Inhibition of Ornithine Carbamoyltransferase by Arginase among Yeasts: Correlation with Energy Production, Subcellular Localization and Enzyme Synthesis

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Thirty-two yeast species belonging to twelve genera were examined for the occurrence of inhibition of ornithine carbamoyltransferase by arginase (epiarginasic regulation) and related properties. Obligate aerobes were devoid of this regulation. Among fermenting species, *Schizosaccharomyces* and budding genera had different properties: all *Schizosaccharomyces* species were devoid of this regulation whereas all species of budding yeasts showing a weak or absent Pasteur effect had this regulation. Strains showing a strong Pasteur effect and taxonomically related to *Saccharomyces* (*Kluyveromyces*) had the regulation, whereas species classified in genera that include species which are obligate aerobes did not.

We confirm that the absence of epiarginasic regulation is correlated with a mitochondrial localization of ornithine carbamoyltransferase but not with the function of the mitochondria in *Schizosaccharomyces japonicus*. In this case, compartmentation could serve to channel anabolic and catabolic functions. Although, in general, the arginase of 'negative epiarginasic' species had no affinity for cytosolic ornithine carbamoyltransferase, one exception was found (*Hansenula anomala*).

Regulation by repression of ornithine carbamoyltransferase and induction of arginase by arginine was the general rule. These types of regulation of enzyme synthesis were only absent in yeasts in which compartmentation is present and could serve as the basis for anabolism–catabolism exclusion.

INTRODUCTION

Saccharomyces cerevisiae has a peculiar regulatory mechanism which excludes interference between biosynthesis and degradation of arginine. Arginase, the first enzyme of the arginine degradative pathway, converts this amino acid into ornithine and urea. This enzyme is able to inhibit ornithine carbamoyltransferase (OTCase), the biosynthetic enzyme which converts ornithine plus carbamoylphosphate into citrulline and phosphate. This inhibition of OTCase was first observed '*in situ*' in cells which were permeabilized, leaving macromolecules inside the cells (Béchet & Wiame, 1965). The nature of the inhibitory molecule was established in cell-free extracts and was shown to be arginase. The binding of the two enzymes is reversible and is affected by arginine and ornithine (Messenguy & Wiame, 1969). The ornithine receptor site is on OTCase and is distinct from the catalytic site. Ornithine at a higher concentration (20 mM) than the K_m value for catalysis (3 mM) provokes a limited inhibition on its own. Arginine acts together with arginase, but alone does not inhibit OTCase. Arginase maintains full catalytic activity when complexed with and inhibiting OTCase (Messenguy *et al.*, 1971; Penninckx, 1975; Penninckx & Wiame, 1976). This system

of regulation, termed 'epiarginasic regulation', is not present in all yeasts (Wiame, 1971), but it has been reported in *Bacillus subtilis* (Issaly & Issaly, 1974).

The OTCases of *Neurospora crassa* and liver are mitochondrial enzymes and thus are not accessible to cytosolic arginase (Weiss & Davis, 1973; Gamble & Lehninger, 1973), making improbable any regulation by association of these two enzymes. However, in *Sacch. cerevisiae* both enzymes are outside the mitochondria (Urrestarazu *et al.*, 1977) and are very likely cytosolic, thus affording the opportunity for regulation by interaction.

The main purpose of the work described in this paper was to try to evaluate the evolutionary process which led to this specific and original regulatory process. The fact that ornithine belongs to both the biosynthetic and degradative pathways creates the possibility of a futile cycle resulting from resynthesis of arginine from ornithine. This would lead to the loss of at least five energy-rich bonds (one for the synthesis of glutamine, two for the synthesis of carbamoylphosphate and two for the synthesis of argininosuccinate). An additional energy-rich bond is necessary to recover ammonia from urea (Roon *et al.*, 1972). The large expenditure of energy in futile resynthesis of arginine would probably be more damaging than the loss of one CO₂ per cycle. The epiarginasic regulation might be a way to avoid this waste. Our attention was then focused on the peculiarity of *Sacch. cerevisiae* in obtaining its energy from an energy-poor process (fermentation) which is retained even in the presence of oxygen when sugar is the energy source (Ephrussi *et al.*, 1956). In this organism, perhaps ATP limitation was an important factor in the original selection of the epiarginasic regulation.

METHODS

Strains. The yeast strains used are listed in Table 1. They belong to the Endomycetales and the Fungi Imperfecti (Lodder, 1970) and came from our own collection (IRC) or from the Centraalbureau voor Schimmelcultures, The Netherlands (CBS). The diploid strain of *Hansenula anomala* was obtained by crossing NRRL Y-366-8 with NRRL Y-2153-4 (Northern Regional Research Laboratory, Ill., U.S.A.).

Media. Yeasts were maintained on complex medium solidified with 2% (w/v) agar (Ramos & Wiame, 1979). Analysis of growth and cell production were performed in medium M, composed of a mineral solution to which separate sterile glucose and sterile vitamin solutions were added. The mineral and vitamin solutions were those previously described by Grenson *et al.* (1966) for medium no. 150. The nitrogen sources used (as indicated in the text) were 0.01 M-ammonium sulphate and/or 1 mg arginine ml⁻¹. The preparation of the mineral solution was slightly modified in later experiments (with *Schizosaccharomyces japonicus* var. *versatilis*) by separate sterilization of the heavy metals and trace elements (Ramos & Wiame, 1979). For *Schiz. octosporus*, 0.1% (w/v) yeast extract was added to the minimal medium. For anaerobic cultures the media were supplemented with 0.1 ml Tween 80 and 1 mg ergosterol (1 mg ml⁻¹ in methanol) per 100 ml medium.

Growth conditions. All the cultures were maintained at 29 °C. Liquid cultures were grown on a rotary shaker in flasks with provision for turbidity estimation. Anaerobiosis was obtained in liquid cultures by two successive evacuations of gas and flushing with nitrogen. Using such conditions, strict aerophiles like *Cryptococcus neoformans* did not grow but microaerophiles like *Schiz. pombe* did. To determine the differential rate of synthesis of an enzyme or of ethanol production, several flasks of the same culture were followed in parallel and harvested at different turbidities. Two qualitative tests of ability to grow anaerobically on solid media were also performed. In the first, cotton plugs were inserted partway into test tubes containing agar (2%, w/v) stabs of the organism. Pyrogallol crystals (0.5 g) and 4 drops of 5 M-KOH were placed on top of the cotton plug, and the test tube was closed with a rubber stopper. The second test used an apparatus applicable to both test tubes and Petri dishes, as described by Brewer & Allgeier (1966) and available commercially (BBL) under the name of GasPak; the tubes were examined after 10 d. For the epiarginasic inhibition test *in situ*, the cells were transferred from one medium to another by filtration of the cultures on Millipore filters, except for *Cr. neoformans* which had to be centrifuged because it warped the filters. The growth in liquid medium was followed by measurement of turbidity at 660 nm. An A₆₆₀ of 1 in the Beckman B spectrophotometer or of 3.4 in the Gilford 2000 spectrophotometer corresponded to 0.53 mg protein (ml culture)⁻¹ as determined with *Sacch. cerevisiae*.

Cell extracts and cell permeabilization. Samples from cultures in the exponential phase of growth were filtered on Millipore filters or centrifuged in the presence of cycloheximide (2 µg ml⁻¹) and washed with cold water. The pellets were stored in ice and subsequently resuspended in the buffer appropriate to the enzyme assay. Cells were broken in a French press precooled to 4 °C, and cell debris was removed by centrifugation at 22000 g for 20 min. The crude extract was desalted on a Sephadex G-25 fine column (Pharmacia) preconditioned and eluted with the buffer appropriate to the enzyme assay.

Table 1. Name and origin of the species (according to Lodder, 1970)

Species	Origin
<i>Brettanomyces lambicus</i> Kufferath & van Laer	IRC L-1-3
<i>Candida tropicalis</i> (Cast.) Berkhout	IRC L-7-4
<i>Candida utilis</i> (Henneberg) Lodder & van Rij ex <i>Torulopsis utilis</i>	IRC L-7-14
<i>Cryptococcus neoformans</i> (Sanfelice) Vuillemin	CBS 132
<i>Debaryomyces hansenii</i> (Zopf) Lodder & van Rij	CBS 767
<i>Debaryomyces hansenii</i> ex <i>Debaryomyces klockeri</i>	CBS 770
<i>Debaryomyces hansenii</i> ex <i>Debaryomyces subglobosus</i>	CBS 792
<i>Debaryomyces hansenii</i> ex <i>Debaryomyces nicotianae</i>	CBS 811
<i>Debaryomyces marama</i> di Menna	CBS 1958
<i>Debaryomyces phaffii</i> Capriotti	CBS 4346
<i>Hanseniaspora valbyensis</i> Klöcker	IRC L-4-1
<i>Hansenula anomala</i> (Hansen) H. & P. Sydow	IRC L-5-6
<i>Hansenula anomala</i> var. <i>anomala</i>	NRRL Y-366-8 × NRRL Y-2153-4
<i>Hansenula wingei</i> Wickerham	IRC L-5-19
<i>Kluyveromyces fragilis</i> (Jørgensen) van der Walt ex <i>Sacch. fragilis</i>	IRC L-3-6
<i>Kluyveromyces lactis</i> (Dombrowski) van der Walt	CBS 683
<i>Pichia dispersa</i> (Dekker) van Rij	CBS 794
<i>Pichia fermentans</i> Lodder	IRC L-5-16
<i>Pichia fluxuum</i> (Pfaff & Knapp) van Rij	CBS 2287
<i>Saccharomyces cerevisiae</i> Hansen	IRC Σ1278b
<i>Saccharomyces fermentati</i> (Saito) Lodder & van Rij	CBS 818
<i>Saccharomyces fermentati</i> ex <i>Debaryomyces dekkeri</i>	CBS 813
<i>Saccharomyces klockerianus</i> van der Walt ex <i>Deb. globosus</i>	CBS 764
<i>Saccharomycodes ludwigii</i> Hansen	IRC L-4-3
<i>Schizosaccharomyces japonicus</i> Yukawa & Maki var. <i>japonicus</i>	CBS 354
<i>Schizosaccharomyces japonicus</i> Yukawa & Maki var. <i>versatilis</i>	CBS 103
(Wickerham & Duprat) Sloof	
<i>Schizosaccharomyces malidevorans</i> Rankine & Fornachon	CBS 5.557
<i>Schizosaccharomyces octosporus</i> Beijerinck	IRC L-0-6
<i>Schizosaccharomyces octosporus</i>	CBS 371
<i>Schizosaccharomyces pombe</i> Lindler	IRC L-0-8
<i>Torulopsis candida</i> (Saito) Lodder	CBS 940
<i>Torulopsis colliculosa</i> (Hartmann) Saccardo	CBS 133

Permeabilization of fresh cells was achieved by treatment with nystatin (Labaz, Brussels, Belgium) as described by Béchet & Wiame (1965) in Tris/HCl buffer (0.1 M, pH 8). Permeabilization of *Schiz. japonicus* strains required a more acid buffer (sodium acetate, 0.05 M, pH 5). In contrast, all the yeasts could be permeabilized with 0.4% (w/v) Triton X-100 (Serva, Heidelberg, Germany) in Tris/HCl buffer (0.1 M, pH 8). All the permeabilization treatments were performed at 30 °C for 40 min with shaking.

For *in vitro* cross-inhibition tests between OTCase and arginase of different origins and the two enzymes of *Sacch. cerevisiae*, all extracts were desalted. Cells cultured on ammonia medium provided OTCase, whereas they were poor in arginase. Cells cultured on arginine medium provided arginase. In the case of *Sacch. cerevisiae*, desalted crude extract was used, whereas for the other species, OTCase was eliminated by the following procedure. Nucleic acids were precipitated by adding protamine sulphate (14–28% of protein weight). The supernatant was desalted on Sephadex G-25 fine preconditioned with sodium maleate buffer (0.001 M, pH 6.5). A sample containing 6000–37000 units of arginase ($\mu\text{mol h}^{-1}$) was applied to an arginyl-Sepharose column as described by Penninckx *et al.* (1974) and eluted with an arginine gradient (0–0.1 M). All operations were performed at 4 °C.

Enzyme assays. Ornithine carbamoyltransferase (OTCase; EC 2.1.3.3) was measured at 30 °C as described in Messenguy *et al.* (1971) except that MgCl_2 was omitted. Tris/HCl buffer (0.1 M, pH 8) was used for the determination of specific activities and *in situ* tests. For the determination of inhibition by excess ornithine, the enzyme activity was measured at 15 °C in Tris/HCl buffer (0.1 M, pH 8.6). *In vitro* inhibition tests were done at 15 °C in Tris/HCl buffer (0.1 M, pH 8), with 0.005 M-arginine and 0.06 M-ornithine as effectors. Arginase (EC 3.5.3.1) was assayed as described by Messenguy *et al.* (1971) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and citrate synthase (EC 4.1.3.7) as described by Urrestarazu *et al.* (1977). Protein concentrations were estimated by the Lowry method with bovine serum albumin as a standard, except that the protein concentrations of chromatography column fractions were estimated by their absorbance at 280 nm. Ethanol was measured as described in Bonnicksen (1963).

Enzyme subcellular localization. We used the technique of stepwise homogenization of spheroplast lysates described by Ryan *et al.* (1973). For this purpose *Schiz. japonicus* var. *versatilis* was grown aerobically in

ammonia medium supplemented with 1% (w/v) yeast extract, 0.001% (w/v) ergosterol and 0.1 M-Tween 80. An isotonic mannitol solution (1.8 M) was added to 25 g fresh yeast (harvested in the exponential phase of growth) to a volume of 35 ml in the presence of 174 mg Zymolase 5000 (Kirin Brewery, Tokyo, Japan) and 4 ml Helicase (I.B.F., Gennevilliers, France). Incubation was at 30 °C for 120 min with continuous shaking. Spheroplasts were then separated by centrifugation in several aliquots and the pellets were processed separately as indicated in the legend to Fig. 6 (for details, see Urrestarazu *et al.*, 1977). Glucose-6-phosphate dehydrogenase and citrate synthase were used as cytosolic and mitochondrial markers, respectively.

RESULTS AND DISCUSSION

Epiarginasic regulation in situ

Epiarginasic regulation was initially discovered by the abolition of OTCase activity when arginine was added to cells growing with ammonia as the sole nitrogen nutrient, OTCase activity being measured in nystatin-permeabilized cells (Béchet & Wiame, 1965). However, further investigations, including the present study, have disclosed two main pitfalls.

First, all yeasts were able to synthesize arginase, which in virtually all cases was induced to a higher level by growth on arginine, but they reacted differently when arginine and ammonia were present in the medium. The main variability lay in their sensitivity to the ammonia effect, even in strains of *Sacch. cerevisiae* which had the potentiality to develop epiarginasic regulation. The presence of ammonia may inhibit arginase synthesis either directly or by inhibiting transport of arginine. A typical ammonia-insensitive strain is strain 1705d in which the phenomenon was discovered; with our usual laboratory strain, Σ 1278b (α), the ammonia effect was strong and the *in situ* epiarginasic reaction was weak (Dubois *et al.*, 1974; Rytka, 1975). Both effects of ammonia may be avoided by shifting cells from ammonia to arginine as the only nitrogen nutrient.

The second pitfall resulted from a difference in permeabilization by nystatin. Some species belonging to genera other than *Saccharomyces* were rather insensitive to nystatin. Triton X-100 could be used in such circumstances; this was the case for *Schizosaccharomyces* species. An unexpected but rare difficulty occurred with *Schiz. japonicus*. In this yeast, addition of arginine to the medium reduced the capacity of the cells to be permeabilized. This capacity was also influenced by aeration, which had other unfavourable effects on this yeast (Wickerham & Duprat, 1945; Bulder, 1963). The lack of permeabilization could be detected by the absence of any enzyme activities after treatment of the cells, as well as by the absence of any leakage of internal amino acids.

Unless stated otherwise, all results (Table 2) are expressed as differential rates, i.e. activity per ml culture versus mass per ml culture, in cultures growing exponentially (Monod *et al.*, 1952).

All species tested belonging to the genus *Saccharomyces* (Lodder, 1970) had a strong positive reaction (inhibition of OTCase activity by arginine). The species tested were *Sacch. bayanus*, *Sacch. bayanus ex oviformis*, *Sacch. bayanus ex pastorianus*, *Sacch. cerevisiae ex turbidans*, *Sacch. chevalieri*, *Sacch. delbrueckii*, *Sacch. diastaticus*, *Sacch. fermentati*, *Sacch. kloederianus*, *Sacch. italicus* and *Sacch. rosei* (Wiame, 1971, 1975). Only *Sacch. cerevisiae* is considered further. Apart from the *Saccharomyces* species, *Saccharomycodes ludwigii*, *Torulopsis colliculosa*, *Kluyveromyces fragilis*, *K. lactis* and *Hanseniaspora valbyensis* (Fig. 1a) gave a positive result *in situ*.

Typical negative results were obtained for many yeasts such as *Candida utilis* (Fig. 1b) and *Schiz. pombe*. Negative results could not be attributed to lack of arginase synthesis (see Table 3): although positive-reacting species were always arginase-rich, some very arginase-rich cells were negative, e.g. *Torulopsis candida*.

Epiarginasic regulation in vitro

A more direct but more laborious test for epiarginasic regulation can be performed by mixing the enzymes (Messenguy & Wiame, 1969). Such *in vitro* tests allowed investigation of

Table 2. Fermentation capacity in aerobic and in anaerobic conditions of growth, epiarginasic regulation and catalytic properties of the OTCases in different yeast species

Species					Epiarginasic regulation			
	OTCase		Fermentation*		<i>In situ</i>	<i>In vitro</i> †		
	K_m for ornithine (mM)	Inhibition by excess ornithine‡	Without oxygen	With oxygen		Test OTCase/ Sacch. arginase (B)	Test OTCase/ test arginase (A)	Sacch. OTCase/ test arginase (C)
OBLIGATE AEROBES								
<i>Cr. neoformans</i>	1.3	++	.	0	—	.	.	.
<i>Deb. hansenii</i> 767	5.1	trace	.	0	—	—	—	—
<i>Deb. hansenii</i> 770	5.3	—	.	0	—	.	.	.
<i>Deb. hansenii</i> 792	6.4	weak	.	0	—	.	.	.
<i>Deb. hansenii</i> 811	3.8	—	.	0	—	.	.	.
<i>Deb marama</i>	17	—	.	0	—	.	.	.
<i>H. wingei</i>	3.4	trace	.	1	—	.	.	.
<i>P. fluxuum</i>	0.9	weak	.	0	—	.	.	.
<i>T. candida</i>	2.1	++	.	0	—	—	—	—
FACULTATIVE ANAEROBES WITH STRONG PASTEUR EFFECT								
<i>C. tropicalis</i>	1	+	27	0	—	—	—	—
<i>C. utilis</i>	1	+	23	1	—	—	—	weak
<i>Deb. phaffii</i>	3.6	—	58	0.5	—	.	.	.
<i>H'spora valbyensis</i>	2.2	++	42	0.5	+	—	weak	—
<i>H. anomala</i> L-5-6	3.3	trace	28	0	—	—	—	+
<i>H. anomala</i> diploid	3.7	trace	19	4	—	—	—	+
<i>K. fragilis</i>	0.7	++	27	0.3	+	—	+	+
<i>K. lactis</i>	0.6	+	27	2	+	+	+	+
<i>P. fermentans</i>	0.7	weak	27	0.1	—	.	.	.
<i>P. dispersa</i>	3.8	+	51	7	—	.	.	.
FACULTATIVE ANAEROBES WITH WEAK PASTEUR EFFECT								
<i>Br. lambicus</i>	0.7	weak	15	12	—	—	—	—
<i>Schiz. japonicus</i> var. <i>jap.</i>	5.4	+	69	75	—§	—	—	—
<i>Schiz. japonicus</i> var. <i>vers.</i>	6.2	+	60	74	—§	—	—	—
<i>Schiz. malidevorans</i>	7.7	weak	52	46	—	.	.	.
<i>Schiz. octosporus</i> L-0-6	.	.	.	.	—	.	.	.
<i>Schiz. octosporus</i> 371	.	.	.	.	—	.	.	.
<i>Schiz. pombe</i>	1.6	weak	42	31	—	—	—	—
<i>Sacch. cerevisiae</i> ¶	3	+	47	34	+	+	.	.
<i>Sacch. fermentati</i> 818	4.5	+	33	13	+	.	.	.
<i>Sacch. fermentati</i> 813	1.6	+	.	.	.	+	+	+
<i>Sacch. kloeckerianus</i>	3.3	+	33	9	+	+	+	+
<i>S'codes ludwigii</i>	1.3	++	68	20	+	+	+	+
<i>T. colliculosa</i>	8.3	+	51	22	+	+	+	+

* Expressed as the ethanol concentration excreted into the medium when the cultures had reached an A_{660} of 0.5 measured with the Beckman B spectrophotometer, corresponding to 0.265 mg protein ml⁻¹ with *Sacch. cerevisiae* as reference.

† 'Test' denotes OTCase or arginase of the yeast under test; 'Sacch.' denotes OTCase or arginase of *Sacch. cerevisiae*. A, B and C correspond to the interactions shown in Fig. 2.

‡ +, Inhibition comparable to *Sacch. cerevisiae*; ++, inhibition more intense than *Sacch. cerevisiae*; weak, inhibition present for ornithine at concentrations ≥ 10 mM.

§ See text for a description of the exceptional behaviour of these yeasts in the *in situ* assay.

|| Growth of *Schiz. octosporus* was very poor on yeast extract medium under anaerobic conditions. In mineral medium there was no growth aerobically or anaerobically; thus the placing of this yeast among the facultative anaerobes is tentative.

¶ Other species of *Saccharomyces* have been considered previously (see text). All showed epiarginasic regulation.

Fig. 1. *In situ* inhibition of OTCase from (a) *Hanseniaspora valbyensis* and (b) *Candida utilis*. The arrow indicates the stage of growth at which cells were shifted from ammonia (○) to arginine (●) medium. Samples of cells were permeabilized with nystatin. Activity is expressed in μmol citrulline formed h^{-1} (ml culture) $^{-1}$. Turbidity was measured with a Beckman B spectrophotometer.

Fig. 2. Designation of cross-reactions between OTCase and arginase of different yeasts and the enzymes of *Sacch. cerevisiae*.

intergeneric or interspecies interactions between the two enzymes (cross-reaction). A partial purification was carried out to separate arginase from contaminating OTCase (see Methods). In the present work cross-reactions were limited to different yeasts in combination with *Sacch. cerevisiae* (Fig. 2). The following categories of results can be defined.

(1) *Full positive couples*. Interactions A, B and C (Fig. 2) occurred, i.e. arginase and OTCase of the tested yeast both exchanged with the *Sacch. cerevisiae* enzymes without qualitative modifications. This was the case for all the *Saccharomyces* species tested. It was also the case for most other yeasts giving a positive result *in situ* (Table 2). Apart from the *Saccharomyces* species, it occurred in *K. lactis* (Fig. 3), *S'codes ludwigii* and *T. colliculosa*.

(2) *Full negative couples*. Interactions A, B and C did not occur. This was the case for most yeasts giving negative results *in situ*. Examples were *Deb. hansenii*, *T. candida*, *C. tropicalis*, *Br. lambicus*, *Schiz. pombe* (Fig. 3) and *Schiz. japonicus* var. *versatilis*.

(3) *Positive specific*. Cross-reactions did not occur (B and C did not occur). The only example found was *H'spora valbyensis* (Fig. 1a, *in situ*; and Fig. 3, *in vitro*). In spite of a weak interaction *in vitro*, the test *in situ* was clearly positive.

(4) *Positive semi-specific*. (4-1) Interaction B did not occur. For example, *K. fragilis* OTCase was insensitive to *Sacch. cerevisiae* arginase, in contrast to the fully positive *K. lactis*. (4-2) No case of interaction C not occurring was found when interaction A occurred.

(5) *Negative for its own enzyme(s) but positive for Sacch. cerevisiae enzyme(s)*. Interaction A did not occur but either interaction B or C did occur. (5-1) *Hansenula anomala* OTCase was insensitive to its own arginase as well as to that of *Sacch. cerevisiae*, but the arginase of *H. anomala* strongly inhibited *Sacch. cerevisiae* OTCase (Fig. 3). Such a reaction did not occur in *H. wingei*, an obligate aerobe. Note that *H. anomala* OTCase is mitochondrial and

Fig. 3. Representative *in vitro* determinations of inhibition of OTCase from different yeasts: cross-reactions between a given yeast OTCase (a) or *Sacch. cerevisiae* OTCase (b) and the arginases from the yeast tested (○) and from *Sacch. cerevisiae* (●). Enzyme activities are expressed in μmol product formed h^{-1} per assay.

would thus be unable to react with its cytosolic arginase (Urrestarazu *et al.*, 1977). (5-2) No case of an OTCase insensitive to its own arginase but sensitive to *Sacch. cerevisiae* arginase was found.

As proposed by Price *et al.* (1978), epiarginasic regulation may be a useful tool for the study of relationships between yeasts. However, too few cases have been analysed to establish

Fig. 4. Double reciprocal plots for ornithine saturation of ornithine carbamoyltransferase in Tris/HCl buffer (0.1 M, pH 8.6) at 15 °C for (a) *Debaryomyces marama* and (b) *Cryptococcus neoformans*.

a definite rule. Usually the species belonging to the same genus interacted similarly, except for *K. lactis* and *K. fragilis*. In other cases, differences paralleled strong biochemical distinctions. The exception of the two *Torulopsis* species is probably an apparent one. Indeed, these yeasts are Fungi Imperfecti and Van Uden & Vidal-Luria (in Lodder, 1970) consider *T. colliculosa* (a full positive species) as the imperfect form of *Sacch. fermentati* (a full positive species), while *T. candida* (a full negative species) is considered as the imperfect form of *Deb. hansenii* or *Deb. marama* (both full negative species). The present study would favour the separation of the two *Torulopsis* species. The difference observed between *H. wingei* and *H. anomala* is worthy of some additional consideration, justified by their additional difference in fermentative capacity. Yeasts which were neither fully positive nor fully negative are the most promising for the refinement of phylogeny or for investigating the fine molecular structure involved in epiarginasic regulation; this is the case for *Kluyveromyces*, *Hansenula* and *Hanseniaspora*. Among these, *H. anomala* (class 5-1) is of particular interest. Its arginase did not react with its own OTCase, which is mitochondrial, but it did react with the cytosolic OTCase of a distantly related organism. This raises the possibility that the difference between the two OTCases is linked with the capacity of OTCase to migrate into the mitochondria.

OTCase interaction with L-ornithine: K_m values and inhibition

Saccharomyces cerevisiae OTCase is partially inhibited by L-ornithine at concentrations three times higher than the K_m value for this substrate (Messenguy & Wiame, 1969). The site for inhibition is distinct from the catalytic site and can be lost by mutation, heating or chemical alteration, with the simultaneous loss of the ability to be inhibited by arginase. Hence partial inhibition of OTCase by ornithine has been proposed to be correlated with epiarginasic regulation (Messenguy *et al.*, 1971). In the present work, we tested various yeast species to determine whether sensitivity to inhibition by arginase was always correlated with sensitivity to inhibition by ornithine at high concentrations (Table 2). The K_m values of different OTCases for ornithine varied from 0.6 to 17 mM, in most cases being from 1–5 mM, i.e. close to the value observed for *Sacch. cerevisiae* (3 mM). Inhibition by excess ornithine was highly variable. Some OTCases showed little or no inhibition, e.g. those of *H. wingei*, *Deb. hansenii*, *Deb. phaffii*, *Deb. marama* (Fig. 4a) and *H. anomala*. Inhibition comparable to *Sacch. cerevisiae* was common. More intense inhibition than *Sacch. cerevisiae* was unusual. There was no relationship between inhibition and K_m value. *Saccharomyces cerevisiae*, *Deb. phaffii* and *T. candida* had similar K_m values but their sensitivity to excess ornithine was intermediate, nil and high, respectively. However, correlation with sensitivity to arginase was absolute: all OTCases sensitive to arginase showed a marked inhibition by ornithine. The reverse was not true: some OTCases were insensitive to arginase but were inhibited by ornithine. This was the case for *C. tropicalis*, *T. candida* and *Cr. neoformans*.

Fig. 5. Effect of aeration on the fermentation capacity of (a) *Kluyveromyces fragilis* and (b) *Schizosaccharomyces pombe*. Cells were grown aerobically (○) and anaerobically (●) in ammonium medium with 3% (w/v) glucose, and the differential rate of ethanol excretion into the medium [expressed as $\mu\text{mol (ml culture)}^{-1}$] was determined. Turbidity was measured with a Beckman B spectrophotometer.

(Fig. 4b), the last two being strongly inhibited by ornithine. Thus, it appears that inhibition by excess ornithine is necessary but not sufficient for the expression of epiarginasic regulation.

Correlation with oxygen interaction

The interaction of oxygen with yeasts is not limited to energy production. Severe anaerobiosis may impair growth in some fermentative yeasts. Some oxygen is required for lipid synthesis in *Sacch. cerevisiae*. Yeasts in which ethanol production is reduced by oxygen by more than 75% have been placed in the class of yeasts with a strong Pasteur effect. *Kluyveromyces fragilis*, *K. lactis* and *Schiz. pombe* grow well in usual anaerobic conditions but not in strict anaerobic conditions (Subik *et al.*, 1974). As our purpose was to evaluate the reliability of the correlation of the origin of energy production and its regulation with epiarginasic regulation, we did not consider *Kluyveromyces* and similar species as obligate aerobes.

In Table 2, yeasts have been classified as obligate aerobes, facultative anaerobes with a strong Pasteur effect, and facultative anaerobes with a weak Pasteur effect. The Pasteur effect was measured by comparing ethanol production in yeasts growing either in a nitrogen-flushed atmosphere or in air (see Methods). We have confirmed the findings of Subik *et al.* (1974) concerning the strict anaerobic growth of *Schiz. japonicus* var. *versatilis* and the less strict growth patterns of *Schiz. pombe* and *K. lactis*. Table 2 shows that the need for a small amount of oxygen is not related to the Pasteur effect. The occurrence of the Pasteur effect reported in Table 2 confirms and extends previous results for *Sacch. cerevisiae* (Swanson & Clifton, 1948; Lemoigne *et al.*, 1954; Ephrussi *et al.*, 1956) and other yeasts (Lafon, 1956; De Deken, 1966). Figure 5 illustrates the presence and the absence of a Pasteur effect.

Clearly the correlation between energy production from respiration and the absence of epiarginasic regulation is followed in many cases but is not complete. One needs to look critically at the exceptions as well as confirmed examples. Was the correlation only apparent and a result of a limited selection of yeasts? Would it be possible to rationalize the exceptions in relation to other properties? Among the obligate aerobes epiarginasic regulation was always absent. This seems well established, especially since we chose representative species from genera which also contained species capable of fermentation, such as *Debaryomyces* and *Pichia*. In addition, some of these epiarginasic negative yeasts had an OTCase strongly inhibited by ornithine, a property which appeared to be required for this regulation.

On the other hand, some fermentative yeasts which did not show the Pasteur effect did not show epiarginasic regulation. *Brettanomyces* had no Pasteur effect but its anaerobic growth was very slow and, in addition, this genus is known to have a peculiar metabolism. Oxygen is

Fig. 6. Determination of the subcellular localization of OTCase and arginase in *Schiz. japonicus* var. *versatilis* by stepwise homogenization of spheroplasts. Enzyme activities were determined in the supernatant of each lysate obtained after 20 min centrifugation at 39 000 g. Each pellet corresponds to about 5 g wet wt cells. Enzymes: OTCase (○); arginase (●); glucose-6-phosphate dehydrogenase (□); citrate synthase (■).

Step 1: the spheroplast pellet was suspended in Tris/HCl buffer (0.05 M, pH 7.4) containing 1 mM-MgEDTA, 0.1% bovine serum albumin and 1 M-mannitol (instead of 1.8 M normally used for protection of spheroplasts). The suspension was homogenized by shaking the tube manually for 1 min and then using a Potter homogenizer for 5 min.

Step 2: same treatment as in step 1 except that the mannitol concentration was 0.6 M.

Step 3: suspension in buffer containing 0.3 M-mannitol; manual homogenization for 2 min, then the mannitol concentration was adjusted to 1 M and homogenization was carried out with a Potter homogenizer for 2 min.

Step 4: suspension in buffer only; 2 min manual homogenization and then the mannitol concentration was adjusted to 1 M.

Step 5: twofold passage of the spheroplast pellet through a French press – 100% activity corresponds to the activity recovered in this supernatant.

involved in some acid production (Custers, 1940). Thus, in this yeast oxygen affects the metabolism by a means other than the usual Pasteur effect.

A puzzling exception was *Schiz. pombe*. Although the Pasteur effect was absent, no epiarginasic regulation was found. This one instance could have been considered minor since *Schiz. pombe* needs at least some oxygen for growth. However, when we investigated other members of the group of fission yeasts, we found that all lacked the epiarginasic effect. *Schizosaccharomyces japonicus* was the most interesting example. Oxygen has adverse effects on its growth and sporulation (Wickerham & Duprat, 1945) and cytochromes *b* and *c* are absent (Bulder, 1963). Its generation time in anaerobic conditions was 3.5 h, whereas active oxygenation led to a generation time of 8 h. In addition, its OTCase was found to be a mitochondrial enzyme (Fig. 6) when tested by the technique of stepwise lysis of spheroplasts (Ryan *et al.*, 1973) previously applied to the study of other yeasts (Urrestarazu *et al.*, 1977). Thus, we conclude that the *Schizosaccharomyces* group as a whole is a well established exception to the correlation between the absence of the Pasteur effect and epiarginasic regulation. It may be important in this connection that fission yeasts and budding yeasts have quite separate taxonomic relationships. One may propose that fission yeasts, which do not show epiarginasic regulation, retain OTCase in the mitochondria. In the case of *Schiz. japonicus* var. *versatilis*, many functions normally associated with mitochondria are absent but OTCase has been located in this organelle (Fig. 6). This last result establishes the independence of diverse mitochondrial functions.

In fermentative yeasts with a strong Pasteur effect, the presence of epiarginasic regulation was variable. However, here again, taxonomic relationships were obvious. *Kluyveromyces* species, which were found to be positive for epiarginasic regulation, are taxonomically related to the *Saccharomyces*, a strong positive genus. Other species in the group which showed a

Table 3. *Specific activity for OTCase and arginase in different yeast species after growth aerobically at 29 °C with ammonia, ammonia plus arginine or arginine as nitrogen nutrient*
Specific activity [$\mu\text{mol formed h}^{-1} (\text{mg protein})^{-1}$] at 30 °C

Species	OTCase			Arginase			Epiarginasic regulation
	Ammonia + Arginine		Arginine	Ammonia + Arginine		Arginine	
	Ammonia	arginine		Ammonia	arginine		
OBLIGATE AEROBES							
<i>Cr. neoformans</i>	19	16	13	9	9	17	—
<i>Deb. hansenii</i> 767	7	3	2.6	7	70	50	—
<i>Deb. hansenii</i> 770	4	1	1	3	30	49	—
<i>Deb. hansenii</i> 792	5	2	2.7	51	39	41	—
<i>Deb. hansenii</i> 811	13	3	2.7	3	19	63	—
<i>Deb. marama</i>	3	0.6	0.6	3	5	17	—
<i>H. wingei</i>	9	13	11	15	62	225	—
<i>P. fluxuum</i>	13	2	0.5	8	74	79	—
<i>T. candida</i>	10	2	1	12	176	670	—
FACULTATIVE ANAEROBES WITH STRONG PASTEUR EFFECT							
<i>C. tropicalis</i>	9	0.6	0.5	2	148	213	—
<i>C. utilis</i>	13	6	7	1	7	247	—
<i>Deb. phaffii</i>	2	1	0.2	3	84	108	—
<i>H'spora valbyensis</i>	14	3	0.3	3	51	262	+
<i>H. anomala</i> L-5-6	9	10	11	12	23	218	—
<i>H. anomala</i> diploid	7	8	8	10	17	243	—
<i>K. fragilis</i>	30	12	2	8	82	372	+
<i>K. lactis</i>	25	10	4	6	42	296	+
<i>P. fermentans</i>	6	1.3	0.8	6	45	149	—
<i>P. dispersa</i>	13	20	14	13	171	294	—
FACULTATIVE ANAEROBES WITH WEAK PASTEUR EFFECT							
<i>Br. lambicus</i>	35	12	1	0.3	1	127	—
<i>Schiz. japonicus</i> var. <i>jap.</i>	18	4	3	67	269	465	—
<i>Schiz. japonicus</i> var. <i>vers.</i>	24	3	2	79	240	213	—
<i>Schiz. pombe</i>	9	6	6	33	92	379	—
<i>Sacch. cerevisiae</i>	30	2	1	6	20	291	+
<i>Sacch. fermentati</i> 818	25	13	11	27	104	370	+
<i>Sacch. kloedenianus</i>	19	15	7	10	25	270	+
<i>S'codes ludwigii</i>	22	10	6	4	39	240	+
<i>T. colliculosa</i>	28	1.5	1.5	12	198	290	+

strong Pasteur effect but no epiarginasic regulation belong to genera which include species that are obligate aerobes and are negative for this regulation.

Regulation of arginase and OTCase synthesis

As the purpose of epiarginasic regulation appears to be to avoid a futile cycle arising from the simultaneous presence of a catabolic enzyme (arginase) and an anabolic enzyme (OTCase), we have investigated the classical regulation of the synthesis of these enzymes. Results are reported in Table 3. Most yeasts showed the classical repression of OTCase and induction of arginase when arginine was added to minimal (ammonia) medium. In addition, they were subject to nitrogen catabolite repression, as judged by the increase in arginase synthesis when arginine was the only source of nitrogen compared with ammonia plus arginine as nitrogen nutrient. In this respect, the behaviour was similar to that of *Sacch. cerevisiae* (Béchet *et al.*, 1970; Dubois *et al.*, 1974). All epiarginasic positive yeasts showed strong arginase regulation.

In some yeasts, e.g. *Cryptococcus*, *Debaryomyces*, regulation was very poor; this was most common in obligate aerobes. From Table 3, it is clear that many species that lack the classical

repression-induction mechanisms have not developed the epiarginasic regulation in compensation. On the contrary, poor regulation of synthesis was more common in obligate aerobes. In yeasts which lack classical regulation, compartmentation of OTCase could again be the basis for anabolism-catabolism exclusion.

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