

RESEARCH LETTER

Expression of Alt a 1 allergen from *Alternaria alternata* in the yeast *Yarrowia lipolytica*

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Abstract

Allergies affect almost 25% of the population in industrialized countries. *Alternaria alternata* is known to be a significant source of aeroallergens and sensitization to this mold is a risk factor for the development of wheezing, asthma, and atopic dermatitis. Diagnosis and treatment of allergies requires the production of large amounts of pure and well defined protein. *Yarrowia lipolytica*, a non-pathogenic ascomycete able to secrete high levels of enzymes that can grow in inexpensive substrates, has been considered a useful host for heterologous gene expression. In the present work, we have developed two vectors for expressing Alt a 1, the most relevant *A. alternata* allergen, in *Y. lipolytica*. One vector is autosomal and one is integrative. With both systems, rAlt a 1 was secreted into the culture medium. The immunological characteristics of the purified recombinant allergen were determined by IgE-blot using sera from 42 *A. alternata*-allergic patients. We have carried out ELISA-inhibition experiments using sera from four patients to compare the IgE-binding capacity of natural and recombinant allergens. Our results show that *Y. lipolytica* is able to produce a recombinant Alt a 1 which is immunochemically equivalent to the natural counterpart and could be used for immunotherapy and diagnostics.

Introduction

Type I allergy, a genetically determined IgE-mediated hypersensitivity, affects almost 25% of the population in developed countries (Gergen *et al.*, 1987). Fungi are associated with allergic diseases, and their major allergic manifestations are: asthma, rhinitis, allergic bronchopulmonary mycosis, and pneumonitis (Burge, 1989; Kurup, 1989; Cramer *et al.*, 2006). *Alternaria alternata* is an important source of aeroallergens and 95–99% of American homes have detectable amounts of *Alternaria* antigens (Salo *et al.*, 2005, 2006). Sensitization to *A. alternata* is an important risk factor for development of wheezing and asthma in children (Halonen *et al.*, 1997; Bartra *et al.*, 2009). Alt a 1 is its major allergen, with a sensitization frequency > 80% and a 29-kDa dimeric structure which dissociates into 14.5- and 16-kDa subunits under

reducing conditions (Achatz *et al.*, 1995; De Vogue *et al.*, 1996).

Allergen extracts prepared from natural source materials are used in the diagnosis and treatment of mold allergies. These extracts are heterogeneous products containing allergenic and non-allergenic proteins. They vary in allergen composition and content, and cross-reactivity of *A. alternata* antigens with antigens from non-related fungi has been described (Schmechel *et al.*, 2008). Therefore, recombinant allergens offer a promising new strategy to replace traditional allergen extracts for diagnosis and allergen-specific immunotherapy.

Escherichia coli, the preferred host for recombinant protein production, contains several bottlenecks, such as incorrect protein folding or production of inclusion bodies that do not appear when the recombinant proteins are expressed in eukaryotic systems. Yeasts offer a

number of advantages as expression systems for complex proteins. As unicellular organisms, they retain the advantages of bacteria in ease of manipulation and growth capacity. But they also have a eukaryotic subcellular organization, which enables them to perform post-translational processing of complex proteins. Additionally, secretion in yeast occurs via a multi-component apparatus, allowing disulfide bond formation, proteolytic maturation, N- and O-linked glycosylation, and other post-translational modifications. Of note, almost all natural allergens are derived from eukaryotic sources and frequently contain intramolecular disulfide bonds as well as post-translationally linked carbohydrates. The yeast most frequently used for allergen expression has been *Pichia pastoris* (Bollok et al., 2009; Pokoj et al., 2010; Stadlmayr et al., 2010) but other yeasts such as *Yarrowia lipolytica* have been found to be attractive alternative host organisms for recombinant protein expression and could be used for allergen expression (Domínguez et al., 1998; Muller et al., 1998).

Yarrowia lipolytica is a hemi-ascomycetous dimorphic fungus that belongs to the order *Saccharomycetales*. The natural habitats of this fungus are oil-polluted environments and foods such as cheese, yoghurt, meat, and poultry products. It naturally produces several enzymes such as proteases, lipases, and esterases (Barth & Gaillardin, 1996) which can be secreted via the co-translational pathway, similar to what occurs in higher eukaryotes (Boisramé et al., 1998). Additionally, *Y. lipolytica* is considered to be non-pathogenic and several processes based on the use of this fungus were classified as 'generally recognized as safe' by the Food and Drug Administration (FDA). Because of the large number of genetic markers and molecular tools available, this yeast is considered an efficient heterologous protein production system (Muller et al., 1998; Gasmi et al., 2011; Rao et al., 2011). Several *Y. lipolytica* promoters have been used for recombinant protein expression (Domínguez et al., 1998; Muller et al., 1998; Wang et al., 1999; Pignède et al., 2000). The copper-inducible bi-directional promoter of *YIMTPI* and *YIMTPII* genes has been characterized previously (García, 1993; Domínguez et al., 2003).

In this work, we report the expression of the major allergen Alt a 1 of *A. alternata* using *Y. lipolytica*. The recombinant allergen shows immunological characteristics similar to those of the natural allergen and could be used for immunotherapy and diagnostics.

Material and methods

Strains, media and growth conditions

The *Y. lipolytica* strains used in this study were E150 (*MatB*, *leu2-270*, *ura3-302*, *his1*, *xpr2-322*) and W29

(*Mata*). The yeast media used were YEPD (yeast extract 1%, peptone 2%, glucose 1%) and Yeast Nitrogen Base (YNB 0.7%, glucose 1%). For allergen production, 50 mL of 0.7% YNB medium (Difco, Detroit, MI) supplemented with 1% glucose, 0.2 mM uracil, and 0.3 mM histidine, was inoculated with an isolated colony from a YNB-agar plate and grown overnight at 28 °C with agitation. Cells were collected by centrifugation at 3000 g for 5 min and resuspended at an OD_{600 nm} of 0.5 in 200 mL of the same medium. When the culture reached an OD of 0.8–1.0, CuSO₄ was added to a final concentration of 0.4 mM, and the culture continued to grow for 24 h. Spent culture medium was concentrated and diafiltered using a 5000-kDa cut-off membrane (Amicon, Austin, TX).

Patients

Sera from 42 patients allergic to *A. alternata* (18 female and 24 male; mean age, 20.5 years; age range, 10–33 years) and 17 control subjects (11 female and six male; mean age, 32.3 years; age range, 14–70 years) were included in the study. Diagnosis of *A. alternata* allergy was based on a clinical history of recurrent rhinitis (four patients), asthma (four patients), rhinoconjunctivitis (12 patients), rhinitis and asthma (12 patients), rhinoconjunctivitis and asthma (10 patients); a positive cutaneous response to a commercial *A. alternata* extract (Bial-Aristegui, Bilbao, Spain); and specific IgE to *A. alternata* extract > 0.35 IU mL⁻¹ according ImmunoCAP (Thermo-Fisher, Uppsala, Sweden). Eight healthy subjects and nine allergic individuals sensitized to different allergenic sources unrelated to *A. alternata*, as demonstrated by negative SPT responses and lack of *A. alternata*-specific IgE, were used as controls.

Genetic techniques

Standard molecular genetic techniques were used (Sambrook et al., 1989). For Southern blotting, genomic DNA was prepared from recombinant yeasts as previously described (Barth & Gaillardin, 1996). Afterwards, DNA was digested, separated on a 0.8% agarose gel, and transferred onto Hybond-N+ nylon membranes (GE-Healthcare, Little Chalfont, Buck, UK). Probes were labeled with [³²P]-dCTP using the MegaPrime Kit (GE-Healthcare).

Construction of pMMR4 and pMMR10 vectors

The autosomal vector pMM4 was used to express the Alt a 1 allergen. The *YIMETII* promoter was obtained from plasmid pSG70 (García, 1993; Domínguez et al., 2003) and cloned between the EcoRI-BamHI restriction

sites of the pBluescript-SK. The Alt a 1-coding gene sequence (Asturias *et al.*, 2003) was cloned after this promoter into the BamHI site, resulting in the pMMR2 vector. As the insertion of the target gene between yeast promoter and terminator sequences produces more efficient expression of heterologous genes in *Y. lipolytica* (Franke *et al.*, 1998), the *YISTE7* terminator was amplified using specific primers and cloned into the SpeI and XbaI-restriction sites of pMMR2, resulting in the pMMR3 plasmid. This plasmid was digested with ClaI and XbaI and the 1.8 kb-fragment containing the fusion of the *YIMTPII* promoter-Alt a 1-*YISTE7*-terminator was purified and inserted into the pINA240 plasmid (Barth & Gaillardin, 1996), giving rise to pMMR4. The correct construction was verified by sequencing.

The construction of the integrative plasmid pMMR10 was performed as follows. To create the pMMR10 plasmid, the 1.8-kb ClaI-SphI fragment from pMMR4 carrying the *YIMTPII* promoter-Alt a 1-*YISTE7*-terminator fusion was cloned into the pINA62 plasmid. The correct construction was verified by sequencing. Plasmid maps of pMMR4 and pMMR10 and sequences of the specific primers used for *YISTE7* terminator amplification are available as Supporting Information (Fig. S1, Table S1).

Purification of natural and recombinant Alt a 1

nAlt a 1 was purified from *A. alternata* CBS 603.78 spent culture medium after 3 weeks of static growth in Czapeck broth at 25 °C. Spent culture medium was concentrated and diafiltrated with a 5000-kDa cut-off. Purification of natural and recombinant Alt a 1 was achieved by immunoaffinity using an immunosorbent column prepared by coupling rabbit anti-Alt a 1 polyclonal antibodies to CNBr-activated Sepharose-4B (GE-Healthcare) (Asturias *et al.*, 2003). Briefly, *A. alternata* and *Y. lipolytica* spent culture media were passed through the immunoaffinity column and after extensive washing with phosphate-buffered saline (PBS), bound protein was eluted using 100 mM citrate buffer, pH 2.7. Fractions were collected in tubes containing neutralizing buffer (1 M Tris-HCl, pH 8.0) and those containing Alt a 1 were pooled, concentrated by ultrafiltration, and stored in aliquots at -40 °C.

Electrophoresis and Western blotting

Alternaria alternata extracts and purified Alt a 1 were separated by SDS-PAGE under reducing and non-reducing conditions (Laemmli, 1970). Protein bands were detected by Coomassie Blue or silver staining. For Western blotting, the separated proteins were transferred electrophoretically to PVDF (Hybond-P; GE-Healthcare). After blocking, membranes were incubated at 4 °C overnight

with human sera or rabbit anti-Alt a 1 antiserum, then washed, and bound antibodies were detected with anti-human IgE or anti-rabbit IgG conjugated to horseradish peroxidase (Dako, Copenhagen, Denmark). The blot was then washed and developed by ECL+ (GE-Healthcare). For IgE-dot blot, 200 ng in 2 µL PBS was dotted onto a nitrocellulose membrane (Hybond-C+; GE-Healthcare) and, after drying, the membrane was blocked and incubated as described above.

ELISA-inhibition assays

ELISA-inhibition assays were carried out on microtiter plates (Greiner, Frickenhausen, Germany) coated with 2 µg mL⁻¹ of nAlt a 1 in 0.1 M bicarbonate buffer, pH 9.6, and blocked with 1% bovine serum albumin (BSA), 0.05% Tween-20 in PBS. Human sera 100 µL (diluted 1 : 20 or 1 : 10), preincubated overnight at 4 °C with the inhibitor, were added to the wells, incubated for 1 h at 37 °C, and developed as previously described (Martínez *et al.*, 1985).

Circular dichroism (CD) analysis of the purified hybrid molecules

Far-UV (190–250 nm) CD spectra at pH 7.0 and 20 °C were recorded with a Jasco J-810 spectropolarimeter equipped with a Jasco PTC-423S temperature controller in cuvettes thermostated at 20 °C. The protein concentration was 0.035 mg mL⁻¹ in 20 mM phosphate buffer and 40 scans were accumulated. All the spectra had the appropriate background subtracted and were then converted to mean residue ellipticity.

Results

Alt a 1 expression in *Y. lipolytica*

Yarrowia lipolytica E150 was transformed with the plasmid pMMR4 and the transformants were grown in YNB medium with and without copper sulfate. At various time intervals, the presence of Alt a 1 in the culture medium was assessed by Western blot showing a band around 15 kDa in reducing conditions (Fig. 1). Optimal production was observed after 24 h incubation in the presence of 0.4 mM CuSO₄. No intracellular Alt a 1 could be detected, suggesting that the natural signal peptide of the Alt a 1 directs the secretion of the allergen to the culture medium. pMMR4 was found to be segregationally stable, as only 30% of cells lost the plasmid after 16 generations in non-selective medium (YEFD).

Nevertheless, to further stabilize expression, integration of the expression cassette into the *Y. lipolytica* genome

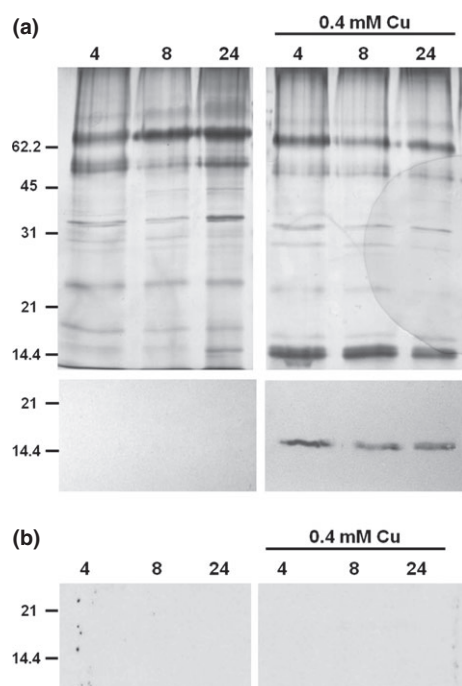


Fig. 1. (a) SDS-PAGE profile (top) and Western blot (bottom) of *Yarrowia lipolytica* culture supernatants harboring the pMMR4 plasmid at 4, 8 and 24 h after inoculation in a medium with and without 0.4 mM of copper sulfate under reducing conditions. Size standards are indicated on the right. (b) Western blot of non-transformed cells under the same conditions. Western blots were incubated with rabbit anti-Alt a 1 antiserum.

was carried out. The integrative vector pMMR10 has a unique NotI site that allows linearization and integration of the vector into the *YILEU2* gene. NotI-digested vector was used to transform *Y. lipolytica* E150 strain and the transformants were selected for a Leu⁺ phenotype on YNB medium. Three transformants (T1, T2 and T3) were analyzed by Southern blot using a fragment of the *YILEU2* gene as a probe. The W29 strain, carrying the complete *YILEU2* gene, was used as a control. Two of the transformants (T2 and T3) showed a single copy of the vector correctly integrated into *YILEU2* gene (Fig. 2). Transformant T2 was used in subsequent experiments.

Transformant cells were grown in YNB media with and without copper sulfate. The time course of the Alt a 1 expression/secretion was examined by SDS-PAGE run under reducing conditions and Western blot (Fig. 3). No recombinant allergen was detected when transformants were grown without copper sulfate. The recombinant Alt a 1 was purified from the culture medium with a yield of approximately 0.5 mg L⁻¹ culture.

nAlt a 1-specific IgE from *A. alternata*-allergic patients recognizes epitopes on the recombinant counterpart

Natural and rAlt a 1 were purified from *A. alternata* and *Y. lipolytica* supernatant culture media, respectively, by immunoaffinity chromatography. A high degree of purity

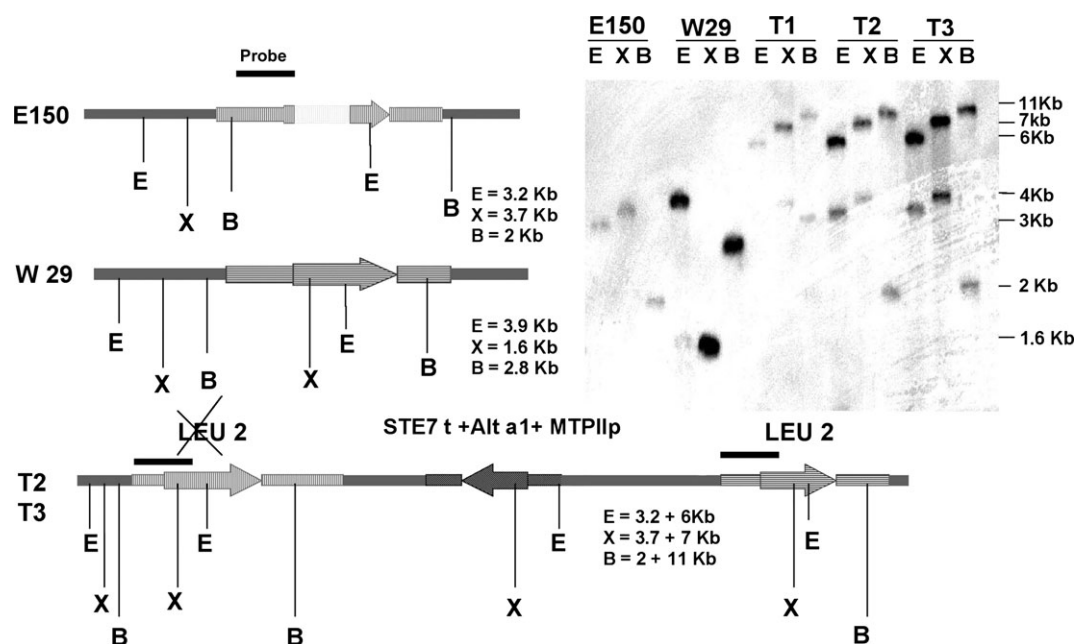


Fig. 2. Verification of the integration of rAlt a 1 in *Yarrowia lipolytica*. Genomic DNA from E150, W29 and transformant (T1, T2, T3) *Y. lipolytica* strains were digested with EcoRV (E), XbaI (X) and BamHI (B). A fragment of the *YILEU2* gene was used as a probe. Size standards are indicated on the right.

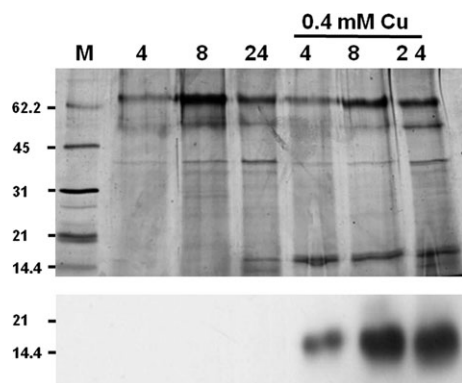


Fig. 3. Time course for the expression of rAlt a 1 in *Yarrowia lipolytica* carrying the integrative plasmid pMMR10. Supernatants from cultures of cell growth with and without copper sulfate were harvested at different times (4, 8, and 24 h) and analyzed by SDS-PAGE run under reducing conditions. Proteins were detected by silver staining and Western blot incubated with rabbit anti-Alt a 1 antiserum. Size standards are indicated on the right. Western blot is shown at the bottom.

was achieved with this single-step purification procedure, as was demonstrated by SDS-PAGE (Fig. 4a).

Comparison of natural allergen from *A. alternata* and the recombinant form produced in *Y. lipolytica* was performed using Western blot, dot blot, and ELISA-inhibition experiments with sera from *A. alternata*-allergic patients. Natural and recombinant Alt a 1 migrate as a 28-kDa protein under non-reducing conditions, but the protein breaks down into 16- and 15-kDa subunits under reducing conditions. Western blotting showed that both natural and recombinant allergen reacted with IgE from the pool of patients' sera, in both reducing (data not shown) and non-reducing conditions (Fig. 4a). Reactivity of natural and recombinant Alt a 1 against rabbit anti-Alt a 1 serum, checked by Western blotting, showed similar results (data not shown).

An IgE-dot blot was performed to confirm recognition by human IgE of non-denatured Alt a 1 proteins using sera from 42 *A. alternata*-allergic patients and 17 control patients. In our population of patients, 41 sera (97.6%) reacted with nAlt a 1 and 37 (88.1%) with its recombinant counterpart (Fig. 4b). None of the sera from control patients reacted with Alt a 1 in the IgE-dot blot assay (data not shown).

IgE binding to Alt a 1 and its recombinant counterpart was quantitatively evaluated by ELISA inhibition experiments performed by coating wells with nAlt a 1 and comparing the IgE binding capacity of nAlt a 1 and rAlt a 1. Sera from four patients (1, 4, 20, and 24) who reacted with both Alt a 1 allergen forms were used for these analysis. Natural and rAlt a 1 displayed the same

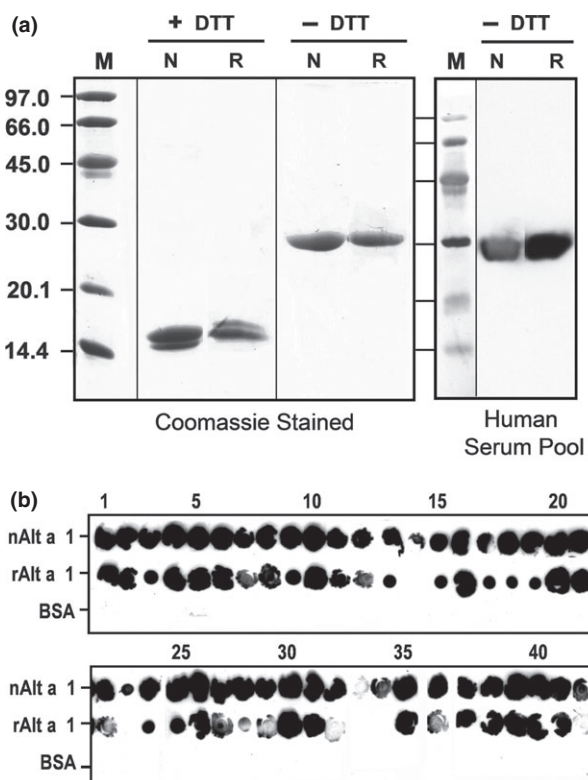


Fig. 4. (a) Immunoblots of *Alternaria alternata* allergens: 0.5 µg of nAlt a 1 (lane N) and rAlt a 1 (lane R) were run in the presence and absence of dithiothreitol (DTT). Western blots were incubated with a pool of sera from patients with *A. alternata* allergy. (b) Dot-blots from nAlt a 1, rAlt a 1, and BSA were spotted onto nitrocellulose membrane and incubated with individual sera from *A. alternata*-allergic patients.

extent of binding inhibition to specific IgE antibodies against Alt a 1, indicating that natural and recombinant proteins share similar allergenic determinants (Fig. 5a).

CD spectra of natural and rAlt a 1 were nearly identical and presented the typical folding pattern of proteins with a high component of β -sheet structures and a low percentage of α -helix (Fig. 5b). The thermal stability of both proteins in reduced and oxidized conditions was also very similar (data not shown).

Discussion

Yeasts are excellent factories for expressing heterologous proteins and the methylotrophic *P. pastoris* is the most popular one (Pokoj *et al.*, 2010; Stadlmayr *et al.*, 2010). It has been used to express a variety of allergens (González *et al.*, 2001; Calabozo *et al.*, 2003) but its expression system can sometimes give problems of low protein secretion levels or hyperglycosylation (Cereghino *et al.*, 2002). In recent years, the yeast *Y. lipolytica* has gained interest as a producer of heterologous proteins such as β -glucanase,

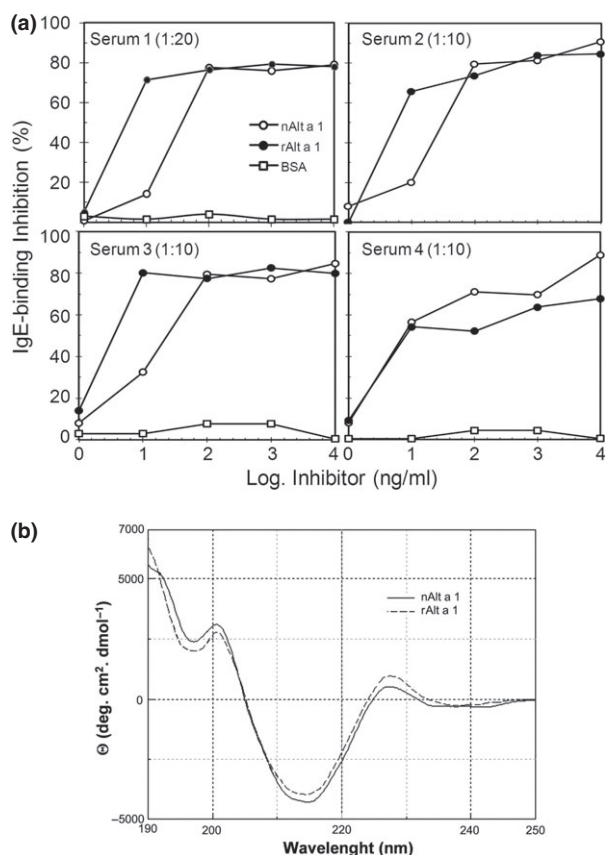


Fig. 5. (a) ELISA-inhibition curves. The binding of specific human IgE from four individual patients (1, 20, 24, and 4, respectively) to nAlt a 1 was inhibited using nAlt a 1 and rAlt a 1. BSA was used as control. Values represent means of three different experiments and error bars are one standard deviation in length. (b) CD spectra of natural and recombinant Alt a 1.

proteases, alginate lyase, and epoxide hydrolases (Madzak *et al.*, 2004; Bankar *et al.*, 2009; Gasmi *et al.*, 2011; Rao *et al.*, 2011) but its use in the expression of allergens has not been reported thus far.

In the present study, we used two different vectors for the expression of the *A. alternata* allergen Alt a 1 in *Y. lipolytica* and demonstrated that both vectors (replicative and integrative constructions) are useful tools for the production and secretion of the recombinant allergen. The yield of rAlt a 1 expression in *Y. lipolytica* was about 0.5 mg L⁻¹ in our experimental conditions. Vectors based on autonomously replicative sequences (ARS), such as those used in the present work, are present in a copy number of 1–10 per cell but they are quite unstable (Vernis *et al.*, 1997). Our results have shown that the integrative vector pMMR10 was stably integrated in the *Y. lipolytica* genome, although one copy per cell was present. However, integrative plasmids, which offer the possibility of multiple integrations (up to 60 copies), have been developed for this yeast (Le Dall *et al.*,

1994; Juretzek *et al.*, 2001) and experiments to assess productivity with these constructions are underway. Although expression of heterologous proteins can be obtained in shake-flask culture, protein levels are typically much higher in fermenter cultures. For example, the yield of the recombinant endoglucanase I of *Trichoderma reesei* produced in *Y. lipolytica* was increased approximately 20-fold when fed-batch fermentation at high-cell density was used (Park *et al.*, 2000). On the other hand, we have demonstrated the use of the *YIMTPI-II* promoter genes to direct the expression of the heterologous protein. To date, many different endogenous *Y. lipolytica* promoters have been used for heterologous expression (i.e. TEF, EXP, FDA, GPAT, GPD, XPR2) (Muller *et al.*, 1998; Nicaud *et al.*, 1989; Damude *et al.*, 2006). One of the strongest promoters is the XPR2 but it has complex requirements for induction that hinder its industrial applications. However, the *YIMTPI-II* promoter has several advantages: (1) it only requires the presence of copper in the culture medium, (2) the inducer is added at the beginning of the process and (3) copper is an inexpensive inducer. Moreover, the expression capacity for this promoter could be increased by designing synthetic hybrid promoters by fusing an upstream activation sequence (UAS) to the core promoter region (Blazek *et al.*, 2011). The combination of using modified promoters, multiple gene integrations, and fed-batch fermentations could improve the production of rAlt a 1 in *Y. lipolytica*.

rAlt a 1 showed a similar secondary structure and immunoreactivity to that of its natural counterpart. Moreover, IgE-dot blot using 42 sera from *A. alternata*-allergic patients and ELISA-inhibition experiments demonstrated that most of the IgE-binding epitopes were retained in the recombinant allergen. Only four of 41 sera from natural Alt a 1-reacting allergic patients did not react by IgE-dot blot with the recombinant allergen. Similar results were found by skin testing in 55 *A. alternata*-allergic patients using natural and recombinant Alt a 1 (Asturias *et al.*, 2005). CD shows that secondary structures of natural and recombinant Alt a 1 are similar but we cannot rule out the existence of some differences between both forms. Additionally, allergen isoforms have been reported with different reactivities in allergic patients (Wagner *et al.*, 2008). To date, no Alt a 1 isoforms produced by a single *A. alternata* strain have been reported, although there is only partial information about *Alternaria* genomes (<http://0-www.ncbi.nlm.nih.gov/ilsprod.lib.neu.edu/Traces/wgs/?val=ACIW>) and therefore the existence of multigene origin of Alt a 1 cannot be ruled out. In any case, the use of recombinant purified recombinant allergens is likely to overcome the problems associated with natural extracts in spite of some decrease in sensitivity (Valenta *et al.*, 2011). IgE reactivity assays using dot-blot were performed with control sera from 17 control patients, eight healthy subjects and nine allergic individuals sensitized to

different allergenic sources unrelated to *A. alternata*. No IgE-binding activity was detected, confirming the high specificity of the assay using purified allergens.

Yarrowia lipolytica could be a useful expression system as it is able to grow in inexpensive media as alkanes, fatty acids, and oil. This is very important when large volume fermenters have to be used. Additionally, inexpensive chemicals, such as the copper sulfate used in this work, could be used as inducers.

In conclusion, heterologous expression of *A. alternata* allergen Alt a 1 was successfully achieved in *Y. lipolytica*. Recombinant Alt a 1 was secreted into the culture medium and detected as a dimer (under non-reducing conditions) similar to the natural allergen. Natural and recombinant Alt a 1 proteins share secondary structure and IgE-binding determinants and skin testing shows a similar reactivity. These results confirm that rAlt a 1 could be an effective candidate for the development of diagnostic and therapeutic approaches and that *Y. lipolytica* has become an attractive host for the expression of complex proteins such allergens.

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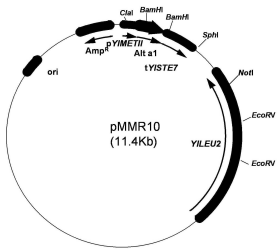
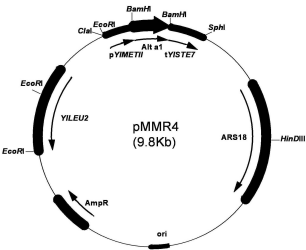
Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Plasmid maps of the ARS-based plasmid pMMR4 and pMMR10.

Table S1. Oligonucleotides used to amplify the *YISTE7* terminator.

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Expression of Alt a 1 allergen from *Alternaria alternata* in the yeast *Yarrowia lipolytica*

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28 **Table S1:** Oligonucleotides used to amplify the *YISTE7* terminator.

Oligonucleotide	Sequence 5'-3'
STE1 f	ttttACTAGTtggtgcatagagtggacgtt
STE1 r	ttttTCTAGAtgccaccagtaacttgaaa

29 *SpeI* and *XbaI*-restriction sites are marked.

30

31 **Figure legend**

32 **Figure S1:** Plasmid maps of the ARS based plasmid pMMR4 and pMMR10.