

Cloning, characterization and expression of complete coding sequences of three IgE binding *Malassezia furfur* allergens, Mal f 7, Mal f 8 and Mal f 9

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Malassezia furfur, formerly known as *Pityrosporum orbiculare* or *P. ovale*, is a yeast that colonizes human skin. Normally, this yeast is nonpathogenic but under the influence of predisposing factors it may induce IgE reactivity in patients with atopic dermatitis. Approximately 40–65% of atopic dermatitis patients have IgE antibodies and/or skin reactivity against *M. furfur* and a higher T-cell response against this yeast is found in atopic dermatitis patients than in healthy individuals. By making a cDNA library displayed on a phage surface, we previously cloned five different IgE-binding proteins, Mal f 5, Mal f 6, MF 7, MF 8 and MF 9, from this yeast. The cDNAs encoding these allergens were sequenced and expressed in *Escherichia coli*. The sequences of MF 7, MF 8 and MF 9 were not full length (missing their 5'-ends) giving only partial gene products. To obtain complete cDNA sequences, we performed RACE-PCR to amplify the 5'-ends of each cDNA. These PCR products were sequenced and analyzed. The coding sequences of Mal f 7, Mal f 8 and Mal f 9 encode proteins with ORFs of 141 (16.2 kDa), 179 (19.2 kDa) and 126 (14.0 kDa) amino-acid residues, respectively. None of the putative proteins showed significant sequence homology with other known proteins in the searched database. The proteins encoded by the complete cDNA sequences were expressed in *E. coli* as recombinant proteins. Immunoblotting and radioallergosorbent test data showed that all of the expressed recombinant proteins have the ability to bind serum IgE from atopic dermatitis patients and furthermore, the *M. furfur* extract could specifically inhibit this IgE binding.

Keywords: allergy; atopic dermatitis; cDNA cloning; *Malassezia furfur*; recombinant allergen.

The yeast *Malassezia furfur*, also denoted *Pityrosporum orbiculare* (*P. ovale*), is an obligatory lipophilic yeast that preferentially colonizes the skin of the head, neck and face region in individuals with atopic dermatitis (AD) [1]. Under certain conditions, this yeast can be involved in the pathogenesis of skin diseases such as pityriasis (tinea) versicolor and seborrhoeic dermatitis [2]. In addition, data from a number of studies suggest the involvement of *M. furfur* in the pathogenesis of AD [2,3]. Earlier studies indicate that between 40 and 65% of AD patients have specific IgE serology and/or positive skin tests to *M. furfur* extract [4–8]. These, and the previously shown data that *M. furfur* extract induces a significantly higher T-cell response in AD patients than in healthy individuals and preferentially with production of T helper (Th)2-like cytokines, suggest that this yeast plays a role in maintaining IgE-mediated skin inflammation in AD [9,10]. In order to gain more information about *M. furfur* and its role in AD, and also to determine the role of individual IgE-binding components, it is important to characterize the IgE-binding

allergens and work with pure allergen preparations from this fungus. Recombinant DNA technology makes this task possible and allows the production of pure recombinant allergens in large quantities [11]. One advantage of recombinant allergen compared with crude allergen extract, which is usually used in the diagnosis of allergy, is that the former are perfectly standardized and highly pure, making them more suitable for immunodiagnosis [12]. Immunoblotting analysis shows that extracts from *M. furfur* contain a variable number of IgE-binding proteins depending on the culture conditions and extraction [13–15]. Four *M. furfur* allergens designated Mal f 1 (36 kDa) [16], Mal f 2 (21 kDa), Mal f 3 (20 kDa) [17] and Mal f 4 (35 kDa) [18] have been sequenced and expressed as recombinant proteins. Using a phage display technique, we made a cDNA library from *M. furfur*, from which we recently cloned five recombinant allergens designated Mal f 5, Mal f 6, MF 7, MF 8 and MF 9 [19]. Mal f 5 showed sequence similarity to Mal f 2, Mal f 3 and to Asp f 3 allergen of *Aspergillus fumigatus*. Mal f 6 showed highest sequence similarity to cyclophilin from *Schizosaccharomyces pombe* [19]. The cDNA clones MF 7, MF 8 and MF 9 were not full length. The aim of this study was to analyze further the cDNA clones Mal f 7, Mal f 8 and Mal f 9, find their full-length sequences, express them in *E. coli* and determine their ability to bind IgE.

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Abbreviations: AD, atopic dermatitis; IPTG, isopropyl thio- β -D-galactoside; PVDF, poly(vinylidene difluoride); RAST, radioallergosorbent test.

Note: O. Rasool and A. Zargari contributed equally to this work.

Note: the novel nucleotide sequence data published here have been submitted to the EMBL sequence data bank and are available under accession numbers AJ011957, AJ011958 and AJ011959 for Mal f 7, Mal f 8 and Mal f 9, respectively.

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MATERIALS AND METHODS

Culturing of *M. furfur*, mRNA purification and cDNA synthesis

Two-day-old cultures of *M. furfur* from the American Type Culture Collection (no. 42132) on glycerol monostearate/olive

Table 1. Sequences of forward (F) and reverse (R) primers used in PCR amplifications or nucleotide sequencing of Mal f 7, Mal f 8 and Mal f 9.

Mal f 7	
1. 7 R	5'-CACAAGCTTAATGATGATGATGATGATGGGCGTTCTTCTTGAGATTGAGC-3'
2. 7 R1	5'-AGGGTGGCTCTGGCAGCGCTC-3'
3. 7 R2	5'-GATACTTGCCACTGTAAAGTGATTG-3'
4. 7 F	5'-GAGCATATGAATAATGAAAAGTACTGTTTCAGTC-3'
Mal f 8	
5. 8 R	5'-CACAAGCTTAATGATGATGATGATGATGGCAGTATTGGCCAGGGATGAC-3'
6. 8 R1	5'-GGTTACCAACACCACCGTTGCCA-3'
7. 8 R2	5'-ACACGCGGGTTGATGCAGCCAG-3'
8. 8 F	5'-GAGCATATGGCCGCCCCCTCTAGCATGGATCGC-3'
Mal f 9	
9. 9 R	5'-CACAAGCTTAATGATGATGATGATGATGCTGAGAGAGAGGCAGGCCAC-3'
10. 9 R1	5'-CAGACCAACCTCTCGATCGAAC-3'
11. 9 R2	5'-GCCCTTGAGGAAGTCTCCTTG-3'
12. 9 F	5'-CACCATATGCGCGCGTTGCTGGTAACC-3'
13. 9 F1	5'-GCGCGAGCGTGAGCACCAATT-3'
14. 9 F2	5'-TGCACGATGCTAAGGACCGTCTGCA-3'

oil (Go) medium (37 °C), had been shown, in our laboratory, to express high levels of allergenic proteins [14]. A cell culture of this age was used to extract poly(A⁺) mRNA by using 'Poly(A) Tract System 1000' (Promega, Madison, WI, USA) or 'Straight A's mRNA isolation system' (Novagen, Madison, WI, USA) kits according to the manufacturers' instructions. cDNA synthesis was performed as described previously [19], using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA, USA) according to the manufacturers' instructions. Briefly, the purified mRNA was converted to double-stranded cDNA using modified cDNA synthesis primer, adaptor and PCR primers [19].

PCR amplification

The primers used in the PCR amplifications are shown in Table 1. All oligonucleotides were synthesized by Medprobe (Oslo, Norway) unless stated otherwise. The 5'-end of each cDNA was amplified by using Marathon cDNA amplification kit (Clontech) following the manufacturer's instructions. The full coding sequences of the cDNA clones were amplified by using 5'-end primers containing a *NdeI* restriction site, 3' primers containing a His₆-tag followed by a *HindIII* restriction site and a stop codon allowing the PCR products to be subcloned into the *NdeI/HindIII* sites of the expression vector (see below). PCR reactions were carried out in a final volume of 50 µL containing 5 µL 10× PCR reaction buffer, 0.2 µM of each primer, 20 µM dNTPs, 1 µL Advantage cDNA polymerase mix and finally either 5 µL of anchor-ligated cDNA (diluted 1 : 100 or 1 : 250 in tricine/EDTA) or 1 µL PCR product (diluted 1 : 50 in H₂O) as template. PCR amplifications were performed in a PE DNA Thermocycler 480 (PerkinElmer, Norwalk, CT, USA) or Eppendorf Mastercycler gradient (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) by using the following conditions as described in the Marathon kit: an initial denaturation at 94 °C for 1 min followed by: five cycles at 94 °C for 30 s and 72 °C for 4 min; five cycles at 94 °C for 30 s and 70 °C for 4 min; and finally, 25 cycles at 94 °C for 20 s and 68 °C for 4 min. To confirm that our cDNA clones are derived from *M. furfur*, we performed PCR (94 °C for 5 min followed by 30 cycles of 94 °C, 60 °C and 72 °C, 1 min each, and finally 10 min of

72 °C) using *M. furfur* DNA and primers amplifying the coding sequences of the cDNA clones. All the primers could specifically amplify the Mal f proteins (data not shown).

Subcloning and sequencing

The PCR products were purified from 1% agarose/ethidium bromide gel using a QIAGEN gel extraction kit (Qiagen GmbH, Hilden, Germany). The RACE-PCR products of Mal f 7 and Mal f 8 were subcloned into PT7 Blue vector using a commercial kit (Perfectly blunt cloning kit, Novagen) and then transformed into *Escherichia coli* XL2-Blue cells (Stratagene, La Jolla, CA, USA) by electroporation. Recombinant clones, containing the RACE-PCR products, were selected, followed by plasmid DNA purification (Qiaprep spin plasmid miniprep kit, Qiagen GmbH) and restriction analysis, to confirm the correct size of each insert. The plasmid inserts were sequenced from both ends using T7 promoter (Novagen), M13 universal (Pharmacia & Upjohn, Uppsala, Sweden) or M13/pUC reverse (Gibco-BRL, Life Technologies, Täby, Sweden) sequencing primers. On the other hand, the RACE-PCR product of Mal f 9, after its purification from agarose gel as described above, was sequenced directly using the following gene specific primers: 9 R1, 9 R2, 9 F1 and 9 F2 (Table 1). Sequencing was performed on an ABI prism 377 sequencer (PerkinElmer) using either of two kits (Dye Terminator or dRhodamine terminator Cycle Sequencing Ready Reaction, PerkinElmer) according to the manufacturer's recommendations. All sequences were analyzed from the BCM Search Launcher using WU-BLASTX 2.0 search of the NCBI nonredundant (nr) protein database.

Expression and purification of recombinant proteins

Expression and purification of the proteins were performed as described previously [19]. Briefly, sequences representing the full ORF of the genes were cleaved with *HindIII* and *NdeI* and ligated into the expression vector PET 17b (Novagen) which was also cleaved with the same pair of enzymes. Ligation products were transformed into the competent *E. coli* XL2 cells (Stratagene) by electroporation and plated on Luria-Bertani medium containing 100 µg·mL⁻¹ ampicillin. Plasmid DNAs,

A			B			C		
1	CGTCTCTGGGCTGGCCACTCTCTACAGACACAACTGTCTACTTAT	48	1	M V A L K F A A V L S V A A	15	1	CAAGTATCAAGAGGTTTTCACACCCGACA	32
49	GCCTCAGGAAAGCATTTTGTGAGTTCCATATTAACCATCTACTGTAC	96	6	AATATGTGTGGCTCCCTCAAGTTTGTCTGTCTGTGAGGTTGTGTCT	53	33	AGGCGGAGGCTGAGGCTCTCAGAGGTTGCTCTGACGCTCTCTCAGAGGCTG	80
97	AAGCATGTCTGCAAAATCACTTACAGATGGCAAGATACAAATGAT	144	16	A V M A A P S S M D R R A S P D	31	81	GCCACAGGGCGGAGGTTTCTCTGCAAGTCTTACAGACCGTCTCTGAGG	128
3	N E K Y C S V A D Y D V K W F K	18	54	GCCTGATGGCGGCGCCCTCTTACGATGGATGGCGGTGTCTGCGCAGAC	101	129	GCCTTGGCGGCGGACGATGACACATGTCATGTCATGTCATGTCATGTC	176
145	AATGAAAGTACTGTCTAGTGGCGGATACAGAGTAAATGTTTATG	192	32	N Q V W V T S A S D Y C L I L P	49	177	TGGTGTGCTGGCTGTGTCGCGGTGTCCTCTCTGCTGCTCTCTGCTG	224
19	E R C Q S H P T D V K T T K W I	34	102	AACCAGGTGTGGGTTTACGAGCGCTCGGACTTACTGCTTAATCTTCT	149	273	TTGAGGCGGCGGCGGCTGTGAGTACCCAGCTCTCTCTGCTGCTGCTGCT	320
193	GAGCGTGGCAGGCGCCCTTACAGATGTGAAAACGACCAAGTGGATC	240	48	R H R E S I G D S E S P G R M R	63	369	AGACCTACTGACCTGCTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT	368
35	A G T D L K I E M D P K E P Y E	50	150	CGTCACCGGAGAGCATTTGGCGACAGCGAGTCCGCGGTGTGATGGC	197	417	ACCAAGATGTGACAGACCTTGGCTCTCTGCTGCTGCTGCTGCTGCTGCT	464
241	GCCTGGACGACCTGAGCATGTGATGATGATGATGATGATGATGATG	288	64	S F C S K P Y D S S Q G Q I N P	79	465	GTCAATGATTTGAGGAGGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT	512
51	L Y C F N Y Y T T F G N L P D A	66	198	TCCTTCTGTCTTACCGGTATGACTCTCTGCTGCGAGGTCAGATTAACCT	245	513	TGCTGAGCAGATTCATCTGCGGCTGTGCTGCTGCTGCTGCTGCTGCTGCT	560
289	TTGTACTGTTCATATATATATACAGTGTGGGAACTTCTGGATGCC	336	80	G F W K E V H F K K T K N Y V Q	95	609	AGGTGCAAGCTTACACTGACAGGAGGCTGCTGCTGCTGCTGCTGCTGCT	656
67	G A K E L D D A T K K A C S A	82	246	GGCTTCTGGAAGAGTGCCTTCAAGAGACCAAGACTATGTTCAG	303	5	A G N P K D T F T Q A A I D R S	20
337	GGTGGCAACCAACTGACGATGATGATGATGATGATGATGATGATG	384	96	L T G C I N P R V Q S T L L S H	111	657	CTGGTAAACCCAGGACACTTTCATCTCAGGCTGCTGCTGCTGCTGCTGCT	704
83	L K S G K Y Q S D P K K S C R	98	304	CTTACTGGCTGCAATCAACCGCGGTGTCAGAGTACCTTCTGTCCAC	351	21	V I D K G E T V R E I V H H I	36
385	CTTACAGTGGCAATTACAAAGTGAACCGAGAGAGAAATGCTCCAG	432	112	D D G G Q Y D S N G N G V G N	127	705	TGATGACAGAGGCTGAGCTGTGCTGCTGCTGCTGCTGCTGCTGCTGCT	752
99	M D K K D I D Q F K E Q C S Q Y	114	352	GATGATGGTGTGCTAGTACGACTCCCAATGGCAAGCGGTGTGTGTGTAAC	399	37	H N I V Q P I I E K E T H E Y H	52
433	ATGCAACAGAGGACATTCACACAGTTTACGACGACCAATGCTCAGTAT	480	128	P E G S V C L G Y S S Y V E L V	143	753	ACAACATTTGTGAGCCCATCATATTTGAGAGGAGACTCATCAGATTAAC	800
115	Q P S D R P P Y G D W S A G T S	130	400	CCTGAGGGTCTGTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT	447	53	R I R T T I P T T H I T H E A P	68
481	CAGCCCTGGATPAGCCCTCTATGAGATTTGCTGCTGCTGCTGCTGCT	528	144	E P S D G K A C I R C C V N D K	159	801	GCATCCGACTTACTTATTTCTTACTCATCATCATCATCATCATCATCAT	848
131	L N V V L L K K N A *	141	448	GAGCCCTCAGACGGCAAGCCCTGCAATCGCTGCTGCTGCTGCTGCTGCT	495	69	I V H E S T A H Q P I R K E D F	84
529	CTCAATGTGGTCTCAATCTCAAGACAGAGCCCTCAATGGAGCGTCCA	576	160	Y C D V G H D E D G C E A V I P	175	849	TTGTGACAGAGTGTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT	896
577	GCAGGACAAACGATATATGATGATGATGATGATGATGATGATGATG	624	496	TACTGCGATTTGTGTCAGATGAGGACGCGCTGTGAGGCGCTCATCCT	543	85	L K G G V L T S T T R S I E E	100
625	TAGTATATCGAGTATGATGATGATGATGATGATGATGATGATGATG	672	176	G Q Y C *	179	897	TCAAGGCGCGCGGTGTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT	944
673	ACTGTGCGAGTGTGATGATGATGATGATGATGATGATGATGATGATG	720	544	GGCCATATCTGCTAAAGTGAACGTCAGACTATTTTCCCATCAACTTGA	591	101	V G L L N L G N N Q R T V E G E	116
721	GAAATTTCTGTGGTGGGCAAAAAA	754	592	TATCACTAATTTGTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT	639	945	TTGGTCTGTGTAACCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT	992
			640	TTTATCTTAAGACATTTTTCATTTGTATTTTGTGCTGCTGCTGCTGCT	687	117	T Y T G G L P L S Q *	126
			688	TATTTGGCGCCCTGTCTATTTGTGTAAGATTTTCTTCTTCTTCTTCT	735	993	CTTACACTGTGGGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT	1040
			736	AGTTGTGCGACTATTTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCT	773	1041	CGCATGCGCAATTAATTTAGCTTCTTCTTCTTCTTCTTCTTCTTCTTCT	1088
						1089	AAAAA	1103

Fig. 1. Complete cDNA nucleotide sequences and deduced amino-acid sequences of three purified *M. furfur* allergens. (A) Mal f 7, (B) Mal f 8, (C) Mal f 9. Asterisks indicate the stop codons.

prepared from different clones, were analyzed by restriction digestion and DNA sequencing. PET vectors containing correct constructs were transformed into *E. coli* expression strain BL 21 DE 3 pLysS (Novagen) to produce C-terminal tagged hexahistidine fusion proteins. The transformed strains were cultured on Luria–Bertani medium containing ampicillin ($100 \mu\text{g}\cdot\text{mL}^{-1}$) and chloramphenicol ($30 \mu\text{g}\cdot\text{mL}^{-1}$) and were grown to $D_{600} = 0.6$ at 37°C . Then, 0.4 mM of isopropyl thio- β -D-galactoside (IPTG) was used to induce expression of the recombinant proteins. The cultures were incubated for another 2 h, after which the cells were collected and resuspended in Tris/HCl (20 mM , pH 7.4) and NaCl (200 mM). The suspended cells were then frozen and thawed, sonicated and centrifuged (15 min , $9000 g$). TALON metal affinity resin (Clontech) was used to purify the recombinant proteins from the pellet (inclusion bodies) solubilized in a solution containing (6 M guanidinium chloride, 20 mM Tris/HCl pH 8 and 100 mM NaCl). Proteins were eluted in a solution containing Tris/HCl (20 mM , pH 8.0), 100 mM NaCl (100 mM) and imidazole (100 mM ; Sigma, St. Louis, MO, USA) and dialyzed against NaCl/ P_i for 3 days at 4°C . The concentrations of the purified proteins ranged from 0.4 to $2.5 \text{ mg}\cdot\text{mL}^{-1}$.

Patient sera and myeloma IgE

The sera used were from AD patients, of both sexes (median age 39 years, range 5–82 years), sent to the Division of Clinical Immunology at the Karolinska Hospital (Stockholm, Sweden) for IgE antibody analysis between January 1997 and February 1998. A panel of 25 sera, with positive values in the Pharmacia CAP System (referred to as RAST; Pharmacia & Upjohn Diagnostic AB) to *M. furfur* whole extract ($\text{m}70$) with RAST classes 1–5, median 3 (0.7 – $57 \text{ kU}\cdot\text{L}^{-1}$, median $14 \text{ kU}\cdot\text{L}^{-1}$) and total IgE values ranging from 70 to $14\,900 \text{ kU}\cdot\text{L}^{-1}$ (median $2911 \text{ kU}\cdot\text{L}^{-1}$), was used for the immunoblotting and RAST analysis to rMal f 7 and rMal f 9.

A different panel of sera, from 25 other AD patients with *M. furfur* RAST classes 1–4, median 2 (0.4 – $24 \text{ kU}\cdot\text{L}^{-1}$, median $6.5 \text{ kU}\cdot\text{L}^{-1}$) and total IgE levels between 53 and $16\,200 \text{ kU}\cdot\text{L}^{-1}$ (median $1996 \text{ kU}\cdot\text{L}^{-1}$), was used to perform the IgE reactivity analysis to rMal f 8. This was due to the fact that rMal f 8 was obtained at a later time point, and that the 25 sera used for the analysis of the rMal f 7 and rMal f 9 did not have a sufficient volume also for the rMal f 8 IgE reactivity study. For negative controls, one serum with a total IgE value of $969 \text{ kU}\cdot\text{L}^{-1}$ but a negative RAST response to *M. furfur* whole extract was chosen. A human myeloma IgE (DES) diluted in normal serum to $5000 \text{ kU}\cdot\text{L}^{-1}$ was used as an additional control [20]. The study was approved by the ethics committee at the Karolinska Hospital.

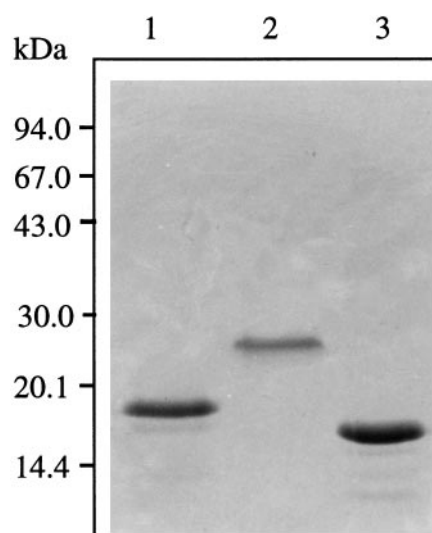


Fig. 2. SDS/PAGE and Coomassie Brilliant Blue staining of expressed recombinant proteins. Lane 1, rMal f 7; lane 2, rMal f 8; lane 3, rMal f 9. Molecular mass markers (Amersham Pharmacia Biotech) are indicated on the left.

RAST to rMal f proteins

The specific IgE-reactivity to the *E. coli* produced rMal f 7, rMal f 8 and rMal f 9 proteins was measured with Pharmacia CAP System RAST®FEIA under standardized conditions, as recommended by the manufacturer (Pharmacia & Upjohn Diagnostics AB). The recombinant allergens were covalently coupled to the cellulose solid phase (ImmunoCAP). A positive RAST result was defined as a value of at least $0.35 \text{ kU}\cdot\text{L}^{-1}$.

Immunoblotting and inhibition study

The purified recombinant allergens ($2 \mu\text{g}$ per mm gel) were denatured by boiling under reducing conditions and run on 15% SDS/PAGE and either stained with Coomassie Brilliant Blue or blotted onto poly(vinylidene difluoride) (PVDF) membranes (Millipore Corp., Bedford, MA, USA). For the immunoblotting analysis, each membrane was cut into strips and incubated with the serum samples diluted 1 : 5 in NaCl/ P_i containing 0.2% BSA and 0.1% Tween 20. Blotting and detection of IgE-binding proteins were performed as described previously [13].

For the inhibition analysis, three serum samples were selected, with *M. furfur* and rMal f 7, rMal f 8 and rMal f 9 positive RAST values. The sera were incubated at room temperature for 1 h with 1.5 mg of the *M. furfur* extract or

Table 2. Results of RAST and immunoblotting to *M. furfur* extract, rMal f 7 or rMal f 9 for 25 sera from patients with AD. The values of RAST class ≥ 4 , 3, 2 and 1 are ≥ 17.5 , 3.5 , 0.7 and $0.35 \text{ kU}\cdot\text{L}^{-1}$, respectively.

	No. of patients positive in RAST (class) to <i>M. furfur</i> whole extract	No. (%) of patients with positive RAST to		No. (%) of patients with positive immunoblotting to	
		rMal f 7	rMal f 9	rMal f 7	rMal f 9
	9 (≥ 4)	8 (89)	4 (44)	6 (67)	4 (44)
	12 (3)	6 (50)	4 (33)	3 (25)	1 (8)
	4 (1–2)	1 (25)	1 (25)	1 (25)	1 (25)
Totals	25	15 (60)	9 (36)	10 (40)	6 (24)

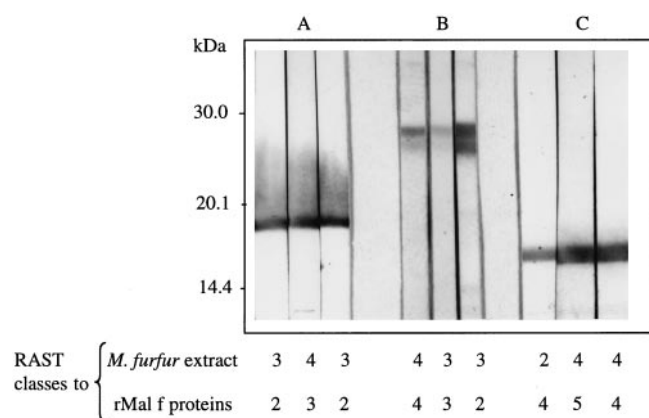


Fig. 3. Immunoblot analysis of purified recombinant allergens using sera from AD patients with different RAST classes to *M. furfur* whole extract and rMal f proteins. (A) rMal f 7, (B) rMal f 8, (C) rMal f 9. Data from three positive sera are shown for each recombinant allergen. Molecular mass markers are indicated on the left.

3 mg BSA (negative control). To detect elimination of the IgE binding to the recombinant proteins, preincubated sera were used in immunoblotting as described previously [13].

RESULTS

Amplification, subcloning and sequencing of the cDNA clones

To obtain the complete cDNA sequence of each clone, RACE-PCR, nucleotide sequencing and standard cloning techniques were performed. By designing primers in the available sequences of the cDNAs, we performed RACE-PCR to amplify the 5' end of each cDNA. The primers 7 R1, 8 R1 and 9 R1 (Table 1) were used to amplify the 5' ends of MF 7, MF 8 and MF 9, and gave PCR products 300, 450 and 950 bp in size, respectively (data not shown). The specificity of the PCR products was determined by performing nested PCR with the following primers: 7 R2 (Mal f 7), 8 R2 (Mal f 8) and 9 R2 (Mal f 9), together with the adaptor primer 1 (AP 1) of the Marathon kit (see Materials and methods). The PCR products of Mal f 7 and Mal f 8 were subcloned into the PT7 blue vector and sequenced from both ends using primers inside the vector (see Materials and methods). In contrast, the 950 bp RACE-PCR product of Mal f 9 was sequenced directly from both ends using the gene-specific primers 9 R1, 9 R2, 9 F1 or 9 F2. Nucleotide sequence analysis revealed that the full sequences of Mal f 7 (754 bp), Mal f 8 (773 bp) and Mal f 9 (1103 bp)

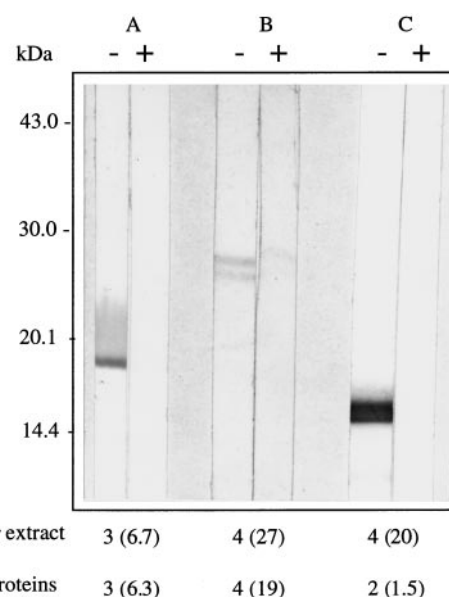


Fig. 4. Inhibition of IgE binding of *M. furfur* RAST positive sera to purified recombinant allergens by *M. furfur* whole extract. The sera were preincubated with NaCl/P_i (-) or *M. furfur* extract (+) and further incubated with either rMal f 7 (A), rMal f 8 (B) or rMal f 9 (C), blotted onto membranes. Molecular mass markers are indicated on the left.

encode ORFs of 141, 179 and 126 amino-acid residues, respectively (Fig. 1A–C). This corresponds to a calculated molecular mass/pI value of 16.2 kDa/7.51 (Mal f 7), 19.2 kDa/5.30 (Mal f 8) and 14.0 kDa/6.35 (Mal f 9). Database searches showed no significant homology to earlier published genes.

Protein sequence analysis of Mal f 8, revealed the presence of a region of predominantly hydrophobic residues, which may represent a signal sequence [21] (Fig. 1B). The cleavage site of the signal sequence is most likely between the amino-acid residues at positions 19 and 20 [22], giving a mature protein with a calculated molecular mass of 17.4 kDa. The primer pairs (7 F + 7 R) (8 F + 8 R, excluding the signal sequence) and (9 F + 9 R; Table 1), were used to amplify the sequences coding for the presumptive mature proteins of Mal f 7, Mal f 8 and Mal f 9, respectively.

Expression of the complete cDNA clones

The amplified coding sequence of each cDNA was subcloned into the expression vector pET 17b and expressed as a highly pure 6-histidine-tagged protein. The expressed proteins were present mainly in the inclusion bodies. The recombinant proteins showed apparent molecular masses of 19 kDa for

Table 3. Results of RAST and immunoblotting to *M. furfur* extract or rMal f 8 for 25 sera from patients with AD. The values of RAST class ≥ 4 , 3, 2 and 1 are ≥ 17.5 , 3.5, 0.7 and 0.35 kU·L⁻¹, respectively.

	No. of patients positive in RAST to <i>M. furfur</i> whole extract	No. (%) of patients with positive RAST to rMal f 8	No. (%) of patients with positive immunoblotting to rMal f 8
	3 (≥ 4)	3 (100)	3 (100)
	11 (3)	10 (91)	5 (45)
	11 (1–2)	5 (45)	2 (18)
Totals	25	18 (72)	10 (40)

rMal f 7 and 17 kDa for rMal f 9 (Fig. 2), which were in agreement with their corresponding calculated molecular masses. There was, however, a difference between the calculated and the apparent (26 kDa) molecular mass of rMal f 8 (Fig. 2).

IgE reactivity

To test if sera from AD patients, RAST positive to *M. furfur* extract, could recognize the recombinant allergens, serum samples were analyzed with RAST and IgE immunoblotting. The recombinant allergens showed positive RAST values of 60% for rMal f 7, 72% for rMal f 8 and 36% for rMal f 9 (Tables 2 and 3). The IgE-binding frequency was 40% for both rMal f 7 and rMal f 8, and 24% for rMal f 9 in immunoblotting (Tables 2 and 3). Examples of sera recognizing the recombinant allergens are shown in Fig. 3. Immunoblotting of rMal f 8 showed the presence of two bands (see Discussion). Neither the myeloma IgE nor the serum with negative RAST to *M. furfur* extract recognized any of the recombinant allergens (data not shown). Among the 25 sera used for IgE reactivity analysis to rMal f 7, nine (36%) were positive both in RAST and in immunoblotting. The corresponding values for rMal f 8 and rMal f 9 were 10 (40%) and six (24%) of the serum samples, respectively. Furthermore, 24 and 8% of the serum samples recognized both rMal f 7 and rMal f 9 with the RAST and immunoblotting test, respectively.

Inhibition analysis

Whole extract of *M. furfur* (1.5 mg·mL⁻¹), preincubated with *M. furfur* RAST positive sera from AD patients, could specifically inhibit the ability of the sera to recognize the recombinant allergens (Fig. 4). BSA did not inhibit binding of serum IgE to the recombinant allergens (data not shown).

DISCUSSION

Using a phage display library, we previously reported cloning and expression of five new *M. furfur* allergens [19]. Two of these (Mal f 5 and Mal f 6), showed complete cDNA sequences, whereas the other three (MF 7, MF 8 and MF 9) were not full length. In this study, we report the cloning and expression of complete sequences of the cDNA clones Mal f 7, Mal f 8 and Mal f 9, and characterized the expressed recombinant proteins from them. The ORFs of Mal f 7, Mal f 8 and Mal f 9 code for proteins of 16.2, 19.2 and 14.0 kDa, respectively, and moreover, they were able to bind serum IgE from AD patients. There was an agreement between the calculated and the gel-estimated molecular masses of rMal f 7 and Mal f 9. However, the observed discrepancy between the molecular masses of rMal f 8 could be due to its abnormal migration on gel, as described for other proteins [23].

Database searches revealed no significant homology to any other known proteins, and therefore, these allergens seem to be unique in their sequences as shown previously for Mal f 1 [16]. The ATG translational start codons of Mal f 7, Mal f 8 and Mal f 9 were most probably at nucleotide positions 139, 9 and 180 of the cDNA clones, respectively. This was supported by the fact that several PCR amplifications and sequencing of the 5' ends of Mal f 7, Mal f 8 and Mal f 9 did not show presence of another in-frame ATG codon upstream of the above-indicated start codons. Furthermore, in Mal f 8, the subsequent sequence following this ATG, codes for a stretch of hydrophobic amino-acid residues which form a signal sequence.

The importance of disulfide bonds in maintaining the antigenic structure of allergens and their IgE reactivity has been shown previously [24]. The Mal f 7 and Mal f 8 proteins contain several cysteine residues, which may form disulfide bonds. It will be interesting to investigate the role of these cysteines in these mechanisms. Because none of the recombinant proteins contain N-glycosylation sites, together with the previously shown data from our group indicating the expression system in *E. coli* to be a good choice for expressing Mal f 1, Mal f 5 and Mal f 6 [19,25], the purified allergens in this study were also expressed in *E. coli*. The IgE binding data showed that 60–72% (based on RAST analysis) and 40% (based on immunoblotting) of the tested patient sera, could recognize the purified rMal f 7 or rMal f 8, whereas, the number of sera recognizing the rMal f 9 was lower. The discrepancy between the RAST and the immunoblotting result could be that the analyzed sera were undiluted in RAST, whereas, they were diluted (1 : 5) in the immunoblotting. In addition, it cannot be excluded that differences in the conformation of the recombinant proteins due to their coupling to RAST compared with their transfer onto PVDF membrane could influence the result. The inhibition study showed the ability of the *M. furfur* extract to completely inhibit the binding of serum IgE to the recombinant allergens, which indicate that all the recombinant allergens contain the IgE binding epitopes of their natural counterparts in the extract. In Figs 3 and 4, immunoblotting of the rMal f 8 revealed the presence of two bands, which were blocked equally by *M. furfur* extract in the inhibition study. Therefore, it is likely that the smaller protein band represents a degradation product of the larger protein.

The IgE binding data of rMal f 7 and rMal f 8 indicate the importance of these allergens and, together with other previously cloned rMal f proteins [16–19]; they may help improve diagnosis of AD based on recombinant allergens, as shown for the diagnosis of fungal allergy to *A. fumigatus* in a recent study [11]. At present, IgE reactivity to different allergens is based mainly on crude allergen extracts which can vary in allergen content and may contain heterogeneous mixtures of proteins [14]. Furthermore, some allergens may be degraded during preparation of the extract which is difficult to standardize.

Therefore, a cocktail of recombinant allergens has many advantages and may, in future, replace crude extracts in diagnosis of allergy. We are now investigating the clinical and immunohistochemical skin prick test and atopy patch test responses to purified rMal f proteins of *M. furfur* in a multicenter study of patients with AD. In addition, recombinant allergens can be used as tools to investigate the role of IgE binding proteins in the pathogenesis of AD [12].

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