Reconstitution of a bacterial/plant polyamine biosynthesis pathway in *Saccharomyces cerevisiae*

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Polyamine synthesis in most organisms is initiated by the decarboxylation of ornithine to form putrescine via ornithine decarboxylase (ODC). Plants, some bacteria and some fungi and protozoa generate putrescine from arginine, via arginine decarboxylase (ADC) and agmatine ureohydrolase (AUH) or agmatine iminohydrolase. A polyamine-requiring strain of *Saccharomyces cerevisiae* with a mutation in the gene encoding ODC was transformed with plasmids bearing genes encoding *Escherichia coli* ADC and AUH. Transformants regained the ability to grow in the absence of exogenous polyamines and contained enzyme activities consistent with the presence of both prokaryotic enzymes. Similar results were obtained when a plasmid containing a gene encoding oat (*Avena sativa* L.) ADC was substituted for the *E. coli* gene. These data demonstrate the successful complementation of a yeast biosynthetic polyamine synthesis defect by genes encoding an alternative pathway found in bacteria; they also show that plant ADC can substitute for the bacterial enzyme in this pathway. The recombinant yeast provides a tool for the study of the functional properties of these enzymes and for discovery of compounds that specifically inhibit this pathway.

Keywords: polyamines, arginine decarboxylase, agmatine ureohydrolase, yeast

INTRODUCTION

Recombinant micro-organisms have many uses in biotechnology. Of most interest to us is the utility of recombinant microbes in drug discovery (Klein & Geary, 1997). One example is the use of a recombinant strain of yeast to screen for compounds that could specifically block nematode polyamine biosynthesis by inhibiting ornithine decarboxylase (ODC; Klein *et al.*, 1997). In this case, a strain of *Saccharomyces cerevisiae* mutated in the ODC locus (*spe1*) was complemented with a cDNA encoding ODC from *Haemonchus contortus*; assay of test compounds in the presence or absence of exogenous polyamines provided a simple screen for ODC inhibitors. Polyamine synthesis is a legitimate chemotherapeutic target for many infectious pathogens (Marton & Pegg, 1995). Among parasitic

organisms, the protozoa *Trypanosoma cruzi* (Majumdar *et al.*, 1992) and *Cryptosporidium parvum* (Keithly *et al.*, 1997) have been shown not to synthesize polyamines through ODC, but through an arginine decarboxylase (ADC)/agmatine ureohydrolase (AUH) pathway typically found in some bacteria (Tabor & Tabor, 1985; Panagiotidis *et al.*, 1987) and plants (Slocum, 1991). To determine whether a micro-organism complemented for these genes could be used to screen for ADC/AUH inhibitors, experiments were designed for the functional reconstitution of a bacterial/plant polyamine biosynthetic pathway in yeast.

METHODS

General methods. DNA-modifying enzymes, media and reagents were as described previously (Klein & Roof, 1988). Nucleic acid sequence analysis was performed with an ABI 377 automated sequencer (Perkin-Elmer) according to the manufacturer's instructions. Oligonucleotides used for PCR or sequence analysis were purchased from Genosys. PCR

Abbreviations: ADC, arginine decarboxylase; AUH, agmatine ureohydrolase; DFMA, DL- α -difluoromethylarginine; DFMO, DL- α -difluoromethylornithine; ODC, ornithine decarboxylase.

conditions, the purification of gel fragments, and their subcloning into the plasmid pCRII (Invitrogen) were as described previously (Klein *et al.*, 1991, 1992). Yeast and bacterial transformations were performed by reported techniques (Kaiser *et al.*, 1994; Sambrook *et al.*, 1989) and *Escherichia coli* strain DH5 α (Bethesda Research Laboratories) was used in experiments requiring a bacterial host for subcloning.

Strains and media. The polyamine-dependent mutant strain of *S. cerevisiae*, Y357 (*LEU2::SPE1 ura3-52 his6*), which contains a disruption of *spe1*, the locus encoding ODC, was a gift from C. W. Tabor (NIH, Bethesda, MD, USA). The *spe1* mutation and the mutant Y357 were formerly described as *spe10* and 262d-1, respectively (Cohn *et al.*, 1980; Xie *et al.*, 1990). Strain YPH499 (a *ura3-52 lys2-801 ade2-101 trp1-63 his3-200 leu2-1*; Skorski & Hieter, 1989) was obtained from Stratagene and served as a wild-type control. Yeast strains were maintained on enriched medium (YEPD) as described previously (Kaiser *et al.*, 1994) or yeast minimal medium (YMM) supplemented with the appropriate amino acids or spermidine (10 $\mu\text{g ml}^{-1}$). Polyamine-deficient medium was YMM, which is similar to the H medium described by Cohn *et al.* (1980). Sporulation medium was as described by Kaiser *et al.* (1994).

Strain constructions. Yeast strains containing the *LEU2::SPE1* knock-out and multiple auxotrophic mutations were constructed using the random spore analysis procedure as described previously (Kaiser *et al.*, 1994) with modifications. Yeast strains Y357 and YPH499 were streaked onto independent YEPD plates and incubated at 30 °C for 24 h. An impression of the Y357 plate was made on a velveteen pad and pressed onto the YPH499 plate at right angles to the streak. This mating plate was incubated for 24 h at 30 °C. To remove unwanted haploids, the mating plate was replica-plated to a YMM plate supplemented with uracil and permitted to grow at 30 °C for 24 h. Colonies were scraped from the intersection of the strains and mixed with 200 μl sterile water. Serial dilutions of 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} and 10^{-5} were made and 100 μl of each dilution was spread on YMM plates supplemented with uracil, and these were incubated overnight at 30 °C. Fourteen individual colonies were picked from the 10^{-4} and 10^{-5} dilution plates, patched to a grid on sporulation medium and incubated at 30 °C for 48 h. The formation of asci was monitored microscopically. Cells transferred using a 100 μl inoculation loop from one of the sporulation grids were suspended in 200 μl 1 M sorbitol in a 12 $\times$ 100 mm glass tube. Approximately 4 μl (100 U) lyticase solution (Sigma) and 100 μl washed, sterile glass beads (0.45–0.5 mm) were added, and the tubes were then placed on a Cell-Production Roller Drum (Bellco) for 1 h at 30 °C. Sterile water (1 ml) was added, followed by vigorous vortexing for 1 min. Asci disruption was monitored microscopically, and the process was continued until all the asci were released. To induce the growth of mutant yeasts, 3 ml sterile water was added to each tube and 100 μl aliquots of serial dilutions of 10^0 , 10^{-1} and 10^{-2} were spread on YMM plates lacking leucine, but supplemented with adenine, histidine, lysine, spermidine, tryptophan and uracil. Plates were incubated at 30 °C for 48 h and colonies were further characterized.

Individual colonies were patched to a grid on YMM plates supplemented as above and were incubated at 30 °C for 24 h. This grid was replica-plated to YMM plates supplemented either with adenine, histidine, lysine, spermidine or uracil (to identify *trp1* mutants and confirm the Leu⁺ phenotype) or adenine, histidine, lysine, spermidine and tryptophan (to identify *ura3-52* mutants). Dependence on exogenous poly-

amines was scored as follows. Strains that were Leu⁺, Ura⁻ and Trp⁻ were replica-plated to YMM plates supplemented with adenine, histidine, lysine, tryptophan and uracil, with or without 10 μM spermidine. Slight growth was seen on the plates lacking spermidine due to endogenous polyamine pools. To deplete endogenous polyamines, cells from these plates were used to inoculate 10 ml liquid cultures of YMM supplemented with adenine, histidine, leucine, lysine, uracil and tryptophan, but lacking spermidine. Cultures were incubated at 30 °C overnight, diluted to an OD₆₀₀ of 0.3 cm⁻¹ and a 10 μl aliquot of this dilution was spread on to YMM plates containing the appropriate nutrients, with or without spermidine. Plates were incubated at 30 °C for 48 h. Strains that were identified as Spe⁻ were then characterized for histidine, lysine and adenine auxotrophy. One strain, termed yASG1-8 (*LEU2::SPE1 ura3-52 trp1-63*), was used in further studies to confirm that transformations were plasmid-mediated.

Plasmid constructions. To test for complementation of polyamine biosynthesis using heterologous genes, several yeast vectors were constructed. The yeast expression plasmid pYcDE8 has been described (Klein & Roof, 1998) and pYcDE8NotI is a modification of it. A sense PCR primer, 5' GGCGCATGCAACTTCTTTCTTTTCTTTTCTTTTCTCT 3', designed to the 5' region of the ADHI promoter in pYcDE8, includes a unique *SphI* site. An antisense primer, 5' GGTACCGAGCTCGAATTCGCGGCCGCCCTTCATTGTATGCTTGGTATAGC 3', was designed to the region of the multiple cloning site (MCS) in pYcDE8 and included a *NotI* site immediately 5' to the *EcoRI* site. Using these primers and pYcDE8 as the substrate, a 450 bp fragment was amplified by PCR, gel-purified and cleaved with *SphI* and *EcoRI*, releasing a larger fragment containing the ADHI promoter. This reaction mixture was fractionated through an agarose gel and the larger fragment was purified. The 450 bp PCR product was then cloned into this linearized vector to yield plasmid pYcDE8NotI. The yeast expression vector Yep24ADHNotI was constructed by cleaving pYcDE8NotI with *SphI* and *EcoRV*, to release a fragment containing an expression cassette consisting of the ADHI promoter, MCS and the CYC1 terminator region. This fragment was then gel-purified. The yeast vector Yep24 was cleaved with *SphI* and *SmaI*, to produce a 200 bp fragment and the linearized vector. The *SphI*–*EcoRV* fragment containing the ADHNotI expression cassette was cloned into the linearized Yep24 plasmid to create Yep24ADHNotI.

The gene encoding oat ADC (pADC2; Bell & Malmberg, 1990) was also cloned into the yeast vector pYcDE8. When pADC2 was treated with *EcoRI*, a 2124 bp fragment containing the ADC ORF was released. This fragment was gel-purified and cloned into the *EcoRI* site of pYcDE8, to create the plasmid pYcDE8-ADC. The orientation of the insert was determined by restriction enzyme and nucleotide sequence analysis.

Cloning the bacterial *speA* and *speB* genes. Using *E. coli* K-12 genomic DNA as substrate, the ORFs for both ADC (*speA*) and AUH (*speB*) were amplified by PCR. A 2000 bp product encoding *speA* (GenBank accession no. M31770) was amplified using primers based on the published sequence (Moore & Boyle, 1990), and its identity was confirmed using internal sense and antisense primers located in the centre of the gene. This amplicon was gel-purified, cloned to yield pCRII-*speA*, and both its identity to *speA* and orientation were confirmed by DNA sequence analysis. A 940 bp product encoding *speB* (GenBank accession no. M32363) (Szumanski & Boyle, 1990) was amplified, gel-purified and cloned to yield pCRII-*speB*; its

identity was confirmed in an identical manner. Plasmids pCRII-speA and pCRII-speB were cleaved with *EcoRI* to release inserts which were then isolated by gel purification and cloned into the *EcoRI* site of pYcDE8 to produce plasmids pYcDE8-speA and pYcDE8-speB. Orientation of the *speA* and *speB* ORF was designed so that the *NotI* site of the vector was 5' to the amino terminus, and the *BamHI* site 3' to the carboxy terminus. Fragments containing *speA* or *speB* were gel-purified after digestion with *NotI* and *BamHI*, and both were then cloned into Yep24ADHNotI at these enzyme sites to yield plasmids Yep24ADHNotI-speA and Yep24ADHNotI-speB.

The yeast strain yASG1-8 expressing only bacterial polyamine genes is designated pbADC/pbAUH, whereas that expressing oat ADC combined with bacterial AUH is termed poADC/pbAUH.

As a control, the yeast vector pYHcODC, which encodes nematode ODC (Klein *et al.*, 1997), was transformed into yeast strain Y357. The vector used for this construction is a derivative of the galactose-inducible vector pSEY68 (a gift from Scott Emr, University of California at San Diego, La Jolla, CA, USA).

Harvesting and cell breakage. Recombinant yeast pellets were prepared as described previously (Klein *et al.*, 1997). Briefly, yeast pellets were disrupted three times at -4°C in a French press under 18000 p.s.i. (124 MPa). The homogenates were mixed with 0.5 mm glass beads (approx. 1/3 vol.) and subjected to 3×30 s bursts at 5000 r.p.m. on a bead breaker (BioSpec Products) with cooling between bursts. Beads were removed by centrifugation for 60 s at 11300 r.p.m. and the homogenate was removed and stored at 4°C . Protein concentration was estimated using the Lowry method.

Enzyme analysis. ADC was assayed in incubations containing 7 μCi (259 kBq) [^{14}C]arginine [327 mCi (12 GBq) mmol^{-1} ; Dupont], 10 mM Tris/HCl, pH 8.0, 60 μM pyridoxal phosphate, 1 mM 2-mercaptoethanol and varying amounts (0.06–2.00 mM) of arginine. The $^{14}\text{CO}_2$ released in 60 min was trapped on benzethonium-hydroxide-soaked filter paper and counted by scintillation (Smith, 1983). AUH was assayed by two techniques. The first was a coupled assay measuring the decarboxylation of [^{14}C]arginine and trapping the $^{14}\text{CO}_2$ released as a measure of ADC activity; the supernatant left was assayed by HPLC for quantification of [^{14}C]agmatine present. A saturating amount of arginine (25 mM, 31 times the ADC K_m for arginine) was added. Hence, the amount of agmatine produced at steady state depends upon the rate constant of ADC (k_1). The actual amount of agmatine (Ag) detected in the supernatant then reflects the amount of substrate present at steady state after consumption by AUH. Thus we arrive at the following formula: $k_1 - \text{Ag} = V_{\text{AUH}}$. AUH was also analysed by measuring the amount of urea formed in a reaction mixture containing 10 mM Tris/HCl, pH 8.0, 5 mM agmatine, 2 mM Na_2HPO_4 and 2 mM DTT. After 30 min the reaction was stopped by the addition of 10% (v/v) HClO_4 and the amount of urea formed was determined using diacetyl monoxamine, as described by Boyde & Rahmatullah (1980).

ADC inhibition kinetics. Time-dependent, irreversible inhibition of ADC was determined as described previously (Bitonti *et al.*, 1982; Yarlett *et al.*, 1992). Extracts of the poADC/pbAUH yeast strain were incubated at 37°C with various concentrations of DL- α -difluoromethylarginine (DFMA). At selected times, 1 μCi (37 kBq) L-[^{14}C]arginine was added to the flask and the assay was incubated for a further 30 min before stopping with 1 ml 40% (v/v) TCA. Released $^{14}\text{CO}_2$ was trapped on benzethonium-hydroxide-soaked filter paper

and the percentage remaining activity was compared to controls lacking DFMA.

HPLC analysis. Amino acids and diamines were separated by reverse phase HPLC using a series LC 410 pump (Perkin-Elmer) coupled to a C-18 10 μm column (4.5×250 cm; 100 μl injection loop) at a flow rate of 1 ml min^{-1} . The method employed a 70 min discontinuous gradient starting with 85% buffer A (2.5 g lithium citrate l^{-1} , pH 2.65, containing 0.22 g octanesulfonic acid l^{-1}) and ending with 85% of buffer B (acetonitrile) (Yarlett & Bacchi, 1988). Standards and samples were precolumn derivatized using 0.8 g *o*-phthalaldehyde l^{-1} (2:1) dissolved in 3 ml methanol and added to a solution of 30.9 g boric acid l^{-1} containing 24 g KOH l^{-1} (pH 10.4) and 1 ml 2-mercaptoethanol. Dual analysis was performed using a Perkin-Elmer fluorescence detector (λ_{excit} 320 nm, λ_{em} 455 nm) (North American Instruments Division), coupled to a β -RAM flow-through model 1B radiometric detector (IN/US Systems) which mixed three parts scintillant (IN FLOW ES) to one part sample. Signals were recorded and integrated using β -RAM model 1, version 1.62 software (IN/US Systems) and concentrations of metabolites were determined relative to standards.

RESULTS

Expression of *speA* and *speB* in yeast

The use of the polyamine-dependent mutant *S. cerevisiae* strain Y357 required the introduction of additional auxotrophic markers. Therefore, strain yASG1-8 was constructed to contain mutations in the *URA3* and *TRP1* loci. As mentioned previously, the bacterial genes *speA* and *speB* were introduced

Fig. 1. Growth results. Yeast strain yASG1-8 containing plasmid Yep24ADHNotI-speB was transformed with (top panel) (1) pYcDE8, (2) pYcDE8-ADC, (3) pYcDE8-speA or (bottom panel) (1) pYcDE8-ADC, (2) pYcDE8-ADCrev (construction in which the cDNA insert was inserted in the opposite orientation). Yeast were grown on polyamine-deficient medium supplemented with spermidine (left-hand side) or not supplemented (right-hand side).

Table 1. Growth of yeast strains cloned in this study

0, No detectable growth; +, detectable growth.

Yeast/cloned gene*	Spermidine	
	10 μ M	0 μ M
Y499 (wild-type)	+++	+++
Y357 (ODC ⁻)	+++	0
Y357/pODC	+++	+++
YASG1-8	+++	0
/pYcDE8	+++	0
/Yep24ADHNotI	+++	0
/pbADC	+++	0
/pbAUH	+++	0
/poADC	+++	0
/pbADC+pbAUH	+++	+++
/poADC+pbAUH	+++	+++

* pODC, *H. contortus* ODC gene in pYHcODC; pbADC, *E. coli* arginine decarboxylase gene; pbAUH, *E. coli* agmatine ureo-hydrolase gene; poADC, *Avena sativa* ADC gene.

into yASG1-8 on plasmids pYcDE8-speA, pYcDE8-speB, Yep24ADHNotI-speA and Yep24ADHNotI-speB. Transformants were tested for growth in media lacking exogenous polyamines and appropriate nutrients (uracil and/or tryptophan) to permit selection of each plasmid or pair of plasmids. Only yeast containing both plasmids grew on medium lacking spermidine (Fig. 1, Table 1). These plasmids were rescued and characterized from the complemented yeasts, and then reintroduced into yASG1-8 to confirm that the Spe⁺ phenotype was plasmid-mediated. Growth rates for the complemented strain pbADC/pbAUH were identical to those of wild-type yeast (data not shown).

Strain yASG1-8 containing only the plasmid Yep24ADHNotI-speB was used as a host in experiments with pYcDE8-ADC to determine if oat ADC could also complement the Spe⁻ phenotype. Only transformants containing both pYcDE8-ADC and pYcDE8-speB grew in polyamine-deficient medium (Fig. 1, Table 1). To

verify that this phenotype was plasmid-mediated, plasmids from individual clones were rescued into *E. coli* and retransformed into strain yASG1-8 containing only Yep24ADHNotI-speB.

Enzyme analysis

ADC activity was measured in each of the transformed yeasts (Table 2). The activity obeyed Michaelis–Menten kinetics and varying the substrate concentration gave typical sigmoidal curves which resulted in linear plots upon Hanes–Wolf analysis. The K_m for arginine was similar for both complemented yeast strains, 78 μ M and 65 μ M for poADC/pbAUH and pbADC/pbAUH, respectively (Table 2). However, strain poADC/pbAUH had 26-fold greater ADC activity than yeast strain pbADC/pbAUH (Table 2). These activities were unaffected by the addition of 1 mM ornithine, and no discernible liberation of ¹⁴CO₂ was detected from 1-[¹⁴C]ornithine, thus confirming the lack of ODC activity in these strains (see Klein *et al.*, 1997). In addition poADC/pbAUH-transformed cells were incubated for 30 min with 100 μ M of either DFMA or DL- α -difluoromethylornithine (DFMO), and then assayed for ADC activity. The results demonstrate that DFMO has no effect upon ¹⁴CO₂ released from arginine, whereas DFMA inhibits ¹⁴CO₂ released by 86% (not shown), which is similar to the results obtained by Bitonti *et al.* (1987), who found that 100 μ M DFMA caused 75% inhibition of oat ADC. Kinetic analysis of DFMA inhibition of ADC activity showed that inhibition was time-dependent (Fig. 2a). The time for 50% inhibition of the ADC activity with various concentrations of DFMA was determined from plots of the log of percentage activity remaining at various times (10–40 min) in the presence of fixed concentrations of DFMA (Fig. 2a). From plots of the half-life of the enzyme activity versus the inverse of the DFMA concentration it was possible to determine an apparent dissociation constant (K_i) of 200 μ M and the half-life ($t_{1/2}$) of the enzyme activity at an infinite concentration of DFMA of 3 min (Fig. 2b). These values are comparable to those determined for the oat ADC (Bitonti *et al.*, 1987).

Table 2. Kinetic constants for the transformed yeast strains poADC/pbAUH and pbADC/pbAUH

One unit of enzyme activity is defined as the conversion of 1 nmol substrate to product in 1 h using 1 mg protein.

Yeast strain	ADC		AUH		Reference
	Activity	K_m (μ M)	Activity	K_m (μ M)	
poADC/pbAUH	7.3	78	5.6	24	Present study
pbADC/pbAUH	0.26	65	5.0	18	Present study
<i>E. coli</i>	2580	30	16500	1200	Boyle <i>et al.</i> (1984)
					Satishchandran & Boyle (1986)
Oat	40	30	–	–	Smith (1983)

Fig. 2. Time-dependent irreversible kinetics of inhibition by DFMA of ADC in extracts of yeast strain poADC/pbAUH. (a) The log of percentage remaining activity with time of preincubation with DFMA was plotted for 0 (●), 25 (■), 50 (▲), 75 (▼) and 100 (◆) μM DFMA. (b) The reciprocal of each DFMA concentration used is plotted against the time taken to reach 50% of the remaining activity ($t_{1/2}$) from which the K_i is extrapolated from the intercept on the abscissa. Similar plots were obtained in three separate experiments. The K_i for DFMA on ADC was determined to be 200 μM .

AUH activity was determined by measuring the amount of urea formed in the presence of varying amounts of agmatine. Urea formation increased in proportion to added agmatine, and the enzyme obeyed Michaelis-Menten kinetics resulting in linear plots by Hanes-Woolf analysis. The calculated activity of the crude enzyme was similar for extracts prepared from both the poADC/pbAUH- and pbADC/pbAUH-transformed cell lines (Table 2). The K_m for agmatine of both transformed cell lines was also found to be similar, 24 μM and 18 μM for poADC/pbAUH and pbADC/pbAUH, respectively (Table 2). AUH activity was

confirmed by measuring the formation of agmatine from arginine. The amount of agmatine formed by ADC in 60 min in these experiments was predicted to be 7.3 nmol, based on the ADC activity measured in the presence of a saturating arginine concentration of 25 mM. The amount detected in extracts from these incubations was 2.8 nmol. As agmatine is only metabolized via AUH to urea, the lower amount of agmatine detected must be accountable by the proportions metabolized to urea by AUH, which results in a calculated activity of 4.5 nmol h^{-1} (mg protein) $^{-1}$. This value is comparable to the activity determined by urea formation, 5.6 nmol h^{-1} (mg protein) $^{-1}$. This result also confirms that the urea is not being further metabolized to CO_2 and NH_3 by urease in these yeast.

DISCUSSION

The polyamines spermidine and spermine, and their diamine precursor putrescine, are multifunctional molecules with several vital roles; for instance they have structural elements that stabilize phospholipids, nucleic acids and proteins (Feurstein *et al.*, 1990; Marton & Morris, 1987). The intracellular concentration of these molecules is highly regulated by the polyamine metabolic pathway, which influences the synthesis, degradation, uptake and excretion of these cations (Marton & Pegg, 1995; Ha *et al.*, 1997). In the majority of the cells of mammals, protozoa, yeast, fungi and most bacteria, ODC is the first and rate-limiting step of polyamine biosynthesis. However, in plants, cryptosporidia and some bacteria, polyamine biosynthesis is initiated by ADC, which produces agmatine: this is further degraded to putrescine via the action of AUH (bacteria) or agmatine iminohydrolase (AIH) (plants).

It has previously been shown that yeast ODC mutants can thrive in a polyamine-deficient medium through the expression of a heterologous ODC (Klein *et al.*, 1997). The complemented yeast served as the basis for a nutrient-dependent viability assay; candidate nematode ODC inhibitors were identified by their toxicity in the absence, but not the presence, of spermidine (Klein *et al.*, 1997). Measuring the viability of recombinant microorganisms under selective conditions has proven to be a flexible, robust and highly efficient path to drug discovery in many therapeutic categories (for review see Klein & Geary, 1997).

Polyamine availability provides a powerful selection for yeast. *S. cerevisiae* contains a constitutive ODC, with an activity of 0.33 nmol min^{-1} (mg protein) $^{-1}$ (Tyagi *et al.*, 1983); the rate of polyamine biosynthesis is controlled by this rate-limiting enzyme. Strains in which polyamine synthesis is absent (Spe1 phenotype) do not grow in the absence of added polyamines (Tabor & Tabor, 1985). However, even very minor amounts of polyamines existing as contaminants in culture reagents can result in detectable growth (Cohn *et al.*, 1980). This polyamine-deficient phenotype made it feasible to attempt complementation of the ODC mutation with genes

encoding an entirely foreign pathway for polyamine biosynthesis, since even low-level production of polyamines would be functional in this system. The ADC/AUH pathway is functional in plants and some bacteria, and although this pathway is not present in yeast cells, these enzymes successfully complemented the yeast polyamine biosynthetic pathway and restored normal growth in the absence of exogenous polyamines. The inability of yeast mutants expressing only one of the plasmids to grow in the absence of added polyamines demonstrates the inability of agmatine to replace putrescine in function, and the requirement for both enzymes to be successfully transcribed by the cell for normal growth to occur.

The rate of putrescine biosynthesis by the transformed yeast cell lines is dependent upon the rate of agmatine formation by ADC and its utilization by AUH. This rate is approximately the same for both the pbADC/pbAUH and poADC/pbAUH transformants, but is approximately threefold lower than the rate of putrescine production by the *Spe1*⁺ phenotype expressing ODC. The rate of polyamine biosynthesis of the poADC/pbAUH and pbADC/pbAUH transformed cell lines is sufficient to meet the organism's requirement for normal growth. The crude activity of ADC from the poADC-transformed cell line was approximately 25-fold greater than the enzyme derived from the prokaryotic genome, despite the fact that the *E. coli* enzyme has a crude activity of 43 nmol min⁻¹ (mg protein)⁻¹ (Boyle *et al.*, 1984) compared to 0.68 nmol min⁻¹ (mg protein)⁻¹ for the oat ADC (Malmberg & Cellino, 1994). This probably reflects the easier processing of the eukaryotic-derived gene by the yeast cell. The complementation of the bacterial ADC gene with the eukaryotic genome to produce a functional protein may be facilitated by the observation that the *S. cerevisiae* ODC (encoded by the *spe1* gene) and the *E. coli* biosynthetic ADC (encoded by the *speA* gene) are from the same taxonomic tree (Sandmeir *et al.*, 1994). However, there is no equivalent yeast protein to the bacterial AUH. The prokaryotic-derived AUH activity was comparable in crude activity from both transformed cell lines, irrespective of the origin of the ADC. The activity of this enzyme was considerably lower than the activity observed for this enzyme from *E. coli*, reflecting the lower transcription of the yeast pbAUH and the tight control over polyamine biosynthesis by the yeast cell. The oat ADC is reported to require proteolytic cleavages from a 66 kDa precursor to produce 42 and 24 kDa proteins (Malmberg & Cellino, 1994): presumably this also occurs in the poADC-transformed yeast cells. It is clear from the enzyme activity determinations performed, using the poADC/pbAUH and pbADC/pbAUH transformed yeast cells, that the rate of polyamine biosynthesis is tightly controlled, despite the fact that the inserted proteins are capable of much greater activities, as inferred from activity determinations using crude lysates of the donor cells.

The importance of polyamines to normal cell growth and development can be inferred from the use of

inhibitors such as DFMO and DFMA which are specific irreversible inhibitors of ODC and ADC, respectively. These inhibitors block polyamine biosynthesis, slowing growth and eventually resulting in cell death (Bitonti *et al.*, 1987; McCann & Pegg, 1992). In common with the host-expressed enzyme, the expressed pbADC and poADC were irreversibly inhibited by DFMA, with time-dependent inhibition constants similar to those calculated for the enzyme in the derived host cells.

This work confirms an earlier study showing *S. cerevisiae* expression of pea (*Pisum sativum*) ADC (Perez-Amador *et al.*, 1995) and extends it by showing complete restoration of polyamine biosynthesis in a yeast transformed cell line by a pathway uncommon to it. It also highlights the potential of this technique for exploring metabolic pathways as potential chemotherapeutic targets from cells not easily cultured in the laboratory.

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