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EXPRESSION OF ACTIVE MUCOR MIEHEI ASPARTIC PROTEASE IN MUCOR CIRCINELLOIDES

by

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The gene encoding the aspartic protease in the mold Mucor miehei was isolated and a vector, pMA67, was constructed to allow expression of this gene in Mucor circinelloides. pMA67 contained the Amp^r gene for selection in E. coli, a Leu^+ gene for selection in M. circinelloides and the M. miehei aspartic prepro-protease gene. A leucine requiring strain of M. circinelloides was transformed with pMA67. It was shown by rocket immunoelectrophoresis that transformants secreted M. miehei aspartic protease. The excreted protease is active and has the same apparent molecular weight as the aspartic protease produced by M. miehei. This paper reports on heterologous gene expression and protein secretion in a member of the commercially important genus Mucor, and it opens up the possibility to use pMA67 for the expression of other important proteins.

1. INTRODUCTION

A few examples of heterologous gene expression in filamentous fungi have been described, e.g. in Aspergillus nidulans (3, 12, 14, 27) – including the expression and secretion of Mucor miehei protease (14) –, in Aspergillus niger (10, 20, 22, 27), Neurospora crassa (9, 13), Ustilago maydis (4), Phycomyces blakesleeanus (28) and Cochliobolus heterostrophus (36). Filamentous fungi are appealing expression systems, since they excrete large amounts of proteins, in contrast to yeast and Escherichia coli. The filamentous fungus Mucor is an excellent target for genetic engineering since it is already widely used in industrial processes. M. miehei, for example, is being used in cheese production, because it produces and secretes an aspartic protease, EC 3.4.23.6 (MmAP) which substitutes for bovine chymosin as a milk-clotting enzyme (17, 26). M. circinelloides also – like all

Abbreviations: Amp = ampicillin; bp = base pair(s);EDTA = ethylene diamine tetraacetic acid, sodium salt; kb = 1000 bp;IPTG = isopropyl β-D-thiogalactoside; McAP = Mucor circinelloides aspartic protease;MmAP = Mucor miehei aspartic protease;SDS = sodium dodecyl sulphate;TRIS = tris(hydroxymethyl)-amino methane;X-gal = 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

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other Mucor species so far investigated (1, 5, 8, 20, 26) produces a protease (McAP) capable of clotting milk (21). The protease of M. circinelloides has an apparent molecular weight lower than that of M. miehei and it does not cross-react with antibodies raised against MmAP (unpublished observation). We are exploring the potential of using Mucor in the production and secretion of large quantities of industrially useful proteins. In this report we describe the isolation, transfer and expression of the M. miehei aspartic protease gene in M. circinelloides. The expression vector, as well as the aspartic protease gene promoter and signal sequence, may have a general utility in the heterologous gene expression and secretion of foreign proteins in Mucor.

2. MATERIALS AND METHODS

2.1. Strains and plasmids

The Mucor strain CBS 370.65 was used to isolate the aspartic protease gene. The leucine auxotroph M. circinelloides R7B (30) derived from the parent strain M. circinelloides f. lusitanicus CBS 277.49 (syn. M. racemousus ATCC 1216b) was the recipient in transformation experiments. The vector pUC 13 (37) was used in all cloning steps. The bacterial host for recombinant plasmids was Escherichia coli K12 strain TBI, obtained from Dr. T. BALDWIN at Texas A&M University. The vector pME2, containing the partial MmAP gene, was a gift from Dr. G. GRAY, Genencor Inc.

2.2. Enzymes and chemicals

Restriction enzymes, T4 DNA-ligase and E. coli DNA-polymerase I were purchased from Boehringer Mannheim. Nucleic acid grade Agarose NA was from Pharmacia. Nitrocellulose membranes from Schleicher and Schuell, and the Genescreen Plus membranes from NEN Research Products.

2.3. DNA isolation from Mucor

Total genomic DNA was isolated from M. miehei as previously described (19).

2.4. Isolation of plasmid DNA

Minipreparations of plasmid DNA were made following the alkaline lysis protocol described by MANIATIS et al. (24). Large scale plasmid DNA preparations were made according to CLEWELL and HELINSKY (11), or by the alkaline lysis procedure described by BIRNBOIM and DOLY (6).

2.5. Isolation of DNA fragments

DNA fragments were separated by electrophoresis in an agarose NA gel with TRIS-acetate running buffer (40 mM-TRIS-acetate, pH 8.0; 1 mM-EDTA). The desired fragments were recovered from the gel by electrophoresis into dialysis bags as described by MCDONNELL et al. (25). The DNA was further purified by extraction with phenol and chloroform:isoamylalcohol (24:1) and precipitated with ethanol.

2.6. Nucleic acid hybridizations

All hybridizations were carried out in aqueous solutions at 63 °C. Hybridizationbuffer contained: 1 mM-EDTA; 5×SSC (0.75 M-NaCl; 0.75 M-Na-citrate); 5×Denhardt's solution (0.1% bovine serum albumin; 0.1% ficoll; 0.1% polyvinylpyrrolidone); 1% SDS; 50 mM-Na_2HPO_4 pH 6.5; 0.5 mg/ml denatured salmon sperm DNA.

2.6.1. Genomic southern blots

2.5 µg DNA were digested with an excess of restriction enzyme and subjected to electrophoresis on a 0.6% agarose gel. The DNA fragments were transferred to Genescreen plus membranes by the capillary blot procedure as described in the manufacturers' manual with the following modifications: denaturation and neutralization of the gel were done for one hour each, the denaturation solution consisted of 0.5 M-NaCl, 1.5 M-NaOH, and the membrane was rinsed in 2×SSC after the transfer was completed (approx. 24 hours) and dried at 80 °C for 1.5 hours. The filter was pretreated in hybridization buffer (0.25 ml/10 cm^2 membrane) for 3 hours and hybridized with denatured ^{32}P-labelled DNA (New England Nuclear) as a probe in a

fresh hybridization solution (0.125 ml/10 cm^2) for 12-24 hours. The membrane was washed at room temperature in several changes of 2×SSC; 0.1% SDS, followed by washes at more stringent conditions (as indicated) for 8-10 hours.

2.6.2. Colony hybridization

Colony hybridization was carried out according to GRUNSTEIN and HOGNESS (15). After hybridization was completed, the filters were washed at 62 °C in 1×SSC; 0.1% SDS, for 4-6 hours.

2.7. DNA sequencing

DNA sequencing was performed by the dideoxy chain terminating method (32).

2.8. Transformation of E. coli

E. coli strain K12 TBI cells were induced to competence according to MANDEL and HIGA (23) and stored frozen in 15% glycerol at -80 °C. Right before use, the cells were thawed on ice and the transformation was carried out as described (16). Clones containing recombinant plasmids were identified as white colonies on X-gal/IPTG indicator plates.

2.9. Growth and transformation of Mucor

The transformation of M. circinelloides R7B was as described (19). The transformants were grown on solid wheat bran medium: 1 g of wheat bran was carefully mixed with 1 ml of H_2O in a 100 ml Erlenmeyer flask plugged with gauze and cotton and autoclaved. The bran was inoculated with 100 µl of a suspension of sporangiospores harvested from isolated transformants.

2.10. Analysis of transformants

After 3 or 7 days of growth at room temperature, the mycelia were washed with 10 ml H_2O and incubated at 5 °C for 24 hours. The supernatants were tested for the presence of extracellular MmAP by rocket immunoelectrophoresis (2) using monospecific antibodies raised in rabbits against purified MmAP. The total milk-clotting activity was measured in reconstituted skim-milk of pH 6.5 or pH 6.0 as described by ROTHE et al. (31)

MmAP and McAP were separated by ion-exchange chromatography on Whatman DE-52 cellulose (0.9×10 cm) equilibrated with 0.02 M-piperazin-HCl pH 5.3 with sodium chloride added to a conductivity of 5.6 mS. A sample containing both MmAP and McAP was dialyzed against the above mentioned buffer and applied to the column. McAP runs through whereas MmAP is bound. The column was then washed with 0.02 M-piperazin-HCl pH 5.3, sodium chloride added to give a conductivity of 11.4 mS, and the MmAP was eluted with 0.2 M-NaCl in 0.02 M-piperazin-HCl pH 5.3.

Fractions were analyzed on 11-15% SDS polyacrylamide gradient gels by a standard immunoblotting procedure (35).

3. RESULTS

3.1. Isolation of the M. miehei aspartic protease gene

Total genomic DNA isolated from M. miehei was cut with different restriction enzymes, subjected to agarose gel electrophoresis and transferred to Genescreen Plus membranes. It was probed with a genomic clone (pME2), containing a partial sequence of the MmAP gene. pME2 contained a 2.8 kb HindIII fragment covering most of the coding sequence for acid protease: amino acids no. 36 through 395 (numbered relative to the startcodon no. 1 of the putative signalpeptide) and a large portion (1.7 kb) of the 3'-flanking sequence (14). It lacked the extreme 5' codons as well as the promotor region. The insert of pME2 was isolated with HindIII and BamHI digestion, thereby reducing the large portion of 3' untranslated sequence to about 700 bp. This 1.9 kb HindIII/BamHI fragment (pME2-HB) was labelled with ^{32}P-dCTP and served as a probe in the Southern blots. When M. miehei DNA was digested with either EcoRI or BamHI, a single band of 13 kb hybridized in each case to the 1.9 kb insert (Figure 1). The fragment hybridizing in a double digest with BamHI and EcoRI was only 2.4 kb in size, thus a more convenient size for cloning. The restriction map of the MmAP gene sequence (14)

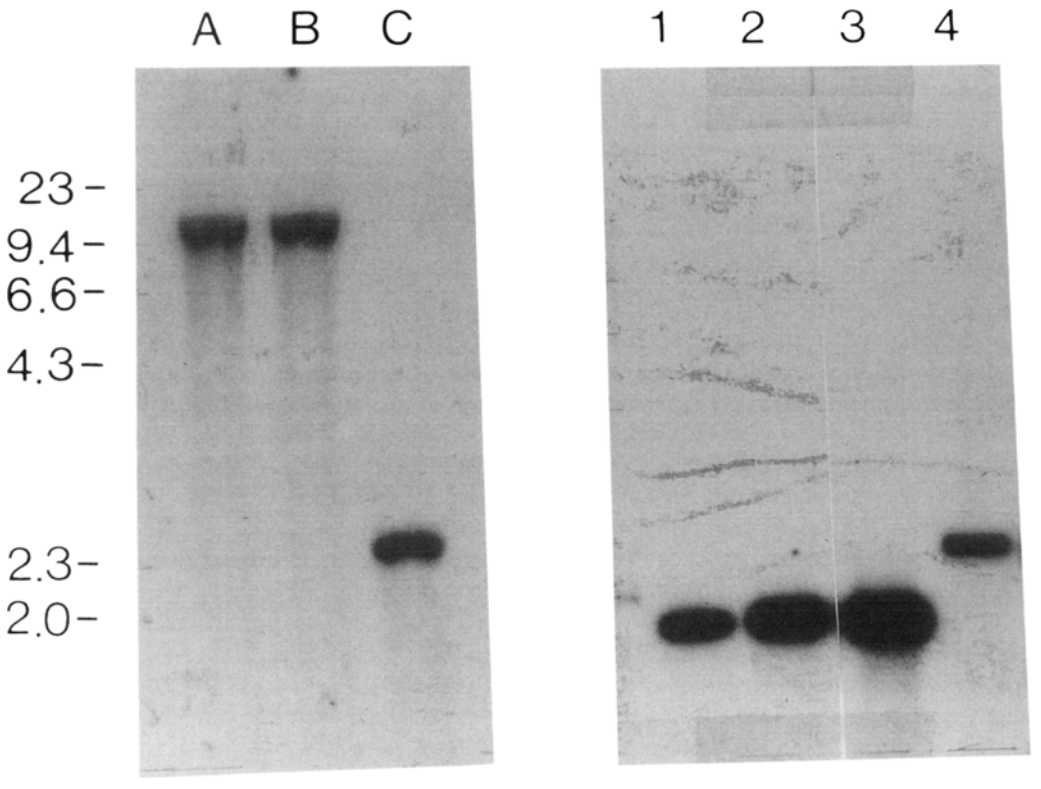

Figure 1. Southern hybridizations with M. miehei DNA.

The probe was the ^{32}P-labelled insert of pME2-HB. Lane a: EcoRI digest, lane B: BamHI digest, lane C: double digest with EcoRI and BamHI. Lanes 1, 2 and 3 contained 1, 2 and 5 copy equivalents of the MmAP gene (519 pg pME2-HB insert corresponds to 1 copy-equivalent. Lane 4: M. miehei DNA digested with EcoRI and BamHI. Hybridization was done at a stringency of Tm-33 °C and the filters were washed at Tm-8 °C. The sizes are shown in kilobasepairs.

confirms the absence of BamHI and EcoRI sites in the coding sequence and in the 5' and 3' flanking regions.

The protease gene of M. miehei is present at a frequency of one per haploid genome as shown by genomic reconstructions (Figure 1). It is interesting to note that pME2-HB, containing acid protease gene sequences from M. miehei, does not hybridize to any restriction fragments of genomic DNA isolated from M. circinelloides (data not shown). This together with the absence of a cross-reaction between antibodies raised against MmAP and the protease produced by M. circinelloides thus indicates, that the acid protease genes of these two species are not highly homologous.

Since the size of the Mucor genome is rather small (10^7 bp per haploid genome (34)), we attempted to isolate the acid protease gene directly from a BamHI/EcoRI digest of total genomic DNA from M. miehei. 12 µg of total DNA were digested with BamHI and EcoRI and separated on a 0.6% agarose gel. The region containing fragments in size between 2.2 and 2.5 kb was purified from the gel and subcloned into pUC 13. The clones containing protease gene sequences were identified by colony hybridization. Two clones out of a total of 170 white colonies tested gave a strong hybridization signal when probed with pME2-HB. Characterization with restriction analysis revealed that both clones harboured a plasmid containing a 2.4 kb

BamHI/EcoRI insert and that their restriction maps were identical. One clone, pMA6, was chosen for further studies. pMA6 was identified as a plasmid containing the MmAP gene by the following criteria: 1) the restriction map of the insert of pMA6 was identical to the map of the protease gene published by GRAY et al. (14), 2) the insert of pMA6 hybridized intensely to pME2-HB at a high stringency condition (Tm-8 °C – data not shown), 3) sequencing of a 500 bp region at the 5'-terminal part of the pMA6 insert revealed a perfect homology to published data (14). The insert of pMA6 contains a 480 bp 5' flanking region, the about 1.3 kb coding sequence and the 600 bp 3' flanking sequence. Two conserved sequences involved in the promotion of eucaryotic gene transcription are present in the 5' flanking region. A TATA box and a putative CAAT box are located at -50 and -148 upstream of the translation initiation codon (data not shown).

The main goal of this study was to determine whether the isolated genomic clone contained a functional acid protease promoter which might be used to direct expression of foreign genes in the fungus M. circinelloides. To assess this possibility, an expression vector with pMA6 was constructed and is was determined whether the MmAP gene could be expressed in M. circinelloides under the control of its own promoter.

3.2. Construction of a Mucor expression vector

Detection of a vector allowing the expression of foreign genes in M. circinelloides required a

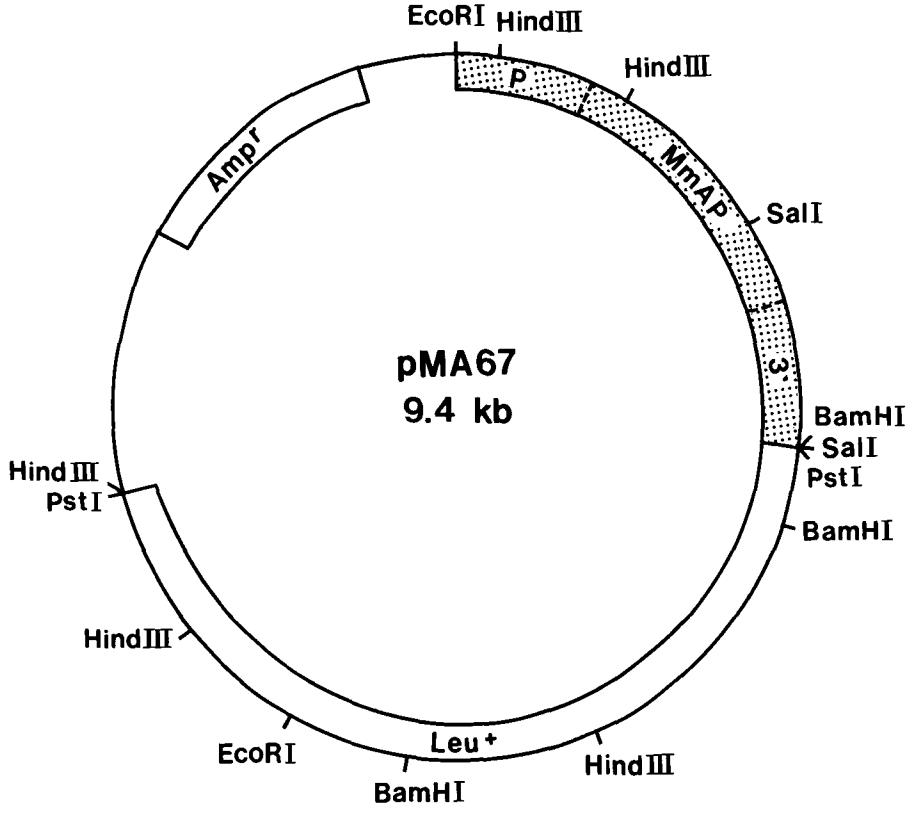

Figure 2. Genetic map of the Mucor expression vector pMA67.

The MmAP gene and the Leu+ gene from M. circinelloides wild-type were cloned into the multiple cloning site of pUC13. The Leu$^+$ gene relieves the auxotrophic requirement for leucine in a mutant recipient strain of M. circinelloides (R7B). The Amp^r gene is from pUC13. The hatched box represents the MmAP gene. P = promoter, 3' = 3' flanking region.

Table I. Milk-clotting activities and extracellular levels of MmAP in selected transformants.

The milk-clotting activity was measured enzymatically and the levels of MmAP were determined immmunologically as described in section 2 (Materials and Methods). CHU: Christian Hansen Unit (16 CHU correspond to 1 mg of purified MmAP from CBS 370.65 and approximately 1 mg of purified M. circinelloides protease). The control was a transformant with pMCL006.

Transformant	total activity (CHU/ml)		MmAP concentration (μg/ml)	
	3 days	7 days	3 days	7 days
11	0.9	1.2	6	8
15	1.0	1.2	7	8
23	0.9	1.1	10	10
25	1.0	1.0	3	3
26	0.9	1.0	3	1
28	0.8	0.6	3	5
29	1.0	0.9	4	5
30	0.8	1.0	4	3
32	0.8	0.9	1	9
34	0.7	0.8	2	1
37	0.8	0.9	2	8
40	1.0	0.9	9	12
control	0.9	1.0	0	0

specific marker permitting the selection of transformants. HEESWIJCK and RONCERO (19) reported the successful transformation of a leucine dependent mutant strain of M. circinelloides to a Leu^+ phenotype with recombinant DNA from a genomic library of a wild-type strain. The gene complementing the mutant allele has been isolated. It has been shown, that the DNA necessary for complementation is located on a 4.4 kb PstI fragment isolated from pMCL1302, a vector used in the transformation (18). By cloning this 4.4 kb PstI fragment into the unique PstI site of pMA6, a Mucor – E. coli shuttle vector, pMA67, was made (Figure 2), which carried the Leu^+ gene as a selectable marker in Mucor, the MmAP gene and the β-lactamase gene for selection in E. coli.

3.3. Transformation of M. circinelloides

Viable protoplasts of M. circinelloides strain R7B were incubated with 1 to 10 μg of pMA67 in the presence of polyethyleneglycol and $CaCl_2$ (19), and plated on minimal medium. After 2-3 days of incubation at room temperature, prototrophic colonies appeared. The frequency of transformation was 200 colonies/μg DNA, which was in the same range as that obtained with pMCL006 in a parallel control experiment where pMCL006 is pBR322 containing the Leu^+ gene on a 4.4 kb PstI fragment (18). No prototrophic clones were obtained when the DNA was omitted. Individual colonies were transferred to fresh minimal medium to determine whether the transformation was stable. 40 clones that exhibited continued growth were chosen for further analysis. The mycelia were allowed to grow on wheat bran medium for several days.

3.4. Analysis of transformants

The supernatants obtained after washing the mycelia were tested enzymatically for total milk-clotting activity which means the sum of the two milk-clotting enzymes M. miehei aspartic protease (MmAP) and M. circinelloides aspartic protease (McAP). The total milk-clotting activity measured is shown in Table I for selected transformants. The total activity ranged between 0.7 and 1.0 CHU/ml after 3 days growth. The values changed only slightly after 7 days

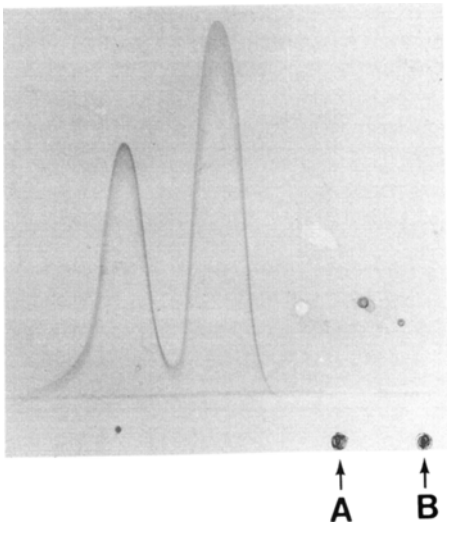

Figure 3. Tandem crossed immunoelectrophoresis of MmAP produced by M. miehei and by transformants of M. circinelloides.

A and B indicate points of application of protease. Left precipitate (from A): MmAP, 4 µl containing 156 µg/ml produced by M. miehei; right precipitate (from B): MmAP, 4 µl containing 250 µg/ml, produced by M. circinelloides R7B transformed with pMA67. The immunological identity between the two MmAP's is demonstrated by fusion of the two precipitates and by the identical precipitate rocket morphology. 80 µl of unpurified antiserum against MmAP were used.

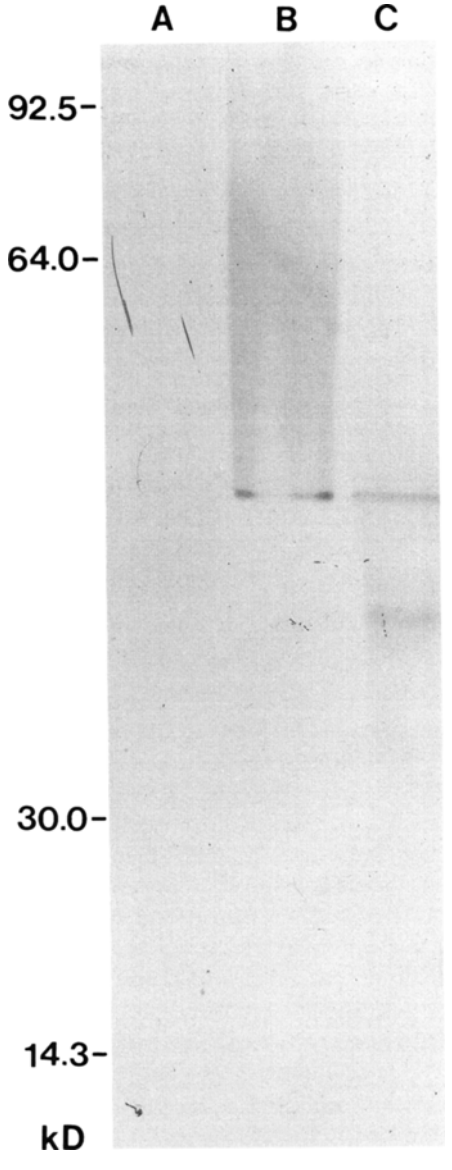

Figure 4. Immunological comparison of MmAP from M. circinelloides with MmAP from M. miehei. Polypeptides separated by SDS-gel electrophoresis, transferred to nitrocellulose filter and probed with antibodies.

Cultural supernatants from: A: R7B transformed with pMCL006 (control); B: authentic MmAP (from M. miehei grown on wheat bran); C: R7B transformed with pMA67. Numbers on the left indicate sizes of protein molecular weight standards in kilodaltons.

growth for the majority of the transformants. The presence of extracellular MmAP was measured immunologically. 30 clones out of a total of 40 produced extracellular MmAP at a concentration of 1 µg/ml or higher (Table I). It was shown that the MmAP produced by M. circinelloides was identical to that from M. miehei by tandem crossed immunoelectrophoresis (7). Figure 3 demonstrates the identity by showing complete fusion between the two precipitates and identical precipitate morphology. In addition the mobilities of the two proteins are identical as judged by SDS-polyacrylamide gel electrophoresis (Figure 4). The MmAP produced in M. circinelloides is active after separation from McAP by ion-exchange chromatography. The milk-clotting activity of the isolated

Table II. Experiment demonstrating inhibition by MmAP-antibodies of enzyme activity of MmAP produced by M. circinelloides.

McAP and MmAP from cultural supernatants of M. circinelloides transformants were separated by ion-exchange chromatography. The milk-clotting activity of the fractions was determined after the addition of MmAP-antibodies (40 µl in 2 ml skim milk, pH 6.0). The activity is expressed as per cent of the activity with no antibodies added.

	incubation with 4 µl of			
sample	water	anti-MmAP batch 1	anti-MmAP batch 2	indifferent antiserum (rabbit-anti-goat-serum)
MmAP from M. miehei (control)	100%	11%	<4%	100%
MmAP from M. circinelloides	100%	16%	<4%	102%
McAP from M. circinelloides	100%	106%	105%	102%

MmAP corresponded to the amount of MmAP found by rocket immunoelectrophoresis. Isolated MmAP was enzymatically active since its specific inhibition by antibodies against MmAP was the same for MmAP isolated from M. miehei and from M. circinelloides transformants, whereas no inhibition was observed for McAP (Table II).

4. DISCUSSION

The complete aspartic protease gene from M. miehei was isolated and used to construct a novel expression vector (pMA67) for use in Mucor. The vector also carries a selectable marker (Leu+) which complements the leucine auxotrophy of the mutant M. circinelloides strain R7B (30) and sequences from pUC13 which allow for its replication and maintenance in E. coli.

M. circinelloides transformants secrete active MmAP of the right size showing immunological identity with authentic MmAP. Apparently the MmAP gene promoter and signal sequences are functional in M. circinelloides. Since authentic MmAP is glycosylated (29), the similar apparent molecular weights of authentic MmAP and MmAP produced by M. circinelloides (Figure 4) indicate that the MmAP polypeptide is glycosylated correctly by M. circinelloides. A. nidulans transformed with the MmAP gene also recognizes the MmAP promoter and secretes the enzyme but fails to glycosylate the polypeptide correctly (14).

The correct recognition and further processing by M. circinelloides probably reflects the phylogenetic relationship between M. miehei and M. circinelloides. However, the two zygomycetous fungi have been considered as members of different genera (33): Mucor miehei together with the other two thermophiles M. pusillus and M. tauricus have been combined into the genus Rhizomucor, while M. circinelloides together with the remaining mesophile species form the genus Mucor. Differences exist between the milk-clotting proteases produced by the two fungi: there is apparently little homology at the amino acid level as shown by the absence of an immunological crossreaction between MmAP-antibodies and McAP as well as at the nucleotide level as demonstrated by the failure of the MmAP gene to hybridize to genomic DNA from M. circinelloides. Finally, there is a difference in the apparent molecular weight of McAP of 34 kD (21) and MmAP with 42 kD. MmAP accumulated over a period of days in

many transformants tested indicating the stability of the transformants.

It is not possible to directly compare the levels of extracellular aspartic protease produced from the transformants with the levels found in its natural host, since the optimal growth conditions for the two species are different. M. circinelloides produces much smaller amounts of extracellular protease in liquid media than on solid medium, in contrast to M. miehei, whose production is maximal in liquid culture (21). However, we judge the levels of MmAP obtained from M. circinelloides transformants (up to 12 µg/ml) to be highly significant and demonstrate heterologous gene expression in a member of the fungal genus Mucor of the class Zygomycetes. The vector pMA67 might prove useful in further attempts to genetically engineer Mucor.

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