

Using Human Sera to Identify a 52-kDa Exoantigen of *Penicillium chrysogenum* and Implications of Polyphasic Taxonomy of Anamorphic Ascomycetes in the Study of Antigenic Proteins

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Received: 30 October 2008 / Accepted: 23 June 2009
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Abstract We are interested in isolating and identifying antigenic fungal proteins from species that grow on damp building materials. The indoor clade of *Penicillium chrysogenum*, the so-called Fleming clade, is the most common species of *Penicillium* on moldy building materials. We have identified a 52-kDa marker protein for the indoor clade of *P. chrysogenum* not present in a taxonomically diverse selection of fungi. It is found in high concentrations in protein extracted from the fungus grown on paper-faced gypsum wallboard. During this process, we illuminated the variability in response to patient sera and of strains of the fungus collected over a wide geographic area. From a collection of sera from all over the USA, 25 of the 48 patients reacted to the 52-kDa protein from this prescreened collection of sera. Most strain/antibody combinations had proportionate ELISA response associated with the presence of the target. However, approximately 25% of the strain/patient serum combinations included people who responded to many common allergens from the Penicillia. All the *P. chrysogenum* strains tested produced the target protein. However, there was considerable variability in patient IgG response to 32-, 30-, and 18-kDa antigens and in their production by the various clade 4 strains.

The target protein was not found in spores or culture extracts of a wide selection of relevant fungi. It appears that the previous studies have been conducted on strains of the fungus from the three clades not those associated with the built environment.

Keywords Exoantigen · *Penicillium chrysogenum* clade 4 · Paper-faced gypsum wallboard · Patient response

Introduction

The association between respiratory illness and indoor fungal contamination is consistent based on studies that depend on occupant reports for both symptoms and, with few exceptions, inadequate exposure assessments [1]. On the basis of that information, expert panels from the United States National Academy of Science in 2000 and 2004 and from Health Canada in 2004 found that mold and dampness were associated with increased upper respiratory disease and with increased asthma in mold-sensitive asthmatics [2–4]. Mold and dampness comprise a mixture of contaminants in addition to mold, notably house dust mites. Accumulations of endotoxin are also common in houses along with traffic pollutants [5].

In a study of 403 children, a 12–50% relative increase in symptom prevalence associated with

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reported mold contamination and excessive moisture remained significant both before and after adjusting for subject characteristics as well as dust mite antigens and endotoxins in settled dust [6]. In these houses, measured concentrations of *Aspergillus* and *Penicillium* in settled dust were higher when mold or mildew was reported than when not mentioned ($P = 0.01$). A comparison of a subset of the homes was made, half with higher areas of visible mold and moisture damage (1.2 m^2) and half from the lowest group (0.42 m^2). This difference in visible mold and moisture damage was associated with a 25-fold increase in colony-forming units/g (CFU/g) dust on malt extract agar (MEA) and a 10-fold increase in the proportion of *Aspergillus* plus *Penicillium* species to phylloplane species in the dust [7, 8].

Indoor moisture problems and the fungi that grow in those environments are at least partially responsible for many of the symptoms observed [6]. It has been essentially impossible to determine the attributable risk for building-associated fungi within the context of home dampness. For asthma, the proportion associated with mold has been estimated between 12–30% from population based-data [9, 10]. This is similar to estimates for exposures in the nonindustrial workplace [11]. However, it is also thought that allergy to mold is a very important risk factor for severe asthma [12, 13]. Unlike other contaminants including endotoxin, house dust mite, and various animal allergens, fungal exposures cannot be quantitatively measured and the only method currently envisaged needs to be based on allergens or antigens [2]. We are interested to identify proteins that occur on building materials that are antigenic in humans for the purpose of such exposure assessments [14, 15]. Most of the known fungal allergens come from phylloplane fungi, and there are serious problems with the specificity of some of the antibody-based detection systems even for the most familiar of these, *Alternaria alternata* [16, 17].

Penicillium chrysogenum is one of the most commonly isolated fungi on moldy building materials in the US and Canada and the most commonly detected *Penicillium* species. According to a compendium of datasets from a number of large commercial laboratories in Canada and the USA, *P. chrysogenum* was observed as a biodeteriogen on insulation, paper-faced gypsum board, wood, manufactured wood, and ceiling tiles [18]. It is also an important biodeteriogen on wooden

artifacts [19], frescos [20], photographic film [21], and archival papers [22] among other artifacts. It is a salt- and metal-tolerant, moderate xerophile with a minimum water activity (aw) in the range of 0.78–0.81 [23]. It can gain nutrition from different grades of paper [19, 23], soft wood [24], sawdust [19], gelatin [21], different formulations of polyethylene film, synthetic rubber and natural rubber [25], phenol [26], possibly lignin components [19] among others.

In a recent monograph of the subgenus *Penicillium*, Frisvad & Samson [27] placed *P. chrysogenum* in the section Chrysogena series Chrysogena, all of which produce penicillin. *P. chrysogenum* and *P. flavigenum* have similar metabolite patterns, but the remaining species have apparently distinct principal metabolite patterns. Other species included were *P. flavigenum*, *P. diposomyis*, and *P. nalgiovense*. Although *P. chrysogenum* is a well-accepted morphospecies [27], it is reliably known that it comprises four morphospecies, only one of which, clade 4 is common in the built environment [28, 29]. Strains of this clade from mainly Canadian buildings produced penicillin G, roquefortine C, and unexpectedly, mel-eagrins in high yield. Additionally, there appeared to be strains differentiated by their ability to accumulate xanthocillin X [28]. The Fleming clade shows evidence of the loss of a sexual phase [30].

Several studies have found case-report level associations between building-related illness and the *P. chrysogenum* contamination [31–33]. A population study of allergy to fungi in Germany in response to *P. chrysogenum* was most common of the building-material-associated fungi [32]. A study in Norway found that IgE levels in response to *P. chrysogenum* were associated with signs of mold and moisture [33]. In addition, exposure to *P. chrysogenum* resulted in an allergic response in a murine model [34, 35]. Considerable cross-reactivity between some *P. chrysogenum* IgE allergens and other fungi has been reported. For example, the allergens Pen ch 13 and 18 (molecular weights of 34 and 32 kDa, respectively) were shown to cross-react with extracts of *P. citrinum* and *P. brevicompactum* [36]. Phylogenetically, the former species is far from the same group within the genus. *P. brevicompactum* and *P. chrysogenum* are both in the subgenus *Penicillium* but in genetically divergent sections [37]. Evidently, these allergens are rather spread across the genus. Further IgE-cross-reactivity has been reported

between serine proteases from *P. chrysogenum*, *A. fumigatus*, and *C. herbarum* in other studies [38–40].

Currently, five different *P. chrysogenum* allergens (all IgE reactive) have been accepted by the International Union of Immunological Societies Allergen Nomenclature Sub-committee only three of which have been published [41]. These allergens presented were isolated from various *P. chrysogenum* strains, primarily cultured from Taiwan. There is no information whether these IgE allergens are common in clade 4 strains of *P. chrysogenum* in North America or about from which clade or strains they were found.

Culture conditions have a large effect on the production of proteins that might be used to produce antibodies for measuring fungal biomass. Proteins that might be present in spores, spore fragments, and mycelia in nature are not necessarily those first or easily accumulated in culture. This is why we have placed such weight on using collections of pre-screened human sera from a clinical testing laboratory with a large catchment area [14].

This report concerns the identification of a protein excreted by *P. chrysogenum* clade 4 antigens in humans, which accumulates in spores and is excreted in high abundance when the fungus grows on paper-faced gypsum board.

Materials and Methods

Human Polyclonal Antibodies

Sera from 48 patients were obtained from a clinical laboratory that serves the United States and Canada for routine and nonroutine allergen testing (IBT Reference Laboratory, Lenexa, KS). During diagnostic testing, these sera were tested using the Pharmacia ImmunoCAP® reagents for their response to various fungi [42].

Cultures

Isolates of *P. chrysogenum* from visibly moldy building materials or indoor air samples, selected for a geographic representation across the USA and Canada, were obtained from Paracel Laboratories (Ottawa, Ontario). Strains from single-spore isolates representing 40 genotypes were derived from mass cultures and transferred to 2% MEA slants (malt extract agar; Difco, Lawrence, KS). Their clades, chemotypes, and culture collection numbers are given in Table 1 [28]. Each strain was transferred onto sterile 2% malt extract agar tubes or 2% MEA plates, stored at 5°C, and then macerated in sterile ultrapure water. A 5% (v/v) aliquot was then used to inoculate

Table 1 *P. chrysogenum* strains used in this study

DAOM	Origin	Substrate	Meleagrín	Roquefortine	Penicillin G	Xanthocillin x	Clade
155627	CCI, Ottawa, ON	Paper	+	+	—	—	4
155628	CCI, Ottawa, ON	Paper	+	+	+	—	4
215336	Edmonton, AB	Wood studs	+	+	+	—	4
234051	Saskatoon, SK	Indoor air	+	—	+	+	4
234052	Ottawa, ON	Pipe wrap	+	—	+	+	4
234053	Ottawa, ON	Indoor air	—	+	—	+	1
234054	Calgary, AB	House dust	—	+	—	+	4
234056	Calgary, AB	Gypsum wallboard	+	+	+	+	4
234057	Deseronto, ON	House dust	+	—	+	+	4
234058	Saskatoon, SK	Indoor air	—	—	+	—	4
234059	Surrey, BC	Insulation	—	+	+	+	4
234064	Ottawa, ON	Gypsum wallboard	—	+	—	—	1
234065	Ottawa, ON	Building material	+	+	—	—	4
234066	Sudbury, ON	Air sample	—	+	—	+	4
234067	Ottawa, ON	Indoor air	+	+	—	+	4

2.8-L Fernbach flasks containing 560 mL of Enniatin medium or 2.0-L Erlenmeyer flasks containing 400 mL of Enniatin medium. The medium comprised 50 g maltose, 8 g peptone, 5 g yeast extract, 0.75 g KH_2PO_4 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.067 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ /L ultrapure water. The liquid cultures were put on a rotary shaker (3.81-cm throw) at 220 rpm at 25°C for 48 h in the dark. All liquid cultures were filtered through cheese cloth, separating the culture filtrate from the mycelium. The culture filtrate was pooled and stored at 4°C for later protein extraction. The mycelium was rinsed with ultrapure water, frozen at −20°C, and then freeze-dried. Cultures of other fungi analyzed were produced under the same conditions.

Spores were produced on rice cultures following the method developed by Murad et al. [43]. Uncle Ben'sTM converted long-grain rice (50 g) was placed in a 500-mL wide-mouth Erlenmeyer flasks with 30 mL of ultrapure water. The flasks were autoclaved for 30 min. The rice cultures were inoculated and then incubated at 25°C in the dark for 3 weeks, then air dried in an oven at 30°C. The spores were carefully removed from the rice grains with a spatula and stored in vials at −20°C.

Strains of other fungi tested for possible cross-reactivity included *A. alternata* 27-1 (Paracel Laboratories number), *Aspergillus fumigatus* 31, *A. niger* 5869-6, *A. penicillioideus* 5053-15, *A. versicolor* DAOM 235361 (National Mycological Herbarium, Ottawa), *Cladosporium cladosporioides* 48-1, *Epicoccum nigrum* 51-2, *Paecilomyces variotii* DAOM 235357, *Penicillium brevicompactum* DAOM 234049, *P. crustosum* DAOM 215343, *P. decumbens* 5566.8, *P. funiculosum* L6321-3, *P. spinulosum* 12-7-1, *P. thomii* L6322-3, *Scopulariopsis brevicaulis* 5869-3, *Stachybotrys chartarum* DAOM 235364, and *Wallemia sebi* 12-7-2.

Extracellular and Spore Protein Extraction

Protein concentrations were determined with the Lowry method, Micro BCA protein assay (Pierce, product # 23235, Rockford IL) or Bradford protein concentration assay (BioRad 500-0002, Hercules CA).

To remove nonprotein materials from the culture filtrate, one volume of ice-cold acetone (Caledon, ON) was slowly added to and mixed with one

equivalent volume of culture filtrate, resulting in a 50% acetone solution. The 50% acetone/culture filtrate was kept at −20°C overnight to allow precipitation of nonproteinaceous material, which was removed by centrifugation (Beckman Coulter Avanti J-E) at 17, 400×g for 5 min at −8°C. The supernatant was again mixed at a 1:1 ratio with ice-cold acetone; this 75% v/v acetone/culture filtrate solution was left at −20°C overnight, then centrifuged for 5 min as above. The protein precipitate was harvested and left for 30 min in a fume hood to allow the remaining acetone to evaporate. The pooled protein was dissolved in 50-mM Tris-HCl buffer, pH 7.5. The acetone-precipitated proteins were further precipitated into 0–50, 50–70, and 70–80% ammonium sulfate (AS) fractions. Each of the three AS protein fractions was dissolved in 50-mM Tris-HCl buffer, then desalted, and concentrated with a 10-kDa cut-off Amicon Ultra centrifugal filter (Millipore Corp, MA).

Spore extracts were produced as follows: One milligram of harvested spores was weighed with a Mettler 163 analytical balance (± 0.02 mg) and then placed in a vial with a polystyrene bead. The vial with the spores was milled for 30 min with a Spex-Certiprep mixer mill (model 5100, Metuchen, NJ). The crushed spore fragments were then dissolved in 0.1 mL of phosphate-buffered saline-0.05% (v/v) Tween 20 (PBST) for 2 h, while shaking at 4°C.

ELISA

MaxiSorp microplate (Nunc-Immuno) wells were coated with 100 μL (20 μg) 75% acetone protein precipitate prepared in 50 mM carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C. Subsequent manipulations were done at room temperature (RT). The plate was washed 3 × 5 min with 150- μL PBST buffer between all incubations. The coated plate was blocked with 100 μL of 2% bovine serum albumin (2% BSA)/PBST buffer (blocking buffer) for 1 h, washed, and then incubated for 1 h with 100 μL of the human sera antibodies (1:1,000). The plate was then incubated with 100 μL of anti-human IgG (γ -chain-specific peroxidase antibody produced in goat (Sigma A-8419) diluted 2,000-fold in 1% BSA/PBST for 1 h. The color was developed by adding 100 μL of 3,3',5,5' tetramethylbenzidine liquid substrate for ELISA (TMB; Sigma T0440) shaking for

10 min, and then stopped by adding 50 μ L of 0.5 M H₂SO₄. The optical density of each sample well was read with a microplate spectrophotometer at 450 nm (Max340PC, Molecular Devices, CA).

Electrophoresis

All electrophoresis experiments were performed as follows unless stated otherwise. The proteins were separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE; 8.5 cm $\times$ 8 cm vertical slab gels) on 10% acrylamide gels using the Laemmli running buffer system [44]. Electrophoresis was performed at 100 V for 20 min followed by 200 V for 50 min using a mini VE Vertical Electrophoresis System (GE Healthcare, San Francisco, CA) connected to a Power Pac 1000 gel electrophoresis power supply (Bio-Rad, Hercules, CA). Gels were stained with GelCode Blue Stain (CBB, Pierce, Rockford, IL) or PlusOne silver staining kit (GE Healthcare). Molecular weight (MW) was assessed with a low-molecular-weight calibration kit for SDS electrophoresis (GE Healthcare). Unless stated otherwise, 30 μ g of protein was applied to each lane of the gels used for separation and membrane transfer.

For immunoblotting, the proteins were transferred to a Hybond-PVDF membrane (Amersham Biosciences) with a blot module (GE Healthcare) at a constant current of 400 mA for 30 min in an ice bath. After the transfer, the membrane was washed with 0.1% Tween tris-buffered saline (TBST) buffer, pH 7.4, for 5 min, blocked with 25 mL of 1% (w/v) BSA/TBST for 1 h at RT, and then incubated in either strongly positive human serum (response at a 1:2,000 dilution measured with ELISA) or anti-*P. chrysogenum* goat serum at a dilution of 1:2,000 in BSA/TBST overnight at 4°C. Washing with TBST was carried out between incubations. Anti-human IgG or rabbit anti-goat IgG (Sigma, Oakville, ON) was used as the secondary antibody at dilutions of 1:10,000 and 1:2,500 in BSA/TBST, respectively, shaking at RT for 1 h. The proteins with immunological activity were visualized with 5 mL of 5-Bromo-4-chloro-3-indolyl phosphate dipotassium/nitrotetrazolium blue chloride (BCIP/NBT) substrate (Sigma, Oakville ON).

Initially, sera from 48 patients were screened on ELISA for response to the 75% acetone protein

precipitate from the culture filtrate (hereafter “culture filtrate extracts”) of 17 strains of *P. chrysogenum* clade 4 [28]. From these data, eight sera and eight strains were chosen to screen the strain proteins by immunoblot.

IgG-reactive protein bands were documented and the molecular weights were compared to the IgG and IgE allergens/antigens from *P. chrysogenum* documented in the literature to ensure that a previously known allergen was not selected. Two proteins were chosen based on their IgG reactions to many sera and consistently produced by clade 4 strains, with electrophoretic molecular weights of 40 and 52 kDa. The latter protein was chosen for all further work.

Enhanced Anti-*P. chrysogenum* Clade 4 Polyclonal Antibody

A goat anti-*P. chrysogenum* clade 4 polyclonal antibody (GpAb) was produced according to the procedure described by Xu et al. [14]. Briefly, a goat was injected with 5 mg of spores from *P. chrysogenum* DAOM 234056. After 23 days, a boost comprising 1 mg of the target protein (52 kDa) was identified with the human sera and obtained by electrophoresis. The electrophoretic gel was CBB stained, the target band removed with a sterile scalpel, de-stained with 40% methanol solution, and freeze-dried. The goat boosting was done at Cedarlane Laboratories, Ltd. (Hornby, Ontario; meets the requirements of the Canadian Council on Animal Care). The above protocol ensures that the target is present in spores and increases the specificity of the resulting polyclonal antibody [14].

For polyclonal antibody purification, a protein G column (BioLynx Inc., Brockville, ON) was used to separate and purify the IgG antibodies from the goat anti-*P. chrysogenum* sera. The first step of the antibody purification involved a 50% AS saturation of the serum, which was then left shaking overnight at 4°C. The following day the sample was centrifuged at 17,400 $\times g$ for 10 min, this removed the lipids and other unwanted materials. The pellet was dissolved in 1 mL of phosphate buffered saline (PBS pH 7.4). The remaining AS was removed with 10-kDa cut-off ultrafiltration filter (Millipore). After desalting, the antibody solution was brought back to a 1 mL volume with PBS buffer and applied to the protein G column that was pre-equilibrated in the same

buffer. After incubation of the column on a shaker for 2 h at RT, the bound IgG antibody was eluted with 0.1 M glycine-HCl, pH 2.7, and collected in 1 mL per test tube containing 50 μ L of 1.5 M Tris-HCl buffer, pH 8.8.

The goat anti-*P. chrysogenum* polyclonal antibody was titrated using pure protein by indirect ELISA. Indirect ELISA was carried out as follows. The 52-kDa antigen was prepared to a desired concentration (ca. 100–1,000 ng/mL) in 50-mM sodium carbonate-bicarbonate buffer, pH 9.6 (Sigma, Oakville ON). Aliquots of 100 μ L of the protein preparation were coated to individual microtiter plate wells with the buffer acting as the blank control. The plate was sealed and incubated overnight at 4°C. The unbound antigen was removed by washing three times with PBST, pH 7.4, and each well was refilled with 200 μ L of blocking buffer containing 1% BSA/PBST. After a 1-h incubation at RT, the blocking solution was discarded. Wells were washed well three times with PBST between incubations. A volume of 100 μ L of primary antibody (GpAb) prepared in serial twofold dilutions with BSA-PBST was added to each well. The plate was then incubated for 1 h. Subsequently, 100 μ L aliquots of mouse anti-goat IgG (whole molecule) horse radish peroxidase conjugate (Sigma, A5420, Oakville ON) prepared in BSA-PBST (1:2,000) were added to individual wells. After incubation for 45 min, the color reaction in wells was developed at 37°C for 10 min with 100 μ L TMB (Sigma, Oakville, ON), terminating the reaction with 50 mL of 0.5 M sulfuric acid added to each well. The absorbance was read with a microplate reader (Max340PC, Molecular Devices, CA) at 450 nm.

Growth on Wallboard

P. chrysogenum (DAOM 234056) was grown on paper-faced gypsum board as detailed as follows. Gypsum board (12.7 mm; CSA-A82.27), which had been stored under air-dry conditions for 1 year from manufacture, was cut into 10 cm $\times$ 20 cm sections, put into a plastic box, and autoclaved for 12 min. Sterile tap water was added to the container until the autoclaved gypsum board had become saturated ($\geq 80\%$ w/w). The panels were each inoculated with a piece of culture actively growing on MEA under aseptic conditions. The plastic box with the

inoculated gypsum board was then left for 3 weeks at 25°C in the dark.

Fungal growth was visible on the wallboard paper by the end of the growth period and inspection of the material confirmed that other fungi or bacteria were not present. The colonized material was then allowed to air dry under safe conditions. The moldy paper sections (4.0 g) were then peeled off the wallboard and washed for 2 h with 50-mM Tris-HCl buffer (pH 7.5; 1:10 w/v). The protein in the solution was then extracted with acetone precipitation and the concentration determined by the Bradford method. The amount of the target protein was determined by running five twofold dilutions, followed by silver staining. This was estimated based on the minimum detection limit of silver-stained protein (ca. 0.1 μ g).

Results

Identification of Target Protein

From the 48 human sera tested based on ELISA and immunoblot response to culture filtrate extracts, eight strains and eight sera were selected for further immunoblot analysis of the crude extracts. Representative data are shown in Figs. 1 and 2. Although various proteins reacted on the immunoblots according to the strain, the consistent presence of 40- and 52-kDa protein antigens in *P. chrysogenum* clade 4 (Figs. 1, 2, arrows) were documented. Three strains did not produce IgG responses to the 32- and 16-kDa observed antigens, two without responses to the 34- and 16-kDa antigens, and the remaining strains did not produce IgG responses to either 34- or 16-kDa antigens. Since immunoblots using anti-human IgE secondary antibodies were not used, we cannot speculate that those IgG antigens observed (34, 16, 32 kDa) in a selection of the strains were proteins of similar MW (Pen ch 13, Pen ch 33, Pen ch 18) accepted as allergens by International Union of Immunological Societies [41]. The number of different antigens detected varied considerably between patients (Table 2).

Of the 48 sera that were screened by ELISA against the protein extracts of 16 strains shown to produce the 52-kDa protein, 25 produced an IgG response on immunoblot. A three-dimensional graph was produced comprising (1) ELISA response of the

Fig. 1 Immunoblot comparing the response of HpAb QC2397 (1/1,000 dilution) culture filtrate extracts of *P. chrysogenum* on a 10% SDS gel. The DAOM strains compared were 234052 (lane 1), 234054 (lane 2), 234056 (lane 3), 234064 (lane 4), 234058 (lane 5), 234059 (lane 6), 234053 (lane 7), and 234057 (lane 8). The arrows indicate the 40- and 52-kDa proteins

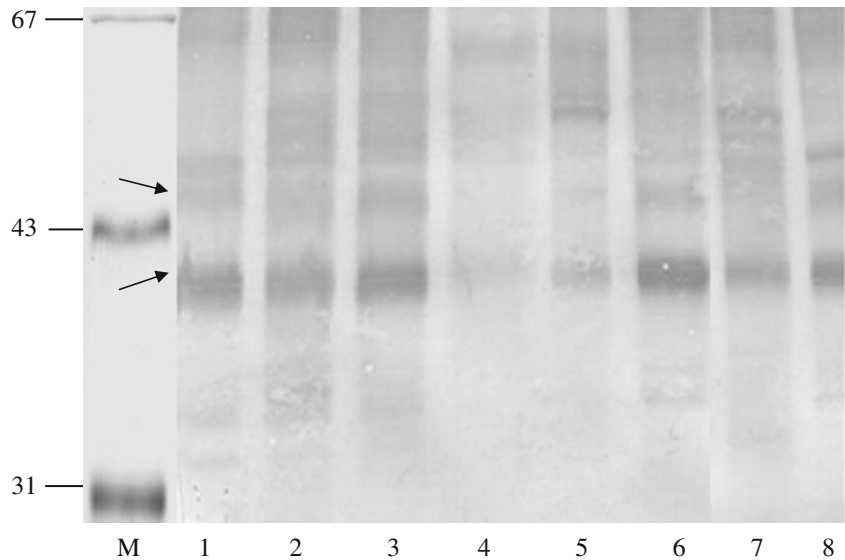
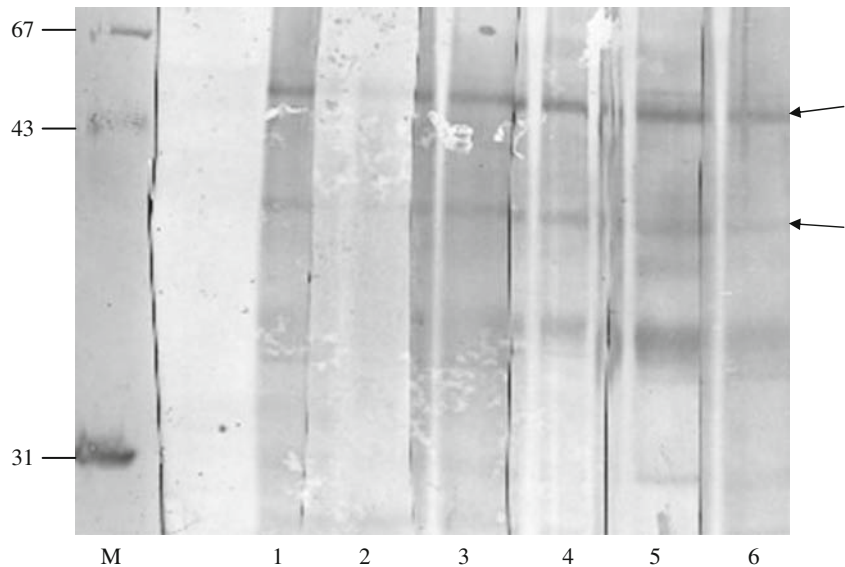


Fig. 2 Immunoblot comparing various HpAbs (1/1,000 dilution) to culture filtrate extracts of DAOM 234056. The HpAbs compared were QC1288 (lane 1), QC1290 (lane 2), QC1303 (lane 3), QC1307 (lane 4), QC2397 (lane 5), and QC2398 (lane 6). The arrows indicate the 52- and 40-kDa proteins



culture filtrate extracts of 16 strains of *P. chrysogenum* known clade and provenance (*X* axis), (2) the response on western graded on an arbitrary scale of 1–3 to the target protein (*Y* axis) and (3) 25 individual human sera (*Z* axis). The different colors in the color image are where these combinations overlap (Fig. 3). The responses fell into two general groups. Most of these strain/antibody combinations had proportionate ELISA IgG responses to the presence of the 52-kDa protein, i.e. the majority of the ELISA response appeared to be explained by the presence of this protein (groupings around immunoblot responses 2

and 3). The second group strain/antibody combinations had a uniformly weaker response on immunoblot but a variable response on ELISA, i.e. from weak to very strong (grouping associated with immunoblot response 1). Culture filtrate extracts of the fungi noted above were screened with these Human polyclonal antibodies (HpAb) for proteins in the region of 52 kDa and none were observed [45].

All the *P. chrysogenum* strains tested produced the target protein. DAOM 234056 had the highest yield of the antigen and was selected for polyclonal antibody production, isolation, and purification

Table 2 *P. chrysogenum* proteins detected on immunoblot with HpAbs from 48 atopic individuals

Protein MW	QC 1288	QC 1290	QC 1303	QC 1307	QC 2397	QC 2398
DAOM 155627						
97						
68			+		+	+
60					+	
52	+	+	+	+	+	+
40			+	+	+	+
34			+			
32						
16						
DAOM 155628						
97						
68			+	+	+	
60						
52	+		+	+	+	+
40			+		+	+
34			+			
32						
16						
DAOM 215337						
97						
68		+			+	+
60					+	
52						+
40						+
34			+			
32		+	+		+	
16					+	
DAOM 234052						
97					+	
68	+	+	+	+	+	+
60			+	+	+	
52	+	+	+	+	+	+
40	+	+	+	+	+	+
34						
32					+	
16					+	

experiments. This strain is found within clade 4 and produces secondary metabolites representative of the clade (Table 1). On immunoblot, the 52-kDa protein is present in the spores of this strain (Fig. 4; among other strains tested, [46]).

Table 2 continued

Protein MW	QC 1288	QC 1290	QC 1303	QC 1307	QC 2397	QC 2398
DAOM 234054						
97					+	+
68	+		+	+	+	+
60	+				+	
52	+	+	+	+	+	+
40	+	+	+	+	+	+
34			+			
32					+	+
16						+
DAOM 234056						
97					+	+
68			+	+	+	+
60	+					+
52	+	+	+	+	+	+
40		+	+	+	+	+
34			+	+		+
32					+	
16						
DAOM 234057						
97					+	
68				+	+	
60					+	+
52		+	+	+	+	+
40			+	+	+	+
34						
32					+	+
16						
DAOM 234059						
97					+	+
68		+		+	+	+
60				+		
52	+	+	+	+	+	+
40	+	+		+	+	+
34						+
32						
16						

Production of Polyclonal Antibodies Against *P. chrysogenum* Spore Antigens

The goats were successfully immunized with spores producing anti-*P. chrysogenum* polyclonal antibodies that responded to many proteins both in the spore

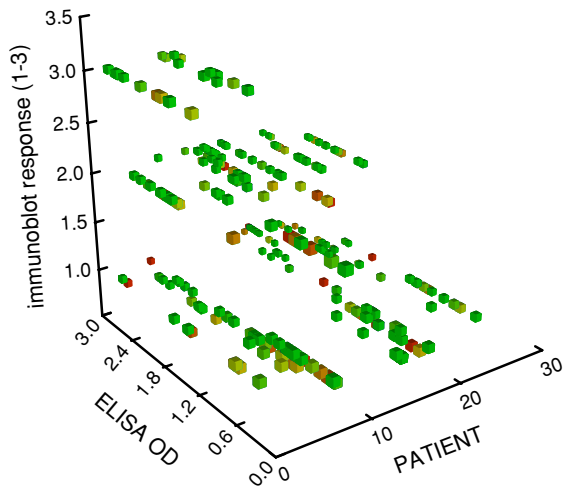


Fig. 3 A three-dimensional graph comprising (1) ELISA response of the culture filtrate extracts of 16 strains of *P. chrysogenum* clade 4 (X axis), (2) the response on western graded on an arbitrary scale of 1–3 to the target protein (Y axis) and (3) 25 individual human sera (Z axis). The different colors in the color image are where these combinations overlap (Fig. 3)

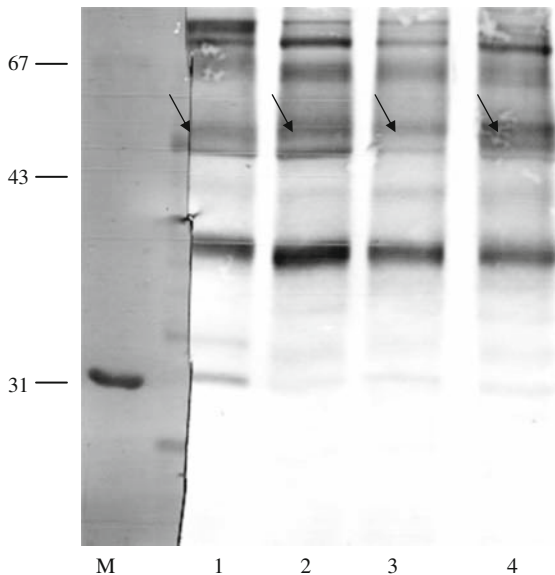


Fig. 4 Immunoblot comparing *P. chrysogenum* DAOM strains culture filtrate reactions (lanes 1–3) and *P. chrysogenum* spore protein extract reaction (lane 4; DAOM 234056). The DAOM identification numbers for the culture filtrate protein samples are 234054 (lane 1), 234056 (lane 2), and 234057 (lane 3). The antigens were probed with human sera QC2397 (1/1,000 dilution). The arrow indicates the 52-kDa antigen, which can be seen in all four samples

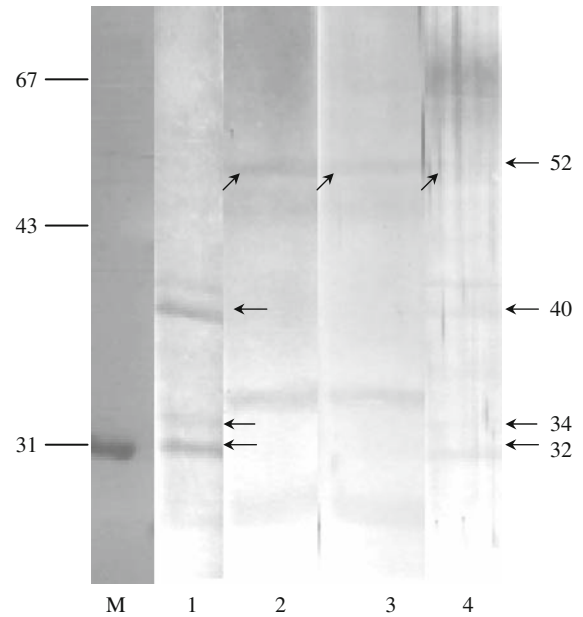


Fig. 5 Immunoblots comparing the spore-immunized goat polyclonal antibodies (lanes 3 and 4) to the 52-kDa target protein boosted polyclonal antibodies (lanes 1 and 2). The spore extract (lanes 1 and 4) and 50–70% AS protein fraction (lanes 2 and 3) are from DAOM 234 056. A reaction to the 52-kDa target protein (arrow) and the decreased response to other antigens are indicated (32, 34, and 40 kDa arrows). The spore-immunized serum was diluted 1/500 and the antigen boosted serum was diluted 1/1,000

samples and in the culture protein extract, including the target protein (Fig. 5, lane 2). Response to the target protein from spore extracts was just discernable before boosting, to clear after the boost (Fig. 5, lane 4). In addition, there was a decrease in antibody titer to nontarget antigenic proteins at 32 kDa, 34 kDa, and 40 kDa observed in our preliminary work (Fig. 5, lane 1–4; [46]). Immunoblotting experiments were performed examining the GpAb response on immunoblot of culture extracts from a collection of related and unrelated species in comparison to *P. chrysogenum* (representative data shown in Fig. 6a, b; see also [45]). None of the culture filtrate extracts tested showed evidence of the 52-kDa protein. A 47-kDa protein reported from *A. fumigatus* was detected on the immunoblot.

The GpAb was titrated by indirect ELISA using pure protein by ELISA. The antibody produced specific binding to the protein with a low, nonspecific backgrounds (<0.05 optical density (OD) units) in control wells. The OD value reached maximum at

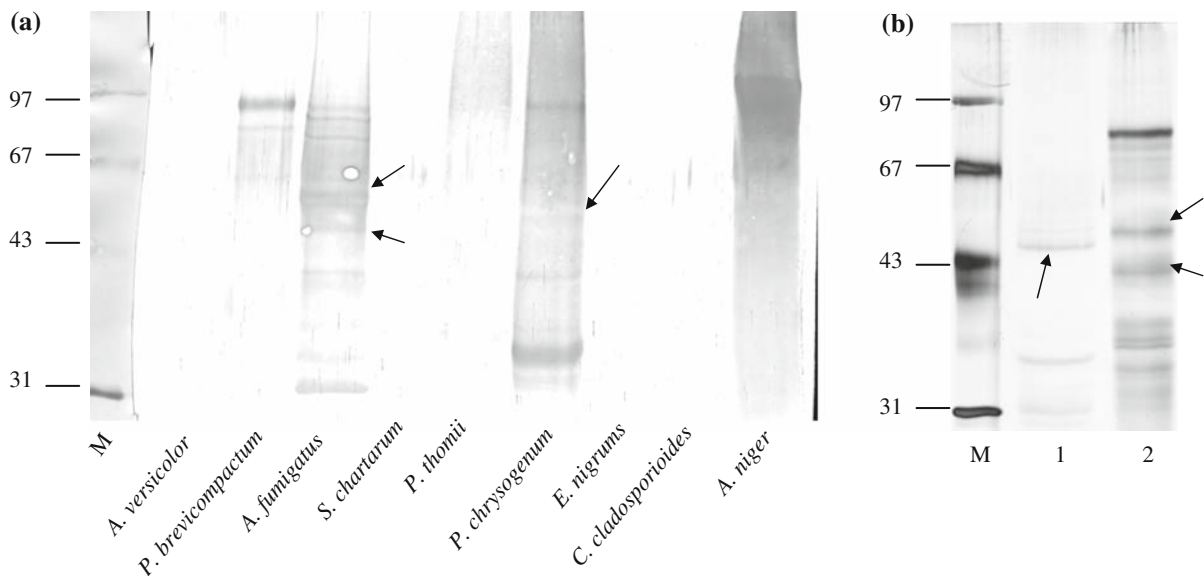


Fig. 6 **a** An immunoblot analysis of IgG-purified anti-*P. chrysogenum* GpAb (1/2,000 dilution) cross-reactivity against a selection of fungi. An arrow indicates the GpAb reaction to the *P. chrysogenum* 52-kDa protein antigen. Two arrows indicate the *A. fumigatus* proteins of similar molecular weight

recognized by the GpAb. **b** A silver-stained SDS-PAGE gel comparing the protein extracts from *P. chrysogenum* and *A. fumigatus*. One arrow indicates the 52-kDa protein antigen; the others indicate the bands that appear close on immunoblot

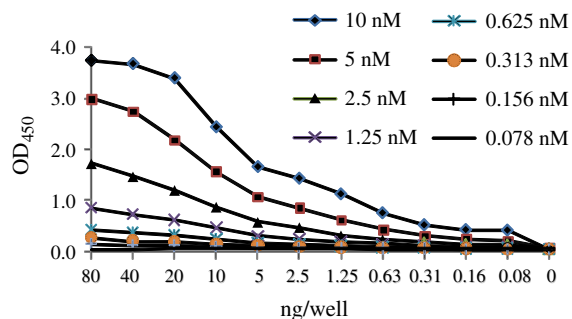


Fig. 7 Titration of goat polyclonal antibody against Pch52 antigen by indirect ELISA. The 52 antigen added in doubling dilutions was coated to a 96-well microplate; antibody was added at various concentrations as shown in the plot legends

dilutions of 10 nM GpAb with 20–40 ng antigen per well (Fig. 7).

Presence of Protein on Paper-Faced Gypsum Wallboard

The presence of a 52-kDa antigen in spores/mycelium was confirmed from extracts of mold growth on a susceptible building material. At the end of the incubation period, mycelium and spores were visible on the paper. The protein extract as described above

was analyzed by immunoblotting and silver stained after SDS-PAGE. A 52-kDa protein antigen was detected with purified IgG goat anti-*P. chrysogenum* pAbs, as well as with the silver-stained gels (Fig. 8). The total protein content extracted from the inoculated and dried gypsum board paper (4.0 g) was 685 μ g. The 52-kDa antigen represented an estimated 1% of the total protein content of the paper extract (based on the response to dilution to extinction experiments and the fact that paper has negligible amounts of protein).

Discussion

There had been a recognition that fungi such as *A. alternata* and *A. fumigatus* resulted in allergy and asthmatic symptoms from the 1930s [46, 47]. However, serious work on isolating specific allergens from fungi did not begin until much later. This work was conducted on fungi for which there is near universal exposure such as *A. alternata*, *A. fumigatus*, and *C. herbarum* [48–50]. Among other implications, study of these common allergenic fungi is associated with inherently large numbers of patients available as

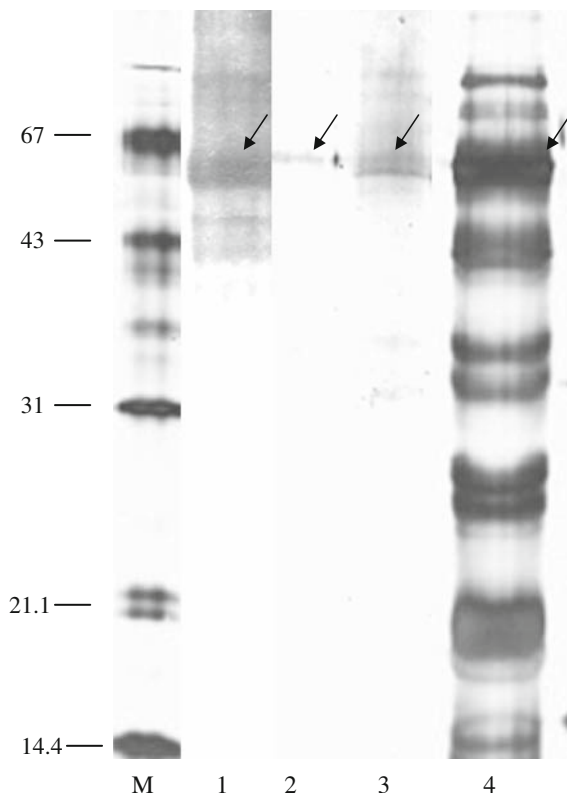


Fig. 8 Immunoblot and silver stain of protein extract of *P. chrysogenum* damaged the gypsum wallboard. Extract of *P. chrysogenum* (lane 1) and wallboard extract (lane 2) with purified IgG GpAbs; silver-stained gel of the same extracts (lanes 3, 4, respectively). Arrows indicate the 52-kDa protein

sources of sera for the discovery process. For the fungi associated with damp building materials, exposures are more recent on a population basis and highly variable. This means that sera from atopic patients must be collected over a large geographic area for some time to enable detection. We have been investigating the presence of fungal antigens from damp building-material-associated fungi in some of the most common fungi [18] and representative of different ecological groups. In doing this work, we have been aided by the availability of sera from a large clinical laboratory serving the US and Canada. For example, with the *Stachybotrys chartarum sensu lato* antigen, we found approximately 10% of the prescreened sera we studied responded to the protein. The protein was shown to be an exoallergen produced on the preferred natural substrate of the fungus and on paper-faced gypsum wallboard.

In the case of the indoor clade of *P. chrysogenum*, just over 50% of the patients were shown to react to the 52-kDa protein from this prescreened collection of sera. This indicates that as in Germany and Norway [32, 33], responsiveness to this fungus by atopic patients mainly from the USA is uncommon but more common than with *S. chartarum* [14]. This is consistent with the observation that *P. chrysogenum* is the most common of the *Penicillia* growing in damp building materials. Our data also show that large collections of sera and large collections of strains are required to properly evaluate the nature of response to building-material-associated fungi. Most strain/antibody combinations had proportionate ELISA response to the presence of the ultimate target, the 52-kDa protein. The number of different IgG antigens detected varied considerably between patients (Table 2). Similarly, all the *P. chrysogenum* strains tested produced the target protein. However, three strains did not produce IgG-responding proteins similar in MW to Pen ch 18 [51] and Pen ch 33 [51], two did not produce an IgG response to proteins close to the MW of Pen ch 13 [51] and 33, and the remaining strains did not produce either Pen ch 13, or Pen ch 33. It has proven useful to screen with many human sera and strains of well-characterized fungi from across Canada and the USA. The target protein was not found in a taxonomically diverse selection of fungi whether spores or culture extracts are based on a human antibody response.

A second component of the process described here is to assure that the protein once isolated is accumulated in spores. We found that the *S. chartarum* antigen while produced in culture and found on the mycelium is concentrated on the spore surface [52]. Similarly, we found that the *A. versicolor* antigen we are currently studying is present in much higher concentrations on spores (~10,000 w/w; [53]) albeit the absolute amount is small.

Aside from its use in further research, the production of a polyclonal antibody demonstrated that the 52-kDa protein present in culture filtrate of the medium used was present in unwashed, ungerminated spores, and spore/hyphal fragments (Fig. 5). Boosting the goat with 1 mg of this protein was found to expand one or more of the most specific antibodies induced by the spores, which enhanced the IgG response to the 52-kDa target protein. This confirmed that the protein involved was present in fungal cells

grown on autoclaved rice and a common substrate resulting in potential human exposure, paper-faced gypsum wallboard, and strains collected across USA and Canada. The target 52-kDa protein was present as approximately 1% of the total protein recovered from the moldy paper (Fig 8) [45].

In view of the large amount of work on *P. chrysogenum* allergens, it is surprising that this protein has not apparently been reported as an antigen or allergen. Since IgG antibodies represent approximately 75% of circulating immunoglobulins and have a much longer half-life than IgE antibodies [54, 55], we have attempted to use this to our advantage for recognition and the detection of this protein, as a marker for the all indoor strains of *P. chrysogenum* from clade 4. Human immune response to the presence of an antigen elicits an almost immediate IgM antibody response, followed shortly by production of IgG antibodies. In our research, we have shown that aside from positive reactions to human sera, the 52-kDa protein produced a detectable reaction to IgG antibodies in immunized goat sera, and thus is producing specific IgG antibodies to an epitope found on this protein in the natural environment. Without continued exposure, the IgE antibody serum titer declines quickly, whereas IgG antibodies remain in the serum for much longer [56]. Also, the previous studies have apparently involved strains from clades not associated with the built environment. As we found for *S. chartarum sensu lato* [14, 15], it is important that strains isolated from the built environment be used and that these are thoroughly characterized. Also consistent with the previous work on *S. chartarum*, the importance of having access to a fairly large collection of human sera cannot be overestimated. The characterization of the 52-kDa protein will be described in a companion paper [57].

Acknowledgments We are grateful for advice from Dr. Jiangping Xu and the expert technical help of Don Belisle, Natacha Provost, and Shari Levac. Useful suggestions from an unknown reviewer are also appreciated. This work was supported by an NSERC IRC to JDM.

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