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Isolation, characterization and transformation, by autonomous replication, of *Mucor circinelloides* OMPdecase-deficient mutants

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Abstract Pyrimidine auxotrophs of *Mucor circinelloides* were isolated after mutagenesis with nitrosoguanidine and selected for resistance to 5-fluoroorotate. These mutants were genetically and biochemically characterized and found to be deficient either in orotidine-5′-monophosphate decarboxylase (OMPdecase) activity or in orotate phosphoribosyl transferase (OPRTase) activity. Different circular DNA molecules containing the homologous *pyrG* gene were used to transform a representative OMPdecase–deficient strain to uracil prototrophy. Southern analysis, as well as mitotic stability analysis of the transformants, showed that the transforming DNA is always maintained extrachromosomally. The smallest fragment tested that retained both the capacity to complement the *pyrG4* mutation and the ability to be maintained extrachromosomally when cloned in a suitable vector is a 1.85 kb *M. circinelloides* genomic DNA fragment. This fragment consists of the *pyrG* coding region flanked by 606 nucleotides at the 5′ and 330 nucleotides at the 3′ ends, respectively. Sequence analysis reveals that it does not share any element in common with another *M. circinelloides* genomic DNA fragment which also promotes autonomous replication in this organism, except those related to transcription. Furthermore, it differs from elements which have been shown to be involved in autonomous replication in other fungal systems. An equivalent plasmid harbouring the heterologous *Phycomyces blakesleeanus pyrG* gene yielded lower transformation rates, but the transforming DNA was also maintained extrachromosomally. Our results suggest that autonomous replication in *M. circinelloides* may be driven by elements normally present in nuclear coding genes.

Key words *Mucor circinelloides* · *pyrG* gene · OMPdecase · Transformation · Autonomous replication

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Introduction

Members of the genus *Mucor* are being used in both basic and applied areas of biological research (for a review see Orlowski 1991). The genetics of *M. circinelloides* is poorly characterized, but the successful application of molecular techniques compensates in part for the lack of sexual genetic analysis in this fungus. The high transformation efficiencies achieved and the preference for the autonomous maintenance of the transforming DNA have allowed the isolation of two nuclear genes of the fungus by direct cloning (Van Heeswijck and Roncero 1984; Anaya and Roncero 1991). The *leuA* gene was isolated within a 4.4 kb *Pst*I genomic DNA fragment and used as a selectable marker in transformation experiments. Since this fragment is maintained extrachromosomally in the transformants when self-ligated, and confers this capacity on different plasmids when cloned in them, it has been proposed that a homologous autonomously replicating sequence (ARS) is linked to the structural *leuA* gene (Van Heeswijck 1986; Roncero et al. 1989). General application of this transformation system is limited by the difficulty of finding mutant strains specifically affected in the *leuA* gene. This limitation could be overcome by using the positive selection system based on the use of 5-fluoroorotic acid (5-FOA), initially described in *Saccharomyces cerevisiae* (Boeke et al. 1984), and

successfully applied to a number of fungi. This procedure allows the isolation of mutants altered either in the gene encoding orotidine-5′-monophosphate decarboxylase (OMPdecase) or in the gene encoding orotate phosphoribosyl transferase (OPRTase), making them available as recipient strains in the establishment of efficient transformation systems (Razanamparany and Bèqueret 1986; Van Hartingsveldt et al. 1987; Mattern et al. 1987; Oakley et al. 1987; Díez et al. 1987; Goosen et al. 1987; Ruiter-Jacobs et al. 1989; Gruber et al. 1990; Bergès and Barreau 1991; Smit and Tudzynski 1992). Aiming at this final goal, we have previously reported the cloning of the *M. circinelloides pyrG* gene and its use in preliminary transformation experiments (Benito et al. 1992).

In fungi, transformation occurs mainly by integration of the transforming DNA into the recipient's genome. Attempts to isolate functional elements promoting autonomous replication have been made in various fungal systems, but with very limited success (Fincham 1989). In *S. cerevisiae*, replicative vectors have been developed based upon the 2 μm DNA plasmid origin of replication (ori) or upon ARS elements from the genome of *S. cerevisiae* (Stinchcomb et al. 1979; Chan and Tye 1980). In the ascomycetous yeasts *Schizosaccharomyces pombe* and *Kluyveromyces lactis*, ARS elements have also been reported (Das and Hollenberg 1982; Sakai et al. 1984). Among filamentous Ascomycetes and Basidiomycetes there are only a few well documented examples of transformation by autonomous replication mediated by homologous ARS elements. In *Ustilago maydis*, a genetic element, UARS1, has been characterized, that promotes high frequency transformation and confers autonomous replication capacity on the plasmids which harbour it (Tsukuda et al. 1988). An element showing similar capacities, designated AMA1, has been isolated in *Aspergillus nidulans* (Gems et al. 1991). Recently, a genomic DNA fragment involved in replicative transformation has been isolated from a plasmid generated through in vivo recombination in the basidiomycete *Pleurotus ostreatus* (Peng et al. 1993). This fragment has been shown to contain a number of general features characteristic of replicons.

Although integrative transformation has been reported in members of the class Zygomycetes (Arnau et al. 1988; 1991; Burmester et al. 1990; Arnau and Strøman 1993), autonomous replication appears to be the preferential mode of transformation in these fungi. In *Phycomyces blakesleeanus* autonomous replication seems to be driven by elements selected in *S. cerevisiae* (Suárez 1985; Revuelta and Jayaram 1986; Suárez and Eslava 1988). In *Absidia glauca* vectors capable of being maintained extrachromosomally have been described (Wöstemeyer et al. 1987), and an element of chromosomal origin, SEG1, has been shown to promote stable mitotic segregation of plasmids (Burmester et al. 1992). In *M. circinelloides* experimental evidence suggests the presence of ARS elements associated with structural genes, since the two genomic DNA fragments isolated by direct complementation confer the ability to be maintained as autonomous elements on those plasmids in which they are cloned (Roncero et al. 1989; Anaya and Roncero 1991). The nature of these elements is unknown at present. The analysis of further homologous transformation systems in this organism, and comparison of the sequences of the elements involved, will probably give more information about the factors that promote autonomous replication of plasmids in *M. circinelloides*.

In this paper we present the isolation of *M. circinelloides* pyrimidine auxotrophs and their genetic and biochemical characterization. We also describe the transformation by autonomous replication of a *M. circinelloides* OMPdecase-deficient strain with plasmids harbouring either the homologous marker or the *P. blakesleeanus pyrG* gene.

Materials and methods

Strains and growth conditions

Escherichia coli strain DH5α [F^- *endA1*, *hsdR17* (r_k^- m_k^-), *supE44*, *thi-1*, λ^-, *recA1*, *gyrA96*, *relA1*] was used for all the cloning experiments and for propagation of plasmids. It was grown under previously described conditions (Sambrook et al. 1989).

The *M. circinelloides* strains used in this work are listed in Table 1. The minimal medium (YNB) and rich medium (YPG) used for their growth have been previously described (Lasker and Borgia 1980). The minimal medium was supplemented with leucine (200 μg/ml) or uracil (400 μg/ml) as needed. When colonial growth was required, the pH of the medium was adjusted to pH 3.2.

Nitrosoguanidine mutagenesis

Vegetative spore suspensions (10^6 spores/ml) of *M. circinelloides* strain CBS 277.49 or strain R7B were treated with *N*-methyl-*N'*-nitro-N-nitrosoguanidine (NG) at a final concentration of 0.1 mg/ml following the procedures described by Eslava et al. (1975) for *P. blakesleeanus*. Aliquots of 10^6 NG-treated spores (survival ranged

Table 1 *Mucor circinelloides* strains used in this work. (*NG* N-methyl-N′-nitro-N-nitrosoguanidine)

Strain	Genotype	Origin
CBS 277.49	Wild type	Centraalbureau voor schimmelcultures, Baarn, The Netherlands
R7B	*leuA1*	CBS 277.49 by UV mutagenesis
MS4	*pyrG1*	CBS 277.49 by NG mutagenesis
MS5	*pyrG2*	CBS 277.49 by NG mutagenesis
MS6	*pyrF1*	CBS 277.49 by NG mutagenesis
MS12	*leuA1 pyrG4*	R7B by NG mutagenesis
MS13	*leuA1 pyrG5*	R7B by NG mutagenesis
MS14	*leuA1 pyrF2*	R7B by NG mutagenesis
MS15	*leuA1 pyrF3*	R7B by NG mutagenesis

from 5% to 20%) were spread on plates of the appropriate minimal medium for each strain, supplemented with 2.5 mg/ml 5-FOA and 400 μg/ml uracil. Spores were also spread on rich medium plates containing 400 μg/ml uracil, allowed to complete a full vegetative growth cycle and harvested as independent recycled spore pools. Aliquots of 5×10^5 recycled spores were plated as before on 5-FOA selection medium. Colonies appearing after 10 days of incubation at 28° C were tested for uracil requirement. True uracil auxotrophic mutants were purified from single spores.

Preparation, fusion and transformation of *Mucor* protoplasts

Protoplasts of *M. circinelloides* were prepared and fused as described by Van Heeswijck (1984). Transformation of protoplasts was performed essentially according to Van Heeswijck and Roncero (1984).

Preparation of cell-free extracts

Spores were inoculated at 10^5 spores/ml in liquid YNB medium supplemented with uracil and leucine. After 24 h incubation at 28° C in an orbital shaker (200 rpm) the mycelia were harvested by filtration and mixed with Ballotini glass beads of 0.5 mm diameter (Braun Melsungen) and 0.05 M TRIS-HCl pH 7.8 in a ratio of 5 ml per g of mycelium (wet weight), and then homogenized in a Braun MSK. The broken cells were centrifuged at $20000 \times g$ for 30 min and the supernatant was filtered through glasswool and stored at 4° C. The extract was used to measure enzyme activities and protein concentrations.

Enzyme assays

OMPdecase activity was measured according to the method described by Lieberman et al. (1955). One unit of activity is defined as the amount of protein which catalyzes the decarboxylation of 1 μmol of orotidylate to UMP per min under standard assay conditions. OPRTase activity was determined using the method described by Silva and Hatfield (1978). One unit of enzyme is defined as the amount of protein which catalyzes the conversion of 1 μmol of orotic acid to orotidine monophosphate per min under standard assay conditions. Protein concentration was determined according to Bradford (1976) using bovine serum albumin as standard.

Plasmids

The recombinant plasmids used in transformation experiments are shown in Fig. 2. Details of their construction are given in Results.

Standard recombinant DNA procedures

General procedures for plasmid DNA preparation, cloning and transformation in *E. coli* were according to Sambrook et al. (1989). DNA fragments for subcloning or labelling were recovered from agarose gels and purified using the Geneclean kit (BIO 101, La Jolla, Calif).

Electrophoresis procedures and transfer of DNA to nylon filters were performed as described by Sambrook et al. (1989). Labelling, hybridization and immunological detection were carried out using the non-radioactive labelling and immunological detection kit (Boehringer Mannheim) according to the conditions recommended by the manufacturer. All the hybridizations were performed under high stringency conditions (66° C).

Isolation of total DNA from *M. circinelloides* strains

Liquid cultures of the appropriate minimal medium were inoculated with fresh spore suspensions at a density of 10^5 spores/ml. Mycelia were harvested by filtration after 3 days incubation at 28° C on a rotary shaker (200 rpm), dried between paper towels and frozen in liquid N_2. Total DNA was prepared following the procedures described by Möller et al. (1992).

Mitotic stability analysis of *Mucor* transformants

Transformants were transferred from the transformation plates to fresh selective medium plates (YNB supplemented with leucine) and allowed to complete a full vegetative growth cycle. Spores were then harvested and the percentage retaining the *ura*$^+$ phenotype was estimated by parallel plating of appropriate dilutions onto selective (YNB supplemented with leucine) or non-selective (YPG) medium. At the same time a new vegetative growth cycle was initiated by plating spores from this pool onto selective and non-selective media. Spores from each of three successive vegetative growth cycles in both media were analyzed.

Results

Isolation of pyrimidine auxotrophs of *M. circinelloides*

Mutants of *M. circinelloides* strain CBS 277.49 and strain R7B resistant to 5-FOA were isolated after mutagenic treatment of vegetative spore suspensions with NG. No resistant colonies were found when mutagenized spores were plated directly on 5-FOA medium (5×10^6 spores tested). When spore suspensions (5×10^5 spores per plate) from the recycled spore pools were plated on the selection medium, 20–30 survivors per plate were obtained (a similar number of abortive colonies which did not grow after transfer to fresh selection medium was also found). Among these, 5% were pyrimidine auxotrophic mutants. To ensure that none of the mutants were represented more than once after the recycling, only one uracil auxotroph derived from each plate, and thus from each pool of recycled spores, was selected. A total of seven uracil auxotrophs, three derived from strain CBS 277.49 and four derived from strain R7B, were isolated and are listed in Table 1. All these strains showed reversion rates lower than 5×10^{-8}.

Genetic and biochemical characterization

Protoplasts from these seven uracil auxotrophs were prepared and fused in all possible pairwise combinations in order to determine the number of complementation groups. The fusion rates obtained in these experiments ranged from 0.002% to 0.3%. This complementation analysis, summarized in Fig. 1, indicates that there are two complementation groups among the uracil auxotrophic strains tested: strains MS4, MS5, MS12 and MS13 belong to one group, and strains

	MS5	MS6	MS12	MS13	MS14	MS15
MS4	–	+	–	–	+	+
MS5		+	–	–	+	+
MS6			+	+	–	–
MS12				–	+	+
MS13					+	+
MS14						–

Fig. 1 Complementation analysis of *Mucor circinelloides pyr*$^-$ strains. +, complementation (growth in minimal medium); –, failure to complement (no growth in minimal medium)

Table 2 Orotate phosphoribosyl transferase (*OPRTase*) and orotidine-5'-monophosphate decarboxylase (*OMPdecase*) activities of the R7B, MS12 and MS14 *M. circinelloides* strains

Strain	Specific activity (U/mg of protein)[a]	
	OPRTase	OMPdecase
R7B	0.047	0.028
MS12	0.054	0.000
MS14	0.005	0.023

[a] One unit of activity is defined as the amount of protein which transforms 1 μmol of substrate per min under standard assay conditions

MS6, MS14 and MS15 to another group. Strains MS12 and MS14 were chosen as representative of each complementation group for further analysis.

To identify the block in pyrimidine biosynthesis in the *pyr*$^-$ strains, whole cell extracts of strain R7B and a representative strain of each of the complementation groups found (strains MS12 and MS14) were prepared and assayed for OMPdecase and OPRTase activities. These results are shown in Table 2. Strain MS12 is deficient in the activity of the enzyme OMPdecase, carrying a mutation in the gene we called *pyrG*. Strain MS14 is deficient in the activity of the enzyme OPRTase, carrying a mutation in the gene we called *pyrF*.

Homologous transformation of *M. circinelloides* strain MS12

M. circinelloides strain MS12 was used as recipient in transformation experiments. In preliminary experiments this strain had been transformed to uracil prototrophy with plasmid pEPM11, which includes the homologous *pyrG* and *leuA* genes. The transforming DNA was maintained extrachromosomally in the transformants (Benito et al. 1992). Since the 4.4 kb *Pst*I *M. circinelloides* genomic DNA fragment cloned in this plasmid has been proposed to contain not only the *leuA* gene but also a homologous ARS, it was assumed that the autonomous replication capacity of plasmid pEPM11 resided on this homologous ARS. To test whether or not the 3.2 kb *Bam*HI genomic DNA fragment containing the *pyrG* gene itself conferred any autonomous replication capacity, this fragment was purified and used to transform *M. circinelloides* strain MS12 in either linearized or self-ligated form. Transformants were obtained only with the ligation mixture, although at low transformation efficiency (see Fig. 2). Total DNA from two different transformants obtained with the ligation mixture, L1 and L2, were analyzed by Southern hybridization using as probe the 3.2 kb *Bam*HI fragment (Fig. 3). Besides the resident genomic copy, two novel bands were detected in both transformants, L1 (lanes B and E) and L2 (lanes C and F). These two bands showed the same electrophoretic mobility when the DNA was undigested (lanes B and C) or digested with an enzyme which did not cut within the transforming DNA (lanes E and F). These results indi-

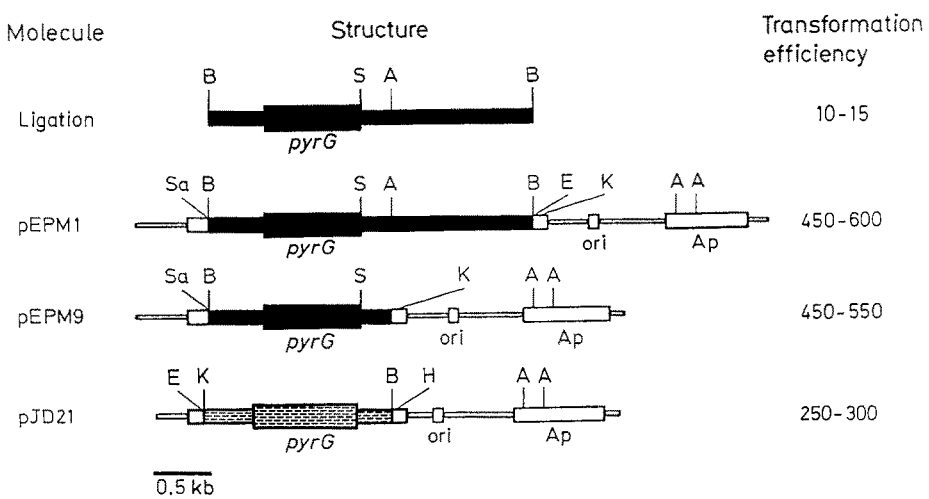

Fig. 2 Linear representation of the molecules used to transform *M. circinelloides* strain MS12. Transformation efficiencies achieved are expressed as the number of transformants per μg of DNA and 10^6 viable protoplasts (ranging values from three different transformation experiments). ■ *M. circinelloides* wild-type genomic DNA. ▨ *Phycomyces blakesleeanus* wild-type genomic DNA. □ Vector sequences (pBluescript KS + in plasmids pEPM1 and pEPM9 and pUC19 in plasmid pJD21). Abbreviations for restriction enzymes are: *A Ava*II, *B Bam*HI, *E Eco*RI, *H Hin*dIII, *K Kpn*I, *S Sal*I, *Sa Sac*I

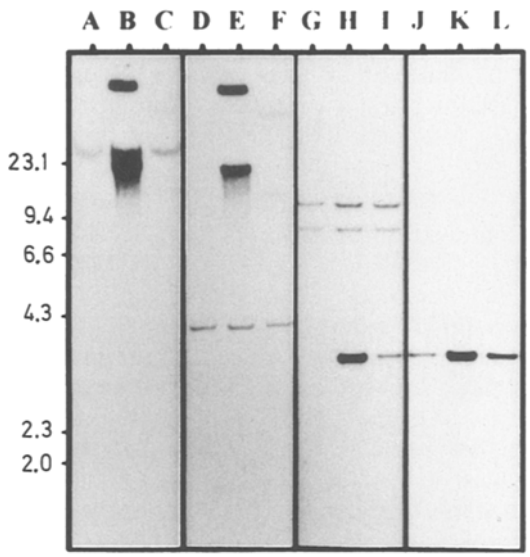

Fig. 3 Southern hybridization analysis of *M. circinelloides ura*$^+$ transformants obtained with the self-ligated 3.2 kb *Bam*HI fragment containing the *pyrG* gene. Total genomic DNA (5 μg) from the non-transformed recipient strain MS12 (lanes A, D, G and J) or from two different transformants obtained with the ligation mixture, L1 (lanes B, E, H and K) and L2 (lanes C, F, I and L) was electrophoresed in a 0.6% agarose gel and transferred to a nylon membrane either undigested (lanes A, B and C), or digested with *Eco*RI (lanes D, E and F), *Sal*I (lanes G, H and I) or *Bam*HI (lanes J, K and L). The membrane was probed with the 3.2 kb *Bam*HI fragment labelled with digoxigenin-dUTP. Numbers on the left correspond to positions of size markers in kb (*Hin*dIII-digested λ DNA)

cate the presence of extrachromosomally maintained circular DNA molecules in the transformants. Densitometric comparison of the hybridizing *Bam*HI fragments in the untransformed strain and in transformant L2 (lanes J and L, respectively) indicates the presence of one copy of the transforming molecule per haploid genome in transformant L2. A similar comparison of the two transformants, L1 and L2 (lanes K and L, respectively), shows that transformant L1 must contain six copies of the 3.2 kb monomer per haploid genome. These molecules could replicate as independent monomers or be assembled in a single hexamer (or several smaller multimers). If the replicative form is a multimer, all the components are arranged in the same orientation, as shown by the hybridization pattern obtained when DNA from transformants L1 and L2 was digested with *Sal*I (lanes H and I, respectively). The multimetric structure of the transforming DNA in transformant L1 is further supported by the reduced mobility of the undigested hybridizing molecules (lanes B and E), but it could not be determined by Southern analysis whether the transforming molecule is a single hexamer present in a low copy number or dimers or trimers present in higher copy numbers.

In order to establish an efficient transformation system in *M. circinelloides* based on the *pyrG* marker, the 3.2 kb *Bam*HI fragment was cloned in the *Bam*HI site of plasmid pBluescript KS + and the resulting plasmid, pEPM1, was used to transform *M. circinelloides* strain MS12. High transformation efficiencies were achieved (Fig. 2). Two different transformants obtained with this plasmid, M1-1 and M1-2, were analyzed by Southern hybridization, giving identical signals. The hybridization pattern obtained with genomic DNA from transformant M1-1, chosen as representative, is shown in Fig. 4. Two discrete hybridization bands were detected in the undigested genomic DNA from the transformant (Fig. 4A, lane b1) when the filter was probed with the digoxigenin-dUTP-labelled plasmid pBluescript KS + . These two bands were also detected when the DNA was digested with an enzyme which does not cut within plasmid pEPM1 (Fig. 4A, lane B1), indicating that the transforming DNA did not integrate into the recipient's genome. Digestion of DNA with an enzyme having a unique restriction site within plasmid pEPM1 resulted in the detection of one fragment (Fig. 4B, lane B1) of the same size as the linearized plasmid pEPM1 (Fig. 4B, lane B). This fragment was also detected when the last filter was probed with the digoxigenin-dUTP labelled 3.2 kb *Bam*HI genomic DNA fragment which includes the *pyrG* gene, together with the resident genomic copy of the probe used (Fig. 4C, lane B1). This observation confirmed that plasmid pEPM1 remained unaltered as extrachromosomally maintained circular molecules in the transformants.

Plasmid pEPM1 was digested with *Ava*II. The 3096 bp *Ava*II fragment was purified, treated with the Klenow fragment of DNA polymerase I and digested with *Bam*HI. The 1.85 kb *Bam*HI blunt-ended fragment which includes the *pyrG* gene was cloned in plasmid pBluescript KS + digested with *Bam*HI and *Hin*dII. With the resulting plasmid, pEPM9, the transformation efficiencies achieved were very similar to those obtained with pEPM1 (Fig. 2). Southern analysis performed with two pEPM9 derived transformants, M9-1 and M9-2, revealed the same behaviour for pEPM9 as for pEPM1. Both transformants gave the same hybridization pattern, but only results from M9-1 are shown (Fig. 4). Since both plasmids, pEPM1 and pEPM9, behave in the same way and have similar transformation efficiencies, it may be assumed that the elements which are needed not only for complementation of the *pyrG4* mutation but also for their extrachromosomal maintenance in the transformants, are located within the 1.85 kb *Bam*HI-*Ava*II *M. circinelloides* genomic DNA fragment.

Sequence analysis

The 1.85 kb *Bam*HI-*Ava*II fragment cloned in pEPM9 has been sequenced (Benito et al. 1992). Its sequence was compared with the sequence of the 4.4 kb *Pst*I *M.*

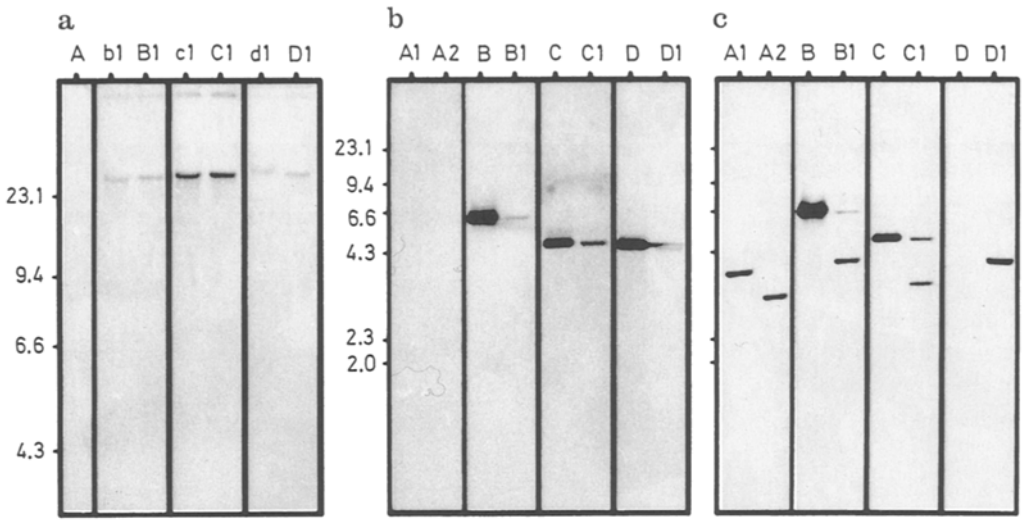

Fig. 4A–C Southern hybridization analysis of *M. circinelloides ura*$^+$ transformants obtained with plasmids pEPM1, pEPM9 and pJD21. DNA was electrophoresed in a 0.6% agarose gel (**A**) or in a 0.7% agarose gel (**B, C**), transferred to nylon membranes and probed with the *Eco*RI linearized plasmid pBluescript KS + labelled with digoxigenin-dUTP (**A, B**) or with the 3.2 kb *Bam*HI *M. circinelloides* genomic DNA fragment which contains the *pyrG* gene labelled with digoxigenin-dUTP (**C**). DNA samples in **A** were from the following sources: 5 µg untransformed strain MS12 (lane A), 5 µg transformant M1-1 (lanes b1 and B1), 5 µg transformant M9-1 (lanes c1 and C1) and 10 µg transformant D21-1 (lanes d1 and D1). DNA was undigested (lanes in *lowercase letters*) or digested with an enzyme which does not cut within the original transforming plasmid; *Sph*I in lanes B1 and C1 and *Cla*I in lane D1. In **B** and **C**, DNA samples were from the following sources: 5 µg untransformed strain MS12 (lanes A1 and A2), 3 ng plasmid pEPM1 (lane B), 5 µg transformant M1-1 (lane B1), 3 ng plasmid pEPM9 (lane C), 5 µg transformant M9-1 (lane C1), 3 ng plasmid pJD21 (lane D) and 10 µg transformant D21-1 (lane D1). DNA samples were digested with an enzyme which cuts once within the transforming plasmid in each case: *Eco*RI in lanes A1, B, B1, D and D1, and *Bam*HI in lanes A2, C and C1. Numbers on left of each panel correspond to positions of size markers in kb (*Hin*dIII-digested λ DNA)

circinelloides genomic DNA fragment which includes the *leuA* gene and promotes autonomous replication in *M. circinelloides*, focusing on the 222 bp *Bam*HI- *Ava*II fragment in the *leuA* promoter region where a homologous ARS element has been proposed to be located (Roncero et al. 1989). No significant sequence similarity was detected between these two sequences, except for the presence of a CAAT box in the promoter regions and the polyadenylation signal sequence in the terminator regions of the *pyrG* and *leuA* genes.

The sequence of the *pyrG* gene-containing fragment was also scanned for similarities with elements which have been shown to be involved in autonomous replication in other fungal systems. It does not include any AT-rich sequence resembling the consensus sequence (A/TTTTATA/GTTTA/T) for *S. cerevisiae* ARS elements (Williamson 1985). There are no obvious sequence similarities with the elements promoting autonomous replication isolated from *U. maydis* (Tsukuda et al. 1988) and *P. ostreatus* (Peng et al. 1993). These elements have been shown to include a number of putative active sequences, such as dispersed AT-rich and GC-rich sequences, direct and inverted repeats and hairpin-loop-forming stretches of high free energy value. Such features are not prominent in the *pyrG* gene-containing fragment.

Heterologous transformation of *M. circinelloides* strain MS12

To determine the behaviour of plasmids lacking homologous sequences, *M. circinelloides* strain MS12 was transformed with plasmid pJD21, which consists of a 1.95 kb *Sst*I- *Sal*I, Klenow-treated *P. blakesleeanus* genomic DNA fragment containing the *pyrG* gene cloned in the *Sma*I site of plasmid pUC19. Transformants were obtained, although the transformation efficiencies achieved were 50% lower than those obtained with pEPM1 or pEPM9 (see Fig. 2). Hybridization patterns obtained by Southern analysis show extrachromosomally maintained plasmids in the transformants (Fig. 4), although its copy number is lower than the plasmid copy number observed in transformants obtained with plasmids harbouring the homologous marker. Comparison of the intensity of the band representing the unaltered resident genomic copy of the probe used (Fig. 4C, lane D1) with that of the band representing the linearized transforming plasmid (Fig. 4B, lane D1; this band was also detected when the filter was probed with the *P. blakesleeanus pyrG* gene, data not shown) indicates that there are 0.25 copies of the transforming plasmid per haploid genome. This relationship is approximately 0.5 in transformant M1-1 and 1 in transformant M9-1 (Fig. 4C, lanes B1 and C1, respectively).

Mitotic stability of the transforming phenotype in the *ura*$^+$ transformants

In order to confirm the extrachromosomal maintenance of the transforming DNA, the mitotic stability of the transforming phenotype was studied. This was done by determining the percentage of spores retaining the *ura*$^+$ phenotype during three successive generations either in the presence or absence of selective pressure. The data presented in Fig. 5 show that in the absence of selective pressure, the transforming phenotype is rapidly lost in all the transformants: only in transformant L1 does a very small percentage (0.45%) of the third generation spores retain the *ura*$^+$ phenotype (Fig. 5B). Even under selective pressure, all the transformants showed a high level of mitotic instability, especially those transformants obtained with plasmid pJD21 (Fig. 5A).

Recovery of the transforming plasmid from *Mucor* transformants

Dilutions (100 ng –2 μg) of undigested total genomic DNA from *ura*$^+$ transformants were used to transform *E. coli* strain DH5α. Ampicillin-resistant colonies were obtained with DNA from transformants M1-1 and M1-2 at a frequency of 0.2–0.3 transformants per μg, and from transformants M9-1 and M9-2 at a frequency of 0.5–0.7 transformants per μg. Restriction analysis of plasmids recovered from *E. coli* transformants showed no difference from the original transforming plasmids. No ampicillin-resistant colonies were obtained with DNA from transformants D21-1 and D21-2. In these last two cases, the genomic DNA was digested with *Eco*RI, an enzyme for which a unique restriction site exists within plasmid pJD21, and religated and dilutions were used to transform *E. coli* DH5α. No ampicillin-resistant colonies were obtained under these conditions either.

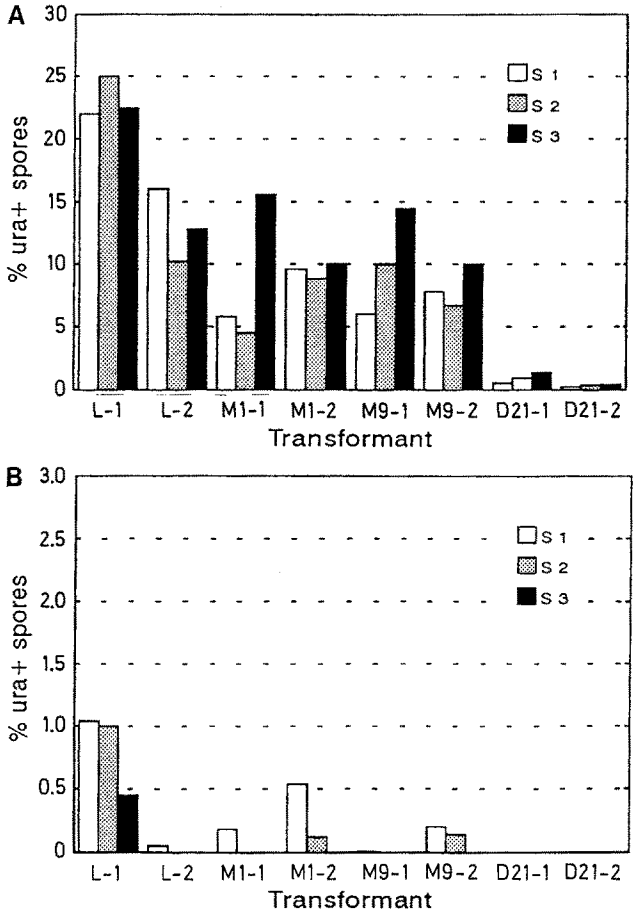

Fig. 5A, B Mitotic stability of the transforming phenotype of the *ura*$^+$ transformants when grown either in selective medium **A** or in non-selective medium **B**. S1, first growth cycle; S2, second growth cycle; S3, third growth cycle

Discussion

We have demonstrated that the 5-FOA based positive selection system for isolating mutants altered either in the gene encoding OPRTase or in the gene encoding OMPdecase is also functional in *M. circinelloides*. However, the procedure for this fungus requires that the mutagenized spore stock complete a full vegetative growth cycle before being exposed to 5-FOA. The nuclei carrying the 5-FOA resistance mutation are most likely to be present in heterokaryosis after mutagenesis, due to the multinucleate nature of the *M. circinelloides* spores. After a recycling process, some spores are homokaryotic for the mutated nuclei, thus allowing the expression of the resistance phenotype. Furthermore, by growing the mutagenized cells in the absence of 5-FOA, the existing pool of pyrimidine biosynthesis gene products is diluted out and the derived spores carrying the mutation for resistance against 5-FOA may be selected afterwards. Similar recycling processes have been indicated in some other fungal species (Goosen et al. 1987; Grimm et al. 1988; Campuzano et al. 1993).

Only 5% of the 5-FOA resistant strains were shown to be true uracil auxotrophic mutants. This proportion is similar to those found in the majority of fungi tested (Razanamparany and Bèqueret 1986; Van Hartingsveldt et al. 1987; Goosen et al. 1987; Díez et al. 1987; Bergés and Barreau 1991; Smit and Tudzynski 1992; Campuzano et al. 1993). The complementation analysis and the biochemical characterization of the uracil auxotrophs revealed two complementation groups: the first includes four strains which are defective in the *pyrG* gene, and the second includes three strains which are defective in the *pyrF* gene.

Transformation of strain MS12 to uracil prototrophy with different circular DNA molecules harbour-

ing either the homologous or heterologous (*P. blakesleeanus*) *pyrG* gene, confirms that this strain is indeed deficient in the activity of the enzyme OMPdecase. The transformation efficiencies varied greatly depending on the transforming DNA. It is interesting to note that no transformant was obtained when transformation was attempted with the linear 3.2kb *Bam*HI genomic DNA fragment containing the *pyrG* gene, while 10–15 transformants per μg DNA were obtained with the self-ligated fragment. Although the transformation efficiency is rather low, this observation suggests an important role for DNA conformation in transformation. The transformation efficiency increased by a factor of 40–50 when pEPM1 or pEPM9 was used. The differences in transformation efficiency may be attributed not only to the purity of the transforming DNA (all the plasmids were purified on CsCl gradients, while the ligation mixture was used directly for transformation), but also to the real number of effective transforming molecules in each case: this is always lower in the ligation mixture since a proportion of the linear fragment forms differently sized circular multimers. The transformation efficiencies achieved with pEPM1 and pEPM9 are in the same range as those obtained in strain R7B with plasmids harbouring the 4.4 kb *Pst*I *M. circinelloides* genomic DNA fragment which contains the *leuA* gene and a putative homologous ARS (Benito et al. 1992; Iturriaga et al. 1992).

Complementation of the *pyrG4* mutation in strain MS12 by transformation with plasmid pJD21 shows that the *P. blakesleeanus pyrG* gene is expressed in *M. circinelloides*. However, the transformation efficiency is 50% lower than those obtained with equivalent plasmids harbouring the homologous marker. The homology at the nucleotide level between these two genes is 76% (96% when conservative deduced amino acid sequence changes are allowed; Benito et al. 1992), but the homology in the promotor regions is not significant. Moreover, both introns in the *M. circinelloides pyrG* gene are bounded by the GT sequence which appears almost invariably in the 5′ extremity of eukaryotic introns. However, the second intron of the *Phycomyces pyrG* gene has the sequence GC at the 5′ cleavage site (Díaz-Mínguez et al. 1990). This variation may reduce the efficiency of intron processing, as has been suggested in other organisms (Aebi et al. 1987), determining a decrease in the expression level of the heterologous marker and, hence, a lower transformation efficiency.

Analysis of total genomic DNA from the transformants by Southern analysis shows that the transforming DNA is maintained extrachromosomally. Consistently, all the transformants show a high level of mitotic instability of the transforming phenotype. The slightly higher percentage of spores retaining the transforming phenotype in transformant L1 supports the hypothesis that the circular transforming molecules in this transformant are dimers or trimers present in a higher copy number. This observation also suggests that the presence of two or three copies of the elements that drive autonomous replication determines a better replication capacity of the circular molecule. Intact transforming plasmids are recovered by transforming *E. coli* with undigested genomic DNA from all the transformants, except for those obtained with plasmid pJD21. This plasmid was also not recovered when the genomic DNA from these transformants was digested with an enzyme that cuts once within plasmid pJD21, religated and used to transform *E. coli*. This is probably due to the low copy number of plasmid pJD21 in the transformants.

Although in filamentous fungi transformation occurs mainly by integration, Zygomycetes, and *M. circinelloides* in particular, seem to be an exception to this rule. In this organism, the 4.4 kb *Pst*I genomic DNA fragment containing the *leuA* gene has been shown to be maintained extrachromosomally either when self-ligated or when cloned in different plasmids (Van Heeswijck 1986; Roncero et al. 1989). It has also been reported that plasmids harbouring a *M. circinelloides* genomic DNA fragment which complements a *met*$^-$ mutation in *M. circinelloides* strain R5A are also maintained extrachromosomally (Anaya and Roncero 1991). From these results the authors concluded that ARS, or elements behaving like ARS, are more abundant and/or easier to isolate in *M. circinelloides* than in other filamentous fungi. Our experiments show that circular molecules harbouring a genomic DNA fragment containing the *pyrG* gene are also maintained extrachromosomally. The smallest fragment tested which drives this capacity is the 1.85 kb *Bam*HI-*Ava*II fragment cloned in plasmid pEPM9, which should include all the elements needed for its autonomous maintenance. Plasmid pJD21 is also maintained extrachromosomally, suggesting that autonomous replication of the transforming DNA does not depend strictly on the presence of homologous sequences in the transforming plasmid. The lower copy number of the transforming plasmid and the higher level of mitotic instability in the transformants, indicates that replication of plasmids lacking homologous sequences is less efficient, but it does indeed occur. It should be noted that a plasmid harbouring only a *P. blakesleeanus* genomic DNA fragment containing the *leu1* gene cloned in pUC19, is also maintained extrachromosomally (Iturriaga et al. 1992).

The fragment cloned in plasmid pEPM9 contains the *pyrG* coding region flanked by 606 nucleotides and 330 nucleotides at the 5′ and 3′ regions, respectively. It shows the general structure of a nuclear coding gene, differing from the structure of the elements which have been shown to promote autonomous replication in other fungal systems. When the fragment is compared with the other proposed *M. circinelloides* ARS containing fragments, no significant similarity is detected, except for the presence of elements related to transcription, suggesting that these common elements may be involved both in the stable maintenance of auton-

omously replicating plasmids and in transcription. Factors affecting transcription and replication have been described recently (for reviews see Guarente 1992; Kornberg and Baker 1992). More extensive comparisons of *M. circinelloides* elements with autonomous replication capacity are needed to support the hypothesis that autonomous replication in this fungus may be driven by elements commonly present in nuclear coding genes.

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