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Abstract

Killer toxin resistant 6 (Kre6) and its paralog, suppressor of Kre null 1 (Skn1) are thought to be involved in the biosynthesis of cell wall β-(1→6)-D-glucan in baker's yeast, *Saccharomyces cerevisiae*. The *Δkre6Δskn1* mutant of *S. cerevisiae* and other fungi shows severe growth defects due to the failure to synthesize normal cell walls. In this study, two homologs of Kre6, namely, K6LP1 (Kre6-like protein 1) and K6LP2 (Kre6-like protein 2), were identified in *Aureobasidium pullulans* M-2 by draft genome analysis. The *Δk6lp1*, *Δk6lp2* and *Δk6lp1Δk6lp2* mutants were generated in order to confirm the functions of the Kre6-like proteins in *A. pullulans* M-2. The cell morphologies of *Δk6lp1* and *Δk6lp1Δk6lp2* appeared to be different from those of wild type and *Δk6lp2* in both their yeast and hyphal forms. The productivity of the extracellular polysaccharides, mainly composed of β-(1→3),(1→6)-D-glucan (β-glucan), of the mutants was 5.1–17.3% less than that of wild type, and the degree of branching in the extracellular β-glucan of mutants was 14.5–16.8% lower than that of wild type. This study showed that the gene disruption of Kre6-like proteins affected the cell morphology, the productivity of extracellular polysaccharides and the structure of extracellular β-glucan, but it did not have a definite effect on the cell viability even in *Δk6lp1Δk6lp2*, unlike in the *Δkre6Δskn1* of *S. cerevisiae*.

Keywords: *Aureobasidium pullulans*; β-Glucan; Polysaccharides; Killer toxin resistant 6; Mutant;

Introduction

β-(1→3),(1→6)-D-glucan (β-glucan) is a universal component of fungal cell walls and plays a key role in forming complexes with other cell wall components such as chitin and mannoproteins. The cell wall β-glucan is known to be an immune stimulator because it is recognized by the innate immune cells in mammals to protect against fungal infections. Previous studies demonstrated the beneficial effects of β-glucans including anti-tumor (Chaung et al. 2009; Liu et al. 2009; Shimizu et al. 2009; Xu et al. 2016; Yoon et al. 2008), anti-infectious disease (Jesenak et al. 2013; Zhou et al. 2009) and anti-inflammatory (Du et al. 2015) activities through the modulation of the immune system. Thus, several cell wall β-glucans extracted from fungi, such as *Saccharomyces cerevisiae*, *Lentinula edodes* and *Ganoderma lucidum*, are used as supplements to improve health.

In *S. cerevisiae*, the most widely used experimental model in fungi, several proteins involved in the synthesis of cell wall β-glucan have been identified. The loss of β-glucan synthesis-related proteins caused physical abnormalities of the fungal morphology and led to critical defects in cell viability, drug resistance, and so on (Teparić and Mrša 2013). Killer toxin resistant 6 (Kre6) and its paralog, suppressor of Kre null 1 (Skn1) are known to be involved in the synthesis of β-(1→6)-linked glucose chains in the cell wall β-glucan (Gilbert et al. 2010; Kurita et al. 2011; Mio et al. 1997; Roemer et al. 1993). The loss of Kre6 led to the reduction of the cell wall β-glucan, resulting in abnormal cell shape and negative effects on cell growth. Skn1 is thought to complement the function of Kre6. Skn1 does not appear to contribute to fungal cell wall synthesis because *Δskn1* showed no apparent phenotype in some fungi (Roemer et al. 1993). The single disruption of *Kre6* or *Skn1* was not critical for the cell viability, whereas *Δkre6Δskn1* showed synthetic lethality or severe growth defects due to the abnormal

biosynthesis of the cell walls.

Some species of fungi, such as *Schizophyllum commune* and *Sclerotium* spp., also produce soluble β-glucan extracellularly in addition to their insoluble cell wall β-glucan (Komatsu et al. 1969; Singh et al. 1974). Such extracellular polysaccharides (EPSs) are viscous and are thought to act as barriers to protect the fungal cells from severe environmental stresses and/or as storages of carbon sources. The chemical properties of soluble and insoluble β-glucans have been analyzed (Kim et al. 2000), but the differences in their biosynthetic pathways are unknown. In addition, the relationship of β-glucan synthesis-related genes with the production of these extracellular β-glucans is still unclear.

A black yeast, *Aureobasidium pullulans,* produces both insoluble cell wall and soluble extracellular β-glucans. *A. pullulans* has been used to produce soluble extracellular β-glucan for industrial purposes because of its high productivity. The soluble extracellular β-glucan showed immune stimulation activities and other beneficial effects similar to those of the insoluble cell wall β-glucans (Aoki et al. 2015a, 2015b; Kawata et al. 2015; Muramatsu et al. 2012, 2014).

In this study, to investigate the biosynthesis of β-glucan in *A. pullulans*, we focused on the functions of the homologs of *S. cerevisiae* Kre6, which plays crucial roles for the biosynthesis of the cell wall β-(1→6)-D-glucans. This research was conducted in the polymorphic fungus *A. pullulans* M-2 because it serves as a model of an extracellular β-glucan-producing fungus. The genes of Kre6-like protein 1 (K6LP1) and Kre6-like protein 2 (K6LP2) in *A. pullulans* M-2 encoding the homologs of Kre6 and Skn1 from *S. cerevisiae* were cloned, and the *Δk6lp1*, *Δk6lp2* and *Δk6lp1Δk6lp2* mutants were generated by genetic recombination. Morphological changes and the productivity of soluble extracellular β-glucans were investigated. In addition, the structure of the soluble

extracellular β-glucans produced by the mutants was elucidated.

Materials and methods

Strains and culture condition

Aureobasidium pullulans M-2 (FERM BP-10014) is a mutant strain obtained from *A. pullulans* M-1 (FERM BP-08615) (Moriya et al. 2013) designated the wild type in this study. Wild type and gene-disrupted mutants were cultivated in 24 g/L potato dextrose broth (PDB, Becton, Dickinson and Company, Sparks, MD, USA) for maintenance and preculture. The β-glucan production was carried out in a rice bran medium (RB) consisting of 15 g/L sucrose and 2 g/L rice bran. After inoculation with each strain, the cells were cultivated at 25°C in a rotary shaker at 150 rpm.

Draft genome analysis of *A. pullulans* M-2

Genomic DNA was extracted from the yeast forming cells of *A. pullulans* M-2 using a Plant/Fungi DNA Isolation Kit (Norgen, Thorold, ON, Canada) and fragmented using the Covaris Acoustic Solubilizer (Covaris, Woburn, MA, USA). A paired-end library was constructed using the TruSeq Nano DNA Library Prep Kit (Illumina, San Diego, CA, USA) and sequenced using the Miseq platform (301-bp paired-end) at the Instrumental Research Support Office, Research Institute of Green Science and Technology, Shizuoka University. The raw read sequences were cleaned up using Trimmomatic (Bolger et al. 2014) by trimming adapter sequences, low-quality ends (quality score, <15), the last 301 bases, and reads less than 150 bp. The resultant 6,582,874 high-quality reads, totaling

1.68 Gbp and corresponds to an approximately 63-fold coverage of the genome, were assembled using SPAdes v3.10.0 (Bankevich et al. 2012) with a default set of k-mer sizes with options (--careful, --only-assembler, and --cov-cutoff auto), and contigs less than 200 bp were eliminated.

Transformation of *A. pullulans*

The transformation of *A. pullulans* M-2 was carried out by electroporation using the Gene Pulser Xcell Electroporation System (Bio-Rad, Hercules, CA, USA) as described previously (De Backer et al. 1999; Thompson et al. 1998) with a slight modification. The cells harvested from an overnight culture of *A. pullulans* M-2 in PDB were suspended in 10 mM Tris-HCl (pH 7.5) containing 5 mM lithium acetate, 10 mM dithiothreitol, and 1 mM EDTA, and the suspension was incubated at 20°C for 1 h. After the cells were washed twice with ice-cold sterile water and once with ice-cold sterile 1 M sorbitol, the cells were resuspended in ice-cold 1 M sorbitol. Approximately 1 μg of DNA was transformed into 40 μL of the cell suspension (5×10^8 cells) by electroporation with a 0.2-cm cuvette at 1.5 kV, 25 μF, and 200 Ω. The cells were recovered in 1 M sorbitol at 25°C for 2 h and plated onto potato dextrose agar (PDA) plates containing 1 M sorbitol and selective antibiotics for 24–48 h at 25°C. The concentrations of hygromycin B (Wako Pure Chemical Industries, Osaka, Japan) and phleomycin (InvivoGen, San Diego, CA, USA) were 50 μg/mL and 10 μg/mL, respectively.

Genotyping of transformants

To confirm the genotype of the transformants, colony direct polymerase chain reaction (PCR) was performed using the KOD FX Neo (Toyobo, Osaka, Japan) and appropriate

genotyping primer sets. Primers 1, 5 and 15, mixed in a reaction, were used in the K6LP1 genotyping PCR, and primers 9 and 14 were used in the K6LP2 genotyping PCR (Fig. 2a & S1, Table S1). The 40 cycles of PCR included denaturation at 98°C for 10 s, annealing at 55°C for 10 s, and extension at 68°C for 1 min/kbp. The positive transformants were reconfirmed by genotyping PCR using their genomic DNA isolated from overnight culture in PDB using the Plant/Fungi DNA Isolation Kit (Norgen) according to the manufacturer's instructions.

Quantification of EPSs

Four pre-cultured strains were each inoculated with 10^3 cells/mL into the RB. These cultures were cultivated at 25°C in a rotary shaker at 150 rpm for 8 days. The EPSs concentrations of the culture supernatants were measured at day-3, 5, 6, 7, 8 by the phenol-sulfuric acid method using D-glucose as a standard (Dubois et al. 1956).

Preparation of EPSs samples from the culture supernatant

High-viscosity culture broth (1 mL) was diluted at least 20-fold and centrifuged at 20,000 × g for 20 min at ambient temperature to remove the fungal cells and residual rice bran particles. The EPSs were recovered from the diluted culture supernatant by ethanol precipitation (repeated 3 times). The precipitated EPSs were dissolved in distilled water and lyophilized.

Structural analysis of β-glucan by methylation analysis

Partially *O*-methylated alditol acetate derivatives were prepared from the purified EPSs samples of 6-day cultures and analyzed by conventional gas chromatography mass

spectrometry with electron ionization (EI-GC/MS) for the linkage analysis of the β-glucan (Hakomori 1964). The samples were analyzed on a Shimadzu GCMS-QP2010 Plus system (Shimadzu, Kyoto, Japan) operating in electron ionization (70 eV) with scan mode (*m/z* 40–300). Samples were analyzed on a DB-5ms fused-silica column (Agilent Technologies, Santa Clara, CA, USA) (0.25 mm internal diameter, 0.25 μm film thickness, 30 m length). The injector was operated in pulsed splitless mode with the injector temperature maintained at 280°C. Helium was used as the carrier gas with a flow rate of 0.8 mL/min and a linear gas velocity of 32.5 cm/sec. The GC-MS oven program had an initial temperature of 60°C followed by ramps of 8°C/min to 280°C for 27.5 min. Data acquisition and processing were conducted with the GCMS solution software (Ver 2.72, Shimadzu). The degree of branching (DB) was calculated on the basis of the peak area of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylhexitol (→3 glucose 1→) and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylhexitol (→3,6 glucose 1→) in the total ion chromatogram (TIC) with the equation (→3,6 glucose 1→)/[(→3 glucose 1→)+(→3,6 glucose 1→)].

Statistical analysis

To check for significant differences between the pairs of data, a two-tailed unpaired Student's t-test was performed.

Results

Identification of the genes encoding the Kre6 and Skn1 homologs in *A. pullulans* M-2

Two proteins that showed high similarities to *S. cerevisiae* Kre6 and Skn1 were identified

as Kre6-like proteins, namely, K6LP1 and K6LP2, in *A. pullulans* M-2 by searching Kre6 (*Saccharomyces* genome database [SGD] ID: S000006363) and Skn1 (SGD ID: S000003375) of *S. cerevisiae* as queries on BLAST against the genome of *A. pullulans* M-2. Thus, the genes encoding K6LP1 (accession number LC271260) and K6LP2 (accession number LC271261) were identified. The amino acid sequence of K6LP1 showed 38.4% identity with Kre6 and 32.5% identity with Skn1, and that of K6LP2 showed 38.9% identity with Kre6 and 36.0% identity with Skn1, and K6LP1 and K6LP2 showed lower sequence identity (44.6%), in contrast to the high sequence identity (66.3%) between Kre6 and Skn1 (Table 1). The glycoside hydrolase family 16 domains and the transmembrane regions of Kre6 and Skn1 from *S. cerevisiae* are conserved in K6LP1 and K6LP2, respectively (Fig. S2). These results suggest that K6LP1 and K6LP2 belong to the glycoside hydrolase family 16 that includes the Kre6 and Skn1 of *S. cerevisiae* and are involved in β-glucan synthesis. Molecular phylogenetic tree was constructed from a multiple alignment of Kre6-like protein sequences of *A. pullulans* M-2, other *Aureobasidium* spp., *S. cerevisiae* and *Candida albicans* (Fig. 1). Each Kre6-like protein sequence of *A. pullulans* M-2 clustered with that of other *Aureobasidium* spp., but these clusters are independent of Kre6/Skn1 clusters of *S. cerevisiae* and *C. albicans*. According to this result, Kre6-like proteins of *Aureobasidium* spp. and Kre6/Skn1 of *S. cerevisiae* are not likely to be in one-to-one correspondence.

Generation of *K6LP* disrupted mutants of *A. pullulans* M-2

To investigate the function of K6LP1 and K6LP2 in *A. pullulans* M-2, we disrupted the genes encoding these proteins and observed changes in phenotypes of the mutants. The gene disruption cassettes were conventionally designed to enable the insertion of these

cassettes by homologous recombination during the transformation of *A. pullulans* M-2 (Fig. 2a & S1). To disrupt *K6LP1*, the cassette containing the promoter of translation elongation factor 1-α (*TEF1*) (Thornewell et al. 1995a, 1995b) from *A. pullulans* M-2 and the hygromycin phosphotransferase gene (*HPT*) with an SV40 terminator was used. In addition, the cassette containing the promoter of *TEF1* and bleomycin resistance gene (*Sh ble*) from *Streptoalloteichus hindustanus* with a *CYC1* terminator was used to disrupt *K6LP2*. Each cassette has the left and right homology arms of the disruption target genes, respectively. First, we generated *Δk6lp1* by homologous recombination and selected the transformants with hygromycin. *K6LP2* disruption using *Sh ble* as a selection marker was conducted against the wild type and *Δk6lp1* to generate *Δk6lp2* and *Δk6lp1Δk6lp2*, respectively. The success of the gene disruption in all strains was confirmed by genotyping PCR (Fig. 2b).

Involvement of K6LP1 and K6LP2 in the cell morphology

The cell morphologies of the wild type and mutants were observed with an optical microscope. The morphology of *Δk6lp1* and *Δk6lp1Δk6lp2* grown overnight in PDB was nearly spherical, while that of the wild type and *Δk6lp2* was elliptical (Fig. 3a). In addition, cell aggregation and hyphal formation were observed in 3-day cultures of *Δk6lp1* and *Δk6lp1Δk6lp2* (Fig. 3a). These morphologies were not observed in the wild type and *Δk6lp2* even after one week of cultivation (data not shown). Alternatively, when the cells were grown in RB, which allows the over-production of β-glucans in *A. pullulans* M-2, their morphology was almost identical (Fig. 3a). When *Δk6lp1* and *Δk6lp1Δk6lp2* were grown on PDA plates, fewer blastospores were observed (Fig. 3b), suggesting that K6LP1 is intimately involved in blastospore production. Although *Δkre6Δskn1* mutants show

synthetic lethality or severe growth inhibition in some fungi (Roemer et al. 2013), *A. pullulans* M-2 *Δk6lp1Δk6lp2* grew normally in liquid media, on PDA and even on selective plates during transformation.

The effects of gene disruption of K6LP1 and K6LP2 on the production of EPSs

A. pullulans, formerly named *Pullularia pullulans*, has long been known as a pullulan producter, meanwhile some strains of *A. pullulans* have been isolated to produce soluble β-glucan as a main component of EPSs with or without pullulan production. From the previous nuclear magnetic resonance (NMR) study, the EPSs from *A. pullulans* M-2 culture supernatant showed a typical spectrum of β-glucan in both ^{1}H-NMR and ^{13}C-NMR (Moriya et al. 2013). The appearance of this EPSs is highly pure β-glucan, composing of a slight amount of pullulan or other polysaccharides. To clarify whether K6LP1 and K6LP2 are involved to produce the soluble β-glucan, the concentration of the EPSs in the culture supernatant of all of the strains was monitored utilizing the phenol-sulfuric acid method. The concentration of the EPSs in the culture supernatant of wild type peaked at day-6 with its highest level, reaching 8.7 g/L. The *Δk6lp1*, *Δk6lp2* and *Δk6lp1Δk6lp2* strains produced 8.1 g/L, 8.3 g/L and 7.0 g/L β-glucan, respectively, with a peak at day-7 or later (Fig. 4). These results suggest that neither K6LP1 nor K6LP2 may be critically associated with the productivity of EPSs in *A. pullulans* M-2.

The effects of gene disruption of K6LP1 and K6LP2 on the structure of the extracellular β-glucan

Unlike the general fungal cell wall β-glucans, the soluble β-glucan of *A. pullulans* M-2 has single glucose branches linked by a β-(1→6)-glycosidic bond (Moriya et al. 2013).

The mechanism of branch formation in soluble β-glucans have been little understood. Hence, the potential functions of K6LP1 and K6LP2 in the branch formation of soluble β-glucan were assessed by analyzing DB of soluble β-glucans from the mutant strains of *A. pullulans* M-2. The DB of each sample was calculated from the result of methylation analysis, which is used widely for the linkage analysis of polysaccharides. In all of the strains, five major peaks of partially *O*-methylated alditol acetate derivatives were identified in the total ion chromatogram (TIC) of GC/MS (Fig. 5). Peaks 1, 2, and 5 derived from β-glucan were 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol (glucose [mannose] 1→), 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylhexitol (→3 glucose 1→) and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylhexitol (→3,6 glucose 1→), respectively. The peaks 3 and 4 that were likely to be derived from other minor polysaccharides were 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylhexitol (→4 glucose 1→) and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylhexitol (→6 glucose 1→), respectively. The calculated DB of the β-glucan derived from the wild type was 0.64 that agrees with previous data (Moriya et al. 2013) and that of the β-glucan derived from *Δk6lp1*, *Δk6lp1* and *Δk6lp1Δk6lp2* was 0.47, 0.50 and 0.49, respectively (Table 2). This decrease of DB in the mutants suggests that both K6LP1 and K6LP2 are partially involved in the side branch formation of extracellular β-glucan. In addition, several unidentified peaks with baseline disturbances were observed in the TIC of *Δk6lp1* and *Δk6lp1Δk6lp2* (Fig. 5b, d). This might be due to the alteration in the extracellular matrix component caused by the loss of K6LP1.

Discussion

In this study, we identified K6LP1 and K6LP2 as the putative Kre6-like proteins from the

draft genome sequence of *A. pullulans* M-2 and generated their gene-disrupted mutants in order to investigate the functions of K6LP1 and K6LP2, especially in soluble extracellular β-glucan production. In *S. cerevisiae*, the double disruption of *Kre6* and its paralog, *Skn1* significantly affected cell growth (Roemer et al. 1993). However, in *A. pullulans* M-2, the disruption of *K6LP1* and *K6LP2* did not severely affect the cell viability even when both genes were disrupted. The disruption of *K6LP1* affected the cell morphology (Fig. 3a). In addition, blastospore production was strongly reduced by the disruption of *K6LP1* (Fig. 3b). Alternatively, the disruption of *K6LP2* had no significant effect on the cell morphology and blastospore production of *A. pullulans* M-2 (Fig. 3). Considering that the unusual cell morphologies were observed only in the *K6LP1*-deficient mutants, K6LP1 might act solely as a major molecule related to the cell wall assembly by synthesizing β-(1→6)-D-glucan rather than acting in concert with K6LP2. In *C. albicans*, functional analysis of the *Kre6* and *Skn1* homologs demonstrated that a deficiency of *Kre6* homolog strongly reduced the β-(1→6)-glucan without affecting the β-(1 → 3)-glucan in yeast forming cells, whereas significant effects on β-glucan biosynthesis were not found in the strain deficient in the *Skn1* homolog (Mio et al. 1997). In addition, the *C. albicans Kre6* was expressed at a higher level than *Skn1* in the yeast forming cells, and *Skn1* expression was induced during its hyphal formation. Although we were unable to identify whether K6LP1 and K6LP2 are homologs for Kre6 or Skn1 in *S. cerevisiae* and *C. albicans*, K6LP1 and K6LP2 might be involved in cell division and life cycle in *A. pullulans* M-2 similarly to that in *C. albicans*. However, according to the molecular phylogenetic analysis as shown in Fig. 1, there seems to be no corresponding relationship between Kre6-like proteins of *Aureobasidium* spp. and already-known Kre6/Skn1. This result agrees with the difference of the viability

phenotype of the double deletion mutant between *S. cerevisiae* and *A. pullulans* M-2, and with the potential taxonomical characteristics of the number of Kre6 homolog between basidiomycetes and ascomycetes (Gilbert et al. 2010).

The EPSs of *A. pullulans* M-2 are composed mostly of the soluble extracellular β-glucan as described above. Therefore, the soluble extracellular β-glucan productivity was estimated by measuring the amount of polysaccharides in the culture supernatants (Fig. 4). The mutant strains showed a decrease in the EPSs production without any conspicuous morphological alterations when grown in the RB. Although the decrease in the production level was not critical, both K6LP1 and K6LP2 could potentially impact the biosynthesis and/or secretion of the soluble extracellular β-glucan. From the view point of the synthetic pathway of β-glucan, it is not clear whether there is any common synthetic pathway between the cell wall β-glucan and soluble extracellular β-glucan. Even if a universally conserved glucan synthase complex, known as Fks1p and Rho1p in fungi (Douglas et al. 1994; Arellano et al. 1996), catalyzes the backbone β-(1→3)-D-glucan synthesis in both insoluble and soluble β-glucans, the branch formation of each β-glucan would be likely controlled independently. Recently, Bgl2p and Gas1p were identified as the key proteins for the branching and elongation of the fungal cell wall β-glucan (Aimanianda et al. 2017). According to the proposed model, these proteins generate the ramified cell wall β-glucan using oligo-β-(1→3)-D-glucans, so that they might not be able to form a single glucose branch as shown in the soluble extracellular β-glucan. In a diatom, vacuolar β-(1→6)-transglycosylases were identified as the Kre6 homolog and were proposed as candidates for β-(1→6)-linked side branch formation in chrysolaminarin, an algal soluble β-glucan that is produced in the vacuoles (Huang et al. 2016). On the other hand, little is known about how the branching occurs in a fungal soluble β-glucan. Considering the calculated

DB of soluble extracellular β-glucan (Fig. 5, Table 2), neither K6LP1 nor K6LP2 seems to play the main role in the branching. The gene disruption of Kre6-like proteins in *A. pullulans* M-2 slightly affected the productivity and structure of the soluble extracellular β-glucan. One of the reason may be an indirect effect caused by, for example abnormal cell wall β-glucan lacking the amount of β-(1→6)-D-glucan. It is necessary to analyze the cell wall β-glucan composition for further understanding of the synthetic pathway of the soluble extracellular β-glucan.

In summary, the morphological changes observed in the PDB culture of *Δk6lp1* and *Δk6lp1Δk6lp2* were caused by the disruption of *K6LP1*. The reduction of the EPSs and the decreased DB of the β-glucan in the RB culture supernatants of the mutants were partly caused by the disruption of *K6LP1* or *K6LP2*. These results indicated that the Kre6-like proteins from *A. pullulans*, K6LP1 and K6LP2 are not essential for cell viability in *A. pullulans* M-2, but they have some effects on the synthesis of both the cell wall and extracellular β-glucans.

Author contributions

Conceived and designed the experiments: H.U., T.K. and E.Y.P. Performed the experiments: H.U. Analyzed the data: H.U. and A.I. Contributed reagents/materials/analysis tools: H.D., T.O., T.K. and E.Y.P. Wrote the paper: H.U. and A.I.

Competing financial interests

There are potential competing interests to declare. Hirofumi Uchiyama and Atsushi Iwai are employees of Aureo-Science Co., Ltd. The β-(1→3),(1→6)-D-glucan-containing *A.*

pullulans-culture broth and its derivatives are marketed by Aureo Co., Ltd., and by Aureo-Science Co., Ltd. There are no other patents, products in development, or marketed products to declare.

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FIGURE LEGENDS

Fig. 1 Molecular phylogenetic tree of the Kre6-like proteins.

The tree was built by MEGA7 software using the neighbor-joining method from a ClustalW multiple alignment of Kre6-like protein sequences from *A. pullulans* M-2, *Aureobasidium melanogenum* CBS 110374, *Aureobasidium subglaciale* EXF-2481, *Aureobasidium namibiae* CBS 147.97, *Aureobasidium pullulans* EXF-150, *S. cerevisiae* S288C and *C. albicans* SC5314 with the *S. cerevisiae* S288C Kre5 as an outgroup. The bootstrap values were shown next to the branches (1000 replicates). The database IDs are as follows; DDBJ accession numbers for *A. pullulans* M-2, GeneBank IDs for the others.

Fig. 2 Generation of *K6LP1*- and *K6LP2*-disrupted mutants of *A. pullulans* M-2

a Schematic representation for the generation of *K6LP1*- (upper panel) and *K6LP2*- (lower panel) disrupted mutants. Left and right homology arms are indicated as L and R, respectively. **b** Genotyping of gene disrupted mutants. Using specific primer sets indicated in panel **a** and Fig. S1, genotyping of gene-disrupted mutants was performed by genomic PCR.

Fig. 3 Morphological changes of *K6LP1*- and *K6LP2*-disrupted mutants of *A. pullulans* M-2

The cell morphologies of the wild type and mutant strains were observed under an optical microscope. The yeast forming cells were grown in PDB and RB (**a**), and the hyphal forming cells were grown on PDA (**b**). Scale bars indicate 20 μm in **a**, and 10 μm in **b**.

Fig. 4 Concentration of the EPSs in the culture supernatants of *A. pullulans* M-2

mutant strains

The mutant strains of *A. pullulans* M-2 were seeded into RB medium at a density of 10^3 cells/mL. The concentration of the EPSs in the culture supernatant was quantified by the phenol-sulfuric acid method at the time points indicated in the figure. The error bars indicate standard deviations calculated by three independent experiments. The asterisk (*) and double asterisks (**) indicate $p<0.05$ and $p<0.01$, respectively.

Fig. 5 Total ion chromatogram of partially *O*-methylated alditol acetate derivatives in EI-GC/MS.

Partially *O*-methylated alditol acetate derivatives of each strain were synthesized from ethanol precipitated fractions of the RB culture supernatants. (**a**) Wild type, (**b**) *Δk6lp1*, (**c**) *Δk6lp2*, (**d**) *Δk6lp1Δk6lp2*. Numbered peaks are as follows; **1**: 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol (glucose [mannose] 1→), **2**: 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylhexitol (→3 glucose 1→), **3**: 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylhexitol (→4 glucose 1→), **4**: 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylhexitol (→6 glucose 1→), **5**: 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylhexitol (→3,6 glucose 1→)

Table 1. Homologies between the Kre6-like proteins of *A. pullulans* M-2 and *S. cerevisiae*

			Similarity			
			1	2	3	4
Identity	1	*A. pullulans* K6LP1	–	58.1	52.3	45.3
	2	*A. pullulans* K6LP2	44.6	–	51.8	49.1
	3	*S. cerevisiae* Kre6	38.4	38.9	–	76.3
	4	*S. cerevisiae* Skn1	32.5	36.0	66.3	–

Table 2. Calculated DB of β-glucan by methylation analysis

Strain	DB
Wild type	0.64
Δk6lp1	0.47
Δk6lp2	0.50
Δk6lp1Δk6lp2	0.49

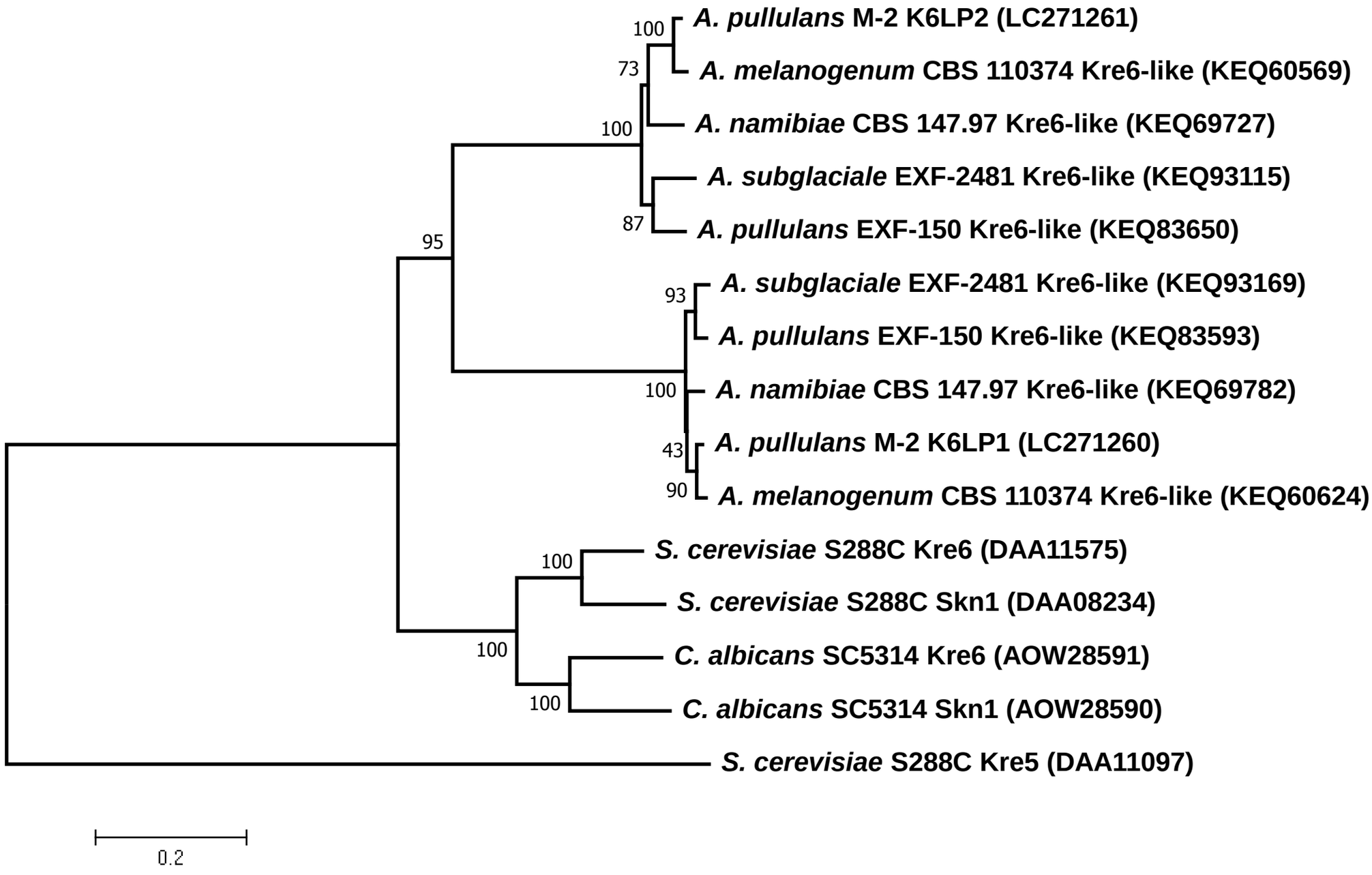
A. pullulans M-2 K6LP2 (LC271261)
A. melanogenum CBS 110374 Kre6-like (KEQ60569)
A. namibiae CBS 147.97 Kre6-like (KEQ69727)
A. subglaciale EXF-2481 Kre6-like (KEQ93115)
A. pullulans EXF-150 Kre6-like (KEQ83650)
A. subglaciale EXF-2481 Kre6-like (KEQ93169)
A. pullulans EXF-150 Kre6-like (KEQ83593)
A. namibiae CBS 147.97 Kre6-like (KEQ69782)
A. pullulans M-2 K6LP1 (LC271260)
A. melanogenum CBS 110374 Kre6-like (KEQ60624)
S. cerevisiae S288C Kre6 (DAA11575)
S. cerevisiae S288C Skn1 (DAA08234)
C. albicans SC5314 Kre6 (AOW28591)
C. albicans SC5314 Skn1 (AOW28590)
S. cerevisiae S288C Kre5 (DAA11097)
100
73
100
87
95
93
100
43
90
100
100
100
0.2

Figure 1

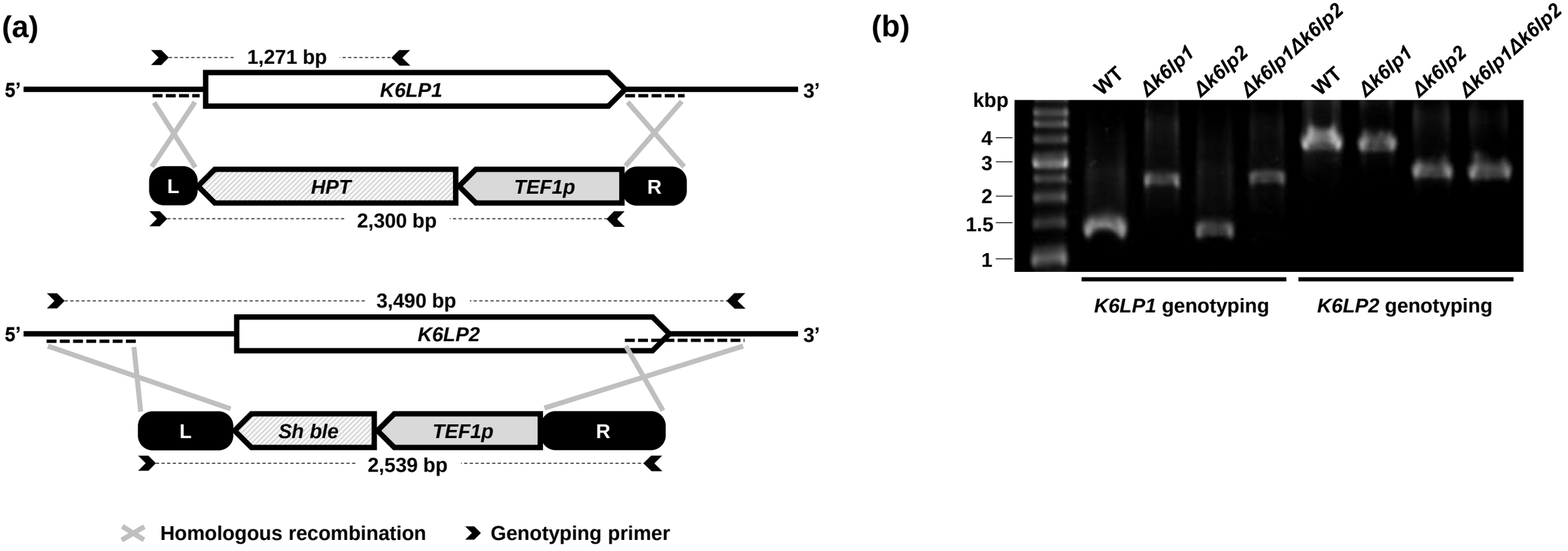
(a)
1,271 bp
5'
K6LP1
3'
L
HPT
TEF1p
R
2,300 bp
3,490 bp
5'
K6LP2
3'
L
Sh ble
TEF1p
R
2,539 bp
Homologous recombination
Genotyping primer
(b)
WT
Δk6lp1
Δk6lp2
Δk6lp1Δk6lp2
WT
Δk6lp1
Δk6lp2
Δk6lp1Δk6lp2
kbp
4
3
2
1.5
1
K6LP1 genotyping
K6LP2 genotyping

Figure 2a, 2b

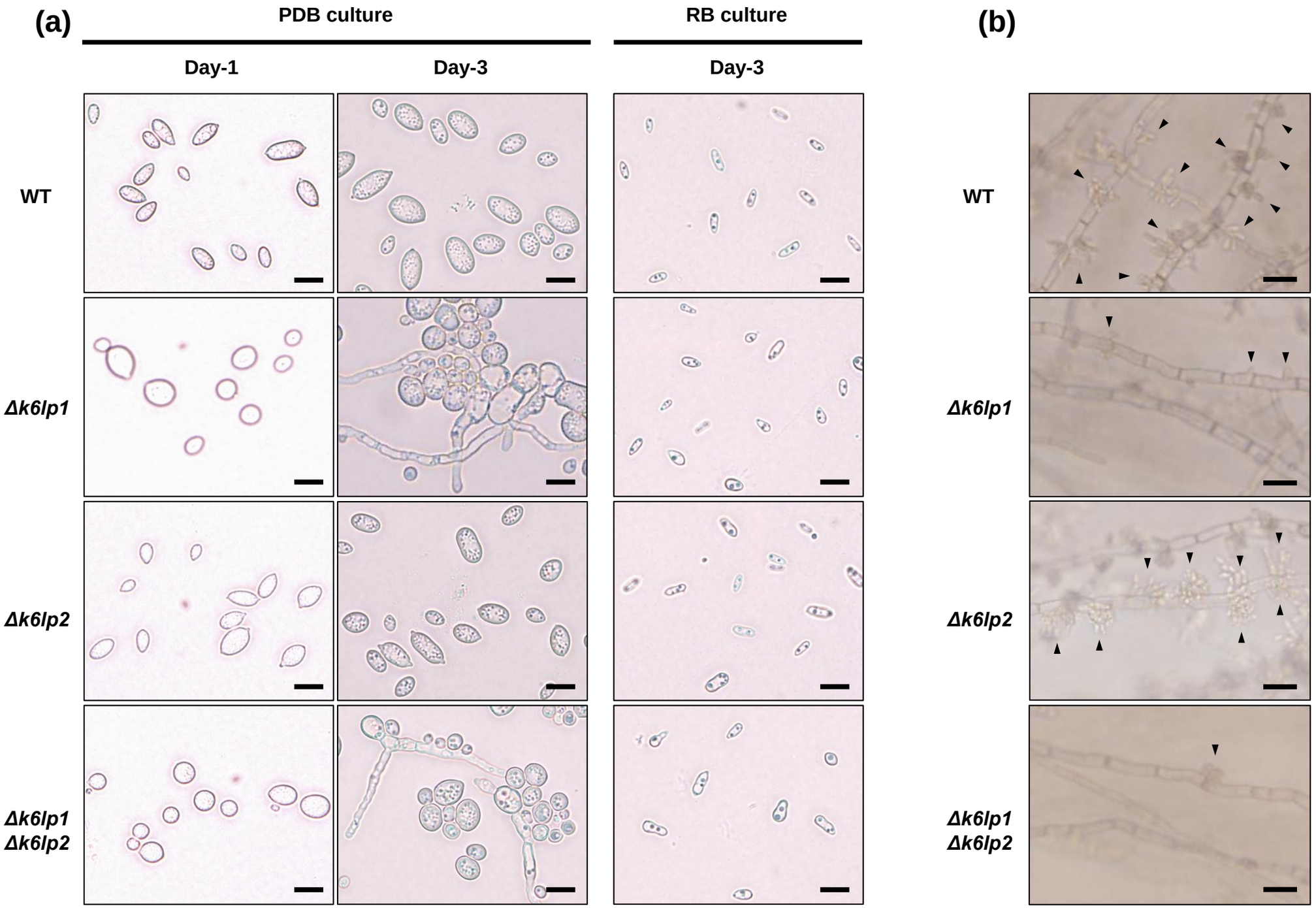
(a)
PDB culture
RB culture
Day-1
Day-3
Day-3
WT
Δk6lp1
Δk6lp2
Δk6lp1
Δk6lp2
(b)
WT
Δk6lp1
Δk6lp2
Δk6lp1
Δk6lp2

Figure 3a, 3b

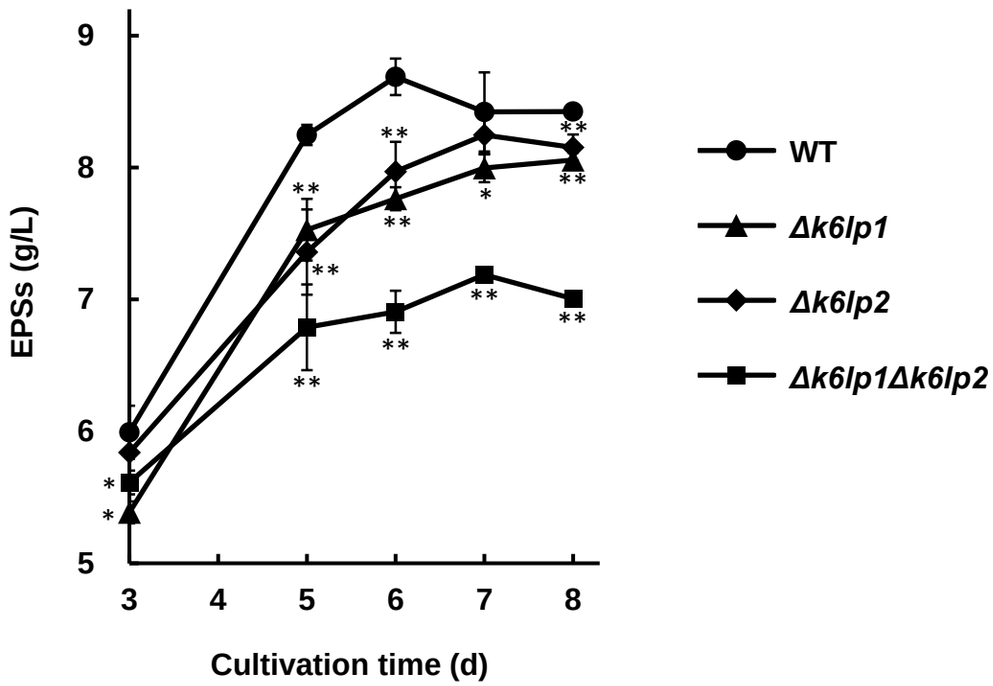
EPSs (g/L)
9
8
7
6
5
3
4
5
6
7
8
Cultivation time (d)
WT
Δk6lp1
Δk6lp2
Δk6lp1Δk6lp2

Figure 4

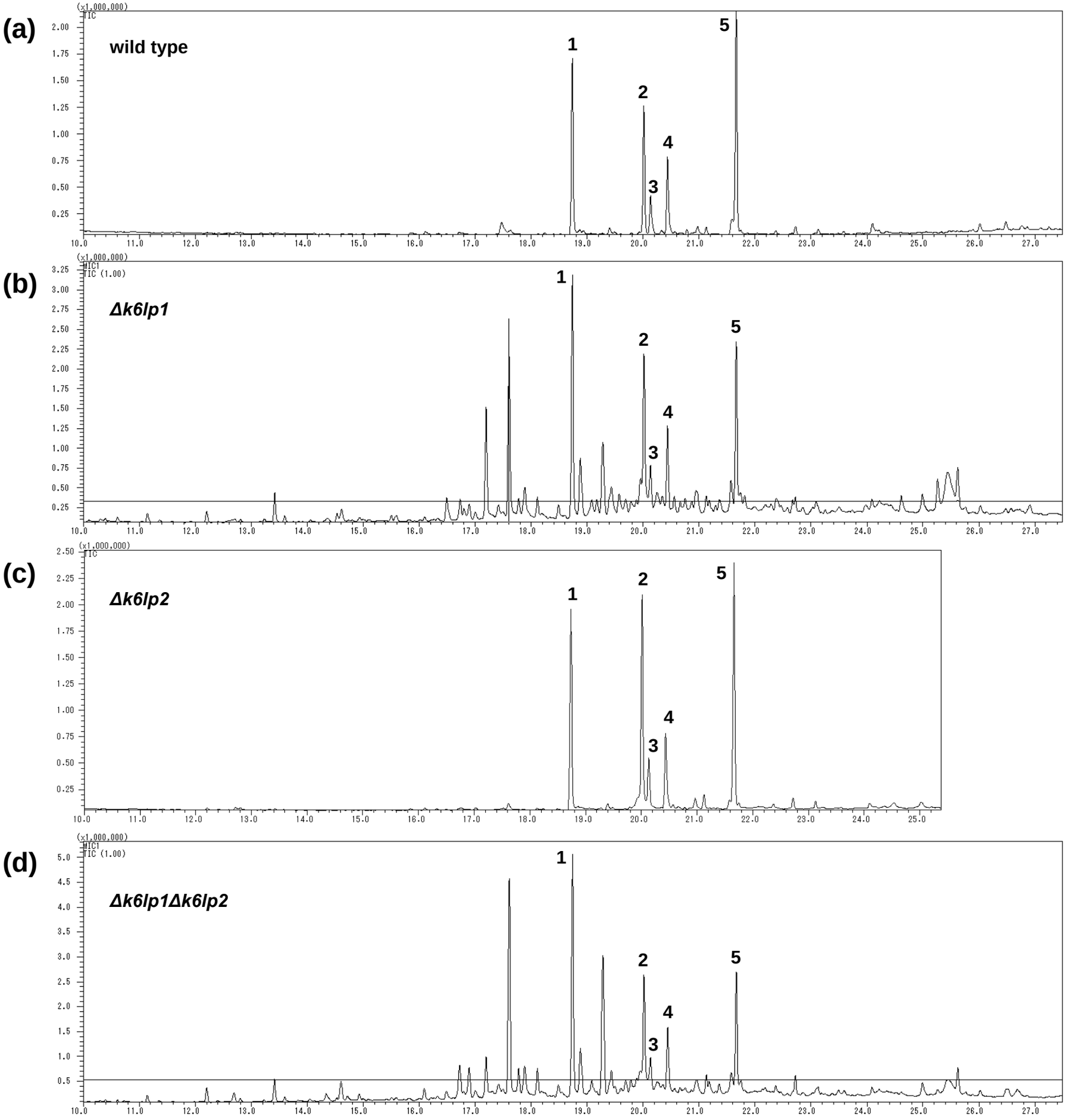

Figure 5

Appl. Microbiol. Biotechnol.

Supplementary information

The effects of gene disruption of Kre6-like proteins on the phenotype of β-glucan-producing *Aureobasidium pullulans*

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Table S1 List of primers used in this study

No.	Primer name	Sequence (5'→3')
1	K6LP1-LHA-F	GTCTCCGCCTCAAAATCATTG
2	HPT-K6LP1-LHA-R	AACTCATCAATGTATCTTATCATGTTGACGAGGTCGTC
3	TEF1p-HPT-F	CCATACACAACCGTCAACATGAAAAAGCCTGAACTC
4	HPT-R	ATAAGATACATTGATGAGTTTGGACAAACCAC
5	TEF1p-F	GGGTAGCAAAGTGGATAGCAAAGG
6	TEF1p-R	GTTGACGGTTGTGTATGGAAG
7	TEF1p-K6LP1-RHA-F	CTATCCACTTTGCTACCCTAACGGCAACGGCAACGAG
8	K6LP1-RHA-R	AATCAGAAGCTCCGTCTTCGTC
9	K6LP2-LHA-F	CACCAAGCCTCAGAAGTCTTAAC
10	Shble-K6LP2-LHA-R	GGCTTTAATTTGCAAGCTAAATCGTACCAATGTTCTGTTCG
11	TEF1p-Shble-F	CCATACACAACCGTCAACATGGCCAAGTTGACCAGTG
12	Shble-R	AGCTTGCAAATTAAAGCCTTCG
13	TEF1p-K6LP2-RHA-F	TTGCTATCCACTTTGCTACCCCTCCTGGGTTTGAAACTACA GAAT
14	K6LP2-RHA-R	CTGTCAAGTCGACCTTCTTTTCT
15	K6LP1-R	GGTATCCCATCATTACATTTGC

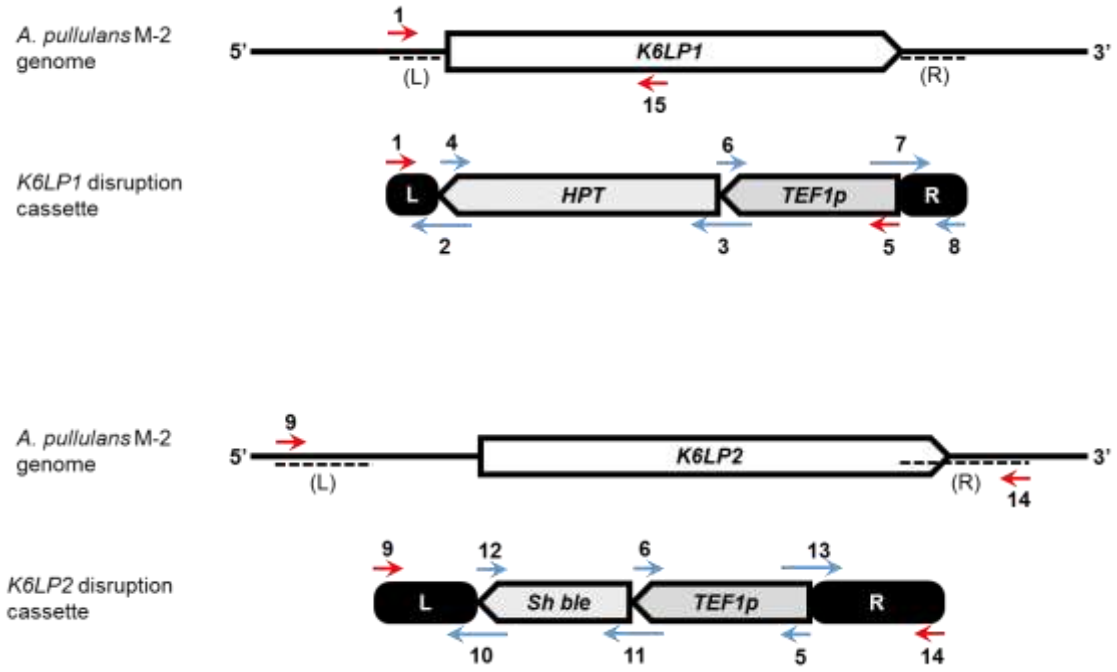

Figure S1 Primers' annealing positions on the genome of *A. pullulans* M-2 and the gene disruption cassettes. The details of the primers are shown in Table S1. Red arrows indicate the primers used for genotyping PCR.

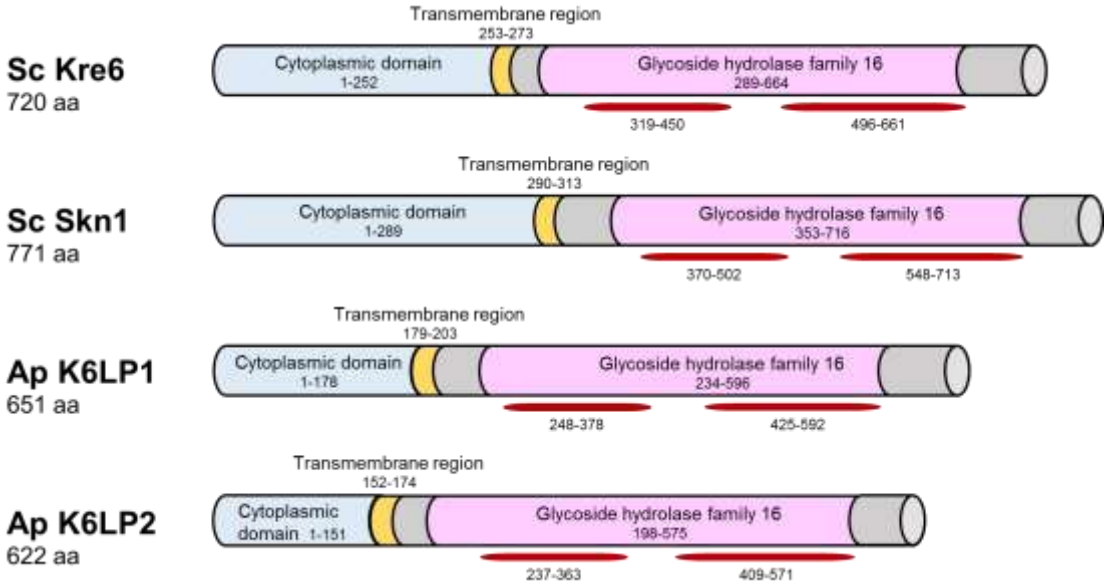

Figure S2 Domain organization of *Saccharomyces cerevisiae* Kre6/Skn1and *Aureobasidium pullulans* M-2 Kre6-like proteins. The red lines under the glycoside hydrolase family 16 domain indicate the Concanavalin A-like lectin/glucanase domain (SSF 49899).