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ORIGINAL RESEARCH PAPER

Use of the *pyrG* gene as a food-grade selection marker in *Monascus*

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Abstract *Ma-pyrG* was cloned from *Monascus aurantiacus* AS3.4384 using degenerate PCR with primers designed with an algorithm called CODEHOP, and its complete sequence was obtained by a PCR-based strategy for screening a *Monascus* fosmid library. *Ma-pyrG* encodes orotidine-5′-phosphate decarboxylase (OMPdecase), a 283-aminoacid protein with 81% sequence identity to that from *Aspergillus flavus* NRRL 3357. A *pyrG* mutant strain from *M. aurantiacus* AS3.4384, named UM28, was isolated by resistance to 5-fluoroorotic acid after UV mutagenesis. Sequence analysis of this mutated gene revealed that it contained a point mutation at nucleotide position +220. Plasmid pGFP-*pyrG*, bearing the green fluorescent protein gene (GFP) as a model gene and *Ma-pyrG* as a selection marker, were constructed. pGFP-*pyrG* were successfully transformed into UM28 by using the PEG method.

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Introduction

The genus *Monascus*, which reproduces both sexually and asexually, belongs to the class *Ascomycetes* and the family *Monascaceae*. It has been used from ancient times in Asian countries as a natural food colorant and flavoring agent or as a medical agent. *Monascus* can produce various secondary metabolites such as monacolin K (Heber et al. 1999), γ-aminobutyric acid (GABA) (Kono and Himeno 1999), pigments and citrinin (Blanc et al. 1995). It also produces industrially and pharmaceutically useful substances.

As *Monascus* spp. are widely used in industrially fermentation and food application, genetically modified microorganisms (GMMs) used must be food-grade in accordance with a general acceptability. Only DNA from the same genus and possibly small stretches of synthetic DNA can be used in food-grade GMMs.

To further optimize *Monascus* for industrial exploitation, genetic modification approaches have been used. But the genetic manipulation of *Monascus* has many potential applications in food safety and in the development of improved food products. Many drug resistance markers, such as hygromycin B (Campoy et al. 2003) and aureobasidin A (Shimizu et al. 2006), have been successfully used in the transformation of

Monascus. However, for safety considerations, antibiotic resistance markers should be avoided for food applications. The safe use of genetically modified *Monascus* requires the development of food-grade transformation systems. Until recently, there is little information available in literature about the food-grade selection marker used for *Monascus* transformation.

Food-grade selection markers are classified into two categories, complementary markers and dominant markers. As complementary markers, *pyrG* gene have been successfully applied to many fungi, since the uridine auxotrophic mutants for these markers can be isolated by direct selection using the pyrimidine analog 5-fluoroorotic acid (5-FOA).

We show here that *pyrG* can efficiently serve in *Monascus* as a food-grade selection marker. We verify this application by constructing a plasmid in *Monascus* carrying *pyrG* and GFP, and we successfully transform the plasmid into *Monasucus* uridine auxotrophic strain by using *pyrG* as a selection marker.

Materials and methods

Strains and growth media

Monascus aurantiacus AS3.4384 was grown on extract/sucrose medium (MES) medium (6 °Bé wort) or Czapek Dox (CD) medium (as g/l 3 $NaNO_3$, 2 KCl, 1 KH_2PO_4, 0.5 $MgSO_4 \cdot 7H_2O$, and 0.02 $FeSO_4 \cdot 7H_2O$, 20 glucose, and, if necessary, 1 5-FOA, 20 uridine were added).

Plasmid construction

A *Sal*I–*Hin*dI fragment (5267 bp) containing a fusion between the GFP expression cassette and pUC18 was isolated from pSGF957 (Kim et al. 2003a, b), resulting in fragment SH-GFP. *Ma-pyrG* containing its own promoter and terminator was amplified by primer S-*pyrG* and H-*pyrG* (S-*pyrG*:5′-ACGCGTCGACCAGATATGTTCAACGATCAGGAT-3′ and H-*pyrG*: 5′-CCCAAGCTTCGAGAGGACTATTCCGAGGGTG-3′), resulting in fragment SH-*pyrG*. This fragment was digested with *Sal*I and *Hin*dI, then inserted into SH-GFP. The resulting plasmid pGFP-*pyrG* contains GFP as a model gene and *Ma-pyrG* as a selection marker.

Isolation of genomic DNA

DNA was isolated from *M. aurantiacus* as described in Jae-Hyuk Yu et al. (2004) in a Mini-BeadBeater-8 (BioSpec).

Amplification of *Ma-pyrG* by CODEHOP PCR

The CODEHOP PCR primers were designed according to the protocol reported by Rose et al. (2003). Ten fungal *pyrG* proteins were selected from GenBank (Table 1) and entered into the BLOCKMAKER program (http://blocks.fhcrc.org/blocks/blockmkr/make_blocks.html), which can generate blocks of similar amino acids. The output from this program, which consisted of aligned blocks of the most highly conserved *pyrG* regions, was next copied and pasted into the CODEHOP algorithm. The CODEHOP program was then run to identify candidate degenerate primers. Two primers were selected from the CODEHOP results, OMP-F: (5′-GCACAACTTCCTGATCTTCgargaymgnaa-3′) and OMP-R: (5′-GAGGCGGGGGTCTGGtaytgytgncc-3′), and used to amplify a fragment of the putative *M. aurantiacus pyrG* gene.

50 ng *Monascus* genomic DNA was used as template DNA for the PCR. Cycling conditions began with 94°C for 5 min; then 35 cycles at 94°C denaturing for 30 s, 60°C annealing for 60 s, and 72°C extension for 60 s, and then a final step of 72°C for 10 min. Taq (TaKaRa) was used as the polymerase and PCRs were performed in a Gene Amp PCR system 2400 thermocycler (Perkin Elmer). The PCR product was TA cloned and sequenced. The sequence

Table 1 Accession numbers of fungal *pyrG* homologs

Species	Accession No.
Aspergillus oryzae RIB40	XP_001826440
Aspergillus terreus NIH2624	XP_001218297
Cladosporium fulvum	Q9HFV8
Penicillium chrysogenum	P09463
Aspergillus fumigatus Af293	XP_755170
Penicillium nalgiovense	Q8J269
Aspergillus niger	P07817
Aspergillus awamori	Q5J2D0
Aspergillus clavatus NRRL 1	XP_001267761
Aspergillus nidulans FGSC A4	XP_663761

was then used in a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) and found to display strong identity to other fungal OMP decarboxylases.

PCR-based screening strategy

We performed a PCR-based screening system for the *M. aurantiacus* genomic fosmid library according to a previous reported method (Kim et al. 2003). In brief, 384 fosmid clones from each filter are pooled and the DNA is purified for the PCR template as a unit. When a filter with positive signal is found, then each constituent 24 columns and 16 rows pool is analyzed individually by the same PCR assay. The location of the positive clone can be identified efficiently. Primers were designed from the obtained *Ma-pyrG* sequence fragment, OMP-S1: (5′-ACACA GTCCAGAAACAGTACCA-3′) and OMP-S2: (5′-CA GCTTATCACCCTTTGACGAG-3′), and used to screen the *Monascus* fosmid library. One of the positive fosmid clones, Q12F9, was subcloned for sequencing.

Sequence analysis

Sequence assembly was performed using the program DNASTAR, and the nucleotide sequence was analyzed using the open reading frame (ORF) Finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). DNA and protein sequence alignments were carried out using the blastn and blastp programs, respectively (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple alignments of the protein sequences were performed using the ClustalW program (http://www.ebi.ac.uk/clustalW/).

DNA sequence accession number

The nucleotide sequence of *Ma-pyrG* has been submitted to GenBank under the Accession No. GU723506.

Selection and confirmation of the uridine auxotrophic strain

The spores of *M. aurantiacus* were collected from the MES medium plate. UV mutagenesis were performed according to standard methods (Gruber et al. 1990). Colonies that were resistant to 5-FOA were also tested for stable uridine auxotrophy by serially replica plating them onto CD agar with and without uridine. One positive colony was obtained and named UM28. The *pyrG* mutation of UM28 was further confirmed by amplifying and then sequencing *Ma-pyrG*.

Complementation of *M. aurantiacus pyrG* mutant with *Ma-pyrG*

Complementation of *M. aurantiacus pyrG* mutant was performed by transforming pGFP-*pyrG* into UM28. Plasmids were introduced into the strain UM28 using the protoplast-PEG method (Fu et al. 2007). The pUC18 empty vector was used as a negative control and the *Aspergillus oryzae* plasmid pAO-*pyrG* was used as a positive control. The *A.oryzae pyrG* gene has previously been shown to be functional (Jacobs et al. 1989).

Fluorescence microscopy

Conidia of pSGF957 transformants were cultured on 50 ml CD medium at 30°C for 36–40 h with shaking. The young hyphae were harvested by centrifugation at 6,000*g* for 5 min, and washed with and resuspended in 500 µl sterile water. Finally, the hyphae were observed using a fluorescence microscope.

Results

Isolation of the *Ma-pyrG* gene

Amplification of *M. aurantiacus* genomic DNA with the primers designed with the CODEHOP program yielded a 430 bp PCR product similar in size to other fungus. The PCR product was cloned and sequenced. A BLAST search with the translated sequences showed a highly significant degree of identity to other fungal *pyrG* homologues.

In order to recover the complete *Ma-pyrG*, a PCR-based screening strategy was used to screen the *Monascus* fosmid library. Specific primers were designed from the putative *Ma-pyrG* sequence fragment. Six *pyrG*-positive clones were obtained from the fosmid library by checking the PCR products. The selected clone Q12F9 was digested with *Eco*RI and *Bam*HI and fragments of 1–5 kb were subcloned for sequencing. The entire sequence of the putative *Ma-pyrG* gene and that of the deduced amino acids of the putative ORF are shown in Supplementary Fig. 1.

There are high similarities with the OMP decarboxylases from *Aspergillus flavus* NRRL3357 (81 % match), *Neosartorya fischeri* NRRL181 (79 %), *Aspergillus fumigatus* Af293 (78 %), *Penicillium chrysogenum* (73 %) and *Aspergillus oryzae* RIB40 (72 %). These high similarities strongly suggest that the cloned gene encodes *M. aurantiacus* AS3.4384 OMP decarboxylase.

Selection and confirmation of a uridine auxotrophic strain

To create *Ma-pyrG* deficient strains, *M. aurantiacus* AS3.4384 was mutagenized by UV radiation. Before the mutagenesis, the susceptibility of *M. aurantiacus* AS3.4384 spores to UV radiation and 5-FOA was estimated. One *pyrG* mutant was obtained from 10^8 spores on 5-FOA selective media. This strain was a uridine auxotroph with a frequency of reverse mutation of less than 10^{-8}, it was designated UM28.

For further confirmation of the mutation, the *pyrG* mutation of UM28 was amplified and then *Ma-pyrG* was sequenced. Sequence analysis of the *Ma-pyrG* gene from the uridine auxotrophic strain revealed a single nucleotide mutation of $C_{220}CT \rightarrow TCT$ caused substitution of Pro to Ser at position 63, with inactivation of OMPdecase function. We concluded that UM28 was a *Ma-pyrG* deficient mutant, and this auxotroph could thus be used in the transformation system with a *pyrG* selective marker.

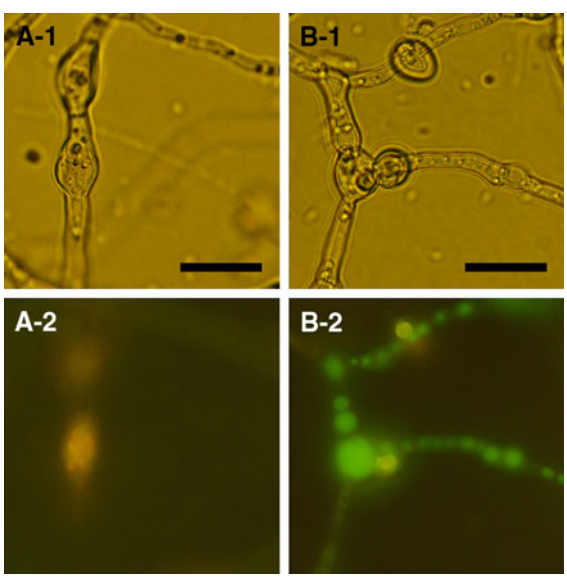

Fig. 1 Fluorescent microscopy of transformants with GFP. The transformants with pGFP-*pyrG* plasmid containing the GFP gene were observed under a microscope. The photographs are of DIC of UM28 (A-1) and transformant (B-1) and those of GFP of UM28 (A-2) and transformant (B-2). *Scale bars* = 20 μm

Functional complementation of *M. aurantiacus pyrG* mutation

To determine whether the *Ma-pyrG* gene encodes a functional protein, we performed a complementation experiment using UM28 as a host. The plasmid pGFP-*pyrG*, containing the *Ma-pyrG* gene and a GFP gene derived from vector pSGF957, was used for transformation. It transformed UM28 to uridine prototrophy, this result showed that the *Ma-pyrG* gene encodes a functional OMPdecase. At the same time, expression of the transformed gene was evaluated using the GFP gene. Hyphae of the recipient strain UM28 appeared red or light green. On the other hand, hyphae of GFP-harboring transformants showed bright green signals (Fig. 1), suggesting that the transformants not only had the GFP gene but also expressed it.

Discussion and conclusions

A new food-grade selection marker is described for transformation system of *M. aurantiacus*: the *Monascus pyrG* gene was isolated by PCR using consensus-degenerated primer design with CODEHOP strategy and its complete sequence was obtained by a PCR-based strategy for screening the *Monascus* fosmid library. A *pyrG* mutant was isolated by UV mutagenesis and identified by the sequencing and plasmid complement results. The *Monascus pyrG* gene can be used as a safe selection marker in molecular breeding.

Although antibiotic resistance has advantages over auxotrophic complementation, as it can avoid constructing an auxotrophic mutant as the host and cloning a complementing gene, it should be avoided for food applications. The production of *Monascus*-fermented products could be improved by genetic engineering to reduce the content of citrinin as well as to increase the production of monacolin K and GABA, and the *pyrG* selective marker will be a safer choice. Furthermore, a *pyrG* marker recycling method was reported (Enfert 1996; Maruyama and Kitamoto

2008), it enables infinite cycles of gene manipulation, and it may be useful for future functional genomic studies.

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