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Uracil-auxotrophic marker recycling system for multiple gene disruption in *Pseudozyma antarctica*

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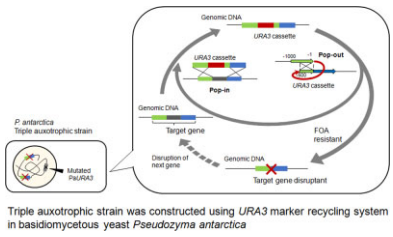
ABSTRACT

The basidiomycetous yeast *Pseudozyma antarctica*, which has multiple auxotrophic markers, was constructed, without inserting a foreign gene, as the host strain for the introduction of multiple useful genes. *P. antarctica* was more resistant to ultraviolet (UV) irradiation than the model yeast *Saccharomyces cerevisiae*, and a Pa*ura3* mutant (C867T) was obtained after 3 min of UV exposure. A uracil-auxotrophic marker (*URA3*) recycling system developed in ascomycetous yeasts and fungi was applied to the *P. antarctica* Pa*ura3* strain. The Pa*LYS12* and Pa*ADE2* loci were disrupted via site-directed homologous recombination of Pa*URA3* (pop-in), followed by the removal of Pa*URA3* (pop-out). In the obtained double auxotrophic strain (Pa*lys12*Δ, Pa*ura3*), Pa*ADE2* was further disrupted, and Pa*URA3* was removed to obtain the triple auxotrophic strain PGB800 (Pa*ura3*, Pa*lys12*Δ, Pa*ade2*Δ). The whole-genome sequence of the PGB800 strain did not contain foreign genes used for genetic manipulation and disrupted Pa*ADE2* and Pa*LYS12*, and removed Pa*URA3*, as planned.

Graphical Abstract



Triple auxotrophic strain was constructed using *URA3* marker recycling system in basidiomycetous yeast *Pseudozyma antarctica*.

Keywords: *Pseudozyma antarctica*, Pa*ura3*, marker recycling system

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Basidiomycetous yeasts have been used as genetic resources for various degrading enzymes; however, due to their ability to produce enzymes, they can also be used as host strains to produce proteins and useful chemicals. A basidiomycetous yeast *Pseudozyma antarctica* (*P. antarctica;* recently renamed *Moesziomyces antarcticus*) secretes lipases (Nielsen, Ishii and Kirk 1999), biodegradable plastic-degrading enzymes (Kitamoto *et al.* 2011), and mannosylerythritol lipids (Kitamoto *et al.* 1990). These materials were selected from their natural environment for their outstanding properties, such as the thermal stability and structural specificity of lipases (Nielsen, Ishii and Kirk 1999), the enzyme with the degradation ability of biodegradable plastics that can functionally replace conventional non-degradable plastics, and the unique properties of mannosylerythritol lipids as surfactants (eg easy production conditions, low toxicity, high biodegradability, and environmental compatibility) compared with chemically synthesized surfactants (Morita *et al.* 2009). If these materials can be mass-produced, they can be applied to the industry and everyday life. When lipases A and B were discovered from *P. antarctica* in the late 1980s, the number of genetically engineered microorganisms was limited. Thus, they were produced by genetically modifying *Aspergillus oryzae* and used for a variety of applications worldwide (Hoegh *et al.* 1995). However, mass production of conventionally produced substances from wild strains is more likely to be successful than that of substances from different organisms. Recently, it has become possible to introduce genes into the *P. antarctica* chromosome in various ways, so that *P. antarctica* itself could be used as a host for mass production of the above useful materials.

In addition, a strain that has been genetically modified using only endogenous genes is known as a self-cloning strain. Self-cloned strains are considered safe for use in Japan (Akada 2002). There are no restrictions on their handling as recombinant microorganisms and many typical existing facilities are available for their production at a low cost. Various bacterial and fungal self-cloning strains are constructed in Japan for use in the production of enzymes, nucleic acids, and amino acids. To construct *P. antarctica* self-cloning strains for the mass production of useful substances, the auxotrophic strain is essential as a host strain because it allows for the introduction of useful genes using its complementary marker genes (Sameshima-Yamashita *et al.* 2019).

Although *P. antarctica* is characterized by the formation of pseudohyphae, *P. antarctica* GB4-(0) isolated from rice husks tends to maintain a single round shape similar to yeasts rather than pseudohyphae (Yarimizu *et al.* 2017). The yeast-like form is advantageous when used as a platform for material production because it increases the stability of the transformed gene in budding cells, enables a stable culture, and is uniformly dispersed in the medium. On the other hand, the mycelium tends to form aggregates, adhere to the apparatus and does not disperse in the medium, making it challenging to establish a stable culture. To increase the variety of transformation markers, a uracil-auxotrophic strain was constructed from *P. antarctica* GB4-(0) by disrupting the orotidine-5′-phosphate decarboxylase gene (Pa*URA3*) using site-directed homologous recombination (HR) of Pa*URA3* and the antibiotic nourseothricin resistance gene (Pa*ura3*Δ::*natMX4*) (Yarimizu *et al.* 2017). An adenine auxotrophic strain with disrupted Pa*ADE2* was obtained using the same method (Pa*ade2*Δ::*natMX4*) (Yarimizu *et al.* 2017). Furthermore, a lysine-auxotrophic strain L1 was obtained via ultraviolet (UV) irradiation and the mutant gene was identified as Pa*LYS12*. Using Pa*LYS12* as a transformation marker, a biodegradable plastic-degrading enzyme-encoding gene under the control of a highly expressed xylanase promoter was introduced, and the mass production of the biodegradable plastic-degrading enzyme using a self-cloning system was demonstrated (Sameshima-Yamashita *et al.* 2019).

Methods for introducing multiple genes into the chromosomes of host microorganisms are often used to create strains suitable for the desired production. The marker recycling system is a method for repeatedly introducing and deleting marker genes and is a powerful tool for performing multiple genetic manipulations using a single marker gene. This has been shown in various ascomycetes, including *S. cerevisiae* (Kaneko *et al.* 2009) and *Aspergillus* filamentous fungi (Maruyama and Kitamoto 2008; Kadooka *et al.* 2016), in which *URA3*/*PyrG* and ATP sulfurylase (*sC*) have been used as marker genes. It has also been applied to a basidiomycetous white-rot fungus (Nakazawa *et al.* 2016).

This study aimed to develop a marker recycling system using Pa*URA3* from *P. antarctica* to produce host strains with multiple auxotrophic phenotypes.

Materials and methods

Strains

The *P. antarctica* GB-4(0) strain was isolated from rice husk (*Oryza sativa*) and deposited in the National Agriculture and Food Research Organization Genebank, Japan (accession no. MAFF306999) (Kitamoto *et al.* 2011). The *P. antarctica* strains PGB028 (Pa*ade2*Δ::*natMX4*) and L1 (Pa*lys12*) were previously constructed from the GB-4(0) strain and were stocked in our laboratory (Yarimizu *et al.* 2017; Sameshima-Yamashita *et al.* 2019). The *P. antarctica* strains constructed in this study are listed in Table 1. We also used the ascomycetous yeast *S. cerevisiae* S288C strain (Mortimer and Johnston 1986). In addition, the basidiomycetous yeasts *Cryptococcus flavus* GB-1 (Kitamoto *et al.* 2011), and *Rhodotorula mucilaginosa* IY-05 (MAFF516139), isolated from rice husks and Italian ryegrass (*Lolium multiflorum*), respectively, were used as yeast species living on plant surfaces.

Media

Yeast strains were cultivated in YM medium (0.3% yeast extract [Becton Dickinson {BD}, New Jersey, USA], 0.3% malt extract [BD], 0.5% peptone [BD], and 1% glucose) or YPD medium (1% yeast extract, 2% peptone [BD], 2% glucose). Also, minimal medium (MM), which was composed of 0.17% yeast nitrogen base without amino acids and ammonium sulfate (BD), 0.5% ammonium sulfate, and 2% glucose was used. 30 mg/L lysine hydrochloride, 1000 mg/L adenine sulfate, and 1000 mg/L uracil (Sigma-Aldrich, St. Louis, MO, USA) were added to each medium, as required. An FOA medium, MM supplemented with 1000 mg/L uracil and 1000 mg/L 5′-fluoroorotic acid (5′-FOA), was used to obtain uracil-auxotrophic mutants, as described previously (Yarimizu *et al.* 2017).

To select the Pa*ADE2*, Pa*LYS12*, and Pa*URA3* complementary transformants obtained after transformation using the protoplast-polyethylene glycol (PEG) method, 0.8 M sucrose-supplemented MM (MMS) containing DO Supplement –Ura (Takara Bio Inc., Shiga, Japan), or MMS supplemented with lysine hydrochloride (30 mg/L), adenine sulfate (60 mg/L), and uracil (1000 mg/L) were used. All yeast strains were cultivated at 30 °C.

Table 1. Strains constructed in this study

Strain	Genotype	Description
Pseudozyma antarctica		
PGB045	P*aura3* (C867T)	Uracil-auxotrophic strain (UV mutant)
PGB776	P*aade2*Δ	Pa*URA3* pop-in strain from PGB045
PGB781	P*alys12*Δ	Pa*URA3* pop-in strain from PGB045
PGB782	P*aura3*, P*aade2*Δ	Pa*URA3* pop-out strain from PGB776
PGB785	P*aura3*, P*alys12*Δ	Pa*URA3* pop-out strain from PGB781
PGB797	P*alys12*Δ, P*aade2*Δ	Pa*URA3* pop-in strain from PGB785
PGB800	P*aura3*, P*alys12*Δ, P*aade2*Δ	Pa*URA3* pop-out strain from PGB797

Table 2. Primers used in this study.

Name	Sequence (5′ → 3′)
PaADE2−1500	CCAATGCAAGCCAGCGCGGGAC
PaADE2−1000	GGCGGTGGATGCAATTCTGTCA
PaADE2+2887c	ACACTGCATTAGAGCGGGGTTG
PaADE2+3366c	CCAGCGCAGGGGTCGGAACGGG
PaLYS12−1500	GACTCTCCTTGGTGTGTTGA
PaLYS12−1000	GCGTACATATAGTTGGCCGC
PaLYS12+2314c	CGAGGACCCCAACACCACCC
PaLYS12+2814c	TACGACGTCATCGCCTTCAT
PaURA3+899c	CCAACCCGCCTTCTTGTACCGC
PaURA3+400	ACTCGTCGGGCGTGCACAAGAT
PaURA3+1	ATGTCGAGCATCACCCTCCAGACGT
PaURA3−300	GCTGCAGCTGCAGCTCTCCAAGCTG
PaURA3+150	GTGCGATGCCGTCGGCCCAGAC
PaURA3−500	CGGCACGGCCGGGTCGAAGCTCGAG
PaURA3+1433c	ACAACAGTAGCGGAAGGGGCAG
PaLYS-500F	ATTTCGGCCCGATCTATAGACTC
PaLYS12+1777c	GTTCACCTCCATGGACGAGCAG
PaADE2−500	TAGCATCGCTCGTCGAAGTGGG
PaADE2+2390c	CAAGCGGCCGACGAAGTGACAC

Evaluation of ultraviolet (UV) tolerance

Yeast cells were cultivated in a 2 mL YPD medium in a test tube, shaking at 150 strokes min^{-1} for 16 h. The cells were then collected via centrifugation at 9000 × *g* for 1 min and resuspended in 1 mL of MM. The concentration of cell suspension was measured using a hemocytometer, and a suspension containing 500 cells was spread onto a YM plate. The plates were exposed to UV light at 50 cm on a clean bench at 1.5 W/m^2 for various periods (0, 15, 30, 60, 180, or 300 sec). After incubation for 2 days, the colony-forming units were counted.

Isolation of the uracil-auxotrophic strains via UV irradiation

The cell suspension containing 1×10^7 cells was spread onto an FOA plate and exposed to UV light for 180 sec, as described above. After incubation for 5 days at 30 °C, colonies were transferred onto YM and MM plates. Cells that could not grow on MM were selected as candidates to establish uracil auxotroph mutants. Among them, strains whose uracil-auxotrophic phenotype was complemented by Pa*URA3* (DDBJ accession number LC193822) transformation were selected as the P*aura3* mutant. Pa*URA3* was amplified via polymerase chain reaction (PCR) using GB-4(0) genomic DNA as a template and the primer pairs listed in Table 2. Sequencing was performed using PaURA3−300 and PaURA3+150 primers.

Plasmids used in this study

DNA fragments for plasmid construction were amplified via PCR using the templates and primer pairs listed in Table S1. The amplified DNA listed in Table S2 was purified via agarose gel electrophoresis and extracted using the Wizard SV Gel and PCR Clean-up System (Promega Corporation, Madison, USA). The method used to construct the plasmids is illustrated in Figure S2. The DNA mixtures were ligated using an In-fusion HD cloning kit (Takara Bio Inc.). pYT155 was constructed to disrupt Pa*ADE2* (DDBJ accession number LC276897) and pYT161 was constructed to disrupt Pa*LYS12* (DDBJ accession number LC431700) (Figure 2a, Table S2). Both ends of Pa*URA3* contain regions −500 to −1 upstream of the target gene and are flanked by regions upstream and downstream of the target gene.

Disruption of Pa*ADE2* and Pa*LYS12* of *P. antarctica* using the protoplast-PEG method

The DNA fragments for Pa*ADE2* disruption were amplified using pYT155 with KOD plus polymerase (Toyobo Co., Ltd., Osaka, Japan) with primer sets PaADE2−1000 and PaURA3+899c, PaURA3+400, and PaADE2+2887c listed in Table 2. Similarly, DNA fragments for Pa*LYS12* disruption were amplified from pYT161 using primers PaLYS12-1000 and PaURA3+899c, PaURA3+400, and PaLYS12+2314c. Purified DNA fragments were mixed with an equal volume of 2 × KTC (1.6 M KCl, 0.1 M Tris-HCl [pH 7.5], and 0.1 M $CaCl_2$) for isotonization. *P. antarctica* was cultured in 2 mL of YM medium and then shaken at 150 strokes min^{-1} for 1 day. Then, 1 µL of preculture was transferred to a 300 mL flask containing 30 mL of YM medium and cultivated while being shaken at 200 rev min^{-1} for approximately 16 h until the optical density at 600 nm (OD_{660}) reached 0.5-0.9. Cells were centrifuged and washed with protoplast buffer (0.5 M sodium tartrate in McIlvaine buffer, pH 6.0). Cells were resuspended in 3.5 mL of protoplast buffer containing 0.5% (w/w) westase (Takara Bio Inc.) and incubated with gentle shaking for 15 min at 30 °C. Protoplasts were harvested at 1000 × *g* and washed twice with wash buffer (0.8 M KCl, 50 mM Tris-HCl [pH 7.5], and 50 mM $CaCl_2$). Aliquots of protoplasts (2.5×10^6 cells/50 µL of KTC) were mixed with 8 µL of isotonic DNA solution, and 17.5 µL of PTC (60% PEG3350, 50 mM Tris-HCl [pH 7.5], and 50 mM $CaCl_2$). After incubation for 20 min at room temperature, 700 µL of PTC was added, and the mixture was further incubated for 20 min at room temperature. Finally, the cell suspension was mixed with 1 mL of 1 × KTC and immediately spread on an

MMS plate containing drop-out uracil amino acids and 2% agar. The plates were incubated at 30 °C for several days.

Confirmation of target gene disruption via HR

The selection of transformants, in which DNA fragments were inserted at designated positions on the chromosome, was carried out via PCR using crude genomic DNA (Lõoke, Kristjuhan and Kristjuhan 2011). The sets of primers are listed in Table 2. Cells growing on the YM medium were suspended in 50 µL of lithium acetate solution (0.2 M lithium acetate, 0.1% sodium dodecyl sulfate [SDS]). After heating to 70 °C, the samples were immediately cooled on ice for 5 min. The lysates were precipitated with 150 µL of 100% ethanol. The precipitates were dissolved in 50 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and centrifuged again to remove cell debris. The supernatants were subjected to PCR as the template DNA. PCR was performed using KOD FX neo (Toyobo Co., Ltd.) with step-down and 3-step methods according to the manufacturer's instructions.

Pop-out of Pa*URA3*

The resultant auxotrophic mutants Pa*ade2*Δ or Pa*lys12*Δ, established in the pop-in step, were cultivated in 2 mL of YPD medium for 24 h and harvested. After adjusting to an OD_{600} of 1.0, 200 µL of cell suspension was spread on FOA plates containing -Ura DO Supplement to select *ura3* strains. The pop-out of Pa*URA3* in the resultant strain was confirmed via colony PCR, using the primers listed in Table 2.

Confirmation of the auxotrophic phenotype of the generated cells

Strains were cultured in 2 mL of YM medium supplemented with 1000 mg/L adenine sulfate, 30 mg/L lysine hydrochloride, and 1000 mg/L uracil in test tubes for 16 h. After harvesting, the cells were washed twice with MM. A total of 2 µL of the cell suspension adjusted to $OD_{600} = 0.1$ with MM were spotted on MM plates and MM plates supplemented with various nutrients. The plates were incubated at 30 °C for 2 days.

Complementation of the auxotrophic phenotype

Pa*ADE2*, Pa*LYS12*, and Pa*URA3* were amplified via PCR using the GB-4(0) genome as a template, and the primer pairs listed in Table 2 (PaADE2−500 and PaADE2+2390c for Pa*ADE2*, PaLYS−500F and PaLYS12+1777c for Pa*LYS12*, and PaURA3−500 and PaURA3+1433c for Pa*URA3*). Cell suspension of each auxotrophic strain treated with 0.1 pmol of each amplified DNA fragment using the protoplast-PEG method was diluted with 1 mL of 1 × KTC and immediately spread over various MMS plates that were not supplemented with adenine, lysine, or uracil. The plates were incubated at 30 °C for 3 days. The strain PGB800 was also transformed with three complementary genes at a time.

Genomic analysis of the triple auxotrophic strain PGB800

Genomic DNA preparation was performed as previously described (Sameshima-Yamashita *et al.* 2019). Briefly, strain PGB800 was cultivated in 30 mL of YM medium at 30 °C at 200 rev min^{-1} for 16 h. Cells recovered from 30 mL of culture were incubated in 10 mL of citrate buffer (150 mM KCl, 580 mM NaCl, 50 mM sodium citrate, pH5.5) containing 20 mg/mL glucanex (Sigma-Aldrich) at 30 °C for 2 h. The protoplasts were harvested and lysed with 1% SDS at 65 °C. The lysate was mixed with 3.75 mL of 4 M potassium acetate, and the genomic DNA of the supernatant was precipitated with 2-propanol. The recovered genomic DNA was further purified using the cetyltrimethylammonium bromide (CTAB) method (Richards *et al.* 1994).

The genomic DNA of PGB800 was sequenced using an Illumina Novaseq6000 (150 base pairs [bp], paired-end) and a PacBio RSII system (Macrogen Japan Corp. Tokyo, Japan). Sequence similarity was determined using the basic local alignment search tool (BLAST) (Altschul *et al.* 1997). PacBio subreads showing similarity (BLAST score ≥50) to "pop-in" fragments of Pa*ADE2* and Pa*LYS12* were extracted. Unitig sequences were generated with the subreads extracted as a result of CANU assembly (Koren *et al.* 2017) to verify the precise excision of Pa*ADE2* and Pa*LYS12*, as expected. Illumina reads were aligned on the GB-4(0) genome sequence using Burrows-Wheeler Aligner (BWA) (Li and Durbin 2009) to examine unexpected deletions in the PGB800 genome. The Illumina reads were also aligned using BWA to the vector sequence (pAG25; Goldstein and McCusker 1999) to amplify the pop-in fragments and investigate the possible insertion of the vector sequence into the PGB800 genome.

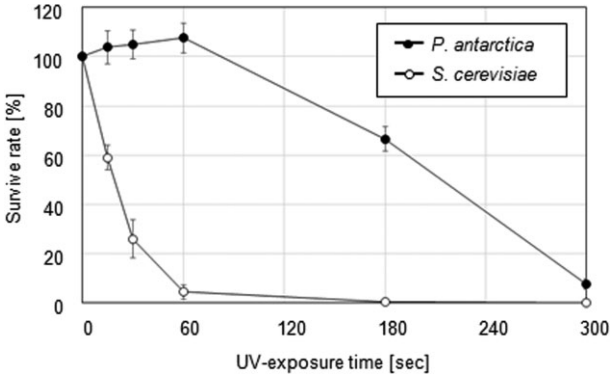

Figure 1. Ultraviolet radiation tolerance of *P. antarctica*. Filled circle, *P. antarctica*; empty circle, *S.cerevisiae*.

Results and discussion

UV resistance of *P. antarctica* and the establishment of the Pa*ura3* mutant via UV treatment

The *P. antarctica* GB-4(0) strain required UV irradiation for 180 sec to achieve 60% survival. The comparable survival rate for the model yeast *S. cerevisiae* cells was 30 sec, which is one-sixth of that required for *P. antarctica* (Figure 1). In contrast, the basidiomycetous yeasts *Cryptococcus flavus* GB-1 and *Rhodotorula mucilaginosa* IY-05, isolated from the plant surface, showed the same UV resistance as *P. antarctica* (Figure S1). All three basidiomycetous yeasts isolated from leaf surfaces tested in this study were more UV-resistant than *S. cerevisiae*. The reason for this is not clear, but it may be related to the fact that plant surfaces inhabited by these yeasts are exposed to sunlight. When obtaining mutants of *S. cerevisiae*, UV irradiation treatment was recommended to achieve approximately half the survival rate (Lawrence 1991); the irradiation length for UV treatment of *P. antarctica* was set to 180 sec in this study.

The composition of the FOA medium used to select uracil-auxotrophic *P. antarctica* strains was determined in a previous study using the Pa*URA3* disruptant (strain Pa*ura3*Δ::*natMX4*) of

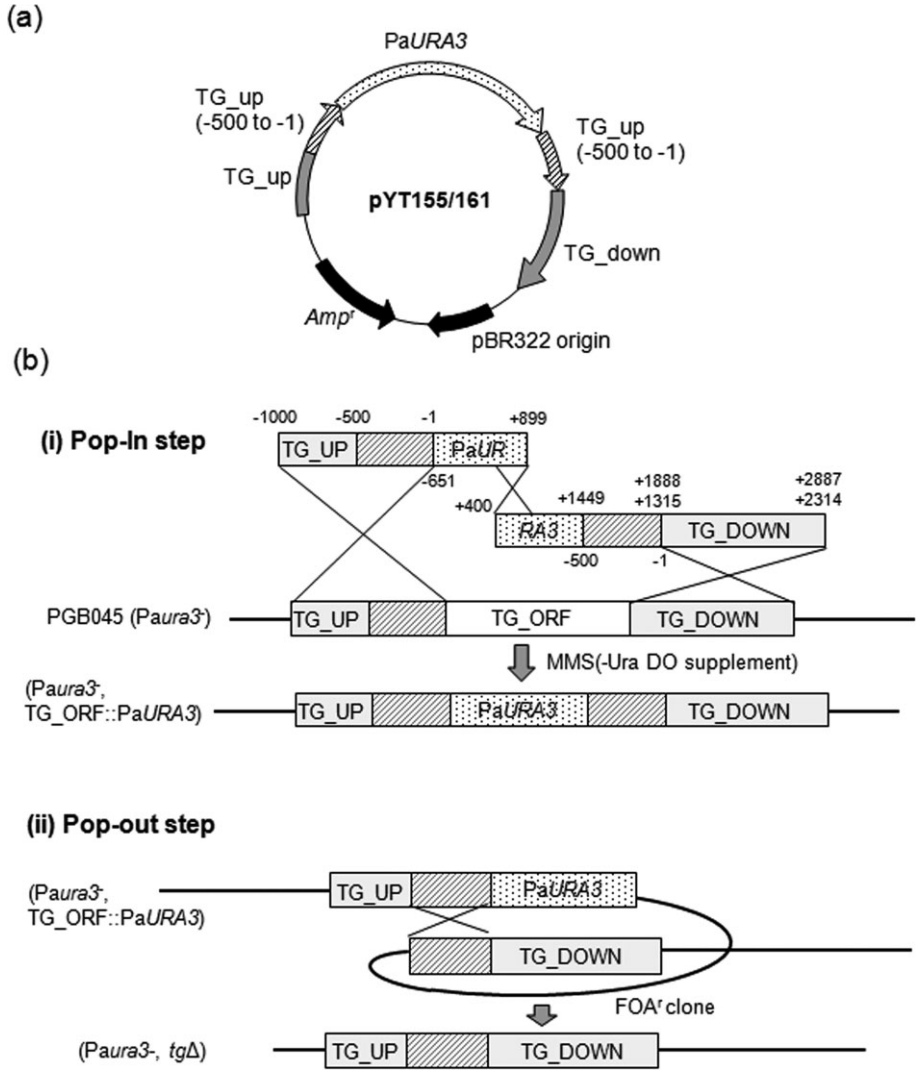

Figure 2. Site-specific gene disruption of *P. antarctica* using the Pa*URA3* marker recycling system. (a) Structures of pYT155 and pYT161 for the disruption of target genes (TGs) using marker recycling system. (b) Homologous recombination via Pa*URA3* pop-in disrupts the target site (i). The cells are then transferred to the FOA medium in which only Pa*URA3* pop-out cells can be grown (ii). The Pa*URA3* cassette in the genomic DNA of FOA-resistant strains was deleted through homologous recombination between −500 to −1 upstream of the target gene located at both ends of the inserted Pa*URA3* sequence.

P. antarctica GB-4(0) (Yarimizu *et al.* 2017). Strain GB-4(0) grew on MM, but Pa*ura3*Δ::*natMX4* did not. Strain Pa*ura3*Δ::*natMX4* grew in a manner similar to strain GB-4(0) when 1000 mg/L uracil was added to MM. The Pa*ura3*Δ::*natMX4* strain, but not GB-4(0), grew on an FOA medium supplemented with MM-uracil (1000 mg/L) and 1000 mg/L of 5′-FOA. In this study, FOA medium was used as the selective medium for Pa*ura3* mutants. *P. antarctica* GB-4(0) cells were grown on the FOA medium to obtain clones that grew after 180 sec of UV irradiation. Among the clones obtained, auxotrophic strains that did not grow on MM were selected. A strain whose auxotroph was complemented by the transformation of the Pa*URA3* fragment was named strain PGB045 (Pa*ura3*). The Pa*URA3* sequence of strain PGB045 was changed from C to T at position 867, resulting in a stop codon.

Acquisition of auxotrophic strains using a marker recycling system

The uracil-auxotrophic mutant *P. antarctica* PGB045 was transformed separately with PCR-amplified fragments from plasmid pYT155/161. The upstream and downstream DNA fragments overlapped by Pa*URA3* were simultaneously introduced (Figure 2b, [i]). This method is reported to increase the efficiency of obtaining the correct transformant in *Kluyveromyces lactis* (Erdeniz, Mortensen and Rothstein 1997) and *Aspergillus nidulans* (Nielsen *et al.* 2006). After incubation on MMS containing DO Supplement -Ura, 60 clones of each candidate Pa*ADE2* or Pa*LYS12* disruptant were transferred to MM and their auxotrophy were examined; seven clones each failed to grow. Then, the genomic DNA of the selected seven candidates each was extracted and the site-specific insertion of the "pop-in" fragment containing Pa*URA3* was confirmed via PCR (Figure 3a[i]). The predicted sizes of DNA fragments "b" and "c" (Table 3) were amplified successfully from the genomic DNA of the three strains. Besides the DNA fragment, which showed the size predicted using the primer sets for DNA fragments ("a" in Table 3), several amplified bands were also detected. It was considered that −500 to −1 upstream of the target gene located at both ends of the inserted Pa*URA3* sequence inhibited the specific amplification by PCR. It was also considered that the "pop-out" of Pa*URA3*

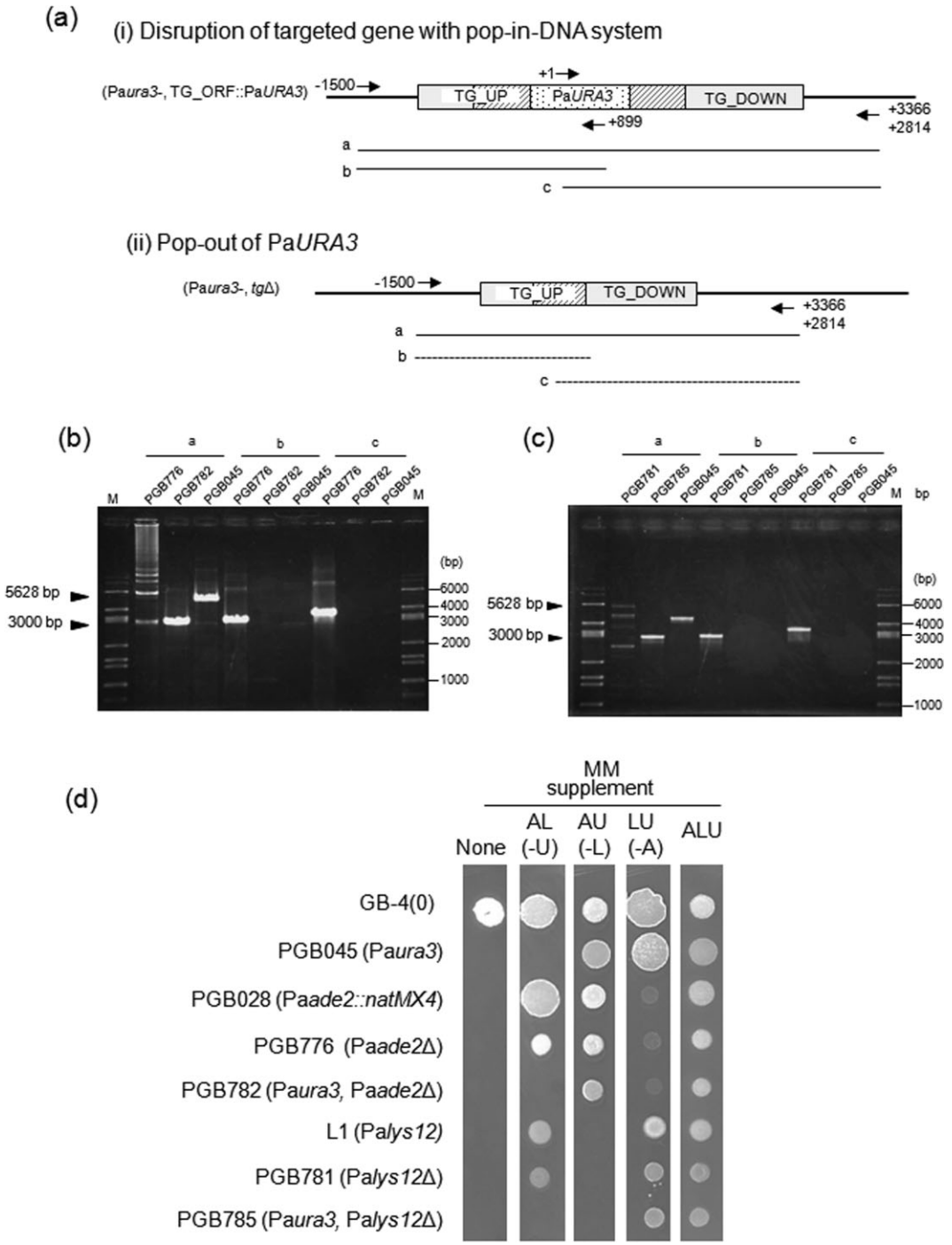

Figure 3. Confirmation of Pa*ADE2* and Pa*LYS12* disruption. (a) Scheme to confirm target gene disruption by the introduction (i) and deletion (ii) of Pa*URA3* using PCR. (b) Confirmation of Pa*ADE2* disruption and Pa*URA3* deletion. (c) Confirmation of Pa*LYS12* disruption and Pa*URA3* deletion. (d) Growth of wild-type and mutant samples on MM plates supplemented various nutrients. AL, supplemented with adenine and lysine; AU, supplemented with adenine and uracil; LU, supplemented with lysine and uracil; ALU, supplemented with adenine, lysine, and uracil; GB-4(0), wild-type strain; PGB045 (Pa*ura3*), host strain. PGB028 (Pa*ade2*Δ::*natMX4*) and L1 (Pa*lys12*) were used as controls.

occurred spontaneously in the chromosomes of the "pop-in" strains when they were cultured in a medium without selective pressure. Therefore, three clones each that could "pop-in" correctly were selected for the disruption of Pa*ADE2* and Pa*LYS12* via HR, respectively. One each of them, PGB776 (Pa*ade2*Δ) and PGB781 (Pa*lys12*Δ), were selected and designated (Figures 3b and c).

Next, the "pop-out" of Pa*URA3* in the "pop-in" strain was tested (Figure 2b [ii]). Consequently, 42 and 11 FOA-resistant strains were obtained from PGB776 and PGB781, respectively. Genomic DNA of the selected six candidates on each auxotrophy was extracted, and the site-specific deletion of Pa*URA3* and the target gene (ORF) was confirmed via PCR (Figure 3a[ii]). The predicted sizes of DNA fragments "a", "b", and "c," which are listed

Table 3. PCR product size for the confirmation of Pop-in/Pop-out

	Primer set	Strain (template)	PCR fragment (bp)
	PaADE2		
a.	PaADE2−1500	PGB045	4866
	PaADE2+3366c	PGB776	5628
		PGB782	3000
		PGB785	4866
		PGB797	5628
		PGB800	3000
b.	PaADE2−1500	PGB045	nd
	PaURA3+899c	PGB776	3087
		PGB782	nd
		PGB785	nd
		PGB797	3087
		PGB800	nd
c.	PaURA3+1	PGB045	nd
	PaADE2+3366c	PGB776	3455
		PGB782	nd
		PGB785	nd
		PGB797	3455
		PGB800	nd
	PaLYS12		
	Primer set	Strain (template)	PCR fragment (bp)
a.	PaLYS12−1500	PGB045	4314
	PaLYS12+2814c	PGB781	5628
		PGB785	3000
b.	PaLYS12−1500	PGB045	nd
	PaURA3+899c	PGB781	3087
		PGB785	nd
c.	PaURA3+1	PGB045	nd
	PaLYS12+2814c	PGB781	3455
		PGB785	nd

Nd, not amplified; bp, base pair.

in Table 3, were amplified successfully from the genomic DNA of all tested strains. Thus, all tested FOA-resistant strains showed Pa*URA3* deletion. One each of them, PGB782 (Pa*ura3*, Pa*ade2*Δ) and PGB785 (Pa*ura3*, Pa*lys12*Δ), were selected and designated (Figures 3b and c). The resulting strains showed no auxotrophic requirements, except for adenine or lysine and uracil (Figure 3d). This result demonstrates that the marker recycling system was functional in *P. antarctica* GB-4(0) and is an effective method for gene disruption.

Construction of the triple auxotrophic strain

Additional Pa*ADE2* disruption using a marker recycling system was established in PGB785 (Pa*ura3*, Pa*lys12*Δ). In a previous study, we found that *P. antarctica* cells with disrupted Pa*ADE2* by the drug resistance gene exhibited a red color as the *ADE2* disruptant of *S. cerevisiae*. After introducing the "pop-in" fragments for Pa*ADE2* disruption, red colored colonies were selected, and 25 colonies that could not grow on MM supplemented with lysine hydrochloride were obtained. Genomic DNA of six candidates was extracted and the site-specific insertion of the "pop-in" fragment was confirmed via PCR. For three strains among six colonies, the predicted sizes of DNA fragments "b" and "c" were amplified successfully from the genomic DNA (Figure 4a). Among them, one strain was selected and designated as PGB797

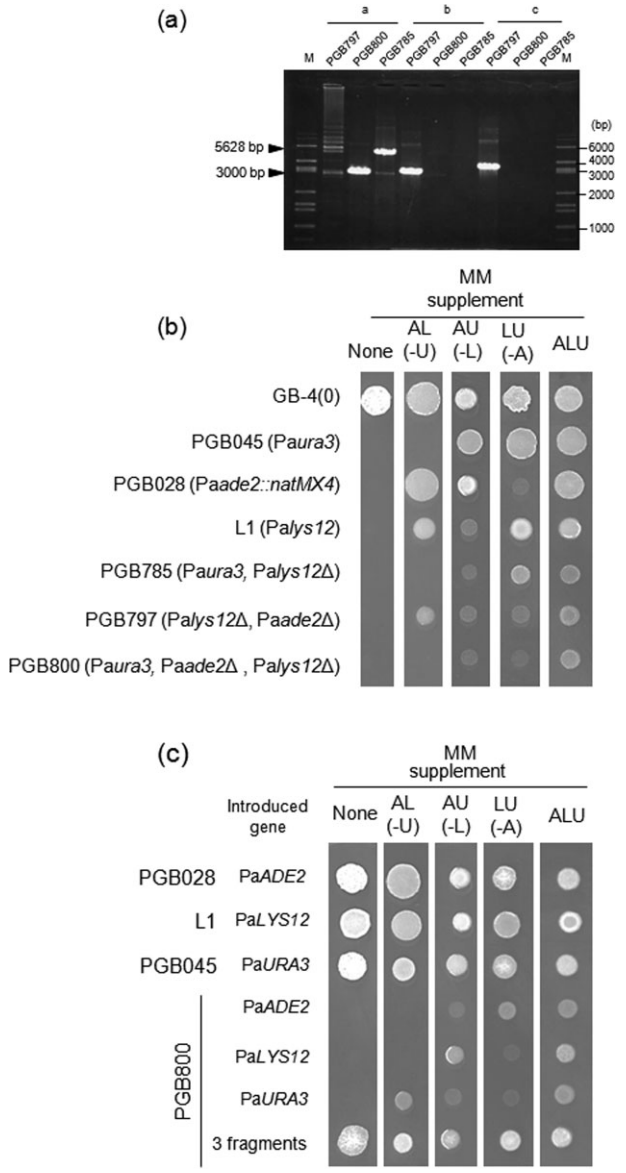

Figure 4. Construction of the triple auxotrophic mutant strain PGB800 and complementation of each gene. (a) Confirmation of Pa*ADE2* disruption and Pa*URA3* deletion. (b) Growth of wild-type and mutants on MM plates supplemented various nutrients. GB-4(0), wild-type strain; PGB785 (Pa*ura3*, Pa*lys12*Δ), host strain. PGB045 (Pa*ura3*), PGB028 (Pa*ade2*Δ::*natMX4*) and L1 (Pa*lys12*) were used as controls. (c) The complementation of adenine, lysine, and uracil auxotroph in strain PGB800.

(Pa*ade2*Δ, Pa*lys12*Δ) (Figure 4b). Subsequently, a "pop-out" of Pa*URA3* was performed for PGB797 on the FOA medium. Among the 30 FOA-resistant strains obtained, genomic DNA of selected six candidates was extracted, and the site-specific deletion of Pa*URA3* and ORF was confirmed via PCR (Figure 4a). The DNA fragments were successfully amplified from the genomic DNA of all the tested strains. Among them, one strain was selected and designated as PGB800 (Pa*ura3*, Pa*ade2*Δ, and Pa*lys12*Δ). The growth of all strains on various media was checked and the resulting strains were found to have no auxotrophic requirements except for uracil, adenine, and lysine, as intended (Figure 4b). This result demonstrates that the marker recycling system is an effective method for multiple gene disruptions in *P. antarctica*. Each complementary gene was introduced separately or

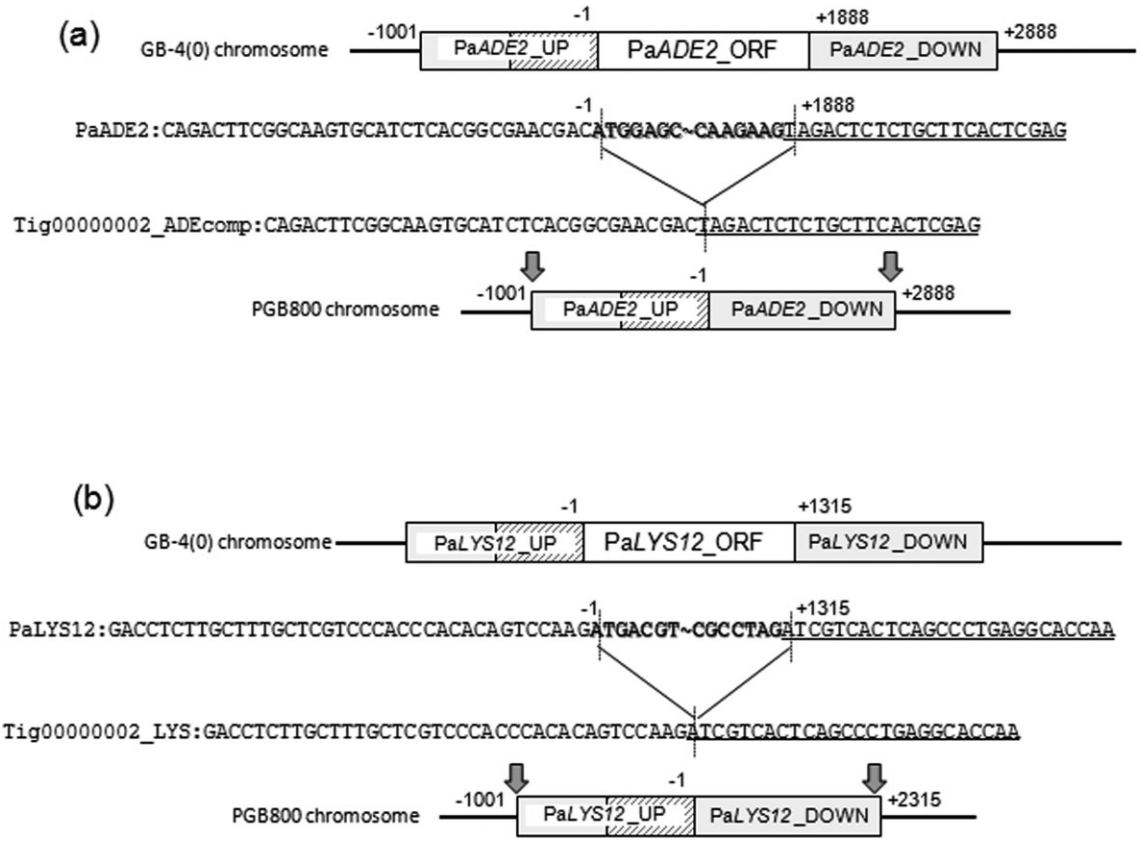

Figure 5. Identification of the nucleotide sequence of the gene disruption site in triple auxotrophic strain PGB800. Open reading frame (ORF) of Pa*ADE2* (a) and Pa*LYS12* (b) was deleted, respectively. Arrows indicate continuity between inserted sequences and original genomic sequences. The nucleotide sequence of the chromosome shows that the target gene has been completely deleted and the surrounding sequence has not changed. The underlined sequences correspond downstream of the target gene, and the bolded sequences correspond within the target gene ORF.

simultaneously into the triple auxotrophic strain PGB800, and each resultant strain was confirmed to lose its auxotrophic trait (Figure 4c).

Examination of the disruption of Pa*ADE2* and Pa*LYS12* via genome sequencing

Genome sequencing of PGB800 was performed to verify the disruption of Pa*ADE2* and Pa*LYS12* via "pop-out," as expected. PacBio sequencing generated 245 870 subreads containing 2520575215 bases. Illumina sequencing generated 21410410 reads, containing 3232971910 bases. The PacBio subreads showing similarity with "pop-in" fragments for Pa*ADE2* and Pa*LYS12* were 722 and 1107, respectively. Pa*ADE2* subreads were assembled into two unitigs. Pa*LYS12* subreads were assembled into three unitigs with 13 unassembled subreads. The assembled sequences of Pa*ADE2* and Pa*LYS12* included single sequences carrying Pa*ADE2* and Pa*LYS12*, the ORFs of which were excised. The ORF excised sequences did not show any other insertions or deletions except for excision (Figure 5).

The remaining assembled and unassembled sequences were matched to the original genomic sequence. Therefore, we concluded that the "pop-out" occurred precisely. Any Illumina reads aligned with the vector sequence used to amplify the "pop-in" fragments were not detected. In addition, none of the PacBio subreads was similar to the vector sequence. Several genomic regions of GB-4(0) did not align with the Illumina reads; however, the uncovered genomic regions had corresponding PacBio subreads (data not shown). Therefore, we concluded that PGB800 did not have any unexpected insertions of external sequences, such as vectors.

In this study, we demonstrate a marker recycling system using the orotidine-5′-phosphate decarboxylase gene functions in the basidiomycetous yeast *P. antarctica*. The triple auxotrophic strain was constructed without the insertion of a foreign gene, which can be used as a host for self-cloning strains to modify their genes using complementary genes as transformation markers.

Previously, we found that the xylanase promoter of *P. antarctica* GB-4(0) strongly induces xylanase gene expression in the presence of xylose (Watanabe *et al*. 2014). The yeast *Phichia pastoris* is commonly used for the overexpression of recombinant proteins using the methanol-inducible AOX1 promoter. However, because methanol is toxic and dangerous, adequate controls for industrial mass production are required. On the other hand, xylose is abundant on Earth as a component of plant hemicellulose. In a high-yield protein production using *P. antarctica* as a host, xylose is intended to be used as a substrate for the xylanase promoter and a carbon source that can be safely used in large-scale culture.

The *P. antarctica* wild-type strain has previously been transformed using a DNA cassette containing a biodegradable plastic-degrading enzyme (PaE)-encoding gene downstream of the xylanase promoter using a foreign drug resistance marker that secretes large amounts of PaE (Watanabe *et al*. 2016). In Japan, the self-cloned cells can be grown in a variety of existing facilities

that have already recouped capital expenditures, thus reducing the enzyme production cost. The lysine-auxotrophic Pa*lys12* mutant that transforms the cassette using Pa*LYS12* markers also secretes large amounts of PaE (Sameshima-Yamashita *et al.* 2019). However, the higher the production, the better the practical use of the enzyme. Multiple auxotrophic host strains can be used to generate self-cloning strains with enhanced productivity of not only PaE but also other useful substances by introducing several cassettes into the chromosome using complementary markers. In addition, by using the Pa*URA3* marker recycling system, a variety of auxotrophic markers other than those presented in this study can be applied (Yarimizu *et al.* 2013). This system can be used for various applications in order to utilize *P. antarctica* as a recombinant protein production platform as well as for functional analysis of *P. antarctica* cells.

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Supplementary material

Supplementary material is available at *Bioscience, Biotechnology, and Biochemistry* online.

Data availability

The data underlying this article are available in the article.

Author contribution

Y.S-Y., T.Y., T.T. and H.K. conceived and designed the experiments. Y.S-Y. and T.Y. performed the main experiments. H.U. and T.T. analyzed the genome sequence data. Y.S-Y., T.Y. and H.K. wrote the paper. All authors discussed the results and commented on the manuscript.

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Disclosure statement

Y.S-Y., T.Y. and H.K. are inventors of the applied patent (Japanese Laid-Open Patent Publication 2018-157 814) relevant to this work.

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