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Development of monoclonal antibodies to recombinant terrelysin and characterization of expression in *Aspergillus terreus*

Ajay P. Nayak[1, 2], Brett J. Green[1], Sherri Friend[3], Donald H. Beezhold[1*]

1. Allergy and Clinical Immunology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, Morgantown, WV 26505.

2. Department of Microbiology, Immunology and Cell Biology, West Virginia University, Morgantown, WV 26505.

3. Pathology and Physiology Research Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, Morgantown, WV 26505.

* Corresponding author.

Ajay P Nayak, M.S.

Allergy and Clinical Immunology Branch,

1095 Willowdale Road.

National Institute for Occupational Safety and Health,

Morgantown, WV 26505.

Phone: (304) 285-5721 Ext 6. Fax: (304) 285-6126.

E-mail: fyg1@cdc.gov, ajnayak1@gmail.com

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ABSTRACT

Aspergillus terreus is an emerging pathogen that mostly affects immunocompromised patients with infections that are often difficult to manage therapeutically. Current diagnostic strategies are limited to the detection of fungal growth using radiological methods or biopsy that often does not enable species-specific identification. As a result, there is a critical need for diagnostic techniques to enable early and specific identification of the causative agent. In this study, we describe monoclonal antibodies (mAbs) developed to a previously described recombinant terrelysin. Sixteen hybridomas of various IgG isotypes were generated to the recombinant protein, of which seven demonstrated reactivity to the native protein in hyphal extracts. Cross-reactivity analysis using hyphal extracts from 29 fungal species, including 12 *Aspergillus* species and 5 different strains of *A. terreus* showed that 3 mAbs (13G10, 15B5 and 10G4) were *A. terreus*-specific. Epitope analysis demonstrated mAbs 13G10 and 10G4 recognize the same epitope ‘PSNEFE’, while mAb 15B5 recognized the epitope ‘LYEGQFHS’. Time course studies showed that terrelysin expression was highest during early hyphal growth and dramatically reduced after mycelial expansion. Immunolocalization studies demonstrated that terrelysin is localized not only within the cytoplasm of hyphae but appeared to be more abundant at the hyphal tip. These findings were confirmed in cultures grown at room temperature as well as at 37 °C. Additionally, terrelysin was detected in the supernatant of *A. terreus* cultures. These observations suggest terrelysin may be a candidate biomarker for *A. terreus* infection.

INTRODUCTION

Bacterial hemolysins have been identified to have a functional role in microbial pathogenesis through lysis of host cell membranes (Bhakdi *et al*., 1996; Chu *et al*., 1991; Seeger *et al*., 1991). Fungal hemolysins have been studied for their pleiotropic functions as virulence factors (Kumagai *et al*., 1999; Malicev *et al*., 2007; Rebolj *et al*., 2007; Zuzek *et al*., 2006), lipoprotein-binding (Kudo *et al*., 2001; Kudo *et al*., 2002), fungal development (Berne *et al*., 2002; Berne *et al*., 2007), and utility as markers of cholesterol micro-domains on eukaryotic cell membranes (Chowdhury *et al*., 2008; Rebolj *et al*., 2006). To date, limited information is available as to the function of these proteins in fungal biology.

Although *A. fumigatus* is the leading cause of invasive aspergillosis in immunocompromised individuals, *A. terreus*, as well as *A. flavus*, *A. niger* and rarely *A. nidulans* have also been identified as etiological agents (Balajee, 2009a; Perfect *et al*., 2001; Segal *et al*., 1998). *A. terreus* is an opportunistic fungal pathogen that has been identified to cause infections including onychomycosis (Hilmioglu-Polat *et al*., 2005), otitis (Tiwari *et al*., 1995), endophthalmitis (Garg *et al*., 2003), peritonitis (Verghese *et al*., 2007), suppurative abscess (Sukroongreung and Thakerngpol, 1985), and osteomyelitis (Natesan *et al*., 2007). Resistance of *A. terreus* to amphotericin B, thermotolerance, and production of accessory conidia have been suggested to aid in the rapid dissemination of the organism during invasive infections (Blum *et al*., 2008; Pore and Larsh, 1967). Collectively, these factors are believed to contribute to the very high mortality rates among immunocompromised patient populations and highlight the need for species-specific diagnostic methodologies.

Fungal hemolysins have been characterized from a variety of fungal species (Ebina *et al*., 1982; Van Emon *et al*., 2003; Vesper and Vesper, 2004; Wartenberg *et al*., 2011). *Aspergillus*

hemolysin (Asp-hemolysin) has been detected in the tissues of mice in an invasive aspergillosis animal model (Ebina *et al.*, 1982). Stachylysin, a hemolysin derived from *Stachybotrys chartarum* was reported to be detectable in sera of exposed animals and patients (Ebina *et al*., 1982; Van Emon *et al*., 2003). In a previous study, we used molecular methods to identify a hemolysin (terrelysin) produced by the opportunistic fungal pathogen, *A. terreus* (Nayak *et al*., 2011a). In this study, we describe the generation of terrelysin-specific monoclonal antibodies (mAbs), and characterize the expression of native terrelysin during fungal growth.

METHODS

Production of monoclonal antibodies against recombinant terrelysin (rTerrelysin)

Three 8-10 week old BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were housed in the animal facility at the National Institute for Occupational Safety and Health (NIOSH), Morgantown, WV. The facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and animals were free of viral pathogens, parasites, mycoplasma, and *Helicobacter* spp. Animals were housed together in HEPA-filtered ventilated polycarbonate cages with autoclaved hardwood Beta-chip bedding and cotton fiber nesting material. The animals were provided with Teklad 7913 rodent chow (Harlan Laboratories, Madison, WI) and autoclaved tap water *ad libitum*. All animal procedures and immunizations were reviewed and approved by the NIOSH Animal Care and Use Committee (ACUC).

Prior to immunization, blood samples were collected from the tail vein and the serum was stored at -20 °C. Each animal was immunized 3 times, every other week with 25 μg of purified rTerrelysin (Nayak *et al*., 2011a) emulsified 50% (v/v) in TiterMax® adjuvant (TiterMax USA, Norcross, GA). The mice were monitored for adverse health effects post immunization. Between immunizations, serum was collected from the tail vein to screen for specific IgG antibody responses.

Following the development of sufficient IgG titers, the animals were euthanized by CO_2 asphyxiation. The spleen was aseptically removed from each mouse and single cell suspensions of the splenocytes produced. Fusion of splenocytes with SP2/0-Ag 14 myeloma cells (ATCC# CRL-1581) was performed as previously described (Köhler and Milstein, 1975). Hybridomas were selected by growing cells in Dulbecco's Modified Eagle Medium (DMEM) (Life

Technologies, Rockville, MD) supplemented with 1 mM sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin, 0.292 mg/ml L-glutamine, 100 mM sodium hypoxanthine, 16 mM thymidine, 10% fetal calf serum (FCS) (HyClone, Logan, UT), and 100 U/ml IL-6 (Boerhinger, Mannheim, Germany). DMEM was also supplemented with azaserine for selective propagation of hybridomas. After 10-14 days of growth, medium from individual wells with hybridoma cell growth was replenished with fresh DMEM medium. The supernatants from individual colonies were tested in a screening enzyme-linked immunosorbent assay (ELISA) to detect antibodies of varying IgG isotypes that reacted with rTerrelysin.

Supernatants of individual clones were tested twice to confirm reactivity of antibodies. Each positive well was then cloned twice by limiting dilution analysis and single positive clones were screened and selected for production of mAbs. Positive clones were frozen in 10% dimethyl sulfoxide (DMSO) and stored at -80 °C for 2 weeks and transferred to liquid nitrogen for long-term storage.

Purification, isotyping and quantification of rTerrelysin mAbs

All rTerrelysin mAbs were concentrated and partially purified by ammonium sulfate precipitation (Andrew and Titus, 1997). Briefly, mAb supernatant was collected from individual hybridomas and centrifuged at 20,000 x *g* for 30 min at 4 °C. The supernatant was collected and saturated ammonium sulfate was slowly added to the supernatant to 45% (v/v) and incubated overnight at 4 °C. The tubes were centrifuged for 45 min at 20,000 x g at 4 °C and the precipitate was collected and resuspended in PBS, pH 7.4. Concentration of purified antibodies and their isotype were determined using methods previously described (Nayak *et al*., 2011b).

Fungal cultures and extracts

For time point assays, fungal cultures were grown by inoculating 50 ml minimal medium (containing glucose, nitric salts and trace elements) (Hill and Kafer, 2001; Nayak *et al*., 2011b), with 2.5 x 10^7 viable *A. terreus* conidia. Viability was determined using the LIVE/DEAD® *Bac*Light™ Viability kit (Molecular Probes, Inc., Eugene, OR) (Chen and Seguin-Swartz, 2002). Following inoculation, flasks were incubated at either room temperature (RT) or 37 °C depending on the specific experimental design. *A. terreus* cultures were grown for up to 12 days with an individual flask representing a 24 h time point. *A. terreus* cultures and mycelial pellets were collected in 50 ml polypropylene tubes and centrifuged at 4,100 x *g* for 10 min. The culture supernatant (CSN) and mycelial pellets were collected and stored at -80 °C and the lyophilized CSN residue was resuspended in PBS, and mycelial pellets were processed using a mortar and pestle in PBS containing Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN). Mycelial slurry was then collected into 15 ml polypropylene tubes and incubated at 4 °C overnight on a shaker to facilitate the release of intracellular proteins into the lysis solution. The next day, mycelial extracts (ME) were centrifuged at 4,100 x *g* for 10 min, and the supernatant collected and stored at -20 °C until analysis.

For cross-reactivity studies, ME were prepared from 29 different fungal species including 12 different *Aspergillus* species using the same method (**Table 1.**) Fungi were grown until mycelial pellets had formed (3-4 days). Protein concentrations of CSN and ME were estimated using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Thermo Scientific, Wilmington, DE) as previously described (Nayak *et al*., 2011b).

Enzyme-linked immunosorbent assays

Screening mAbs. Hybridomas producing anti-terrelysin mAbs were identified by indirect ELISA. In brief, 96-well Immuno MaxiSorp microplates (Nunc, Rochester, NY) were coated overnight with rTerrelysin (1 μg/ml) in 0.05 M carbonate coating buffer pH 9.6 (CCB), and blocked with phosphate buffered saline (PBS) containing 0.5% Tween-20 and 5% nonfat dry milk (PBSTM) for 1h. CSN from each hybridoma was incubated in duplicate wells for 1 h at 37 ^{o}C, washed with phosphate buffered saline (PBS) containing 0.5% Tween-20 (PBST), and detected using alkaline phosphatase conjugated goat-anti mouse IgG antibody (H+L) (Promega, Madison, WI) diluted 1:5000 in PBSTM for 1 h at 37 ^{o}C. The wells were then washed in PBST and developed for 30 min using 4-paranitrophenyl-phosphate substrate (Sigma). Reactivity was determined by measuring the optical density (OD) at 405 nm. To compare the reactivity of identified mAbs, each mAb was purified and used at a concentration of 1 μg/ml and assayed with various dilutions of rTerrelysin bound to the assay plate. The plates were washed 3 times between individual steps of incubation with PBST.

Inhibition ELISA for analysis of native terrelysin. An inhibition ELISA was performed over a period of 2 days. On day 1, the 'assay plate' was coated with 0.1 μg/well of rTerrelysin and a second 'inhibition plate' was blocked overnight with PBSTM to prevent protein binding. On day 2, both plates were washed with PBST and the 'assay plate' was blocked with PBSTM at RT for 2 h. In the inhibition plate, serial dilutions of ME and CSN samples (protein concentration range 5 mg/ml-0.078 mg/ml) and serially diluted rTerrelysin standard (protein concentration range 78 μg/ml-1 mg/ml) were incubated with 40 ng/ml of anti-terrelysin mAb 10G4.. Negative control wells were incubated with PBS and positive control wells were incubated with mAb 10G4 alone. The inhibition plate was incubated on a shaker at RT for 1 h at

200 rpm and for 30 min without shaking. Next, the assay plates were washed and incubated with 100 µl of the mixture from the inhibition plate at 37 °C for 1 h. Plates were washed and incubated with alkaline phosphatase conjugated goat anti-mouse IgG antibody (H+L) diluted 1:5000 in PBSTM for 1 h at 37 °C. Plates were washed and developed using 4-paranitrophenyl-phosphate substrate. Reactivity was measured at OD at 405 nm and terrelysin concentrations determined for each sample by comparing to the standard curve using regression analysis.

Western blot analysis of rTerrelysin and fungal ME

Western blot analysis was performed for screening individual mAbs reactivity to rTerrelysin, native terrelysin (*A. terreus* CSN and ME extracts), and for cross-reactivity analysis. rTerrelysin (500 ng/ml) and ME (2.5 mg/ml) collected from 4 day cultures of *A. terreus* were individually separated using SDS-PAGE on 12% polyacrylamide gels. For cross-reactivity testing, ME (2.5 mg/ml) from 29 different fungal species were separated on 12% polyacrylamide gels. Proteins were transferred overnight to nitrocellulose membranes (0.22 µm, BioRad) and the membranes were blocked using Tris-buffered saline containing 0.1% Tween-20 (TBST) and 3% bovine serum albumin (blocking buffer). Membranes were washed with TBST and transferred to a BIO-RAD Multi Screen apparatus (BioRad, Hercules, CA). Individual lanes were incubated with 1 µg/ml of mAb diluted in blocking buffer and incubated on a rocker for 1 h. Membranes were washed 3X with TBST and incubated with alkaline phosphatase conjugated goat anti-mouse IgG antibody (H+L) diluted 1:5000 in blocking buffer for 1 h on a rocker. Membranes were then washed with TBST and developed for 15-20 min using 1-Step NBT/BCIP (Promega, Madison, WI) substrate solution. The reaction was stopped by washing the membranes with distilled water.

Epitope mapping

Epitope mapping was performed using synthetic peptide synthesized by Sigma Genosys (JPT Peptide Technologies GmbH, Berlin, Germany). For peptide scans, 68 peptides spanning the entire rTerrelysin sequence (including the *Strep*-tag II sequence) were synthesized as linear decapeptides overlapping by 2 amino acids. Peptides were covalently bound to a Whatman 50 cellulose support (PepSpots membrane) at the C-terminus and the N-terminus was acetylated for higher stability. The membranes were processed for epitope mapping per the manufacturer's instructions.

In brief, the PepSpots membrane was rinsed in methanol for 5 min, washed 3X with TBS for 10 min, blocked overnight at 4 °C on a shaker with TBS containing 3% BSA. The membrane was incubated with 5 µg/ml of rTerrelysin mAbs for 3 h at RT on a shaker. The mAb 9B4, an IgG_1 isotype which recognizes a *Stachybotrys chartarum* conidial surface protein served as a negative control (Schmechel *et al.*, 2006). The membrane was washed in TBST 3X for 5 min each and then incubated with goat anti-mouse IgG horseradish peroxidase (HRP) conjugated antibody (Promega, Madison, WI) diluted 1:50,000 in blocking buffer for 1 h at RT on a shaker. The membrane was washed thoroughly in TBST 3X for 5 min each and developed with ECL Western blotting substrate (Promega) as per manufacturer's instructions. After a brief incubation, excess substrate was discarded and the membrane was exposed to CL-XPosure™ clear blue X-Ray film (Thermo Scientific, Rockford, IL) and developed using a SRX-101A tabletop processor (Konica Minolta, Ramsey, New Jersey). For regeneration, the PepSpots membrane was washed twice with water for 10 min each and then incubated with regeneration buffer I (62.5 mM TRIS containing 2% SDS, pH 6.7; 100 mM 2-mercaptoethanol) at 50 °C using four 30 min incubations. The membranes were washed 3X for 20 min with PBS (10X), 3X with TBST, and

3X with TBS for 10 min at RT. The membrane was analyzed to ensure efficient removal of bound primary and secondary antibody prior to analysis of new mAbs.

Microscopic examination of *A. terreus* morphology

In order to correlate the expression of terrelysin to associated growth changes in *A. terreus*, we cultivated *A. terreus* in 24-well plates containing 2 ml of minimal medium. Wells were inoculated with 1 x 10^6 viable *A. terreus* conidia and incubated on a shaker at either RT or 37 °C. Growth of *A. terreus* was monitored at various time points using an Olympus IX70 microscope (Olympus Corporation, Tokyo, Japan) and images were captured using a QImaging Retiga 2000R Fast camera (QImaging, Surrey, Canada) and processed using the SimplePCI6 software (Hamamatsu Corporation, Sewickley, PA).

Confocal Scanning Laser Microscopy localization of native *A. terreus* terrelysin

Immunolocalization of terrelysin was studied using a slight modification of previously described methods (Osmani *et al*., 1988; Xiang *et al*., 1995). Briefly, *A. terreus* FGSC 1156 cultures were grown on alcohol-sterilized cover slips in 6-well tissue culture plates that contained minimal medium. Cultures were incubated at 37 °C under static conditions for 24 h. Cover slips were fixed with 8% formalin buffered saline that contained 50 mM PIPES (pH 6.7), 25 mM EGTA, 1% DMSO and 5 mM $MgSO_4$ for 1 h at RT and the cover slips were then rinsed with MTSB (50 mM PIPES (pH 6.7), 5 mM EGTA and 5 mM $MgSO_4$). Cell wall digestion was carried out for 1 h at RT with an enzyme solution containing 2.5% Driselase® (Sigma, St. Louis, MO), 1% lysozyme from chicken egg white (Sigma) and 2 mM EGTA. Cells were rinsed with H_2O and then treated with 0.1% Triton X-100 in TBS pH 7.4 for 10 min. Cells were then rinsed

in MTSB and TBS once each. Cells were then blocked with 3% bovine serum albumin in TBS (TBSB) overnight at 4 °C with gentle shaking. Next, the cover slips were incubated with mAb 15B5 (3 μg/ml) in TBSB for 3 h with gentle shaking. mAb 9B4 (*S. chartarum* conidial surface protein) served as an negative control and mAb 13E11 that reacts with an *A. terreus* exoantigen leucine aminopeptidase served as a positive control (Nayak *et al*., 2011b). Cells were washed thoroughly in TBST and stained with AlexaFluor® 594 conjugated goat anti-mouse IgG antibody (Molecular Probes Inc., Eugene, OR) diluted 1:50 in TBSB for 1 h at RT. Cells were washed in TBST and cover slips were placed on clean slides with ProLong® Antifade Reagent with DAPI (Molecular Probes Inc.). Cells were observed with a Zeiss LSM-510 Meta Confocal Microscope System (Axioplan 2 Stand) (Carl Zeiss, Thornwood, NY) and the images were acquired with Zeiss software version 3.2 (Carl Zeiss). All settings on the confocal laser microscope remained constant throughout the analysis.

RESULTS

Isotyping and sensitivity screening of rTerrelysin mAbs

Thirty-two hybridomas recognized rTerrelysin during the initial screening. Sixteen clones survived multiple cloning steps and were further analyzed in screening, cross-reactivity, and immunolocalization experiments. Nine clones (2G4, 3B2, 6D2, 7D8, 13G10, 15B5, 16C7, 9F4, 19E3) produced IgG_1 isotype mAbs, 4 (3B7, 10G4, 15C5, 15E4) produced IgG_{2a} isotype mAbs, and 3 (6E4, 10G7, 2D3) produced IgG_{2b} isotype mAbs. These mAbs exhibited variable reactivity to immobilized rTerrelysin in ELISA (**Fig. 1**). mAbs 2G4, 9F4, 19E3, 6E4 and 15E4 showed the least reactivity ($\leq$ 0.25 OD_{405} value) to rTerrelysin (data not shown). mAbs 3B2, 6D2, 7D8 and 16C7 showed moderate reactivity, while mAbs 13G10, 15B5, 3B7, 10G4, 15C5, 10G7 and 2D3 showed highest reactivity to rTerrelysin.

Western blot screening of mAbs to rTerrelysin and native terrelysin

Western blot analysis revealed reactivity to denatured rTerrelysin (16.5 kDa) for 11 of the 16 mAbs (**Fig. 2a**). The higher molecular weight bands observed in the lanes with purified rTerrelysin are likely to be aggregates formed during the storage of the protein at -20 °C. For native terrelysin, 4 of the 11 mAbs (3B2, 6D2, 16C7 and 2D3) did not show any reactivity with *A. terreus* ME (**Fig. 2b**). Seven mAbs (7D8, 13G10, 15B5, 3B7, 10G4, 15C5, and 10G7) reacted to a putative native terrelysin in ME and were chosen for further study.

Cross-reactivity and strain variations

Five strains of *A. terreus* including the pathogenic strain FGSC 1156 and the commonly used environmental strain ATCC 1012 were analyzed with each mAb by Western blot. All

mAbs, except 7D8, exhibited reactivity to a protein of the same molecular weight in ME derived from all strains (**Table 1**). Interestingly, the mAb 7D8 reacted to ME from 3 strains but 2 strains (NIOSH 17-30-31 and NIOSH 35-08-06) did not react.

ME from 11 other *Aspergillus* species including the pathogens *A. fumigatus, A. flavus, A. nidulans* and *A. niger* were also tested. mAb 15C5 showed maximum cross-reactivity among the mAbs tested, exhibiting cross-reactivity to ME from 6 different fungi, including *A. nidulans* and *A. parasiticus*. mAb 10G7 did not cross-react with any tested *Aspergillus* species, but it did react to ME from *E. rostratum*, *P. variotii* and *T. harzianum*. mAb 3B7 cross-reacted to ME from *C. cladosporioides*. No cross-reactivity was observed with any mAbs against ME from 4 *Penicillium* species. 4 mAbs (7D8, 13G10, 15B5 and 10G4) did not cross-react with any of the tested fungal species (**Table 1**).

Epitopes for rTerrelysin-mAbs

Owing to the high specificity determined in cross-reactivity studies, 3 mAbs (15B5, 13G10 and 10G4) were tested to determine their epitopes using overlapping decapeptides that span the entire sequence of rTerrelysin. For mAb 15B5, 2 spots were recognized at positions 20-21 (**Fig. 3**). The sequence of spots 20 and 21 correspond to 'SFLYEGQFHS' and 'LYEGQFHSPE', respectively. These data suggest that the epitope recognized by mAb 15B5 is 'LYEGQFHS'.

After ensuring complete removal of bound mAb 15B5 and regeneration of the SPOTs membrane, the membrane was then scanned using mAb 13G10. Four consecutive spots (52-55) reacted with mAb 13G10 and the resultant epitope was 'PSNEFE' (**Fig. 3**). Subsequently, mAb 10G4 was found to recognize the same epitope as mAb 13G10 based on SPOTscan data (**Fig. 3**).

SPOTscan of the membrane using control mAb 9B4 and secondary antibodies did not result in reactivity to any spots (data not shown).

Time-point kinetics of terrelysin expression

The expression of native terrelysin was examined using an inhibition ELISA to quantify the amount of terrelysin in CSN and ME at 24 h intervals (**Fig. 4a**). In ME, the highest relative concentrations of terrelysin were observed during the first few days of growth ranging between 10-12 μg/mg of total fungal protein. A 10-fold reduction was observed by day 4 - 5 (1 μg/mg of total protein) and after day 6 very little terrelysin was quantified in ME. Lower levels of terrelysin were detected in the CSN with a maximum at day 6 and then declining to nearly baseline by day 12.

The morphological features that were observed at the same time intervals are depicted in **Fig. 4b**. The early time points when expression of terrelysin was the highest, involved the initial stages of conidial germination and hyphal extension (12 h to 24 h). Hyphal aggregation was observed up to 96-120 h, when quantities of terrelysin were observed to decline. Beyond this time interval, the morphological changes could not be differentiated. Collectively, these results suggest that terrelysin expression occurs during initial stages of fungal growth between days 0-4. When hyphal aggregation is complete, a concurrent reduction in the production of terrelysin was observed. Terrelysin was detected in the CSN at each measured time interval with peak concentrations observed on day 6.

As *A. terreus* is thermotolerant, this experiment was repeated at 37 °C to further compare morphological stages with terrelysin production. Again, cultures were grown for 12 days and ME and CSN was collected at 24 h intervals. Overall the growth of *A. terreus* was accelerated and

the morphological stages of conidial germination and hyphal extension occurred earlier compared to RT treatments (**Fig. 5b**). Terrelysin expression was highest on day 1 (12 µg/mg of total protein) and decreased 3-fold by day 2 (4 µg/mg of total protein) (**Fig. 5a**). The concentration of terrelysin was further reduced 8-fold by day 3 (0.5 µg/mg of total protein). In CSN, comparatively higher proportion of terrelysin was detected during initial growth at 37 °C compared to RT; however, there was a decline in the concentration of terrelysin in the culture supernatant at 37 °C.

In summary, we observed that terrelysin expression is concurrent with conidial germination, hyphal extension and aggregation. Terrelysin concentration declined on formation of the mycelial pellets. Lower levels of terrelysin were detected in the CSN.

Immunolocalization of terrelysin

The cellular localization of terrelysin was examined by immunohistochemical staining methods using the terrelysin-specific mAb 15B5. Terrelysin was widely distributed within the hyphae (**Fig. 6**), with staining often of greater intensity near the hyphal tips. This pattern was distinctly different than the positive control (mAb 13E11) that stained a leucine aminopeptidase, a secreted protease, within the extracellular matrix of the hyphae (Nayak *et al.*, 2011b). No staining was observed in the extracellular matrix with mAb 15B5.

DISCUSSION

Aspergillus terreus is an emerging opportunistic pathogen reported to be an etiological agent of fatal disseminated infections in immunocompromised populations (Baddley *et al*., 2003; Hachem *et al*., 2004; Iwen *et al*., 1998). Due to the ability of this species to grow at internal body temperatures and produce accessory conidia, this filamentous fungus is capable of initiating an infection and disseminating within the body. Resistance to antibiotic treatment such as amphotericin B allows for a longer infection time until effective treatment. A combination of these factors facilitates rapid dissemination of the fungus and can result in mortality. To date, very little is known about the overall pathogenesis and involvement of specific virulence factors of *A. terreus* during infection

In *A. fumigatus* infections, Asp-hemolysin was reported in tissues of experimental animals in an infection model (Ebina *et al*., 1982) and a role for hemolysin as potential virulence factors had been previously suggested (Berne *et al*., 2009; Ebina *et al*., 1982; Kumagai *et al*., 1999; Zuzek *et al*., 2006).

Previously, the gene for terrelysin was cloned and used to produce a recombinant protein (Nayak *et al*., 2011a). In this study, rTerrelysin was used as the antigen for the development of terrelysin-specific mAbs, which were then utilized to characterize terrelysin expression during *A. terreus* growth. Four of the 11 mAbs generated to rTerrelysin (3B2, 6D2, 16C7 and 2D3) did not react to native terrelysin in *A. terreus* ME. Two possibilities for this include; a) the mAbs recognized the N-terminal tag associated with rTerrelysin or b) their epitopes may be modified by post-translational processes under natural conditions as there is a putative *N*-glycosylation (N-X-S/T) site in the terrelysin sequence. One antibody (mAb 2D3), reacted with other purified recombinant proteins (rEnolase from *Chaetomium globosum* and rHev b 5 an allergen from

363 *Hevea brasiliensis*) expressed in the same pASK-IBA6 vector (data not shown). It was
364 concluded that mAb 2D3 recognizes an epitope in the N-terminal tag expressed by this vector.
365 Epitope mapping showed that mAb 2D3 recognizes the *Strep*-tag II sequence 'WSHPQFEK'
366 (data not shown). The other 3 mAbs that did not react with native terrelysin did not show
367 reactivity to other recombinant proteins, thus it is possible that their epitopes are conformational
368 or blocked by post-translational modifications to the native protein.

369 In order to be useful as a diagnostic tool for the serological detection of terrelysin, it is
370 essential that the mAb be species-specific. We tested 5 *A. terreus* strains, including the strain
371 used for genomic sequencing (NIH 2624/FGSC A1156). All mAbs tested detected terrelysin
372 expressed in this strain. Interestingly, 7D8, showed reactivity towards 3/5 strains of *A. terreus*
373 tested and while low affinity might explain this inconsistent detection, genetic diversity within
374 the epitope for this mAb is also a possibility. Although additional strains from different clinical
375 and environmental isolates were not tested, the data suggests consistent expression of terrelysin
376 across strains. Recently, a new species *A. alabamensis*, which is phenotypically homologous but
377 genetically different to *A. terreus*, was isolated from immunocompetent patient populations
378 (Balajee *et al*., 2009b). It will be important to test the reactivity of these mAbs to *A. alabamensis*
379 as well as other fungal species within Section *Terrei* such as *A. carneus* and *A. niveus*, which can
380 also cause infections under rare circumstances (Crissy *et al*., 1995; Wadhwani and Srivastava,
381 1984).

382 The terrelysin mAbs did not cross-react to ME from other clinically relevant *Aspergillus*
383 species including *A. fumigatus*, *A. flavus* and *A. niger*. Weak reactivity was observed for mAb
384 15C5 with *A. nidulans* and *A. parasiticus* in Western blots. This reactivity was restricted to
385 several high molecular weight proteins of ~ 50 and ~ 60 kDa and probably represents non-

specific staining. Using cross-reactivity data, we identified 3 mAbs (13G10, 15B5 and 10G4) that did not cross-react with any of the tested species. These 3 highly specific mAbs are important candidates for immunodiagnostic detection of *A. terreus* in clinical samples. Based on epitope mapping studies, we identified 2 different epitopes recognized by these antibodies. mAb 15B5 (IgG_1) recognized the epitope 'LYEGQFHS' while both mAbs 13G10 (IgG_1) and 10G4 (IgG_{2a}) recognized the epitope 'PSNEFE'. These epitope sequences were not identified in homologous hemolysins derived from other fungal species with known aegerolysin sequences (Nayak *et al*., 2011a).

Expression of hemolysin in relation to growth has been studied previously for *Pleurotus ostreatus* and *Pseudomonas aeruginosa* (Berne *et al*., 2002; Berne *et al*., 2007; Rao *et al*., 2008). For *P. ostreatus*, a basidiomycete, the greatest expression of ostreolysin was observed during the initial stages of fungal fruiting. For the bacteria *P. aeruginosa*, it was observed that expression of its hemolysin, PA0122 is highest in the stationary phase of growth (Rao *et al*., 2008). In contrast, limited information is available for the expression of aegerolysins in Ascomycetes. Recent studies have reported high Asp-hemolysin transcript levels in developing hyphae of *A. fumigatus* (Gravelat *et al*., 2008). In the present study, the expression of terrelysin was associated with morphological stages of *A. terreus* growth. The presence of terrelysin was highest during initial growth stages that encompassed conidial germination, hyphal extension and hyphal aggregation ("log phase"). During stationary growth phases, (lack of increase in mycelial pellet size) detection of terrelysin was significantly reduced. This was observed at earlier time intervals when cultures were grown at 37 °C and the presence of terrelysin consistently correlated with the morphological changes. These findings are consistent with studies by Rutqvist which showed that the hemolytic principle could be purified from *A. fumigatus* mycelia only during a limited

409 period of incubation at room temperature and that this period was earlier and shorter at 37 °C
410 (Rutqvist, 1965).

411 Using an inhibition ELISA, terrelysin was detected in CSN even though we did not
412 identify a signal peptide on the terrelysin sequence using SignalP 3.0. This is in contrast to an
413 earlier report where terrelysin was not identified in the secretome of *A. terreus* (Han *et al.*, 2010).
414 Whether detection of terrelysin in CSN is a result of active secretion, hyphal degradation,
415 fragmentation, or due to the involvement of unique secretory processes currently remains
416 unknown and is the focus of future research. Recently, Asp-hemolysin was identified as one of
417 the most abundant protein in the *A. fumigatus* secretome even though the protein does not
418 possess a signal peptide as noted earlier (Wartenberg *et al.*, 2011). Other studies have
419 highlighted secretion of proteins with no predicted signal sequence and have proposed non-
420 conventional secretory mechanisms (Medina *et al.*, 2005; Nickel, 2010; Rubartelli and Sitia,
421 1997). At day 6 in RT cultures and day 4 at 37 °C, small increases in levels of terrelysin were
422 observed in CSN. Hyphal fragmentation was observed at these time points and this could, in part,
423 explain the presence of terrelysin in CSN. Our immunolocalization studies demonstrate a
424 uniform distribution of terrelysin within *A. terreus* hyphal cytoplasm with greater reactivity
425 localized at hyphal tips. The hyphal tips are a region of extensive metabolic activity and consist
426 of higher concentrations of proteins compared to regions that are adjacent to this zone of apical
427 extension. Based on these morphological and immunohistochemical observations, it appears that
428 terrelysin is expressed during early stages of growth, probably during the emergence of hyphae
429 from conidia and active hyphal growth.

430 In summary, seven terrelysin mAbs were characterized and 3 were identified to be highly
431 specific for *A. terreus*. These mAbs were used to show that terrelysin is expressed during

conidial germination and early growth of *A. terreus* hyphae. Since terrelysin was detected in CSN, it is possible that it could be detected in the serum of infected patients; however, this aspect has not been confirmed and remains the focus of future research. Serologic detection of terrelysin would make terrelysin useful as a biomarker, however the early expression of terrelysin in *A. terreus* growth may limit its usefulness for detecting infection. The kinetics of terrelysin production *in vivo* is unknown and more studies are required.

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TABLE AND FIGURE LEGENDS

Table 1. Cross-reactivity profiles of terrelysin-mAbs analyzed by Western blot. As: *Aspergillus*, Ac: *Acremonium*, Al: *Alternaria*, Ch: *Chaetomium*, Cl: *Cladosporium*, Ex: *Exserohilum*, Fu: *Fusarium*, Ne: *Nesosartorya*, Pa: *Paecilomyces*, Pe: *Penicillium*, Sc: *Scopulariopsis*, Tr: *Trichoderma*. +: Positive reactivity.

Fig. 1. Reactivity of mAbs to rTerrelysin. Moderate reactivity is defined as ≤ 1.0 (OD_{405} value) when assayed with 1 μg/ml of mAb concentration. High reactivity is defined as ≥ 1.0 (OD_{405} value) when assayed with 1 μg/ml of mAb concentration.

Fig. 2. Western blot analysis of a) rTerrelysin and b) *A. terreus* mycelial extract with terrelysin mAbs. Lane 1: Molecular weight marker, 2: mAb 2G4, 3: mAb 3B2, 4: 6D2, 5: mAb 7D8, 6: mAb 13G10, 7: mAb 15B5, 8: mAb 16C7, 9: mAb 9F4, 10: mAb 19E3, 11: mAb 3B7, 12: mAb 10G4, 13: mAb 15C5, 14: mAb 15E4, 15: mAb 6E4, 16: mAb 10G7, 17: mAb 2D3. Positive reactivity is identified by presence of an immunoreactive band at ~ 17 kDa.

Fig. 3. Epitope mapping of anti-rTerrelysin mAbs. SPOTs membrane scans for mAbs 15B5, 13G10 and 10G4. Each spot represents a decapeptide of rTerrelysin sequence. Decapeptides were sequential with an overlap of 2 amino acids and spanned the entire sequence of rTerrelysin including the N-terminal purification *Strep*-tag II and the Factor Xa cleavage site.

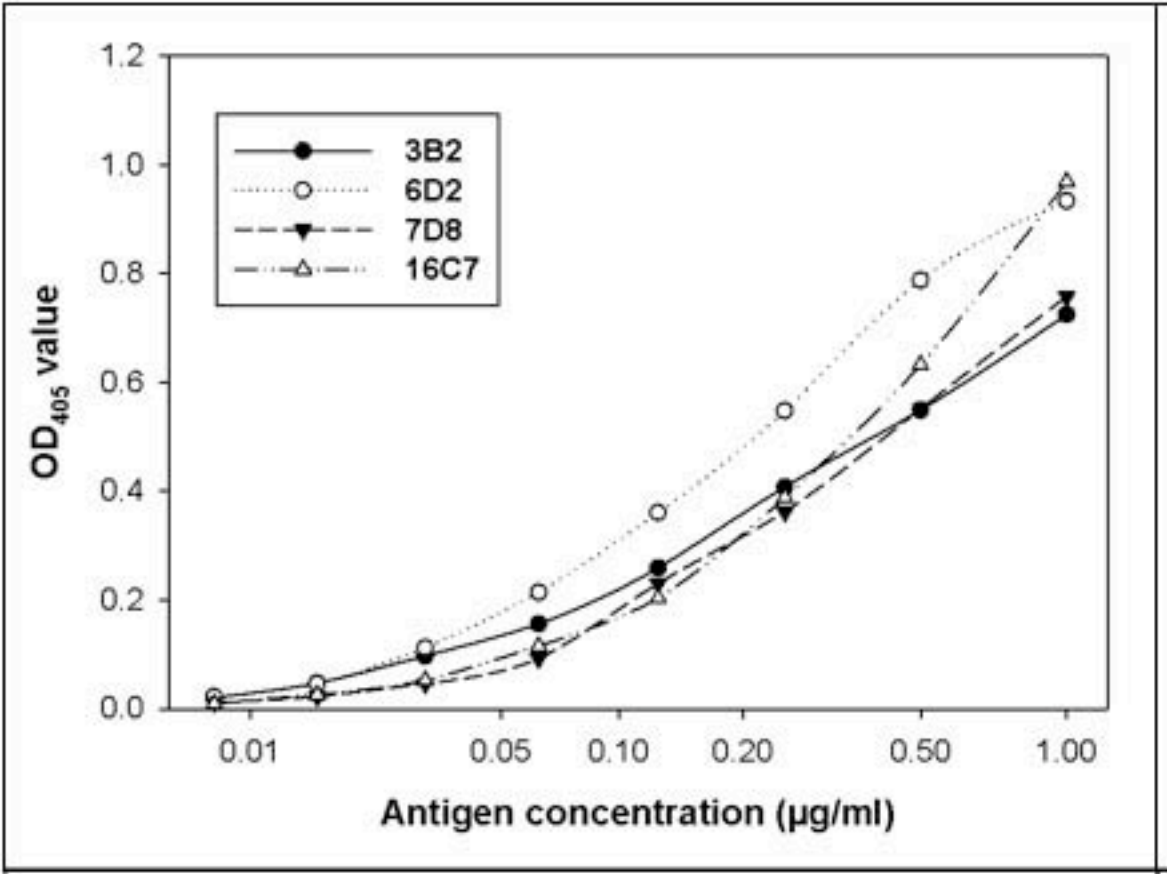
Fig. 4a. Kinetics of terrelysin expression at RT. ELISA inhibition analysis performed using mAb 10G4. Data points represent samples collected on different days after inoculation and error bars represent standard error of the mean calculated from 3 separate experiments.

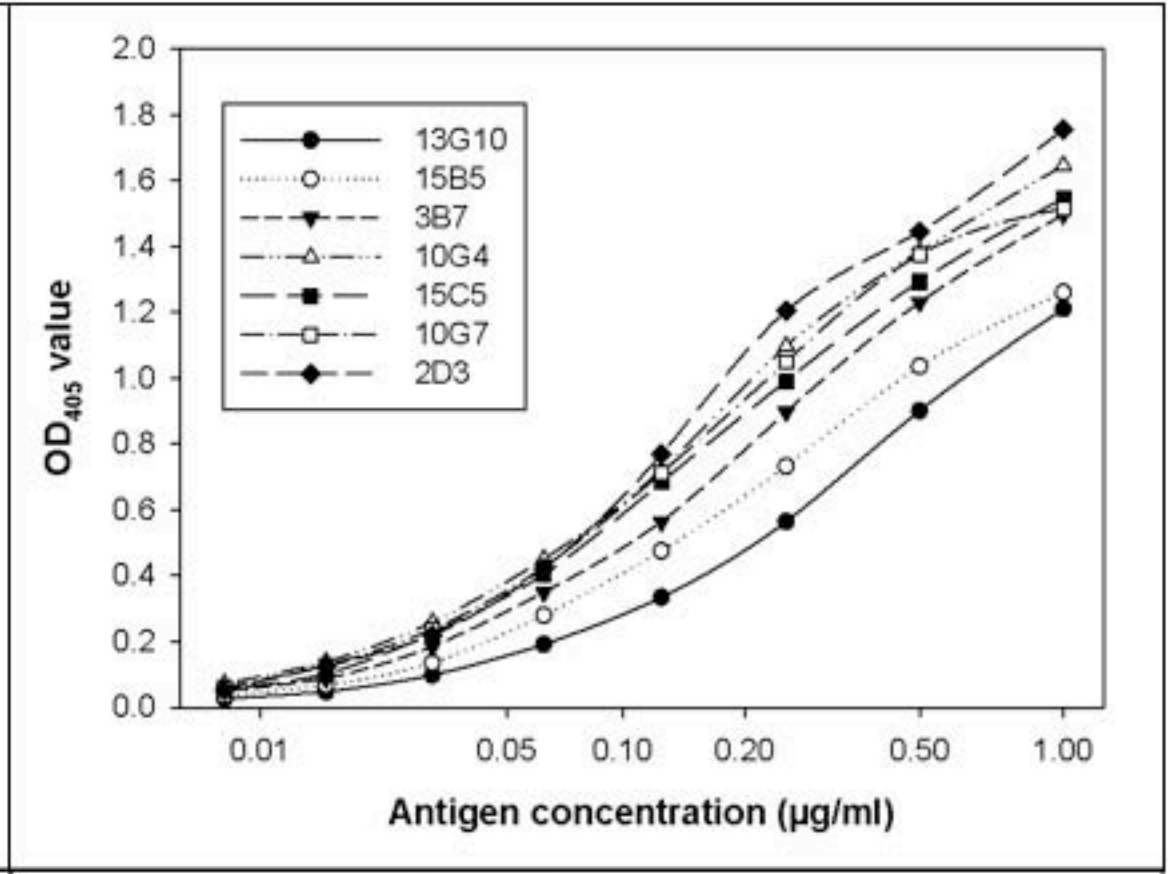
Fig. 4b. Morphological changes and progression of *A. terreus* culture growth at RT. Black arrows point to germinating conidia and hyphal extension in early cultures. Bars represent 10 μm for 0-24 h and 100 μm for 48-120 h.

Fig. 5a. Kinetics of terrelysin expression at 37 °C. ELISA inhibition analysis performed using mAb 10G4. Data points represent samples collected on different days after inoculation and error bars represent standard error of the mean calculated from 3 separate experiments.

Fig. 5b. Morphological changes and progression of *A. terreus* culture growth at 37 °C. Black arrows indicate germinating conidia and hyphal extension in early cultures. Bars represent 10 μm for 12-24 h and 100 μm for 36-72 h.

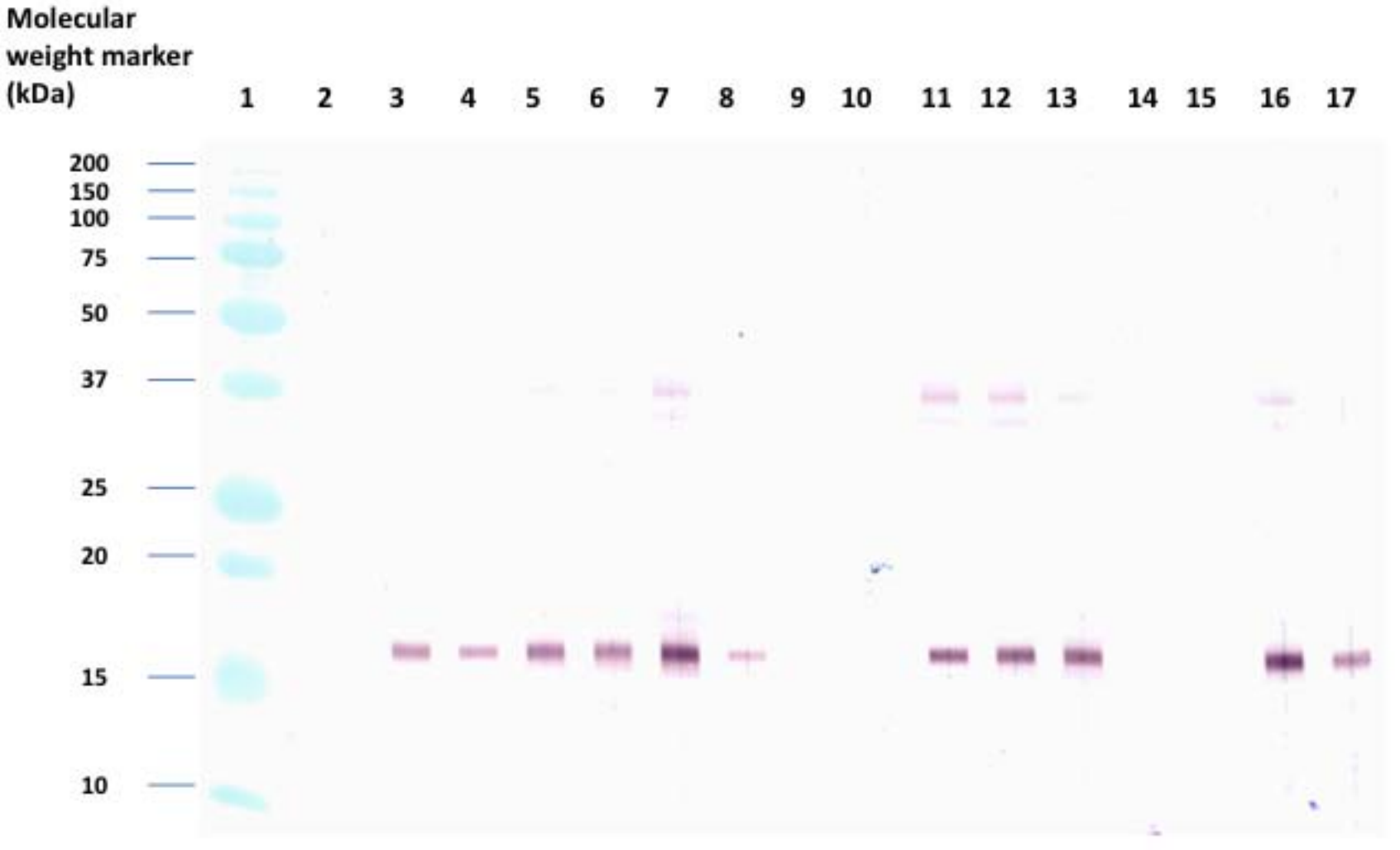
Fig. 6. Immunolocalization of terrelysin in *A. terreus* hyphae. Immunolocalization of the antigens was determined AlexaFluor® 594-labeled goat anti-mouse IgG secondary antibodies (Red) and nuclear staining was identified through DAPI staining (blue). mAb 13E11 served as a positive control and reacts with *A. terreus* leucine aminopeptidase, while *Stachybotrys* specific mAb 9B4 served as a negative control. White arrows indicate staining by anti-terrelysin 15B5 mAb at fungal hyphal tips.





MODERATE REACTIVITY

HIGH REACTIVITY


Molecular weight marker (kDa)
1
2
3
4
5
6
7
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9
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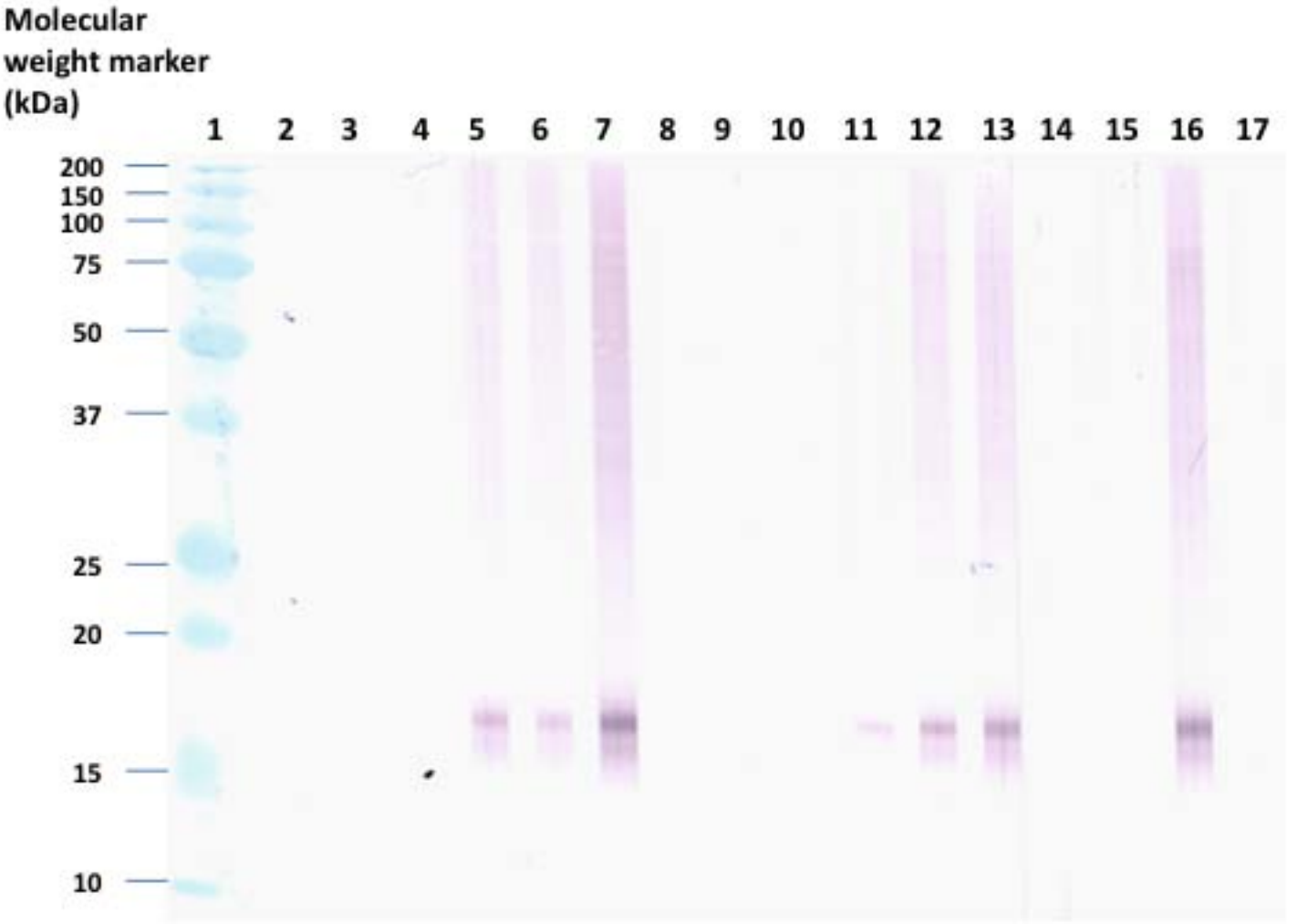
11
12
13
14
15
16
17
200
150
100
75
50
37
25
20
15
10


Molecular
weight marker
(kDa)
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16
17
200
150
100
75
50
37
25
20
15
10

Spot number

15B5

1-20

21-40

41-60

61-68

Spot number

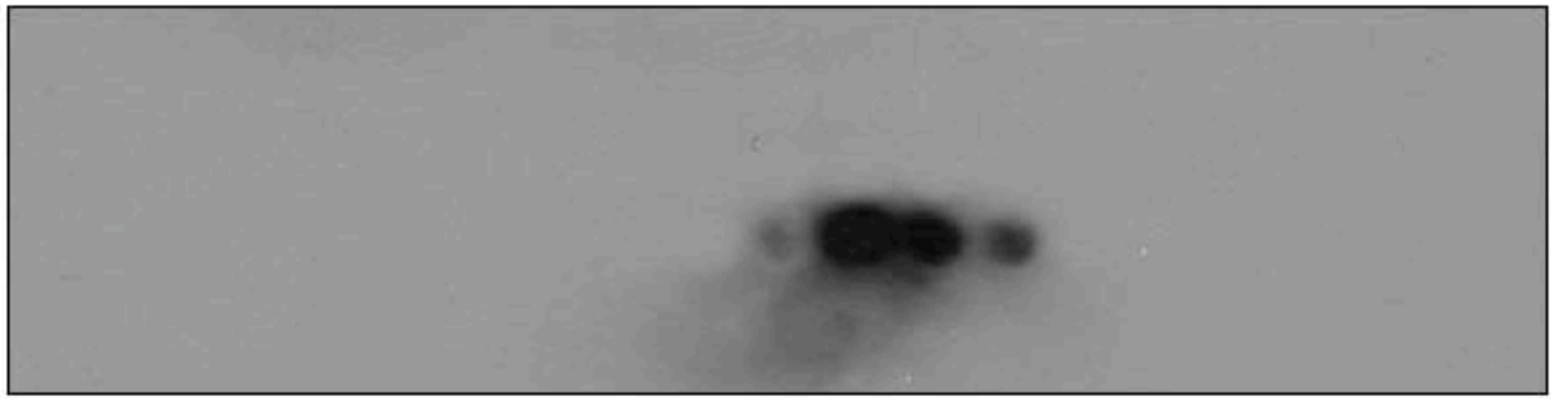
13G10

1-20

21-40

41-60

61-68



Spot number

10G4

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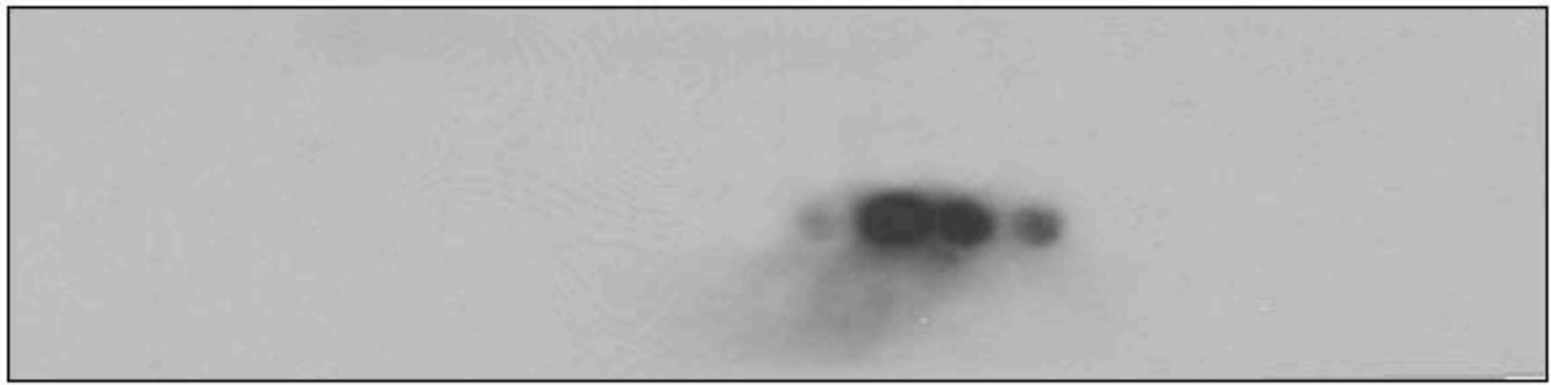
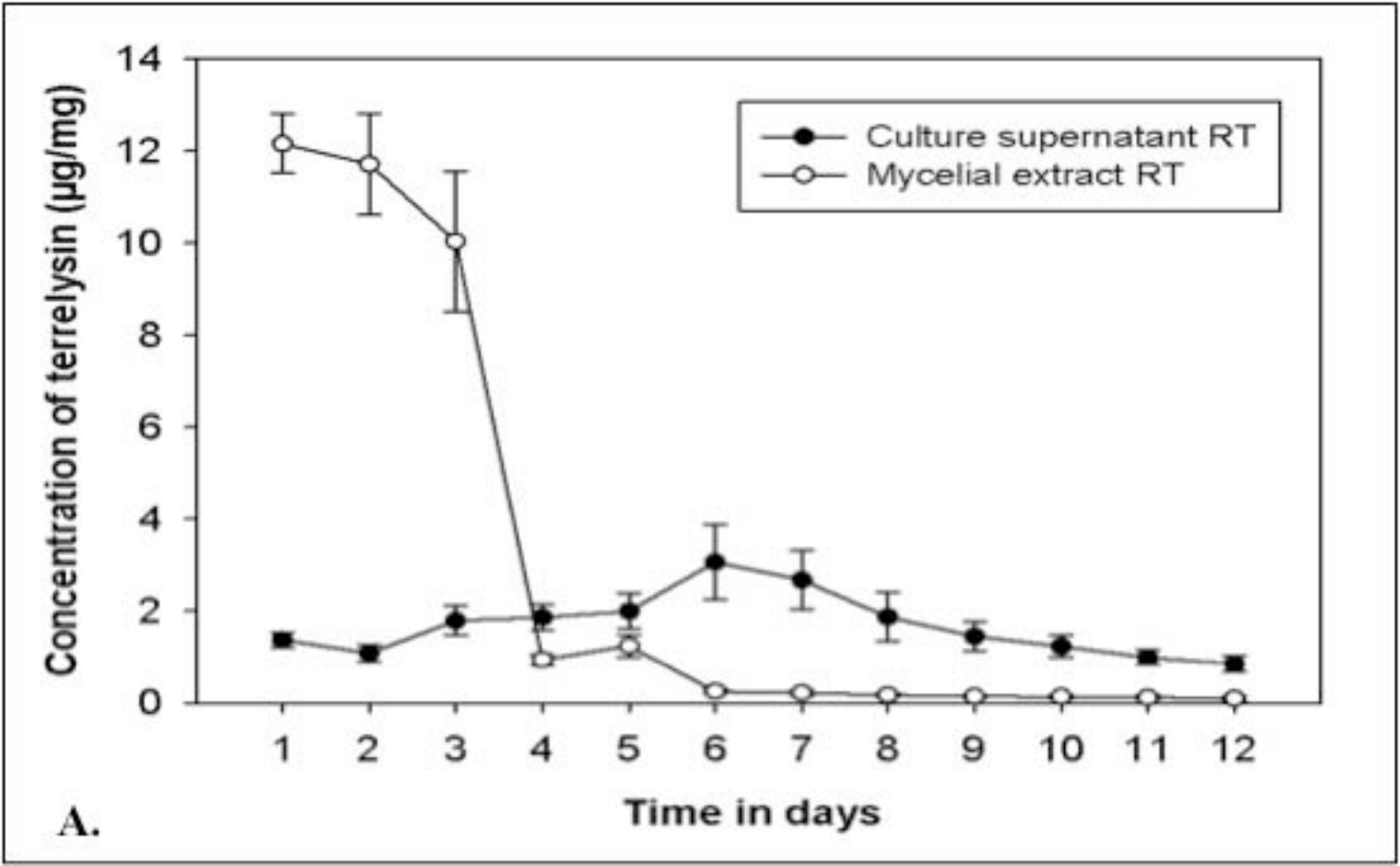
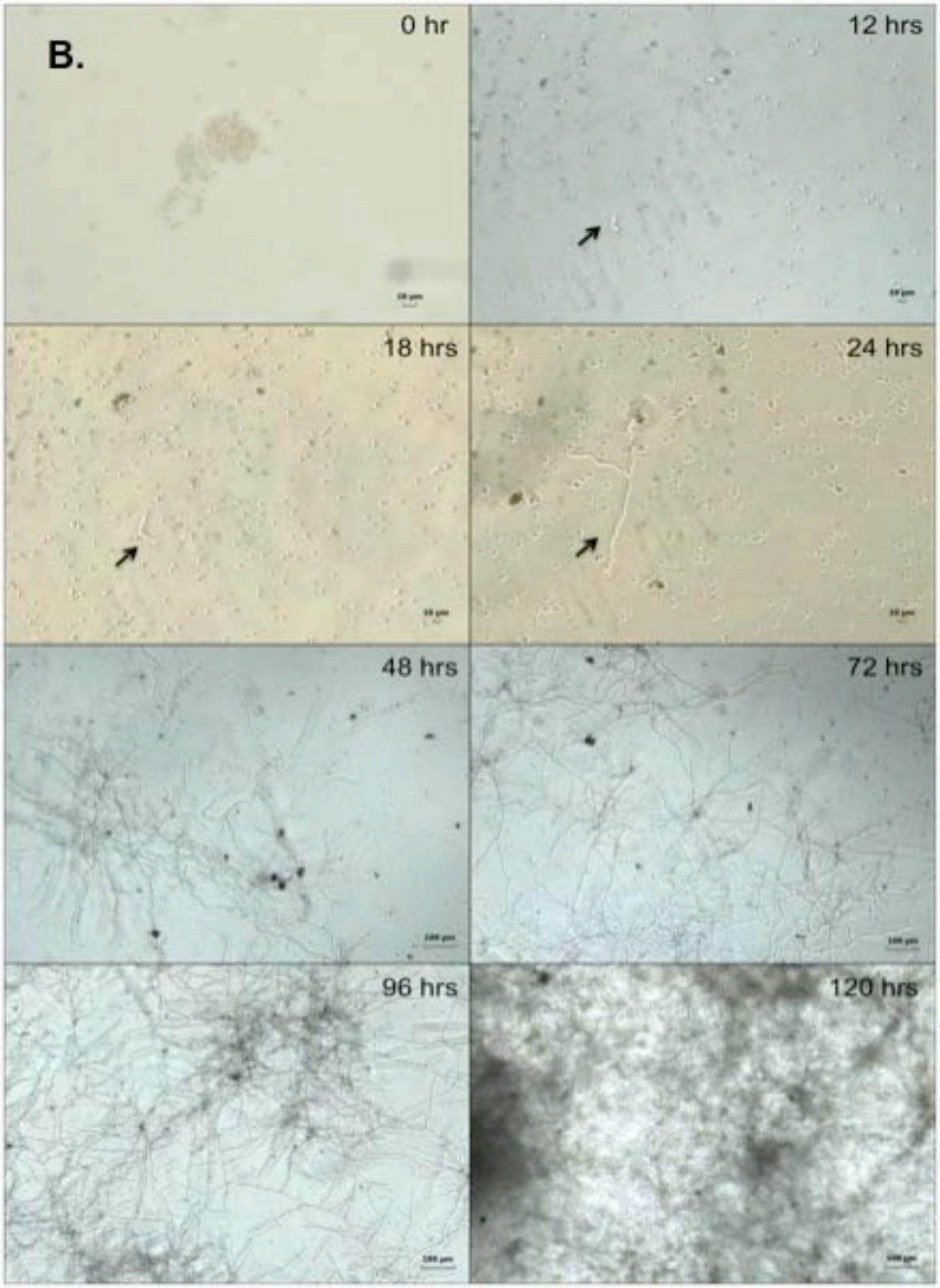
21-40

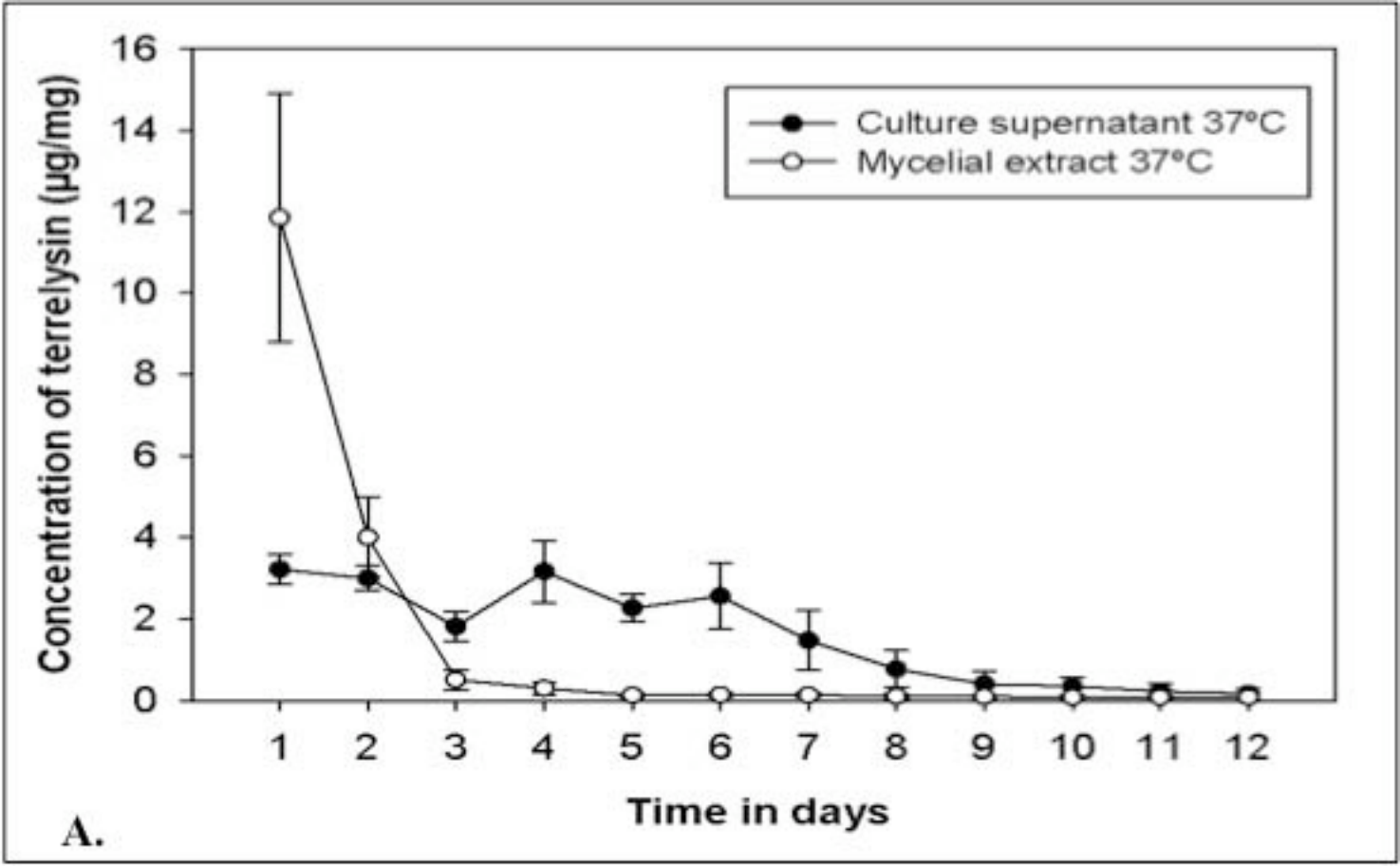
41-60

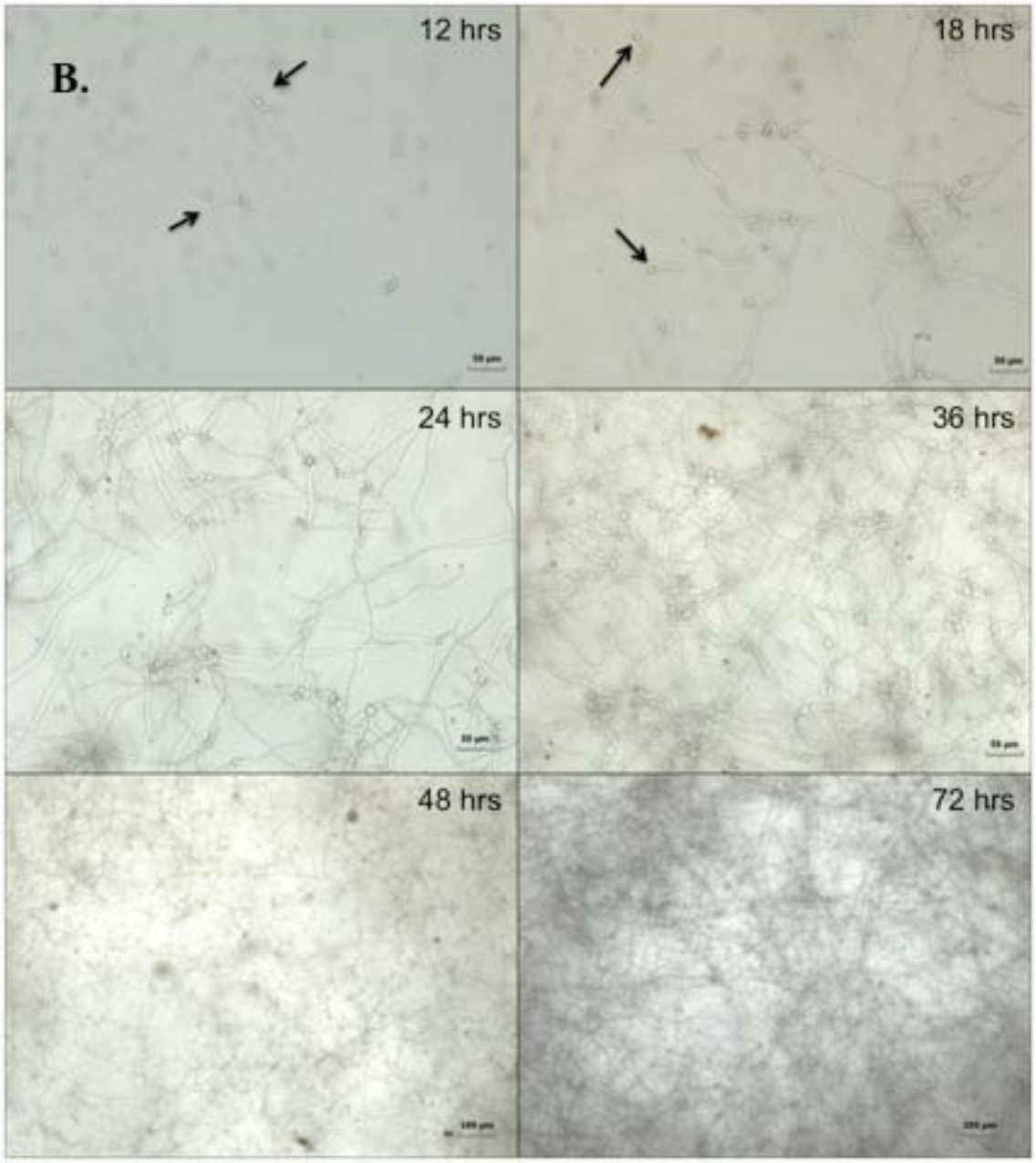
61-68

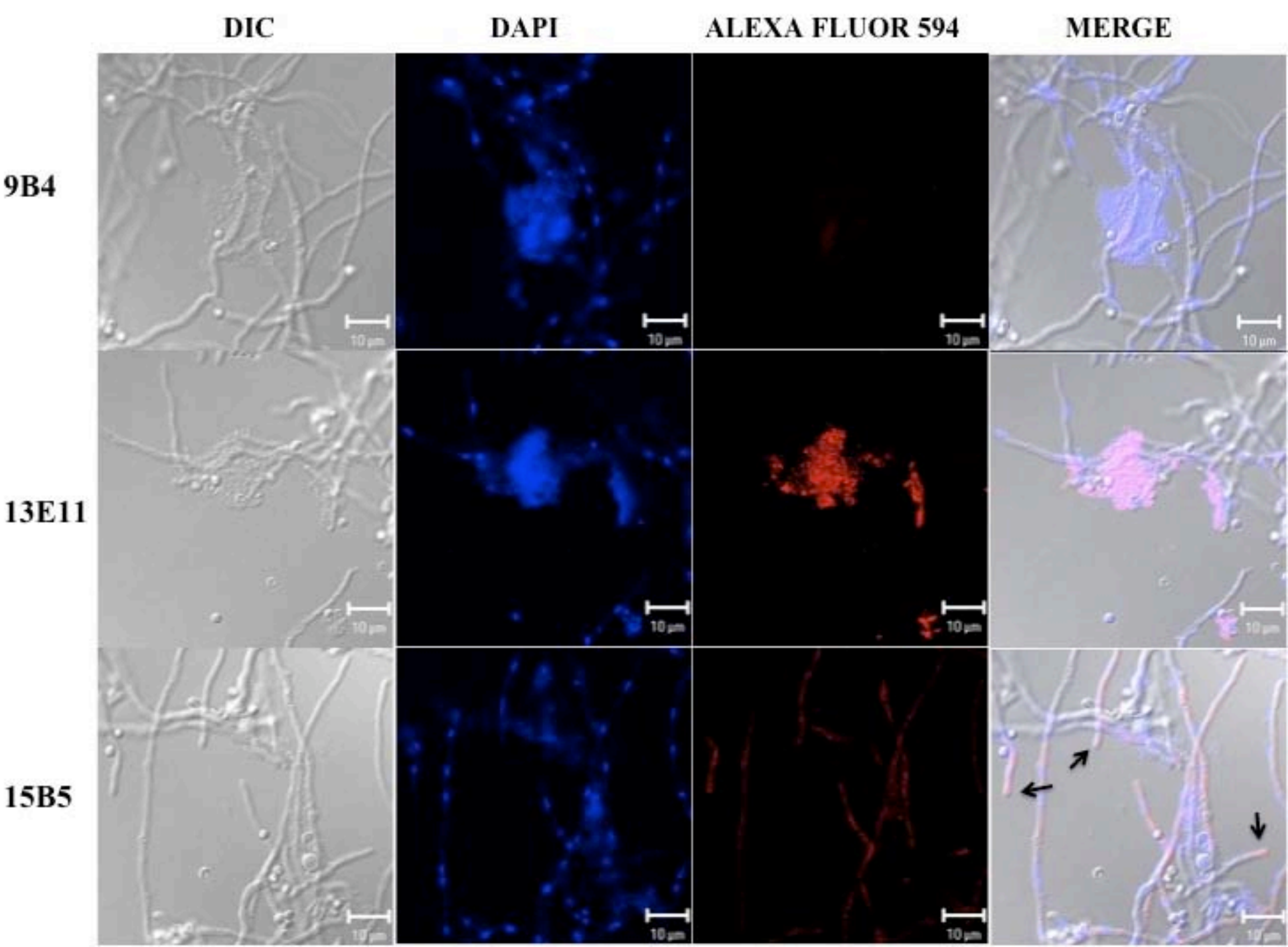



Concentration of terrelysin (µg/mg)
14
12
10
8
6
4
2
0
Culture supernatant RT
Mycelial extract RT
1
2
3
4
5
6
7
8
9
10
11
12
Time in days
A.


B.
0 hr
12 hrs
18 hrs
24 hrs
48 hrs
72 hrs
96 hrs
120 hrs


Concentration of terrelysin (µg/mg)
16
14
12
10
8
6
4
2
0
1
2
3
4
5
6
7
8
9
10
11
12
Time in days
Culture supernatant 37°C
Mycelial extract 37°C
A.


B.
12 hrs
18 hrs
24 hrs
36 hrs
48 hrs
72 hrs


DIC
DAPI
ALEXA FLUOR 594
MERGE
9B4
13E11
15B5
10 µm