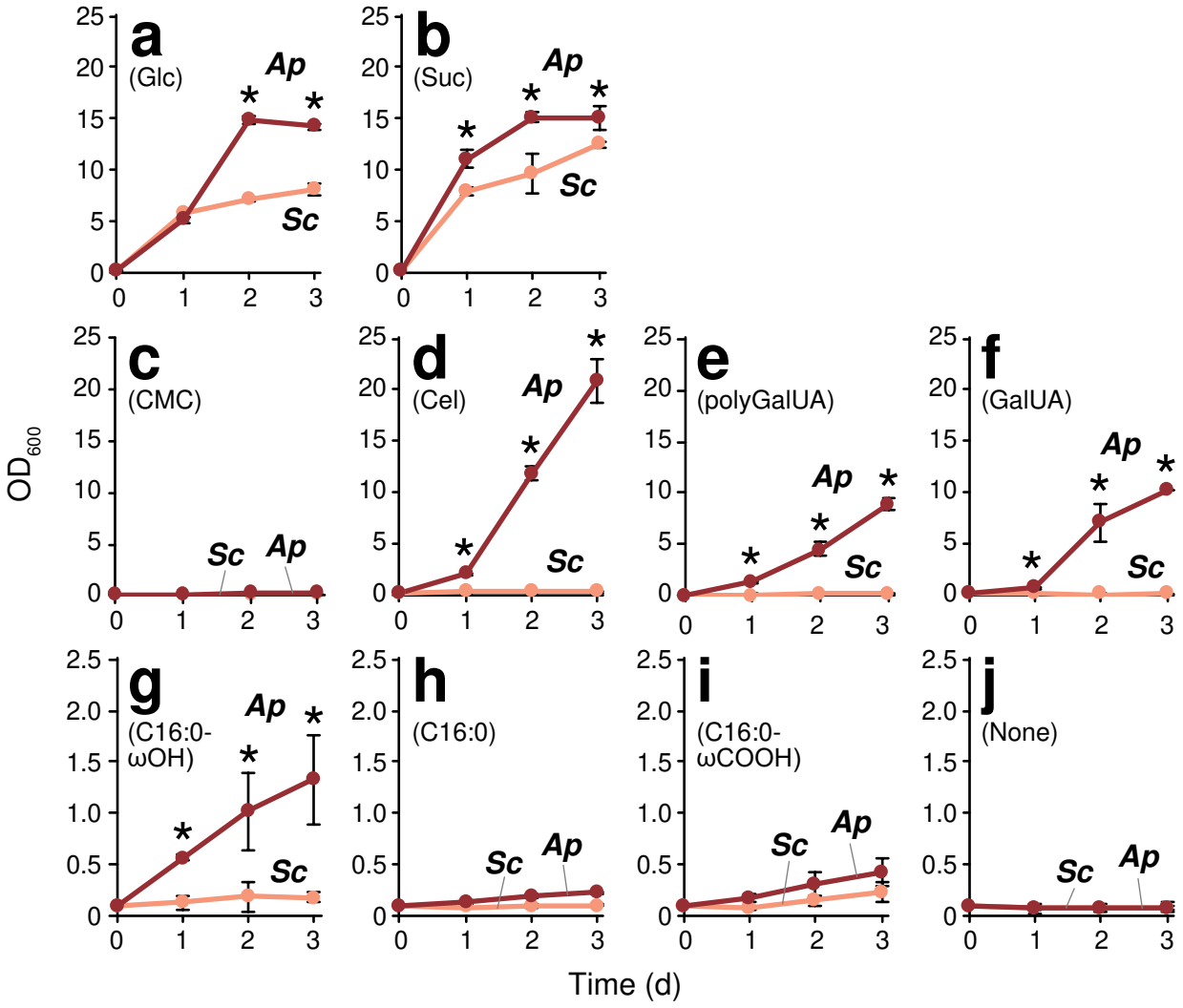

a
b
Carbon: Glucose
Carbon: Intact grapes
CO₂ emission rate (mL/0.5 d)
CO₂ emission (total mL)
Sc
Ap + Sc
Ap
Time (d)

Fig. 5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(Glc)
b
(Suc)
c
(CMC)
d
(Cel)
e
(polyGalUA)
f
(GalUA)
g
(C16:0-
ωOH)
h
(C16:0)
i
(C16:0-
ωCOOH)
j
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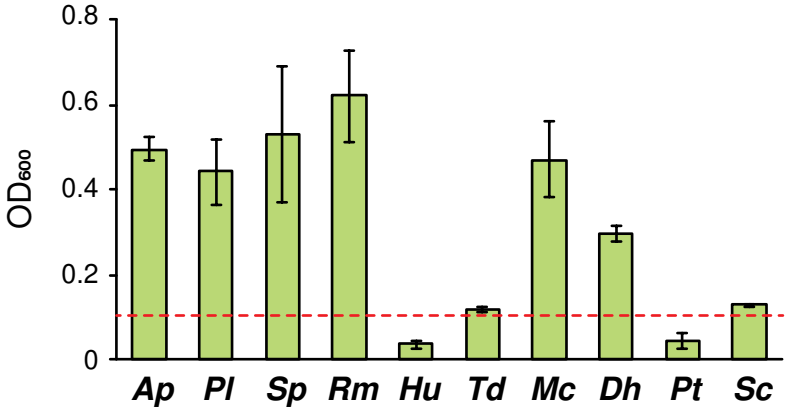
Ap
Sc
*
OD600
Time (d)

Fig. 6



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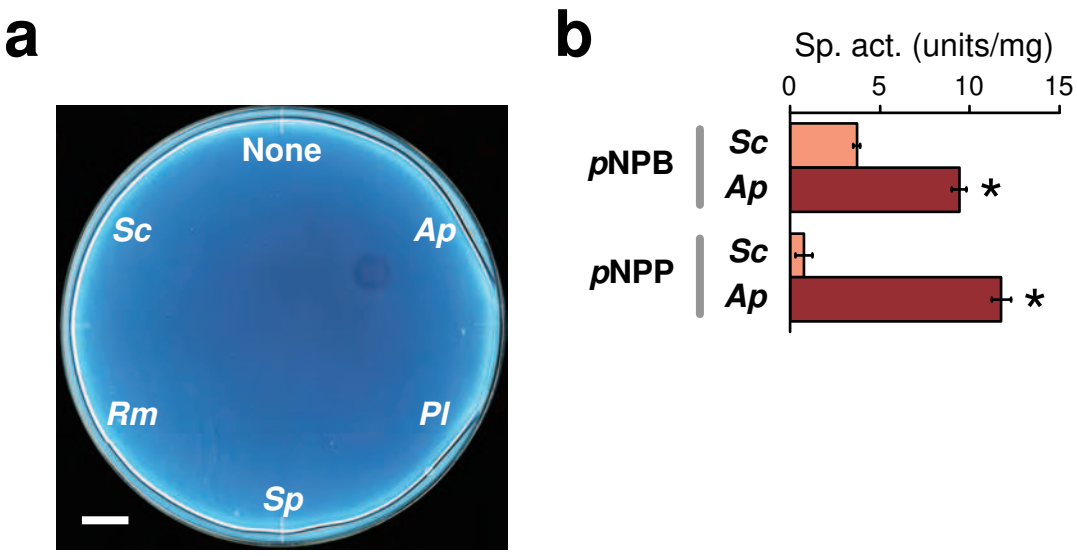
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Pl
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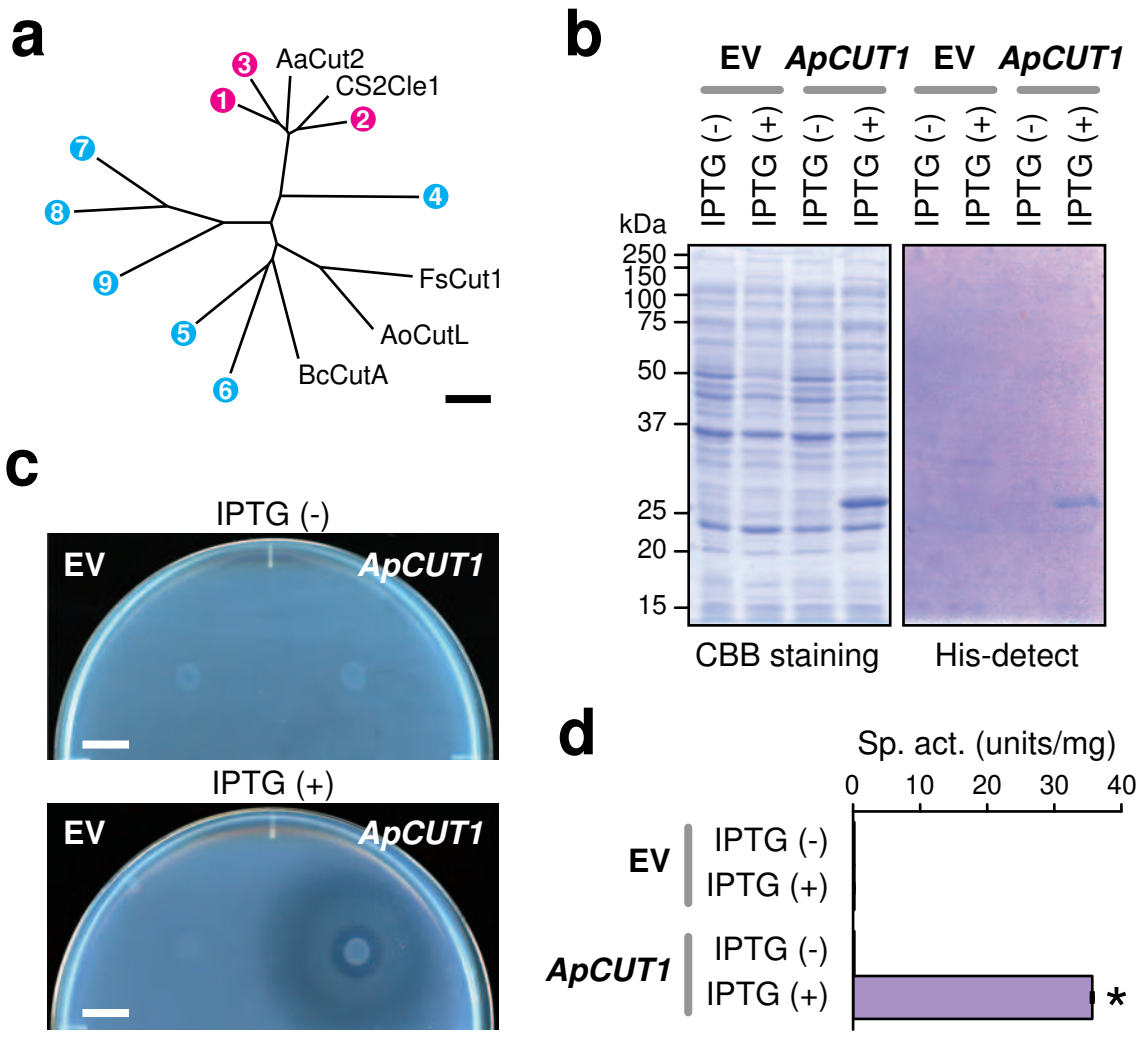
5
10
15
pNPB
Sc
Ap
*
pNPP
Sc
Ap
*

Fig. 8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CS2Cle1
FsCut1
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BcCutA
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EV
ApCUT1
EV
ApCUT1
IPTG (-)
IPTG (+)
kDa
250
150
100
75
50
37
25
20
15
CBB staining
His-detect
c
IPTG (-)
EV
ApCUT1
IPTG (+)
EV
ApCUT1
d
Sp. act. (units/mg)
0
10
20
30
40
EV
IPTG (-)
IPTG (+)
ApCUT1
IPTG (-)
IPTG (+)
*

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