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Construction of a *Pseudozyma antarctica* strain without foreign DNA sequences (self-cloning strain) for high yield production of a biodegradable plastic-degrading enzyme

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ABSTRACT

The basidiomycetous yeast *Pseudozyma antarctica* GB-4(0) esterase (PaE) is a promising candidate for accelerating degradation of used biodegradable plastics (BPs). To increase safety and reduce costs associated with the use of PaE, we constructed a self-cloning strain with high-PaE productivity. A Lys12 gene (Pa*LYS12*)-deleted lysine auxotroph strain GB4-(0)-L1 was obtained from GB-4(0) by ultraviolet mutagenesis and nystatin enrichment. Subsequently, the PaE gene (Pa*CLE1*) expression cassette consisting of GB-4(0)-derived Pa*CLE1*, under the control of a xylose-inducible xylanase promoter with Pa*LYS12*, was randomly introduced into the GB4-(0)-L1 genome. A PaE high-producing strain, PGB474, was selected from among the transformants by high throughput double-screening based on its ability to degrade emulsified polybutylene succinate-*co*-adipate. Quantitative PCR revealed that four copies of the PaE gene expression cassette were introduced into the PGB474 genome. PGB474 produced 2.0 g/L of PaE by xylose-fed-batch cultivation using a 3-L jar fermentor for 72 h.

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Pseudozyma antarctica; PaE; self-cloning; xylanase promoter; jar fermentation

The accumulation of plastic waste is becoming a serious problem worldwide [1,2]. Biodegradable plastics (BPs) with good mechanical strength can replace conventional non-degradable plastics, such as polyethylene. BPs can be degraded completely into water and carbon dioxide by microorganisms in the natural environment. Aliphatic polyesters, such as polybutylene succinate-*co*-adipate (PBSA) and polybutylene succinate (PBS) are often blended with BP products, such as agricultural mulch film since they have similar mechanical properties to polyethylene. The degradation speed of BP products depends not only on the characteristics of the blended polymer but also largely on environmental conditions [3], and is sometimes slower than desired. Since the depolymerization step controlls the rate of degradation of BP polymers [4], acceleration of the degradation of BP polymers through the use of a BP degrading enzyme is one possible solution to reduce the accumulation of used BP products in the natural environment. Enzymes that degrade PBS and PBSA have been isolated from various microorganisms such as yeast *Cryptococcus* sp. S-2 [5], fungus *Aspergillus oryzae* [6], and bacteria *Thermobifida alba* [7]. We previously demonstrated the accelerated degradation of commercially available BP mulch films in agricultural settings through spray-treatment with a culture filtrate of the fungus *Paraphoma* sp. strain B47-9 containing a BP-degrading enzyme (*Paraphoma*-related cutinase-like enzyme, PCLE). The treatment produced many visible cracks on the fresh films 1 day after treatment [8].

The yeast *Pseudozyma antarctica* (*Moesziomyces antarcticus*) secretes an esterase (PaE) that can degrade PBS, PBSA, and other types of BP films faster than PCLE [9–11]. The degradation speed of cut BP mulch film in soil under laboratory incubation conditions was significantly enhanced by pretreatment with PaE containing *P. antarctica* culture filtrate compared to that without pretreatment [12]. Therefore, we considered PaE to be a promising candidate for accelerating the degradation of BPs. *P. antarctica* strains increased the secretion of PaE when cultivated with xylose [13]. Since xylanase was also produced in the culture filtrate in large amounts [14], the xylanase promoter was used to control PaE production. A fragment containing the PaE gene (Pa*CLE1*) of *P. antarctica* type strain JCM10317 was sited between the xylanase promoter and the terminator of strain T-34, and the neomycin resistance

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Supplemental data for this article can be accessed here.

gene of *Escherichia coli* under the control of the *P. antarctica* T-34 homocitrate synthase promoter was transformed randomly into the *P. antarctica* strain GB-4(0) chromosome. The drug-resistant transformant strains were selected on a plate containing geneticin (G418). Using a xylose fed batch jar fermentor, 13.4× concentrated PaE was produced from the transformant strain GB-4(0)-X14 compared to that produced by the wild-type strain [15].

Products created via the use of genetically modified organisms (GMOs) are subject to additional legal restrictions compared to those from traditionally-bred varieties, and over 140 countries have joined the Cartagena Protocol on Biosafety. The safety assessment of microbial enzymes in industrial scale production focuses more on contaminants entering the manufacturing process than on the enzymes themselves [16,17]. Self-cloning microorganisms that involve the insertion of genetic material into a place in the DNA different from that in which the DNA normally occurs are excluded from the requirements of this legislation [18]. Following recognition of the self-cloning status of the microorganism, the strain can be used for industrial production in the same manner as conventional wild-type strains in Japan [19–21]. Since the PaE gene high expression cassette is derived from *P. antarctica*, a host vector system derived from *P. antarctica* can be used to generate a self-cloning strain. Generally, a self-cloning strain is constructed using an auxotrophic mutant as a host and introducing a gene connected with an auxotrophic complementation gene as a selection marker. We constructed *P. antarctica* GMO strains auxotrophic to uracil and adenine by replacing the gene that synthesizes nutrients with an exogeneous drug-resistant marker gene [22]. However, no auxotrophic mutant *P. antarctica* strain without an exogeneous gene has been constructed.

In this study, we aimed to develop a host vector system using auxotrophic mutants and complementary genes of rice seed-derived *P. antarctica* GB-4(0) and construct a self-cloning strain by introducing PaE gene high expression cassette made of GB-4(0) DNA sequences.

Materials and methods

Strains, plasmids, and primers

The wild-type *P. antarctica* strain GB-4(0), isolated from rice seeds [9] deposited in the Genebank at the National Institute for Agrobiological Sciences, Japan (accession no. MAFF 306999) was used as the host strain. The lysine auxotroph mutant GB-4(0)-L1 (Pa*lys12*) and a PGB474 strain that introduced a xylose-inducible PaE gene expression cassette with Pa*LYS12* in GB-4(0)-L1 were constructed in this study. All the yeast strains were cultivated at 30°C. *E. coli* DH5α (Takara, Kyoto, Japan) was used as the host for plasmid construction and amplification. Plasmid pUC19 was used for the construction of a PaE gene expression cassette. Primers used in this study are listed in Table 1.

Isolation of the auxotrophic mutants by UV mutagenesis and nystatin enrichment

P. antarctica GB-4(0) was cultivated in 5 mL of YM medium (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, and 1% glucose) with shaking at 160 min^{-1} for 24 h. The culture (1 mL) and 30 mL of ice-cold sterilized water were mixed in a petri dish (optical density at 660 nm [OD_{660}] was 1.0). The mixture was stirred with a magnetic stirrer and exposed to UV light at a distance of 50 cm for 60 s.

The UV-irradiated cells were collected at 1,000 × *g* for 1 min and resuspended with 5 mL of YM medium. After incubation with shaking at 160 min^{-1} for 3 h, the 1 mL culture was centrifuged at 1,000 × *g* for 1 min. Growth of the recovered cells was suppressed by nitrogen starvation after which the cells were washed twice with sterilized water,

Table 1. Primers used in this study.

Primer name		Sequences
For construction of pPaLYS12XG-Pa*CLE1*		
PLYS12_F1	5′	-ACGGCCAGTGAATTCGTACATATAGTTGGCCGCTGCATAT-3′
TLYS12-TXYN_R	5′	-AAAGAGAACGAGAAGTTCACCTCCATGGACGAGC-3′
PXYN_F	5′	-ACCGAGCTCGAATTCCACGCCACGTTCGATACCGAC-3′
PXYN-PaEG_R	5′	-AACTGCATCGTAAGGTTTGTTTGGCGTTTTGGG-3′
TXYN-TLYS12_R	5′	-ACTTCTCGTTCTCTTTTTGCCAGGTGTTGGACT-3′
TXYN-PaEG_F	5′	-AGGGATAATCCGTCGTCACCGGCTTGCCTGTAT-3′
PaEG_F	5′	-ACCTTACGATGCAGTTCAAGTCGACCTTTGCCG-3′
PaEG_R	5′	-ACGACGGATTATCCCTGAAGAGCCTTGATACCG-3′
For qPCR		
act_4F	5′	-ATGCATCCAGTCTACCTTCGC-3′
act_3R	5′	-TCCAGGTCGCACTTCATGATC-3′
PaCLE_1F	5′	-AATGCCCTTCCTCAGCTTACTG-3′
PaCLE_1R	5′	-CGTTACCATCGACGTTGCAAG-3′
act_1F	5′	-TTTCCGTCTCACGTCTGAAGG-3′
act_1R	5′	-AGTTCAAGAGCGAGTCAGGATG-3′

The *Eco*RI recognition sequences are underlined.

resuspended with 5 mL of nitrogen-deficient-YNBD (0.17% Bacto yeast nitrogen base w/o amino acids and ammonium sulfate [Becton, Dickinson and Company, Franklin Lakes, NJ, USA] and 2.0% glucose) and shaken at 160 min^{-1} for 4.5 h. After centrifugation, the recovered cells were washed twice with sterilized water and resuspended with 4 mL of YNBD containing 0.5% ammonium sulfate. To promote the proliferation of non-auxotrophic cells, these were shaken at 160 min^{-1} for 3 h. Then, the culture was mixed with 500 μL of 0.01% nystatin solution and shaken at 160 min^{-1} for 1 h.

The nystatin-treated culture was diluted 10^3 times with sterilized water and 50 μL were spread on YM medium plate. After incubation for 2 days, apparent colonies were replicated on YM medium and YNBD plates (0.67% YNB w/o amino acids, 2% glucose, and 2% agar) and cultivated. Colonies that did not grow on the YNBD plate were selected as candidate auxotrophic mutants. The auxotrophic mutant type was confirmed by growth on a minimal medium supplemented with a combination of five or four different amino acids and nucleotide bases (adenine, guanine, cysteine, methionine, uracil, histidine, leucine, isoleucine, valine, lysine, phenylalanine, tyrosine, tryptophan, threonine, proline, glutamic acid, serine, alanine, aspartic acid and arginine) in a final concentration of 40 mg/L, respectively [23].

Genomic DNA extraction

Yeast strains were pre-cultivated in 2 mL of YM medium for 24 h. The pre-cultures (300 μL) were added to 300-mL flasks containing 30 mL of YM medium and cultivated with rotary shaking at 200 rpm for 16 h. Then, 30 mL of culture was harvested and incubated for 2 h at 30°C in 10 mL citrate buffer (150 mM KCl, 580 mM NaCl and 50 mM sodium citrate, pH 5.5) containing 20 mg/mL glucanex (Sigma-Aldrich, St. Louis, MO, USA). The protoplasts were harvested, resuspended, and incubated for 30 min at 65°C in 7.5 mL of Tris buffer (50 mM Tris-HCl and 20 mM EDTA, pH 7.5) with 1% SDS. The lysates were 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 purified further using the CTAB method [24].

Genomic analysis of lysine auxotrophic strain GB-4(0)-L1

The genomic DNA of strain GB4-(0)-L1 was sequenced on an Illumina Miseq system according to the manufacturer's instructions using a NEBNext Ultra DNA library prep kit for Illumina (New England BioLabs, Ipswich, MA, USA) and a Nextera mate-pair sample prep kit (Illumina, San Diego, CA, USA). The sequences were compared with the GB-4(0) genomic sequence as a reference.

Construction of the PaLYS12 complementing plasmid harbouring the PaE gene expression cassette

The entire PaLys12 gene of strain GB-4(0), including parts of the 5′ upstream (999 base pairs [bp]) and 3′ downstream (469 bp) regions, was amplified by polymerase chain reaction (PCR) using genomic DNA as the template and primers PLYS12_F-1 and TLYS12-TXYN_R (Table 1). The xylanase promoter region P_{XYN1} (1513 bp) and the terminator region T_{XYN1} (300 bp) were also amplified by PCR using genomic DNA of GB-4(0) as the template and primers PXYN_F and PXYN-PaEG_R for PXYN or TXYN-TLYS12 and TXYN-PaEG_F for TXYN. The PaE gene (675 bp) was also amplified by PCR using genomic DNA of GB-4(0) as the template and primers PaEG_F and PaEG_R. The all PCR products were purified and ligated with *Eco*RI-digested pUC19 in one reaction using an In-fusion HD cloning kit (TaKaRa BIO, Shiga, Japan). The DNA solution was introduced into *E. coli* DH5α and the cells were then plated onto LB agar containing ampicillin and incubated at 37°C. Some colonies were picked and subjected to colony PCR to confirm whether the DNA fragments were ligated in the correct order. A colony possessing the successfully ligated plasmid was selected and the pPaLYS12XG-Pa*CLE1* plasmid was isolated from cells.

Transformation of a lysine auxotrophic mutant with the PaE gene expression cassette

pPaLYS12XG-Pa*CLE1* was digested with *Eco*RI. A 5.3 kb DNA fragment (PaE gene expression cassette with Pa*LYS12*) was purified from the agarose gel and 1 μg of DNA was introduced into lysine auxotroph mutant GB-4(0)-L1 cells by lithium acetate treatment, as described previously [22]. The cells were spread on an YNBD plate. After incubation for 2 days, apparent colonies were replicated on a new YNBD plate and cultivated again. The colonies able to grow on the YNBD plate were selected as transformants and were considered candidates for the PaE high-producing strain.

High throughput screening for isolation of transformants showing high BP degrading activity

The GB-4(0)-L1 transformants containing the PaE gene expression cassette with Pa*LYS12* were inoculated onto 3× FMM-8.0% xylose-emulsified PBSA plates (0.2% $NaNO_3$, 0.06% KH_2PO_4, 0.06% $MgSO_4 \cdot 7H_2O$, 0.3%

yeast extract, 8.0% xylose, 0.5% emulsified PBSA [Bionolle EM-301, Showa Denko K. K., Tokyo, Japan], and 2% agar) using a toothpick and incubated for 3 days. PBSA degradation activity was evaluated by the formation of a clear zone around a colony (the clear zone size was calculated by subtracting the colony diameter from the clear zone diameter). Next, 115 strains showing a relatively larger clear zone and two control [host strain GB-4(0)-L1 and L1 strain transformed with PaLYS12 fragment, L1(Pa*LYS12*)] showing a relatively smaller clear zone were selected for a second screening. The selected strains were pre-cultivated in 800 µL of YM medium using a 96 DeepWell plate (SANPLATEC CORPORATION, Osaka, Japan) at 1,500 rpm for 24 h. An aliquot of the preculture (80 µL) was transferred to 800 µL of YPX medium (1% yeast extract, 2% peptone, and 8% xylose) in a 96 DeepWell plate and incubated at 1,500 rpm at 30°C for 66 h. After centrifugation, the supernatants were transferred to new plates and stored at −30°C until evaluation of BP degradation.

PaE productivity of each of the transformants was evaluated by the PBSA degradation activity of the culture supernatant in the 96 DeepWell plate. There was a good correlation between the turbidity (OD_{660}) and the emulsified PBSA concentration, which occurred in a PBSA concentration range of 0.025–0.15% in 150 µL HEPES buffer (25 mM, pH 7.3 ± 0.1, DOJINDO, Tokyo, Japan) in a 96-well microplate (Supplemental Figure S1). The culture supernatants were diluted to 4× with sterilized water. Each diluted supernatant (10 µL) was mixed with 140 µL of buffer and emulsified PBSA mixture in a 96-well microplate (PROTEOSAVE™; Sumitomo Bakelite, Tokyo, Japan). The reaction mixtures (150 µL containing 25 mM HEPES buffer with emulsified PBSA [turbidity of 0.5 at 660 nm] and 10 µL of sample) were incubated at 900 rpm using a DeepWell Maximizer (TAITEC, Saitama, Japan) at 30°C for 10 min. High PaE production candidates were selected based on the decreased emulsified PBSA turbidity. The selected transformants were used for the subsequent flask cultivation experiments.

Flask cultivation of transformants to select high PaE productivity

The selected transformants were inoculated into 2 mL of YM medium in test tubes (ϕ 18 mm, with cotton plugs) and cultivated by reciprocally shaking at 150 min^{-1} for 24 h. Each preculture (200 µL) was transferred to 20 mL of YPX medium in a 100-mL flask with cotton plugs, and rotary shaken at 200 rpm. After 72 h of incubation, the culture (1 mL) was harvested and centrifuged at 20,000 × *g* for 10 min. The PaE concentration of the supernatant was analyzed as described below. The pellets were dried at 105°C for 6 h and their dry cell weights were recorded to indicate cell growth.

Evaluation of PaE protein content based on PBSA degradation activity

The PaE concentration of the culture supernatants was evaluated based on the PBSA degradation activity by determining the decrease in turbidity of 0.05% (w/v) emulsified PBSA as follows: samples were diluted 4× with sterilized water in a Protein LoBind 1.5 mL tube (Eppendorf AG, Hamburg, Germany). The reaction mixture (1,995 µL) containing 1 mL of 50 mM HEPES buffer (pH 7.3 ± 0.1), 0.05% w/v PBSA (initial $OD_{660} \fallingdotseq 0.55$, Supplemental Figure S1) was added to a glass tube (inner diameter, 10 mm) and 5 µL of diluted sample was added. The reaction mixture was immediately vortexed and incubated for 15 min at 30°C, 150 min^{-1}. OD_{660} was measured before and after incubation. PBSA degradation activity (PaE activity), specific activity, and PaE concentration were calculated according to the following formula:

$$C = \frac{(T_2 - T_1) \times D}{t \times v \times S}$$

where C = PaE concentration [mg/mL], $T_1 = OD_{660}$ (optical path length = 10 mm) before incubation, $T_2 = OD_{660}$ (optical path length = 10 mm) after incubation, D = ratio of pre-dilution of the sample, t = incubation time [min], v = added volume of diluted sample [mL] and S = specific activity (= 13.41) [U/mg].

To determine specific activity, PaE activity was measured using purified PaE that was diluted to 0.02–0.8 mg/mL with sterilized water (0.1–4.0 µg PaE in a reaction mixture). Since there was a linear correlation ($r^2 > 0.99$) between the amount of PaE and PaE activity and the specific activity showed a standard deviation/average of <20%, the range of PaE content in the reaction mixture was set at 0.2–1.25 µg (Supplemental Figure S2). The specific activity of 13.41 ± 1.30 U/mg was calculated according to the following formula:

$$S = \frac{T_2 - T_1}{t \times w}$$

where S = specific activity [U/mg], $T_1 = OD_{660}$ (optical path length = 10 mm) before incubation, $T_2 = OD_{660}$ (optical path length = 10 mm) after incubation, t = incubation time [min], and w = amount of PaE in a reaction mixture [mg].

Quantitative PCR for PaCLE1 copy number determination

Gene copy numbers of Pa*CLE1* and Pa*ACT1* in the respective strains were quantified by qPCR. Primer sequences used are shown in Table 1. Standard samples were generated using the PCR amplicon. Pa*CLE1* (691 bp) was amplified from the PaE gene expression cassette with Pa*LYS12* using PaEG_F-PaEG_R primers and Pa*ACT1* (1405 bp) was amplified from the *P. antarctica* GB-4(0) genome using act_4F and act_3R primers with Q5 High-Fidelity 2× Master Mix (New England Biolabs, Tokyo, Japan). The PCR conditions were as follows: initial denaturation at 98°C for 30 s, followed by 30 cycles of 98°C for 10 s, 55°C for 30 s, 72°C for 30 s (Pa*CLE1*) or 1 min (Pa*ACT1*), with a final extension at 72°C for 2 min. Agarose gel electrophoresis (2.0% agarose gel) confirmed whether the PCR product was a single band. DNA concentrations were determined using a Qubit BR dsDNA Kit (ThermoFisher Scientific, Tokyo, Japan) according to the manufacturer's instructions. qPCR was carried out using SYBR Premix ExTaq II (TaKaRa BIO) with the PaCLE_1F-PaCLE_1R primer pair for Pa*CLE1* and the act1_1F-act_1R primer pair for Pa*ACT1* according to the manufacturer's instructions using the StepOne Plus Real-time system (ThermoFisher Scientific). After normalization using the copy number of Pa*ACT1*, the copy number of Pa*CLE1* in each strain was compared to that in GB-4(0)-L1.

Jar-fermentor cultivation

The PGB474 strain was cultivated in a jar fermentor as previously described [15]. Briefly, 18 mL of preculture was added to the 3-L jar fermentor containing 1.5 L PaE production medium (0.2% yeast extract, 0.2% $NaNO_3$, 0.5% $(NH_4)_2SO_4$, 0.04% KH_2PO_4, 0.04% $MgSO_4 \cdot 7H_2O$, and 2% xylose). Batch cultivation was performed until entire xylose has been depleted (around 24 h). Next, xylose fed-batch cultivation was performed by adding feeding medium (0.3% yeast extract, 0.05% KH_2PO_4, 0.01% $MgSO_4 \cdot 7H_2O$, and 20% xylose) at a rate of 300 mL d^{-1}. The cultivation conditions were as follows: aeration rate 2 LPM, agitation value 500 rpm, temperature at 30°C, and pH was controlled at 6.0 with 14% ammonia solution which also provided a nitrogen source for the culture.

At various times during the cultivation (24, 30, 48, 54, and 72 h), 1 mL of culture was harvested and centrifuged at 20,000 × *g* for 10 min. The pellets were dried at 105°C for 6 h and their dry cell weights were measured to investigate cell growth. At the same time, the amount of PaE in the supernatant was measured as described above.

Accession numbers

The gene accession numbers are as follows: PaE gene (Pa*CLE1*) of *P. antarctica* GB-4(0), LC276896; xylanase gene (Pa*XynG*) of *P. antarctica* GB-4(0), AB901085; putative homo-isocitrate dehydrogenase gene (Pa*LYS12*) of *P. antarctica* GB-4(0), LC431700. The accession numbers have been deposited in GenBank/EMBL/DDBJ.

Results

Isolation of the lysine auxotrophic mutants of GB-4(0)

After the GB-4(0) strain was treated with UV and nystatin, 3.6×10^3 colonies that had grown on the YM plate were transferred to a YNBD plate and growth was investigated. Of these, one colony that did not grow was selected as a candidate auxotrophic mutant. As it was able to grow on a YNBD plate containing lysine, this indicated that it was a lysine auxotrophic mutant. The lysine auxotrophic mutant was tentatively named GB-4(0)-L1 (Figure 1). The frequency of *P. antarctica* GB-4(0) auxotrophic mutants ascertained by this method via UV-mutagenesis and nystatin-treatment was estimated at 2.8×10^{-4}.

Genomic analysis of the GB-4(0)-L1 lysine auxotrophic strain

Using the genome database of *P. antarctica* GB-4(0) as a reference, it was determined that a 7,826 bp region was deleted in *P. antarctica* GB-4(0)-L1. This region contains the 5′ region of the putative Pa*LYS12* (672 bp), a predicted homolog of *Saccharomyces cerevisiae LYS12* which encodes homo-isocitrate dehydrogenase. Complementation experiments introducing the putative Lys12 gene [strain L1(Pa*LYS12*)] proved that the deletion of Pa*LYS12* caused the lysine auxotrophy of *P. antarctica* GB4-(0)-L1 (data not shown). Other genes related to the

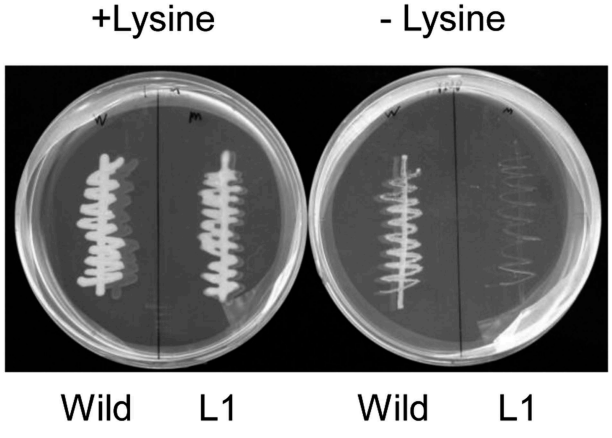

Figure 1. Growth of *Pseudozyma antarctica* GB-4(0) wild type and GB-4(0)-L1 on YNBD plate with (left) or without (right) 40 mg/L lysine.

lysine biosynthesis pathway (putative Pa*LYS1*, Pa*LYS2*, Pa*LYS4*, Pa*LYS9*, and Pa*LYS20*, annotated from homology searches with those of *S. cerevisiae*) were confirmed as being intact (data not shown).

Screening for selection of the PaE high production strain

To create the high PaE-producing self-cloning strains, the pPaLYS12XG-Pa*CLE1* plasmid was designed for the construction of the PaE expression cassette (Figure 2). Pa*LYS12* was used as a selective marker for complementation of the lysine auxotroph. The PaE gene expression cassette with Pa*LYS12* was cut from pPaLYS12-XG-Pa*CLE1* and purified before it was introduced into *P. antarctica* GB-4(0)-L1. Of the colonies that appeared on the YNBD plates as lysine auxotrophic-complemented transformants, 183 clones were randomly picked, and the clear zone formation on 0.5% emulsified PBSA plates was evaluated. Transformants with a relatively larger clear zone (115 strains) were selected from the top for second screening (Figure 3(a)).

In the second screening, 115 strains were cultivated in liquid medium and PBSA degradability of the supernatant was evaluated in HEPES buffer (pH 7.3) in a 96-well microplate (n = 4). The results revealed that strains with larger clear zones on the PBSA plate in the first screening tended to have a larger reduction activity of emulsified PBSA turbidity in the second screening (Figure 3(b), $r = 0.62$, $P = 9.63 \times 10^{-14}$). Next, five strains with the highest PBSA degrading activity in the second screening and the host strain were cultivated in 20 mL of YPX in a flask for 72 h (n = 3). The PaE concentration of the flask cultivation culture supernatant was evaluated in a glass tube (inner diameter, 10 mm), which revealed that all of the transformant strains produced ≥10× PaE compared with that of the host strain. Of these, strain #3 showed the highest productivity (24.5-fold PaE of the host strain). The growth of the tested strains showed no significant difference (Figure 4(a)).

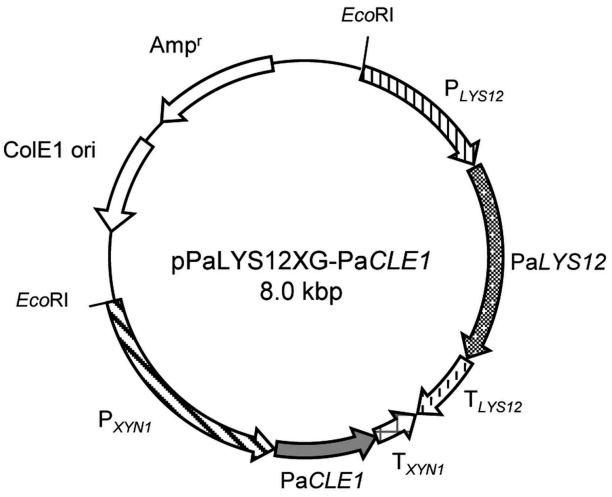

Figure 2. Structure of the PaE gene expression plasmid pPaLYS12XG-Pa*CLE1*. The plasmid was constructed based on pUC19. Pa*LYS12* including a promoter (P_{LYS12}) and terminator (T_{LYS12}) region was used as a selection marker.

Quantitative PCR for PaCLE1 copy number determination

The copy number of Pa*CLE1* in the genomic DNA of each strain (#3, #38, #75, #105, #112, and GB-4(0)-L1) was quantified using qPCR. Since GB-4(0)-L1 contains only one endogenous Pa*CLE1* in its genome, it was estimated that the genome of each of the candidates contained 5, 3, 2, 12, and 4 copies of Pa*CLE1*, respectively (Figure 4(b)). Thus, the number of introduced PaE expression cassettes was determined to be 4, 2, 1, 11, and 3 copies, respectively, by subtracting the endogenous Pa*CLE1*.

PaE production by a self-cloning strain using a jar-fermentor

We renamed #3 as the self-cloning strain PGB474 and evaluated its PaE productivity by xylose fed-batch cultivationusing two 3-L jar fermentors. The average amount of PaE produced after 24, 48, 72 h cultivation was 0.04, 1.04, 1.98 g/L respectively (Figure 5). The growth curve and PBSA degradation activity of the culture supernatant revealed that PaE was produced with cell growth (Figure 5). The secretion of 23-kDa PaE was confirmed by SDS-PAGE analysis of periodically sampled culture fractions from the jar (Supplemental Figure S3).

Discussion

The previously constructed PaE high production strain (GB-4(0)-X14) harbors the PaE gene high expression cassette and an exogenous drug resistant gene as the selection marker. In this study, we aimed to construct self-cloning strains of *P. antarctica* with high PaE productivity that could easily be used in industrial applications in Japan. The nutrient auxotrophic mutants and their complementing genes are generally used for the construction of self-cloning strains. We initially had difficulty with the negative selection of *P. antarctica* auxotrophic mutants after UV mutagenesis. Nystatin, which selectively kills growing *S. cerevisiae* cells under conditions in which non-growing cells survive, was used to enrich for auxotrophic mutants in a population of cells that had been mutagenized [23]. Negative selection of 3.6×10^3 colonies following nystatin enrichment resulted in one auxotrophic mutant (GB-4(0)-L1, Figure 1) that had a partial loss of Pa*LYS12* from its genome. GB-4(0)-L1 transformed with Pa*LYS12*

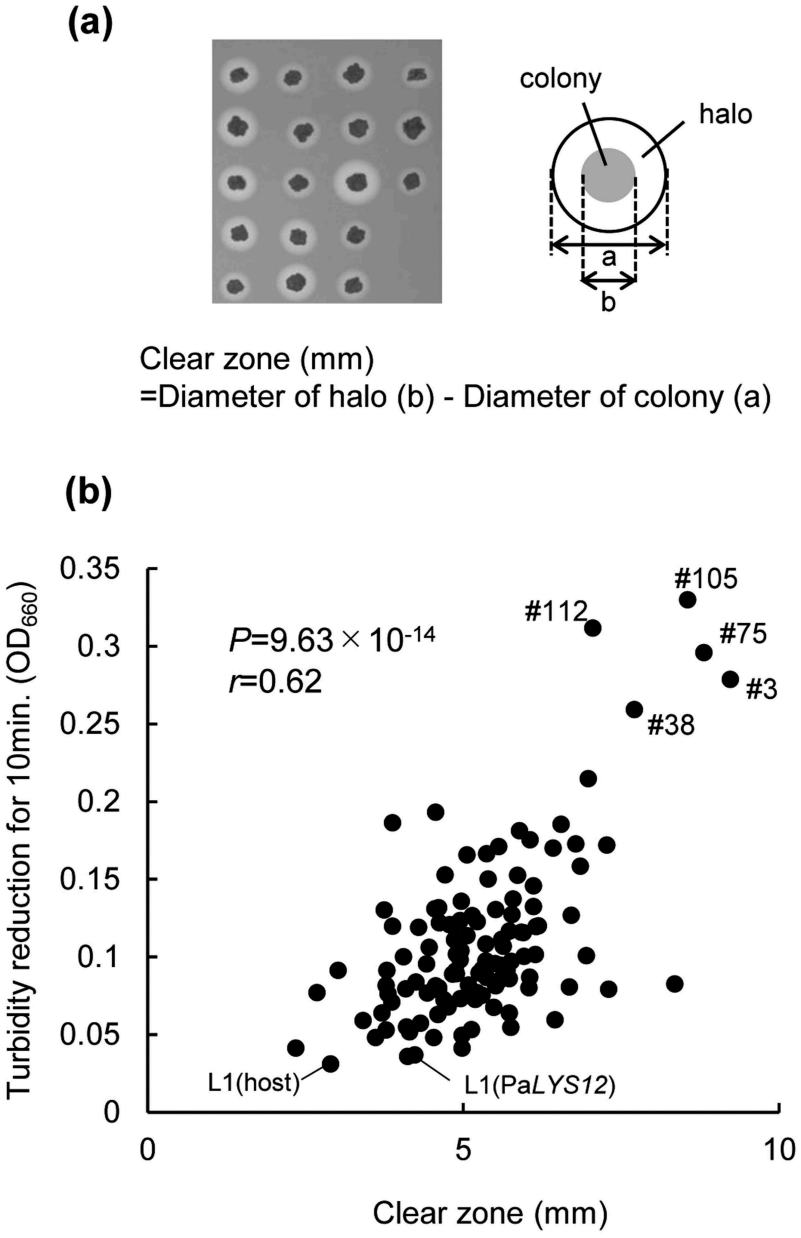

Figure 3. Screening methods for selection of the high PaE-producing strains. (a) Assay for clear-zone formation using 0.5% PBSA emulsion plate. The clear-zone size was calculated by subtracting the colony diameter from the clear zone diameter. (b) Correlation between the clear-zone size (n = 1) and the average of the emulsified PBSA-degrading activity (n = 4). Host strain GB-4(0)-L1 and L1 (Pa*LYS12*) [GB-4(0)-L1 introduced with Pa*LYS12* fragment] were used as a control. *P* represents significant values and *r* represents Pearson's correlation coefficient.

showed recovered growth on a YNBD plate (data not shown). Therefore, we obtained a host and marker gene pair to construct the self-cloning strain of *P. antarctica*.

In repeated experiments, two lysine-auxotrophic mutants were obtained from 1.6×10^3 colonies and the mutations were confirmed to be different from Pa*LYS12* (data not shown). These results suggest that nystatin enrichment is also useful for the selection of *P. antarctica* auxotrophic mutants, although all the obtained mutants were lysine auxotrophs. Sanchez and Demain (1977) modified the nystatin enrichment method in the methanol-utilizing yeast *Hansenula polymorpha* and succeeded in dramatically increasing the mutant acquisition efficiency by careful selection of suitable conditions for mutagens, nystatin concentrations, and the timing of each step [25]. Optimization of the experimental conditions would improve the efficiency of obtaining various auxotrophic mutants in *P. antarctica*.

The previously constructed PaE gene high-expression cassette contains the xylanase (Pa*XYN1*) promoter of *P. antarctica* T-34 isolated from tree sap at Mt. Tsukuba [26] and the Pa*CLE1* gene of the *P. antarctica* type strain (JCM 10317^T = CBS 214.83^T) isolated from Antarctic lake sediment, and these genes were functional in the GB-4(0) strain. The cell morphology of T-34 and JCM10317 was

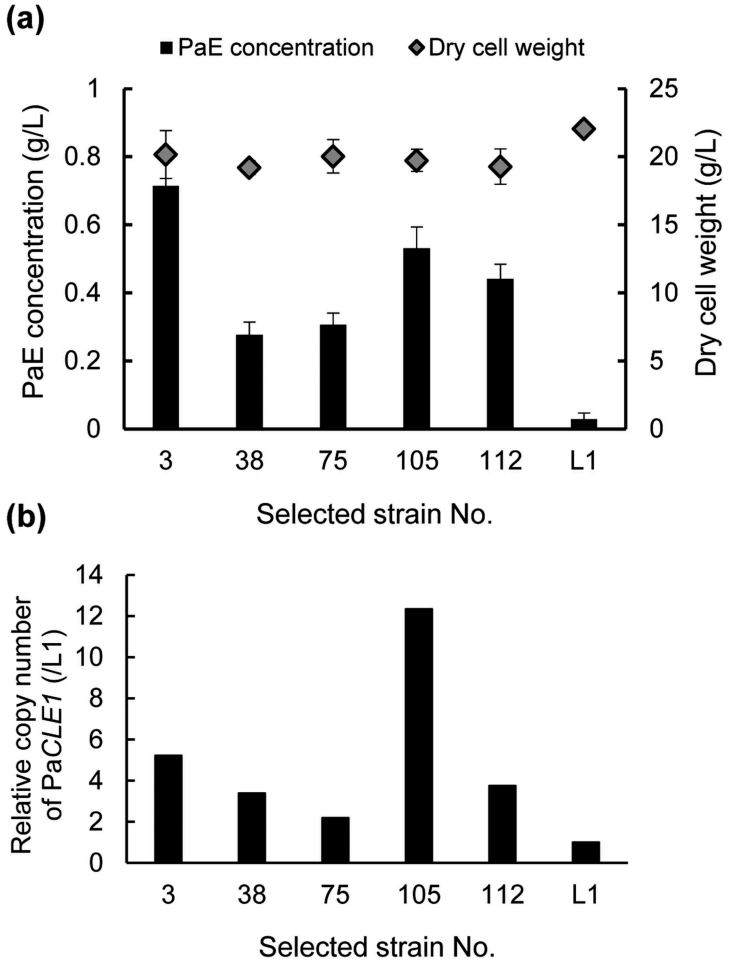

Figure 4. PaE productivity and Pa*CLE1* copy number of the transformants. (a) PaE concentration and growth (dry cell weight) of selected transformants of flask cultivation. The host strain GB-4(0)-L1 was used as a control. The results show the average of three independent experiments. Error bars show standard deviation. (b) Copy number of Pa*CLE1* determined by qPCR of genomic DNA in GB-4(0)-L1 and the selected strain. The copy number of Pa*ACT1* was used as an endogenous control. Results are shown as the relative copy number compared with the number of Pa*CLE1* in the GB-4(0)-L1 genome.

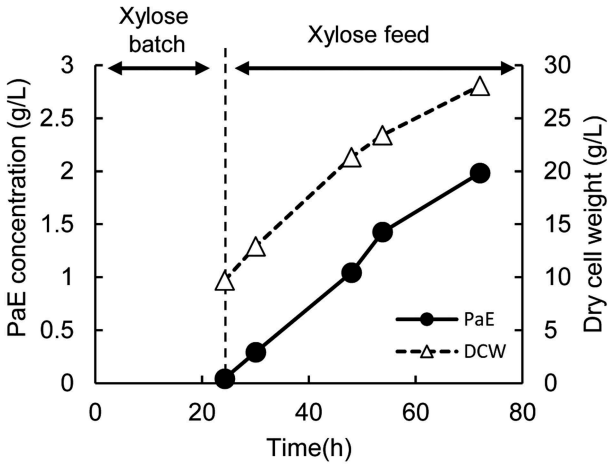

Figure 5. PaE production of strain PGB474 with xylose feeding using a jar fermentor. Cell growth (open triangles) and PaE production (closed circles) with 2% xylose. Xylose feeding commenced after 24 h. The results show the average of two independent experiments.

elongated and grew as hyphae with blastoconidia in chains and branches, whereas strain GB-4(0) cells exhibited a smooth, ellipsoidal yeast-like shape under the same culture conditions [22]. Although leaf-derived *P. antarctica* strains induced PaE production in the presence of xylose, strain T-34 did not [15]. The nucleotide sequence identity of the Pa*XYN1* and Pa*CLE1* promoters of *P. antarctica* T34 showed 87.0% and 89.6% with that of *P. antarctica* GB-4(0), respectively. Furthermore, the nucleotide sequence identity of Pa*CLE1* of *P. antarctica* JCM10317 showed 99.5% with that of *P. antarctica* GB-4(0). Although these strains are identified as *P. antarctica*, their origin, morphology, physiology, and specific gene sequences differed greatly. Regarding the safety issues involved in the use of rice seed-derived strain GB-4(0) to reduce contaminants during PaE production, here, we constructed a new PaE gene high-expression cassette containing only the GB-4(0) genome sequence (Figure 2). Both ends of the PaE gene expression cassette with Pa*LYS12* were designed to have *Eco*RI recognition sequences. For one end, the *Eco*RI recognition sequences were taken from upstream of GB-4(0) xylanase gene, and since there is no *Eco*RI site at

6,000 bp upstream of Pa*LYS12*, the other end was linked with one of the many *Eco*RI recognition sequences on the GB-4 (0) chromosome. Transformants introducing the PaE gene expression cassette with Pa*LYS12* showed high PaE productivity on a xylose medium (Figure 3). This showed that the xylanase promoter of GB-4(0) also acts to produce large amounts of protein in GB-4(0)-L1, similar to the xylanase promoter of T-34.

An efficient screening method was needed to select for the high PaE-producing strain among the cassette-introduced strains. Previously, we isolated the GB-4(0) strain and other plant-derived *Pseudozyma* strains as PBSA degraders on a plate that was designed to detect even weak PBSA degradation activity. This was a double layered plate containing emulsified PBSA and soybean oil as an upper, thin layer [9]. Next, a monolayer plate containing emulsified PBSA and a 2% xylose plate were used to detect *P. antarctica* transformants with a high amount of PaE production under the control of the xylose-inducible xylanase promoter [15]. Here, we modified the plate conditions to identify the higher PaE-producing strains. On 0.5% emulsified PBSA plates, the growth of *P. antarctica* was relatively slow with 8% xylose compared to that with 2% xylose and difference in the clear zone size between the transformants after 3 days incubation was more apparent in media containing 8% xylose than in media containing 2% xylose (Figure 3(a)). Cultivation of the cells and detection of the protein productivity in the supernatant in 96-well plates can also be used for high throughput screening [27]. The 115 selected strains were cultured in YPX liquid culture in a DeepWell plate and the PBSA emulsion degradation activity of the culture supernatant was confirmed in a hydrophilic 96-well microplate that reduced the non-specific binding of proteins (Figure 3(b)). Although both methods produced variable results, there was a significant positive correlation between the single clear zone size and the mean turbidity reduction in four individual trials (Figure 3(b)).

Five strains showed the highest emulsified PBSA activity in the double high throughput screening and the flask cultivation supernatant showed > 10 times PBSA degradation activity than that of host strain. These results showed the efficiency of our screening method.

We analyzed the PaE productivity of the transformants (Figure 4(a)) and the relative copy number of Pa*CLE1* in the genome on PaE production (Figure 4(b)). The result showed that strain #3, which contains four copies of the PaE gene expression cassette and strain #105, which contains 11 copies, showed higher PaE activity compared to strain #38 (two copies) and strain #75 (a single copy). Other studies have reported that multiple copies of the expression cassette caused a high level of expression in yeast [28] and fungi [29]. We assumed that the amount of PaE produced by the original Pa*CLE1* and controlled by the Pa*CLE1* promoter in the transformants was the same as the amount of PaE in the host strain. Next, we calculated the amount of PaE produced by the introduced Pa*CLE1* under the control of the xylanase promoter by subtraction of the amount of the host strain from that of the transformants. The result was divided by the number of Pa*CLE1* introduced and produced a ratio of the produced amount of PaE from the introduced Pa*CLE1*/relative copy number of introduced Pa*CLE1* (Supplemental Table S1). The ratios were between 0.10 and 0.23, except for strain #105 (0.04). The results indicated that the ratio is not exactly proportional, but showed that the greater the number of introduced genes, the greater the production of enzyme. Since we randomly introduced the PaE gene expression cassette into the *P. antarctica* GB-4(0) chromosome, our results indicated that the insertion position on the chromosome influenced expression levels of the PaE gene. Because of the extremely low ratio of #105, this suggests that some of the introduced genes may not contribute to PaE production. Since the PaE productivity of clone #3 (PGB474) was the highest, this strain was selected for subsequent study.

To analyze the introduced cassette in the PGB474 genome, Southern hybridization analysis was performed (data not shown). A probe was used for specific detection of the PaE gene expression cassette with Pa*LYS12*, and the signal patterns suggested that the PaE gene expression cassette with Pa*LYS12* was singly inserted into many unspecified positions along the chromosome and was not ligated in tandem, back-to-back, face-to-face, or in an ipsilateral orientation, although the fragments treated with restriction enzymes were not clear.

The PGB 474 strain showed good growth and PaE productivity in the xylose fed-batch cultivation using a jar fermentor, and 2 g/L of PaE was produced in 72 h (Figure 5). PaE productivity steadily increased in tandem with cell growth and therefore further production could be expected by prolonging the culture time.

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Author contributions

H. Kitamoto, Y. S., and T. W. conceived and designed the experiments. Y. S. and T. W. performed the main experiments. T. T. carried out the enzyme assay. T. Y., S. T., T. M., H. Koike, and K. S. performed the essential experiments and analyzed the data. Y. S. and H. Kitamoto wrote the manuscript. All authors discussed the results and commented on the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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