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Molecules and Cells

Insect Ornithine Decarboxylase (ODC) Complements *SPE1* Knock-Out of Yeast *Saccharomyces cerevisiae*

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Ornithine decarboxylase (ODC) is a rate-limiting enzyme in the biosynthesis of polyamines, which are essential for cell growth, differentiation, and proliferation. This report presents the characterization of an ODC-encoding cDNA (*Slit*ODC) isolated from a moth species, the tobacco cutworm, *Spodoptera litura* (Lepidoptera); its expression in a polyamine-deficient strain of yeast, *S. cerevisiae*; and the recovery in polyamine levels and proliferation rate with the introduction of the insect enzyme. *Slit*ODC encodes 448 amino acid residues, 4 amino acids longer than *B. mori* ODC that has 71% identity, and has a longer C-terminus, consistent with *B. mori* ODC, than the reported dipteran enzymes. The null mutant yeast strain in the ODC gene, *SPE1*, showed remarkably depleted polyamine levels; in putrescine, spermidine, and spermine, the levels were > 7, > 1, and > 4%, respectively, of the levels in the wild-type strain. This consequently caused a significant arrest in cell proliferation of > 4% of the wild-type strain in polyamine-free media. The transformed strain, with the substituted *Slit*ODC for the deleted endogenous ODC, grew and proliferated rapidly at even a higher rate than the wild-type strain. Furthermore, its polyamine content was significantly higher than even that in the wild-type strain as well as the *spe1*-null mutant, particularly with a very continuously enhanced putrescine level, reflecting no inhibition mechanism operating in the putrescine synthesis step by any corresponding insect ODC antizymes to *Slit*ODC in this yeast system.

INTRODUCTION

Polyamines are ubiquitous and essential aliphatic cations for cellular growth, proliferation and differentiation in all eukaryotic cells (Hayashi, 1989; Tabor and Tabor, 1984; Zappia and Pegg, 1988). Their biosynthesis proceeds in two parallel pathways, starting with the following enzymatic reaction steps: 1) ornithine decarboxylase (ODC) converts ornithine to putrescine, the diamine precursor for further synthesis of two major polyamine compounds, spermidine and spermine and 2) *S*-adenosylmethionine decarboxylase (AdoMetDC) decarboxylates *S*-adenosylmethionine (AdoMet) forming decarbylated adenosylmethionine (dcAdoMet), an intermediate in the synthesis of both spermidine and spermine (Balasundaram et al., 1994; Janne et al., 1991; Schipper and Verhofstad, 2002).

ODC is a reliable key and rate-limiting enzyme in the polyamine biosynthesis of yeast, *Saccharomyces cerevisiae*, since the deletion of the *SPE1* gene encoding ODC causes a significant depletion in intracellular polyamine levels and cell growth (Balasundaram et al., 1994; Mangold and Leberer, 2005; Schwartz et al., 1995). Expression of ODC is tightly regulated by the pathway end-products, which are polyamines that activate multiple complex molecular mechanisms, including the expression of the ODC antizyme (ODC Az) and the ODC Az inhibitor (AZI) (Fonzy, 1989; Pegg, 2006).

The ODC family occurs in all types of cells, and its eukaryotic members are divided into four distinct branches: plant, fungal, chordata, and nematode/arthropod. Surprisingly, the closest relationship is shown between fungal and chordata ODC groups, with a bootstrap support of 77% among the four groups (Kidron et al., 2007). Mangold and Leberer (2005) showed that human liver ODC is able to fully complement the function of the yeast homologue of ODC in the *spe1* deletion strain of *S. cerevisiae*, and this report raises the question as to whether an arthropod ODC could complement the deficiency of endogenous ODC in yeast cells, regardless of its corresponding regulatory proteins ODC Az and AZI.

In this paper, we describe the isolation of cDNA encoding ODC from a moth species, the tobacco cutworm (*Spodoptera litura*), the deletion of *SPE1* from *S. cerevisiae*, and the transformation of the *spe1*-deleted yeast strain with the insect ODC cDNA producing full compensation of the depleted ODC function by the expression of insect ODC based on the results of analyses of cell growth rates and intracellular polyamine levels.

MATERIALS AND METHODS

Insect rearing and testis dissection

Larvae of the tobacco cutworm, *Spodoptera litura* (Noctuidae; Lepidoptera), were reared on Chinese cabbage at 25 ± 1°C

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Table 1. Yeast strains and plasmids used in this study

Strain/plasmid	Relevant characteristics	Source / Reference
W303-1A	MATa *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*	Xue et al. (1993)
HNU121	MATa *ade2-1 his3-11,15 leu2-3,112 trp1-1 can1-100 spe1::URA3*	This study
HNU121/p413GAL1-*Slit*ODC	p413Gal1-*Slit*ODC in HNU 121	This study
p413GAL1	*GAL1* promoter, *CYC1* terminator	Mumberg et al. (1994)
pBluescript SK(+)	Cloning vector	Stratagene
pCR2.1	PCR Cloning vector	Invitrogen
pSPE1	pBluescript containing yeast *SPE1*	This study
pSPU3	*spe1::URA3* cassette in pSPE1	This study

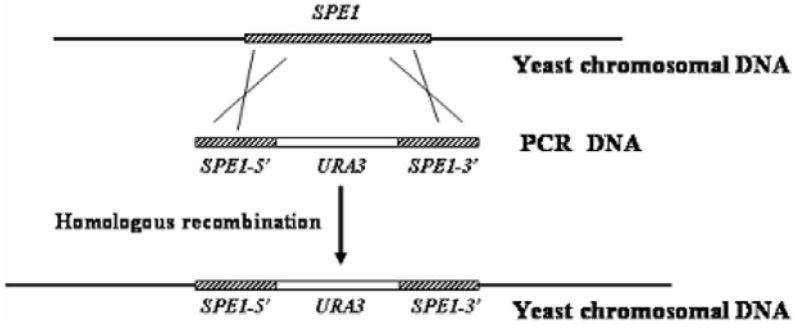

Fig. 1. One-step *SPE1* gene disruption using the *URA3* marker gene

and 60% relative humidity under a 16:8 h light:dark cycle. Male pupae (3-day old) identified by their sexually dimorphic pigmentation pattern were dissected under cold insect saline, and the testes were removed through the dorsal wall of the fourth abdominal segment using fine forceps.

Cloning of ODC-encoding cDNA (*Slit*ODC) from *S. litura*

Ten extirpated testes were immersed in TRIzol (GIBCO/BRL) immediately after dissection, and total RNA was extracted according to the manufacturer's instructions. Double-stranded cDNA was synthesized from total RNA using the Universal Riboclone cDNA Synthesis System (Promega), according to the manufacturer's instructions.

A PCR reaction was performed in the PTC-100 Programmable Thermal Cycler (MJ Research, Inc.) to amplify a 1,110 bp partial domain in the ORF of insect ODC family members using a gene-specific 5′ primer (ODC-P5, GGGCACTGGCTTCGATTGCGC) and a degenerate 3′ primer (ODC-P3, YYAYTTSAGSGCRCAYTCSAC), which were designed according to the ODC-encoding sequence of *Bombyx mori* (Fig. 2). Fifty microliters of PCR reaction containing 100 ng of cDNA template, 0.25 mM dNTPs, 0.2 mM $MgCl_2$, 10 μM 5′ and 20 μM 3′ primers was initiated by the addition of 2 U Taq polymerase (Takara Ex-Taq) at 80°C following an initial 5 min incubation at 95°C and then followed by 39 PCR cycles (95°C for 1 min, 58°C for 1 min, and 72°C for 2 min). The amplified product was analyzed by electrophoresis in agarose gel, eluted to be ligated into the *E. coli* plasmid pCR2.1 using a TA cloning kit (Invitrogen), and then sequenced.

In order to obtain the full-length coding region, two gene-specific RACE primers were designed (ODC-R3 for downstream, TGTCATTCCACGTGGGTAGCG; ODC-R5 for upstream, GGGGAAGTGCTCCTCCAAAGC) (Fig. 2) based on the sequence of the partial domain to amplify the 5′ and 3′ cDNA ends by the RACE procedure (Frohman et al., 1988) using a Marathon cDNA amplification kit (Clontech) and Advantage cDNA polymerase (Clontech), according to the manufacturer's protocols. Fifty microliters of PCR reaction containing 100 ng of cDNA template, 0.25 mM dNTPs, 0.2 mM $MgCl_2$, 10 μM 5′ primer and 20 μM 3′ primer was initiated by the addition of 2 U Taq polymerase at 80°C following an initial 5 min incubation at 94°C and then followed by another heating at 94°C for 30 s. Then, the amplification was carried out by three sequential denaturation-annealing cycles (5 cycles of 94°C for 30 s -67°C for 4 min; 5 cycles of 94°C for 30 s -65°C for 4 min; 35 cycles of 94°C for 20 s -65°C for 4 min) and one extension step (72°C for 5 min). Each of the amplified 5′- and 3′-RACE fragments was gel-purified, cloned into pCR2.1, and sequenced.

Based on the sequences of the two ODC cDNA ends, a pair of gene-specific primers (ODC-5, ATGAAGGTCGTGGAA; ODC-3, TCACTTGAGCGCGCACTC) was designed (Fig. 2) to amplify the full-length ORF cDNA. Fifty microliters of PCR reaction containing 100 ng of cDNA template, 0.25 mM dNTPs, 0.2 mM $MgCl_2$, 20 μM each of 5′ and 3′ primers was initiated by the addition of 2 U *Taq* polymerase at 80°C following an initial 5 min incubation at 95°C and then followed by 39 PCR cycles (95°C for 1 min, 54°C for 1 min, and 72°C for 2 min). The PCR fragment of expected size was isolated and confirmed by DNA sequencing. The full-length coding region of ODC cDNA (*Slit*ODC, GenBank accession no. GQ861425) was assembled into the yeast expression vector p413GAL1 (Mumberg et al., 1994) using *Eco*RI sites in both pCR2.1 and p413GAL1, and the recombinant construct was confirmed by sequencing.

Disruption of *SPE1* gene in yeast

Plasmids and strains used in the disruption and complementation of ODC are outlined in Table 1. The *SPE1* gene of W303-1A was disrupted by the *URA3* marker gene using a one-step gene disruption method (Kim et al., 2009; Rothstein, 1991) (Fig. 1). Plasmid pSPU3, *spe1::URA3* cassette in vector pSPE1,

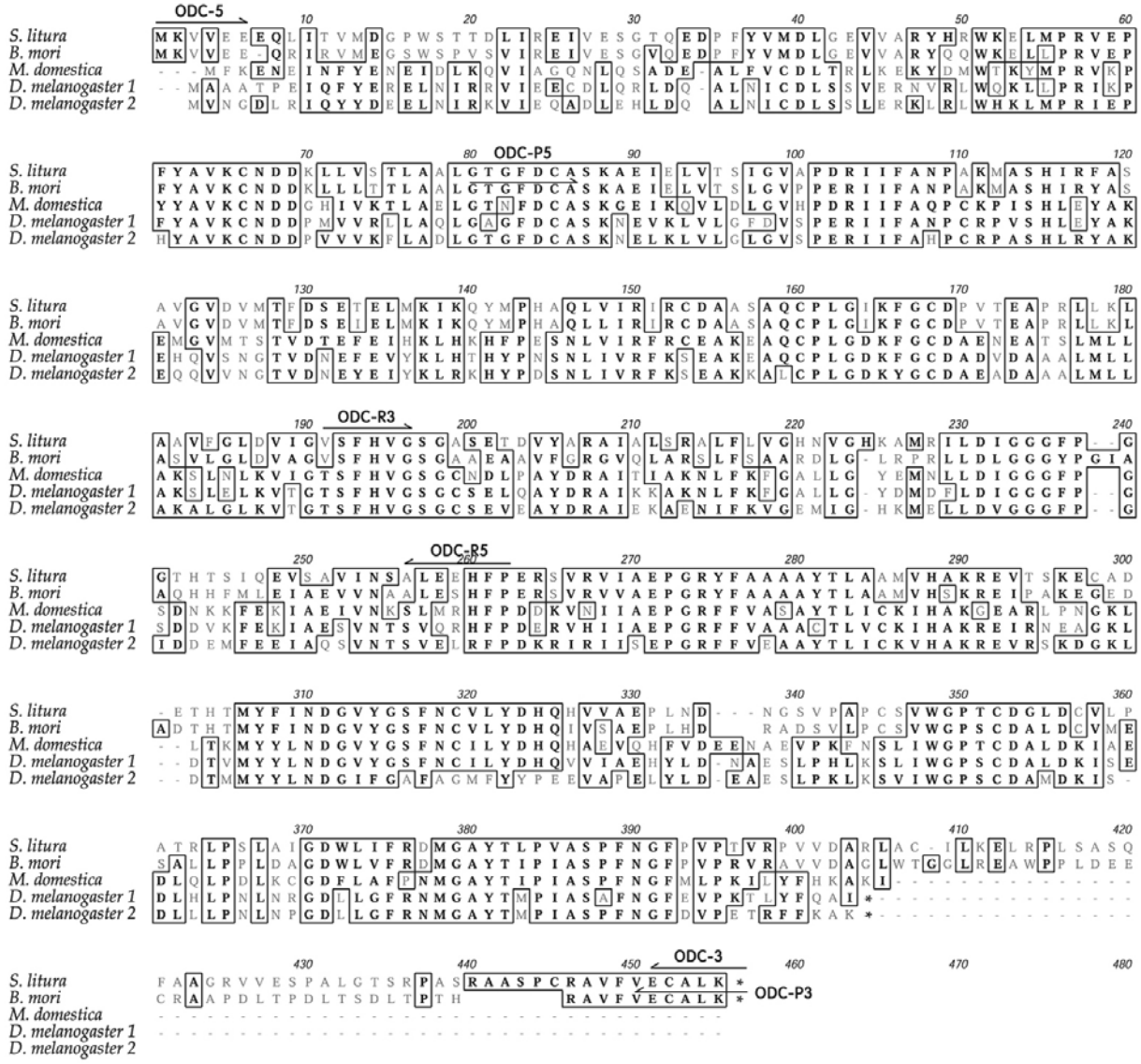

Fig. 2. Alignment of deduced amino acid sequences of *S. litura* ODC cDNA (*Slit*ODC) from this study with previously described insect ODC sequences. Previously reported sequences are as follows: *B. mori* from the silkworm, *Bombyx mori* (unpublished, GenBank accession no. DQ443339), *D. melanogaster* from the fruit fly, *Drosophila melanogaster* (Rom and Kahana, 1993, GenBank accession no. X66601), and *M. domestica* from the house fly, *Musca domestica* (unpublished, GenBank accession no. AF4111043). Identities in the conserved amino acids are framed. Arrows indicate the positions used to hybridize the primers. Alignment was performed using the Clustal W (1.4) algorithm (Thompson et al., 1994) with the MacVector 7.0 software suite (Oxford Molecular Ltd.) with a 10.0 open gap penalty, a 0.1 extended gap penalty, 40% delay divergent, a gap distance of 8, and blosum similarity matrix.

was constructed as follows. The *SPE1* gene was amplified by PCR with a pair of primers (SPE1-5, AAGGATCCTTACTATGTCTAGTACTCAA; SPE1-3, ATCTCGAGCCTTCAATCGAGTTCAGAGTCT) and cloned as a *Bam*HI-*Xho*I fragment into the plasmid pBluescript SK(+) (pSPE1). The *URA3* gene was amplified from yeast chromosomal DNA in a PCR reaction with primers URA3-5 and URA3-3. The 1.3 kb PCR product was digested with *Pvu*II and ligated into pSPE1 digested with *Hin*cII and *Hin*dIII, followed by a Klenow fill-in reaction.

For chromosomal *SPE1* gene disruption, a PCR reaction containing 10 ng of plasmid pSPU3, 200 nM each of SPE1-5 and SPE1-3 primers, 5 μl of 10× PCR buffer, 2.5 μl of 10 mM dNTPs, and 2.5 U *Taq* polymerase was incubated at 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. The amplified fragment containing the *spe1::URA3* was then introduced into W303-1A to generate a *spe1* mutant strain. The *SPE1*-disrupted transformants (HNU121) were selected on SD minimal medium plates lacking uracil. The insertion of *URA3* into the *SPE1* gene was confirmed by PCR amplification with genomic DNA obtained from the Ura$^+$ cells using primers SPE1-5 and SPE1-3.

Expression of *Slit*ODC in *SPE1* null mutant yeast

The full-length cDNA designated as *Slit*ODC was subcloned into p413GAL1, an expression vector with the GAL1 promoter. Leader and tail sequences from the *GAL1* and *CYC1* genes of

the p413GAL1 plasmid, respectively, were fused to the *Slit*ODC coding sequence to provide proper yeast transcription initiation and stop signals. The resulting construct (p413GAL1-*Slit*ODC) was introduced into HNU121 cells whose *SPE1* gene had been disrupted. The transformants (HNU121/p413GAL1-*Slit*ODC) were cultured in a medium containing galactose as a carbon source to induce *Slit*ODC.

Real-time quantitative reverse transcription PCR (RT-PCR) analysis

Reverse transcription of yeast total RNA was performed with the QuantiTech reverse transcription kit (Qiagen). One microliter of the reverse transcription mixture was used for the quantitative PCR with the Applied Biosystems 7500 using the SYBR Premix Ex Taq II kit (Takara). Oligonucleotide primers for quantitative PCR were designed using the Primer Express Software v3.0 (Applied Biosystems). The PCR was performed in a volume of 25 μl containing 12.5 μl of SYBR Premix Ex Taq II, 1 μl of ROX Dye II, and 0.8 μl of 10 μM forward and reverse primers. The instrument settings were: initial enzyme activation at 95°C for 20 s, followed by 40 cycles of 95°C for 10 s, 57°C for 20 s, and 72°C for 34 s. The specific primers were as follows: for the *ODC* gene, forward primer AGTAGCGCCTGACCGCATTAT and reverse primer CGGATGTGAGACGCCATCT; for the 18S rRNA, forward primer CGGCTACCACATCCAAGGAA and reverse primer GCTGGAATTACCGCGGCT. 18S rRNA was used as an internal control, and the relative quantification for the ODC transcript was corrected to the 18S rRNA values. Amplification of specific transcripts was confirmed by melting curve analysis provided by the AB 7500 system at the end of each run. PCR specificity and product length were further checked by agarose gel electrophoresis and ethidium bromide staining to verify that the primer pairs amplified a single product of the predicted size.

Yeast cultivation and determination of growth rate

Yeast media and cultures were prepared according to Adams et al. (1997). YPD medium, for the non-selective propagation of *S. cerevisiae* strains, contained 20 × *g* dextrose, 20 × *g* bacto-peptone, and 10 × *g* yeast extract (Difco) per liter. Synthetic media (SD) contained, per liter, 20 × *g* dextrose or galactose, 6.7 × *g* yeast nitrogen base (Difco) without amino acids, and necessary auxotrophic supplements. New yeast strains were created through lithium acetate transformation (Adams et al., 1997).

Precultured yeast strains in SD-dextrose media were inoculated into 20 ml of SD-galactose media at 1×10^4 cells/ml and incubated for 60 h at 30°C and 250 rpm by the depletion phase. One milliliter of culture taken at every 12 h was diluted appropriately for cell counting using a hemacytometer, and cells were taken in 5 ml at the late exponential phase for cell mass determination.

Analysis of cellular polyamine contents

Polyamine contents were analyzed according to a modification of Corbin et al. (1989). The harvested yeast cells were washed with distilled water by centrifugation at 10,000 × *g* and homogenized in cold 20% perchloric acid. The homogenate was centrifuged at 10,000 × *g* at 4°C for 10 min, and the supernatant was applied to a Bio-Rad AG 50W-X4 cation-exchange resin (H^+ form, 200-400 mesh in a Kontes Econoflex 10-ml column). A polyamine-containing fraction was eluted through the following steps: 2.8 ml of 0.7 M NaCl in 0.1 M sodium phosphate (pH 8.0), 2 ml of deionized distilled water, 3 ml of 1 N HCl, and finally 2.4 ml of 6 N HCl. The final eluate was dried in a rotary vacuum evaporator at 40°C and then dissolved in 400 μl of distilled water for analysis by HPLC. *o*-Phthalaldehyde-thiol (OPT) reagent was prepared by dissolving 10 mg of *o*-phthalaldehyde and 10 μl of 2-mercaptoethanol in 200 μl of ethanol and diluting this with 5 ml of 0.5 mM sodium borate buffer (pH 10.8). The polyamine solution (200 μl) was mixed with 25 μl of OPT reagent, stored overnight under argon to reduce background fluorescence, and reacted for 60 s before injection onto an HPLC column. HPLC was carried out using a Waters system consisting of a 600 controller and 474 scanning fluorescence detector (340 nm excitation/455 nm emission). The OPT-derivatized polyamine solution (100 μl) was injected onto a Altex Ultrasphere ODS column (5 μm, 4.6 mm × 15 cm) and eluted with methanol (solvent A) and 0.1 M phosphate buffer including 0.05 M DCMA (N, N-dimethylcyclohexylamine) (solvent B) using a gradient program (20% A & 80% B for 0-2 min, linear gradient to 80% A & 20% B for 2-10 min, hold for 2.3 min, and linear ramp to initial condition for 12.3-12.5 min).

RESULTS AND DISCUSSION

Cloning of cDNA encoding ODC from *S. litura*

We tried to screen ODC encoding mRNA from 3-day old pupal testes of *S. litura*, since the testis is a typical organ with a high polyamine content (Shubhada et al., 1989; Tosaka et al., 2000), and a high polyamine level is observed in the testis during the period of rapid cell division and differentiation at the early pupal stage in insects (Hamana et al., 1989; Jeong et al., 2001).

In the initial screening using cDNA template synthesized from testis RNA, PCR reactions employing some different combinations of primers to hybridize the several conserved domains in the previously reported insect ODC sequences failed to amplify any corresponding cDNAs. However, we succeeded in designing a pair of competent primers based on the *B. mori* ODC sequence, which consists of a gene-specific 5′ primer (ODC-P5) complementary to highly conserved target sequences on the antisense-strand encoding the GTGFDCA amino acid sequence motif and a degenerate 3′ primer (ODC-P3) complementary to the target sequences on the sense-strand encoding the VECALK amino acid sequence motif, including the termination codon located on the very 3′ end (Fig. 2). A fragment of 1,110 bp, which is 9 bp longer than the expected 1,101 bp, was amplified and then confirmed as the desired domain by alignment with the reported sequences. We next used the RACE procedure to obtain 5′ and 3′ ends of ODC cDNA and amplified 1,347 bp of full-length ORF cDNA (*Slit*ODC) using a gene-specific primer pair (ODC-5 and ODC-3) (Fig. 2).

Sequence analysis of *Slit*ODC

The deduced amino acid sequence of *Slit*ODC was aligned with the four insect ODC sequences described previously (Fig. 2). The first insect ODC sequence was characterized from the fruit fly, *Drosophila melanogaster* (Diptera). Rom and Kahana (1993) isolated two distinct ODC genes (ODC1 and ODC2) from the *Drosophila* genome using the primers designed to hybridize the two highly conserved domains of mouse, yeast, trypanosome, and human ODC amino acid sequences and demonstrated that both of the two *ODC* genes are transcriptionally active. Another Dipteran ODC sequence (GenBank accession no. AF411043) was characterized from the genomic DNA of the house fly, *Musca domestica* (2001, unpublished), and the first lepidopteran ODC sequence (GenBank accession no. DQ443339) was reported from the silk worm, *Bombyx mori* (2006, unpublished). *Slit*ODC (448 amino acids) had a considerable similarity to *B. mori* ODC (444 amino acids), with 71% identity in amino acid sequence, regarding only 72% identity

Table 2. Number of cells of W303-1A (wild-type), HNU121 (*spe1*-deleted), HNU121/ p413GAL1-*Slit*ODC (transformed HNU121 with p413GAL1-*Slit*ODC), and HNU121 /p413GAL1 (transformed HNU121 with the control plasmid p413GAL1) *S. cerevisiae* strains during incubation in SD-galactose media.

Strain	Number of cells/ml				
	12 h	24 h	36 h	48 h	60 h
W303-1A	$8.63 \pm 0.08 \times 10^5$	$1.15 \pm 0.09 \times 10^7$	$8.67 \pm 0.67 \times 10^7$	$9.80 \pm 0.47 \times 10^7$	$9.67 \pm 0.32 \times 10^7$
HNU121	$2.67 \pm 0.09 \times 10^5$	$1.26 \pm 0.28 \times 10^6$	$2.63 \pm 0.30 \times 10^6$	$3.63 \pm 0.40 \times 10^6$	$2.70 \pm 0.26 \times 10^6$
HNU121/p413GAL1-*Slit*ODC	$8.73 \pm 0.15 \times 10^5$	$1.10 \pm 0.20 \times 10^7$	$9.40 \pm 0.36 \times 10^7$	$10.37 \pm 0.83 \times 10^7$	$10.20 \pm 0.84 \times 10^7$
HNU121/p413GAL1	$3.00 \pm 0.20 \times 10^5$	$1.10 \pm 0.26 \times 10^6$	$2.13 \pm 0.14 \times 10^6$	$1.97 \pm 0.12 \times 10^6$	$1.80 \pm 0.11 \times 10^6$

Values are mean ± SD of three replicates.

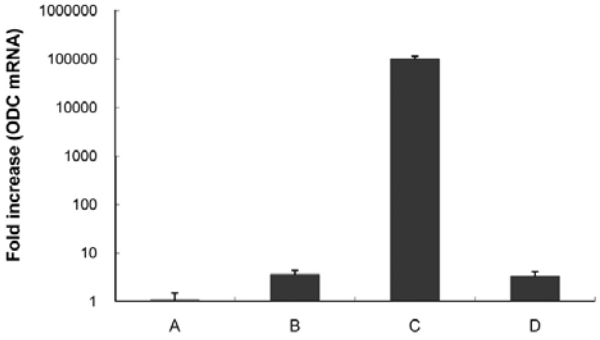

Fig. 3. Expression of *Slit*ODC in yeast strains. Cells were grown in the synthetic minimal medium with 2% glucose until the density reached 3×10^7/ml and were then shifted to the same minimal medium amended with 2% galactose for 24 h. The amount of the ODC transcript was measured by real-time quantitative RT-PCR and normalized to 18S RNA. The results of three experiments are presented as mean ± SD. (A) W303-1A, wild-type; (B) HNU121, *spe1*-deleted; (C) HNU121/p413GAL1-*Slit*ODC, transformed HNU121 with p413GAL1-*Slit*ODC; (D) HNU121/p413GAL1, transformed HNU121 with the control plasmid p413GAL1.

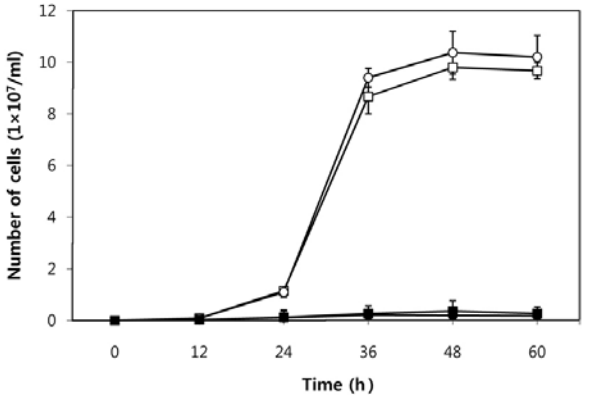

Fig. 4. Comparison of the growth profile between W303-1A (□, wild-type), HNU121 (■, *spe1*-deleted), HNU121/p413GAL1-*Slit*ODC (○, transformed HNU121 with p413 GAL1-*Slit*ODC), and HNU121/ p413GAL1 (●, transformed HNU121 with the control plasmid p413GAL1) *S. cerevisiae* strains during incubation in SD-galactose media.

between ODC1 and ODC2 of *D. melanogaster*, compared with the much lower identity (38-40%) between lepidopteran and dipteran ODC sequences (393-396 amino acids).

Phillips et al. (1987) reported that ODC with a shorter C-terminus showed a slower *in vivo* turnover in its activity, since a shortened C-terminus is more stable to intracellular degradation. The enzymatically active form of ODC is a homodimer, in relation to the regulation of polyamine metabolism; however, when the ODC monomer makes a heterodimer with ODC Az induced by high cellular polyamine levels, its C-terminus is efficiently degraded by a 26S proteasome (Coffino, 2000; Yuan et al., 2001). *Slit*ODC and *B. mori* ODC were found to be significantly larger than the dipteran enzymes, mostly due to their longer C-terminal peptides, suggesting that the lepidopteran enzymes are less stable (Rom and Kahana, 1993) and consequently seem to have more sensitive post-translational regulation related to the interactions with ODC Az and the ensuing degradation process.

Complementation of yeast *SPE1* null mutation by *Slit*ODC

Since the decarboxylation of ornithine by ODC is the only way to synthesize putrescine in *S. cerevisiae* (Tabor and Tabor, 1985), the *spe1* null mutation leads to an incompetency in the biosynthesis of polyamines, showing intracellular polyamine depletion followed by cessation of growth and some morphological and developmental abnormalities (Mangold and Leberer, 2005; Schwartz et al., 1995). When the mutant cells of *S. cerevisiae* that are defective in polyamine production are transferred from YPD medium to an amine-free medium, however, the cessation of growth does not abruptly occur because of the intracellular pools of polyamines remaining from the previous growth in the amine-containing medium. Thus, the growth rate reaches near zero after the progression of about 13 generations in the amine-free medium (Balasundaram et al., 1991; 1994).

The *spe1* mutant (HNU121) of the W303-1A *S. cerevisiae* strain disrupted by *URA3* was able to grow for about 9 generations from the initial level of 1×10^4 cells/ml to about 3×10^6 cells/ml in SD-galactose medium, although the maximum is > 4% of the wild-type strain, revealing the above retarded cessation of growth (Balasundaram et al., 1991; 1994). In contrast, the W303-1A wild strain grew to about 1×10^8 cells/ml at 48 h of cultivation (Table 2, Fig. 4). In order to confirm the absolute requirement of polyamines for the growth of *S. cerevisiae*, Balasundaram et al. (1994) diluted the culture of *spe1* disruption strain by 10-fold with the same fresh amine-free medium when the optical density exceeded 100 times that of the initial inoculation level. In this study, however, we permitted the HNU121 strain to grow somewhat as the above to monitor not only the growth rate but also the intracellular polyamine profile to compare with those from the strain complemented by the insect's ODC. In addition, although a polyamine-deficient *S. cerevisiae* strain was known to grow in anaerobiotic conditions, such as growing under N_2/CO_2 or in the stoppered cuvettes

Table 3. Intracellular polyamine content (nmol/ml culture) of W303-1A (wild-type), HNU121 (*spe1*-deleted), and HNU121/p413GAL1-*Slit*ODC (transformed HNU121 with p413GAL1-*Slit*ODC) *S. cerevisiae* strains during incubation in SD-galactose media

Strain	24 h			36 h			48 h		
	Put	Spd	Spm	Put	Spd	Spm	Put	Spd	Spm
W303-1A	0.75 ± 0.15	0.19 ± 0.03	1.21 ± 0.15	0.18 ± 0.03	1.65 ± 0.19	3.43 ± 0.38	0.62 ± 0.20	4.43 ± 0.25	5.76 ± 0.33
HNU121	0.22 ± 0.06	0.18 ± 0.03	0.28 ± 0.09	0.06 ± 0.01	0.09 ± 0.02	0.23 ± 0.07	0.04 ± 0.01	0.04 ± 0.01	0.21 ± 0.04
HNU121/p413GAL1-*lit*ODC	0.70 ± 0.05	0.70 ± 0.17	1.41 ± 0.11	2.55 ± 0.24	2.20 ± 0.18	6.06 ± 0.43	4.86 ± 0.35	4.96 ± 0.30	7.59 ± 0.54

Put, putrescine; Spd, spermidine; Spm, spermine
Values are mean±SD of three replicates

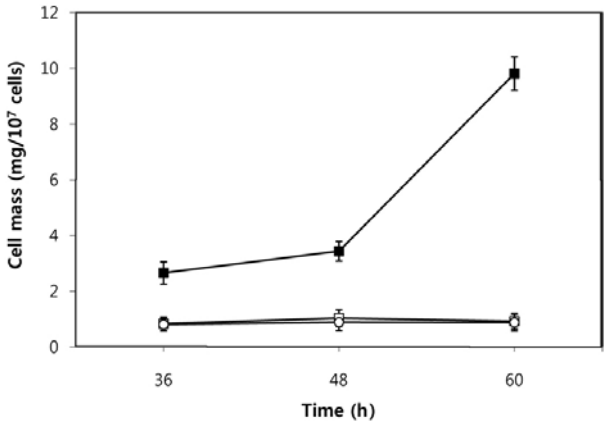

Fig. 5. Changes in the cell mass of W303-1A (□, wild-type), HNU121 (■, *spe1*-deleted), and HNU121/p413 GAL1-*Slit*ODC (○, transformed HNU121 with p413GAL1-*Slit*ODC) *S. cerevisiae* strains during incubation in SD-galactose media.

completely filled with culture without any exogenous polyamine supply (Balasundaram et al., 1991), the growth of HNU121 was not caused by any anaerobiosis, since the culture had been sufficiently aerated in this experiment.

We introduced p413GAL1-*Slit*ODC into the HNU121 and then confirmed by real-time quantitative RT-PCR that the transcription of *Slit*ODC proceeded reliably in the yeast cells (Fig. 3). The HNU121/p413GAL1-*Slit*ODC, a transformant of HNU121 with p413GAL1-*Slit*ODC, which is consequently a mutant possessing the substituted *Slit*ODC for its own deleted enzyme, exhibited a fully recovered growth profile (Fig. 4), showing even a slightly higher growth rate than that of the wild-type strain. In addition, it was verified that this recovery is directly caused by the introduced *Slit*ODC's action, because the growth rate of another strain transformed with the control plasmid p413GAL1 (HNU121/p413GAL1) was about the same as that of the wild-type strain.

On the other hand, based on the results from the cell mass determination (Fig. 5), the ODC-deficient strain, HNU121, showed much heavier cells (2.66-3.44 mg/10^7 cells) than the other two strains with ODC action, wild-type and HNU121/p413GAL1-*Slit*ODC, which had masses of 0.84-1.04 mg and 0.81-0.89 mg/10^7 cells at 36-48 h, respectively. The cell mass, in addition, remarkably increased to 9.81 mg/10^7 cells at 60 h in the ODC-deficient strains, while the other two strains remained as approximately the same mass. This abnormal enlargement of the polyamine-deficient *S. cerevisiae* cells has also been documented morphologically in previous reports. A null mutant *S. cerevisiae* strain for the *SPE2* gene, encoding *S*-adenosylmethionine decarboxylase, which is another rate-limiting enzyme in polyamine biosynthesis, showed a number of microscopic abnormalities, including a striking increase in cell size, varying from slightly larger than normal to > 10 times the normal volume. The abnormally large forms were seen in 25-50% of the haploid and ≈80% of the diploid cells (Balasundaram et al., 1991), showing an exact consistency with the above results from our experiment. Also, a sustained growth of *spe1* haploid mutants in polyamine-free medium led to a reduction in the budding index, G1 arrest, and large and misshapen cells, suggesting that the *in situ* production of polyamines is an important determinant for sporulation and that the cells are not totally defective in their ability to grow (Schwartz et al., 1995).

Although the HNU121 cells were able to grow somehow, their polyamine reserves were gradually exhausted during the incubation in the polyamine-free medium, and they showed remarkably depleted polyamine levels in putrescine, spermidine, and spermine of > 7%, > 1%, and > 4%, respectively, of the levels in the wild-type strain (Table 3). These results were consistent with phenomena in the three ODC-deficient *S. cerevisiae* strains induced by the mutagenetic method using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, which showed significantly depleted intracellular polyamine levels (Cohn et al., 1980). We monitored the polyamine contents as a total intracellular amount per unit volume of culture to examine their actual alteration profiles related to the ODC expression, because the wild-type and *Slit*ODC-transformed strains bear much smaller cells than HNU121, which produces abnormally large cells with an arrested proliferation rate during the incubation period (Fig. 5). The *Slit*ODC isolated from *S. litura* was found to distinctly complement the ODC-deficiency in *spe1*-deleted HNU121 yeast strain with the fully recovered intracellular polyamine contents (Table 3, Fig. 6). The putrescine level, in particular, profoundly increased in HNU121/p413GAL1-*Slit*ODC, showing about 5 times higher levels than the wild-type strain at 48 h.

Mangold and Leberer (2005) transformed a *spe1*-deleted *S. cerevisiae* strain with human liver ODC cDNA and showed that the human ODC complements its abnormal morphological phenotypes, including large and misshapen cells and growth arrest caused by the deficiency of endogenous ODC. They also reported that an overexpression of human ODC antizymes in the transformant strain prevented the ODC-mediated complementation of the growth defect, which was then relieved by the addition of putrescine into the media. In our study, the extraordinary increment of putrescine levels in the *Slit*ODC-transformed strain could be a reflection of no inhibition of action of the corresponding specific insect ODC Az to the introduced *Slit*ODC (Fig. 6).

In conclusion, a null mutation of the *SPE1* gene in *S. cerevisiae* led to a significant depletion in the intracellular polyamine level and a consequent arrest in the cell proliferation rate; however, when it is complemented by insect ODC having 37% iden-

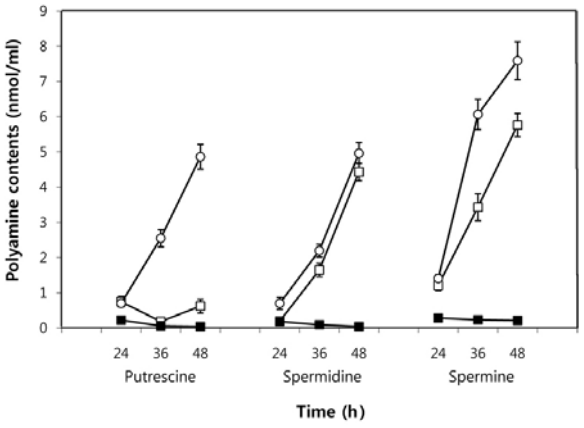

Fig. 6. Comparison in intracellular polyamine contents between W303-1A (□, wild-type), HNU121 (■, *spe1*-deleted), and HNU121/ p413 GAL1-*Slit*ODC (○, transformed HNU121 with p413GAL1-*Slit*ODC) *S. cerevisiae* strains during incubation in SD-galactose media.

tity to the yeast endogenous enzyme, the depleted polyamine level fully recovered and the proliferation arrest was completely relieved. It was of particular interest that the putrescine level was remarkably enhanced in *SlitODC*-complemented yeast, which may be due to the absence of corresponding ODC Az. This result leads to a further investigation on the polyamine profiles in the yeast strain equipped with the insect ODC-ODC Az interaction system.

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