# APPENDIX

## A. Culture Media

### 1. Brain Heart Infusion media (BHI)

This medium was used in the routine culture of yeasts.

| | |
|---|---|
| Brain heart infusion (Oxoid) | 37 g |
| Agar Bacteriological No.1 (Oxoid) | 20 g |
| Distilled water | 1000 ml |

The solution was autoclaved for 15 minutes at 121°C and 15 lb pressure. Media was poured into the Petri-dishes in the same way as SDA and 10 ml of media was poured into 25x150 mm sterile test tubes and allowed to cool and harden on a slant.

For broth culture medium, agar was omitted and 200 ml aliquots were kept in 1000 ml flasks at 4°C before use.

### 2. Sabouraud's Dextrose Agar (SDA)

This medium was used in routine culture and in sterility controls.

| | |
|---|---|
| Peptone (Oxoid) | 10 g |
| D-glucose (Sigma) | 40 g |
| Agar Bacteriological No.1 (Oxoid) | 20 g |
| Distilled water | 1000 ml |

The solution was autoclaved for 15 minutes at 121°C and 15 lb pressure. 15 ml of solution were poured under sterile conditions into 90 mm diameter Petri-dishes (Sterilin, Bibby Sterilin Ltd., Staffordshire, UK) whilst the medium was still hot and allowed to cool and harden at room temperature. Medium was stored after checking for sterility at room temperature and 37°C for a maximum of 4 weeks at 4°C.

### 3. Malt Extract (ME) agar media

| | |
|---|---|
| Malt Extract (Oxoid) | 20 g |
| Peptone | 10 g |
| Glucose | 20 g |
| Distilled water | 1000 ml |

10 ml of solution was poured into 25x150 mm test tubes and then autoclaved for 10 minutes at 110°C and 15 lb pressure. When the medium was still hot, it was cooled and allowed to harden on a slant. The medium was stored (after checking for sterility at room temperature and 37°C) for a maximum of 4 weeks at 4°C. For broth culture medium, agar was omitted and 200 ml aliquots were kept in 1000 ml flasks at 4°C before use.

4. **Minimal medium broth pH 5.5**

| | |
|---|---|
| 15 mM glucose (Sigma) | 2.7 g |
| 10 mM $MgSO_4$ | 2.4 g |
| 29.4 mM $KH_2PO_4$ | 4.0 g |
| 13.0 mM glycine (Sigma) | 0.97 g |
| 3.0 µM vitamin B1 (Sigma) | 0.001 g |

The above components were mixed and the pH was adjusted to 5.5 by adding 1.0 M HCl. Aliquots of 200 ml of the minimal medium in 1000 ml flasks were made and autoclaved for 15 minutes at 121°C and 15 lb pressure. The medium was then kept for a maximum of 4 weeks at 4°C.

5. **ATCC medium: 712 *Acanthamoeba* medium**

| | |
|---|---|
| Proteose Peptone (BD 211684) | 20.0 g |
| Yeast extract | 1.0 g |
| Agar (if needed) | 20.0 g |
| Distilled water | 950.0 ml |

Prepare and sterilize separately each of the following components and add directly to the basal medium as indicated below to avoid precipitation:

| | |
|---|---|
| 0.4 M $MgSO_4 . 7H_2O$ | 10.0 ml |
| 0.05 M $CaCl_2$ | 8.0 ml |
| 0.1 M Sodium citrate . $2H_2O$ | 34.0 ml |
| 0.005 M $Fe(NH_4)2(SO_4)2 . 6H_2O$ | 10.0 ml |
| 0.25 M $Na_2HPO_4 . 7H_2O$ | 10.0 ml |
| 0.25 M $KH_2PO_4$ | 10.0 ml |

Adjust pH to 6.5. Autoclave 25 minutes at 121°C.Add aseptically:

| | |
|---|---|
| 2 M Glucose (filter-sterilized) | 50.0 ml |

# Manuscript

# Interaction of *Penicillium marneffei* and *Candida albicans* with soil amoeba as a model of fungal pathogenesis

Sirida Youngchim[1*], Joshua D. Nosanchuk[2] and Noungnuch Vanittanokom[1]

[1]Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai, 50200, Thailand, [2]Departments of Microbiology and Immunology and Medicine, Albert Einstein College of Medicine, Bronx, New York 10461,USA

*Correspondence*: Dr Sirida Youngchim, Department Microbiology, Faculty of Medicine, ChiangMai University, ChiangMai 50200, Thailand, Tel.: +6653 945332 Ext 412, Fax: +6653 217144

e-mails: syoungch@med.cmu.ac.th and syoungchim@gmail.com

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**ABSTRACT**

*Penicillium marneffei* is an important dimorphic mycosis endemic in Southeast Asia, but the origin and maintenance of virulence in this organism is mysterious. Recently, several pathogenic fungi including *Cryptococcus neoformans, Blastomyces dermatitidis*, *Sporothrix schenckii*, and *Histoplasma capsulatum* were shown to interact with amoebae in similar behavior, applying that fungal pathogenic strategies may arise from environmental interactions with phagocytic microorganisms. In this study, we examined the interactions of *P. marneffei* and *Candida albicans* with the soil amoeba *Acanthameobae castellanii.* Both *P. marneffei* and *C. albicans* were ingested by amoebae, and phagocytosis of fungal cells resulted in amoeba death and fungal growth. Exposure of *C. albicans* to amoebae induced germ tube formation and changed to hyphae at 37°C. These results are consistent with the view that soil amoebae, environmental predators may contribute to the selection and maintenance of certain traits in *P. marneffei* and *C. albicans* that confer on these microbes the capacity for virulence in mammals.

## 1. Introduction

Most of human-pathogenic fungi that are attained from the environment dwell in ecological niches defined by soils, trees, and decaying vegetation. Soils are extreme environments, and soil-dwelling microbes must alter to rapidly changing, harsh conditions. Soil microbes occupy an environment where there must be brutal competition for nutrients. In addition to these nutritional and physical stresses, soil-dwelling microbes must cope with predators in the form of amoebae and other protista, which feed on bacteria and fungi. Consequently, soil-dwelling microbes must develop ways to escape phagocytosis and/or survive ingestion through mechanisms for intracellular survival.

The amoebae are an extremely diverse group of eukaryotic microorganisms that constitute a major class of phagocytic organisms in soils. *Acanthamoeba* is a free-living, ubiquitous ameba that occurs in trophozoite and cyst stages during its life cycle. In addition, *Acanthamoeba castellanii* is a soil amoeba that feeds on bacteria and fungi that was originally isolated from cultures of *Cryptococcus* neoformans, and has been used to study bacteria–amoeba interactions. Both plant and animal pathogenic fungi that reside in soils and vegetation inhabit extreme environments where they must compete with other microbes, endure extremes of humidity and survive predation by amoeboid organisms and small animals such as nematodes. Hence, both share comparable risks and selection pressures. For several human pathogenic fungi it has been demonstrated that determinants of virulence needed for mammalian pathogenicity are also important for surviving predation by amoeba, slime molds, and nematodes (Steenbergen, *et al.*, 2001; 2003; 2004).

*P. marneffei* is unique in its genus in being dimorphism by growing in living tissue or in culture at 37°C as yeast-like organisms (although the latter are more properly described as fission arthroconidia) or in culture at environmental temperatures as a mycelial phase. The ability of *P. marneffei* to grow at 37°C must play a major role in its infectivity (Hamilton, 2003). At 37° C *in vitro*, *P. marneffei* is grown as arthroconidia divided by fission to produce yeast cells. The morphology of yeast cells grown in vitro differs from that found *in vivo* (Cánovas & Andrianopoulos, 2007). It depends on the nutrition conditions of culture media which influence the morphology of *P. marneffei* yeast cells (Tongchusak *et al.*, 2004).

Most common pathogenic fungi are considerate saprophytic because they are free living and do not require an animal host for propagation. Likewise, several dimorphic fungi are important human pathogens, but the origin and maintenance of virulence in these organisms is mysterious, since an interaction with a mammalian host is not a required for fungal survival. For instance, *Cryptococcus neoformans* was shown to interact with macrophages, slime molds (Steenbergen, *et al.*, 2003) and amoebae (Steenbergen, *et al.*, 2001) in a similar manner, suggesting that fungal pathogenic strategies may arise from environmental interactions with phagocytic microorganisms.Since thermally dimorphic fungi are found primarily in the soil, we hypothesized that *A. castellanii*, an environmental phagocytic predators could place selective pressures on *P. marneffei* soil fungi. Here, we investigated the interaction of *P. marneffei* with *A. castellanii.* According to global warming has been assumed to bring about new fungal diseases in the coming century, to understand the mechanisms by which virulence emerges in environmental microbes is necessary (Garcia-Solache, & Casadevall, 2010).

## 2. Materials and methods

### 2.1 Organisms and culture conditions.

*Penicillium marneffei* ATCC 200051, *Candida albicans* ATCC 90028, *A. fumigatus* B5233 and *A. castellanii* ATCC 30324 were obtained from American Type Culture Collection (ATCC). *P. marneffei* and *C. albicans* were maintained on Sabouraud dextrose agar (SDA, Difco) at 25ºC. For experimental use and routine maintenance, *Acanthamoeba castellanii* was cultured as adherent cells in peptone-yeast extract–glucose (PYG) broth (ATCC medium 354) at 28 ºC in the dark (Bozue & Johnson, 1996).

### 2.2 Production of *P. marneffei* and *A. fumigatus* conidia

*P. marneffei* ATCC 200051 was isolated from a bone marrow sample of a patient infected with HIV at Maharaj Nakorn Chiang Mai University, Chiang Mai, Thailand. *P. marneffei* was maintained by monthly subculture onto Malt Extract Agar (MEA; Oxoid). *P. marneffei* and *A. fumigatus* were grown on MEA for 7-10 days at 25° C, and added 5 ml of sterile PBS onto surface growth; conidia were removed by gentle scraping with a cotton swab. The conidia were collected by filtration through sterile glass wool, centrifuge at 5000 g for 15 min, and then washes three times with sterile PBS. *C. albicans* ATCC 90028was cultured on Sabouraud dextrose broth for 24 h at 25° C, and then harvested by centrifugation at 5000 g for 15 min and washes 3 times with PBS.

**2.3 *Acanthamoeba castellanii*.** *Acanthamoeba castellanii* ATCC 30324 was obtained from the American Type Culture Collection and was maintained routinely at room temperature in PYG broth (ATCC medium 354) as monolayers in 75-cm$^2$ tissue culture flasks. *A. castellanii* was harvested by tapping the flasks, centrifuged at 2500 rpm for 10 min, and suspended in fresh distilled water or 0.02 M phosphate-buffered saline (PBS) (0.137 M NaCl, 0.003 M sodium phosphate [pH 7.4]). Cell counts were determined with a hemocytometer with a modified Fuchs-Rosenthal chamber. In addition, *A. castellanii* viability was determined by trypan blue staining, and the initial viability was always greater than 98% (data not shown). Amoebae were subcultured at intervals of 10 days.

**2.4 Phagocytosis Assay.** *A. castellanii* cells were removed from tissue culture flasks (Corning, Corning, N.Y.), washed with PBS, and counted with a hemocytometer. The cells were suspended to $10^6$ cells/ml in PBS and added to 24-well tissue culture plates at $10^6$ cells/well and allowed to adhere for 2 h at 28°C before the addition of fungal cells, *P. marneffei, A. fumigates* and *C. albicans* at a 10:1 effector-to-target ratio. The plates were incubated for 2 h at 28°C and 37°C. The media were aspirated, and the cells were fixed with ice-cold methanol for 30 min at 4°C and washed three times with PBS, stained with Giemsa diluted 1:10 in PBS for 2 h. The plates were viewed with a microscope at 100 magnification, and four wells per experimental condition were used to ascertain the percentage of phagocytic cells. The phagocytic index is the number of *A. castellanii* with internalized yeast per 100 amoebae (Steenbergen *et al.*, 2001).

**2.5 Fungal killing assays.** *A. castellanii* cells were removed from tissue culture flasks (Corning, Corning, N.Y.), washed with PBS, and counted with a hemocytometer. The cells were suspended to $10^5$ cells/ml in PBS, and 100 μl was added to 96-well tissue culture plates. The plates were incubated at 37°C for 2 h prior to adding fungal cells to allow for *A. castellanii* acclimation. *A. castellanii* viability was determined by trypan blue staining, and the initial viability was always greater than 98% (data not shown). *P. marneffei conidia* were washed, harvested, and suspended in PBS, and cell numbers were determined with a hemocytometer. Fungal cells were added to the acclimated cultures of *A. castellanii* at a 1:10 effector-to-target ratio and incubated at 37°C. At 0, 24, and 48 h, the number of viable yeast cells was determined by CFU. At each time interval, the 24-well plates were placed on ice for 10 min to loosen the cells from the bottoms of the plates. The *A. castellanii* cells were lyses by shear stress induced by pulling the suspension through a 27-gauge needle five to several times (Moffat & Tompkins, 1992). Fungal viability was unaffected by this procedure, as determined by comparison of initial hemocytometer determinations and CFU counts. For each well, serial dilutions were plated onto BHI agar plates, which were then incubated at 37°C for 48 h. At each time, a minimum of 4 tissue culture wells per isolate were used to determine CFU, and each experiment was repeated at least one time. Conidial killing assays were performed as described above with two differences.

**2.6 Amoeba killing.** Trypan blue exclusion assays were applied to determine the number of viable *A. castellanii* cells at time interval, 0, 24, and 48 h. Amoebae and fungal cells, *P. marneffei, A. fumigatus* and *C. albicans* were incubated in PBS in 24-well tissue culture plates at a 1:10 ratio. At each time interval, the medium was aspirated and the cultures were incubated with a 1:10 dilution of trypan blue in PBS. The 24-well plates were viewed at a magnification of X100, and the percentage of dead amoebae was determine d by counting the number of amoeba cells unable to exclude the dye per total amoebae counted. At each time interval, five wells per culture condition were counted and experiments were repeated at least one additional time.

**2.7 Germ tube formation**. *A. castellanii* cells were removed from tissue culture flasks, washed with PBS, and counted with a hemocytometer. The cells were suspended to $10^6$ cells/ml in PBS, and 1ml was added to eight-chamber glass culture slides (SPL Lifescience, Korea). The plates were incubated at 37°C for 2 h prior to adding fungal cells to allow for *A. castellanii* acclimation. *A. castellanii* viability was determined by trypan blue staining, and the initial viability was always greater than 98% (data not shown). *C. albicans* were suspended at $10^7$ cells/ml, which confirmed by CFU determination on SDA plates at 28°C. *C. albicans* were added to the acclimated cultures of *A. castellanii* at a 1:10 effector-to-target ratio and incubated at 28 and 37°C for 24 hours. The germ tube germination was calculated by counting the total number of *C albicans* (in both of germination and non germination of yeast cells). Five wells were counted per experimental condition, and each experiment was repeated. *C. albicans* incubated in PBS alone was included in the experiment as negative control.

**2.8 Transmission electron microscopy (TEM).** TEM was used to examine the intracellular compartment of *P. marneffei and C. albicans* within *A. castellanii.* Plastic adherent *A. castellanii* monolayer containing 2 x$10^6$/well in 24-well tissue culture plate was infected with *P. marneffei* or *C. albicans* at a multiplicity of infection (MOI) of 10. After 2 hours of incubation at 37 °C, amoeba infected with *C. albicans* was removed by using rubber policeman and fixed with 2.5 % glutaraldehyde in 0.1 M cacodylate at room temperature overnight. The sample was prepared for electron microscopy by previous described (Steenbergen *et al.*, 2001). The samples were mounted with gold–palladium, and viewed in a Transmission Electron Microscope JEOL JEM-2010.

**2.9 Interaction of *C. albicans* with *A. castellanii* at different temperature.** To investigate the effect of temperature, *C. albicans* was incubated with *A. castellanii* at different temperature, 28°C and 37°C for variable time. Amoebae and fungal cells were incubated in PBS in eight-chamber glass culture slides at a 1:10 ratio. At each time point, the medium was aspirated, wells were washed with PBS, and fixed with 1% paraformaldehyde at 4°C for 30 min. Coverslips were mounted with a mounting solution of 0.1% *n*-propyl gallate and 50% glycerol in PBS, and the slides were viewed at a magnification of X100 at different time, 30 min, 2, 24 and 48 h.

**2.10 Statistical analysis.** Student's *t* test was used for statistical analyses. Both the statistical analysis and the graphs were compiled by two tailed, unpaired Student's t-test using Prism 4 software (GraphPad). A *P*-value $\leq 0.05$ was considered significant.

## 3. Results

### 3.1 Phagocytosis of fungi by amoebae.

The phagocytosis indexes of *A. castellanii* for *P. marneffei*, *C. albicans* and *A. fumigatus* were investigated (Fig. 1,2). *A. castellanii* was capable to phagocytose each of the fungi. *P. marneffei* and *C. albicans* were phagocytosed at significantly higher rates compared to *A. fumigatus* in both 37°C and room temperature ($p \leq 0.001$). In addition, the phagocytosis index of *P. marneffei* conidia was found 80% which significantly higher than other fungi, *C. albicans* and *A. fumigatus* in both temperature (37°C and 28°C). Based on dimorphic fungus, both yeast cells and conidia of *P. marneffei* were determined the phagocytic indexes. *P. marneffei* conidia were phagocytosed by *A. castellanii* at significantly higher rate than yeast cells at 37°C ($p \leq 0.01$). In contrast, the phagocytic indexes of conidia and yeast cells of *P. marneffei* were not different when studied at room temperature (28°C).

### 3.2 Growth of *P. marneffei* and *C. albicans* in presence of *A. castellanii.*

Neither fungi nor amoebae replicated significantly when incubated in PBS alone, probably as a result of nutritional starvation. Incubation of *P. marneffei* with amoeba cells resulted in significant CFU increases ranging between 2- to 16-fold when incubated for 24 to 48 hours (Fig.3A). Increases in CFU for *P. marneffei* in the presence of amoebae at 48 h were significant compared to the fungi alone ($P \leq 0.001$). For *C. albicans*, incubation with *A. castellanii* resulted in threefold increase in CFU compared to PBS-alone condition at 24 and 48 hours (Fig.3B). Initial numbers of CFU changed, since the experiment with all fungi was done simultaneously. Each experiment was done at least twice with similar results.

### 3.3 Amoebae are killed by *P. marneffei, A. fumigatus,* and *C. albicans.*

Trypan blue exclusion assays were used to determine the percentage of amoebae alive after incubation with the fungi. The results, depicted in 3C , have shown that a significant proportion of amoebae exposed to *P. marneffei*, *A. fumigatus*, or *C. albicans* were killed. At the beginning of the assay, 99% of the amoebae were alive and excluded the dye. At 48 h, more than 50% of amoeba cells were no longer viable, as indicated by incapacity to exclude dye. *P. marneffei* and *A. fumigatus* killed 30 to 50 % of *A. castellanii* cells. *P. marneffei* had the highest killing rate of 50.68% compared to other fungi, *A. fumigatus* and *C. albicans*. In addition, *C. albicans* had the lowest killing rate of 32.54 % at 48 h. The amoeba death occurred within the first 24 h for all the isolates and continued to cause amoeba death through 48 h.

### 3.4 Germ tube formation.

Incubation of *C albicans* with amoeba cells resulted in germ tube formation at both room (28°C) temperature and 37 °C (Fig. 3D). The percentages of germ tube production were found 28.42% and 60.79 % at room temperature and 37°C, respectively (Fig.3D). The germ tube production was significantly higher at 37°C compared to room temperature (28°C) ($p \leq 0.0001$). However, *C. albicans* was incapable to produce germ tube in PBS in either 28°C or 37°C (data not shown).

### 3.5 Electron microscopy of amoeba-fungus interactions.

TEM was used to demonstrate that amoebae internalized *P. marneffei* conidia after incubation 30 min and 2 h (Fig. 4A,B). The conidia enclosed in membrane bound vesicles inside *A. catellanii.* In addition, the interaction between *C. albicans* and amoebae was studied by TEM showing multiple contacts with yeast cells and amoeba during phagocytosis (Fig.5). *C. albicans* is phagocytosed and encircled in membrane-bound vacuoles (Fig.5A,B). Several amoebae had more than one internalized yeast cell indicative of either separate phagocytic events or intracellular replication. Within 24 h, yeast cells of *C. albicans* exposed to amoebae were internalized into phagocytic vacuoles (Fig. 5A,B). The internalized yeast cells were starting germination to form germ tube after incubated at 37°C for 2 h (Fig. 5C,D). However, *C. albicans* incubated in PBS in either room temperature or 37°C remained yeast cells. Growth of *C. albicans* correlated with the increasing number of the death amoeba. Surprisingly, germ tube formation in *C. albicans* was found at room temperature when phagocytosed into amoeba, although the percentage of germ tube was lower than found at 37°C.

### 3.6 Interaction of *C. albicans* with *A. castellanii* at different temperature.

Fig 6 showed the interaction of *C. albicans* and amoebae at 37 °C, germ tube formation was seen after incubated for 2 h (Fig. 6B). Then, the germ tube was transformed to short filament and hyphae in 24 h (Fig. 6C) and 48 h (Fig. 6D). In contrast, *C. albicans* incubated with amoebae at room temperature infrequently found germ tube, but exhibited an increase in buds of yeast cells over time (Fig. 7). *C. albicans* incubated in PBS alone was included in the experiment as negative control. For negative control, *C. albicans* incubated in PBS only was included in the experiment (Fig S1).

## 4. Discussion

Amoebae represent a major class of environmental predators in soils and may exert potential selection pressures on environmental populations to generate variants with the potential for mammalian pathogenicity (Mylonakis *et al.*, 2002, Steenbergen *et al.*, 2003). Early studies in the 1970s suggested that a particular type of amoebae, *Acanthamoebae polyphaga*, could be predatory for *C. neoformans,* but the interaction was not depicted at the cellular level (Bunting *et al.*, 1979). In fact, the interaction between *C. neoformans* and *A. castellanii*, a soil amoeba that feeds on bacteria and fungi was analyzed in detail (Steenbergen *et al.*, 2001, 2003, Malliaris *et al.*, 2004 Chrisman *et al.*, 2010). Given the recent observation that other dimorphic fungi, *H. capsulatum, B. dermatitidis,* and *S. schenckii* are capable of nonlytic exocytosis from mammalian phagocytic cells (Steenbergen *et al.*, 2004), we investigated whether similar phenomena occurred following ingestion of *P. marneffei* and *C. albicans* by *A. castellanii. P. marneffei* was readily ingested by *A. castellanii*, and the interaction between the fungal and amoeboid cells resulted in the death of the host cell and proliferation of the fungal cells.

*P. marneffei* is a thermally dimorphic fungus which can convert from yeast to mycelial form depending on temperature. Hence, this fungus believed to survive in the soil as distinct, multibranched, mycelial form, but this has not been directly demonstrated. We investigated the interaction of *A. castellanii* with *P. marneffei* to demonstrate that *A. castellanii* can serve as a host system for *P. marneffei*. Our results found that *A. castellanii* can ingest *P. marneffei* resulted in amoebae death which found 37.4% and 50.68 % at 24 and 48 hours, respectively. The result revealed a reduction in viable amoebae and indicated that *P. marneffei* was competent to kill amoeba and exploited them for food. Additionally, *P. marneffei* can reproduce only in the presence of amoebae which similar to the previous study of other dimorphic fungi, *H. capsulatum, B. dermatitidis,* and *S. schenckii* (Steenbergen *et al.*, 2004).

Electron microscopy revealed that both *P. marneffei* and *C. albicans* were enclosed in a membrane-bound vacuole after ingestion by amoebae. For these fungi, internalized fungal cells can exploit the amoebae after ingestion and possibly gain nutrients by feeding from the remains of the killed host cells. Thus, the mechanism of killing of amoeba cells required contact between the fungal and amoeba cells.

Several human pathogenic yeasts like *C. albicans*, *C. neoformans* may persist as viable organisms in natural environment. Lacks of nutrients in natural ecosystem are often limiting factor for microbial population. In our study was showed C. albicans was able to get nutrients by feeding on amoeba. *C. albicans* can survive in different water including fresh or sea water for a long period which may be explained by their autophagie strategy adopted under starvation condition (Mizushima, 2005). It is unsurprised that the interaction between *C. albicans* and amoeboid cells resulted in the death of the host cell and increase of the yeast cells.

Our results demonstrate that *A. castellanii* can serve as a host system for the *P. marneffei* and *C. albicans*. We propose that phagocytic predators in the environment apply selective pressures, which favor fungal attributes that confer survival advantages in animal hosts. We do not exert that amoebae are the sole selective pressure for the emergence and maintenance of virulence in the environment. Clearly, the ability to grow at 37°C would seem to be an important requirement for mammalian virulence. In fact, previous studies suggest that nematodes (Mylonakis *et al.*, 2002) and slime molds (Steenbergen *et al.*, 2003) could provide additional selection pressures for the acquisition of virulence factors. Bacteria may also contribute to the selection of traits associated with mammalian pathogenesis (Cirillo *et al.*, 1997). Furthermore, we note that there are many types of amoebae, and it is possible that other amoeboid species are able to efficiently kill these fungi. However, the similarity of interactions between amoebae and macrophages and the observation that fungal virulence can be enhanced by exposure to amoebae strongly link these phagocytic predators to the phenomenon of fungal virulence for animals.

In summary, we purpose a possible mechanism by which the need for survival against environmental predator, *A. castellanii* has resulted in the acquisition of characteristics by *P. marneffei* and *C. albicans* that can also function as virulence factors for animals. In addition, the interaction of *A. castellanii* and *P. marneffei* is implied to the evolutionary bases for virulence factor development and maintenance.

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## Figure Legends

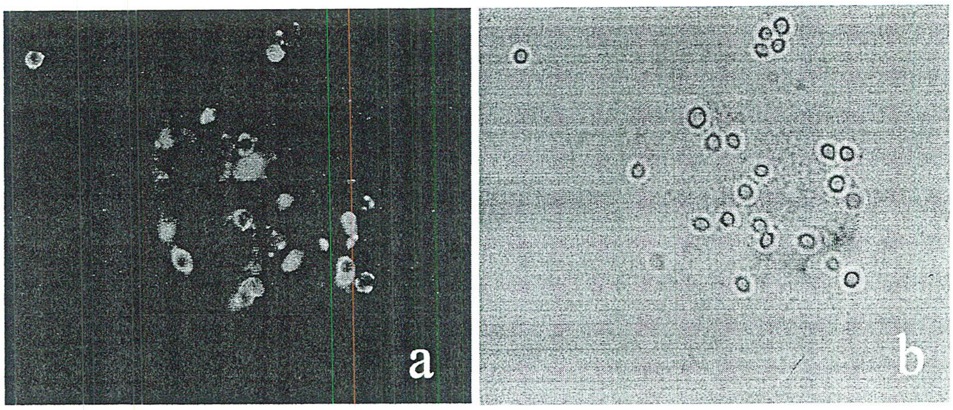



**Fig.1. Corresponding Immunofluorescent(a) and light microscopic pictures (b) of 2 h post-incubation of *A. castellanii* with FITC-labeled *P. marneffei* conidia. (Magnifications: x1000)**

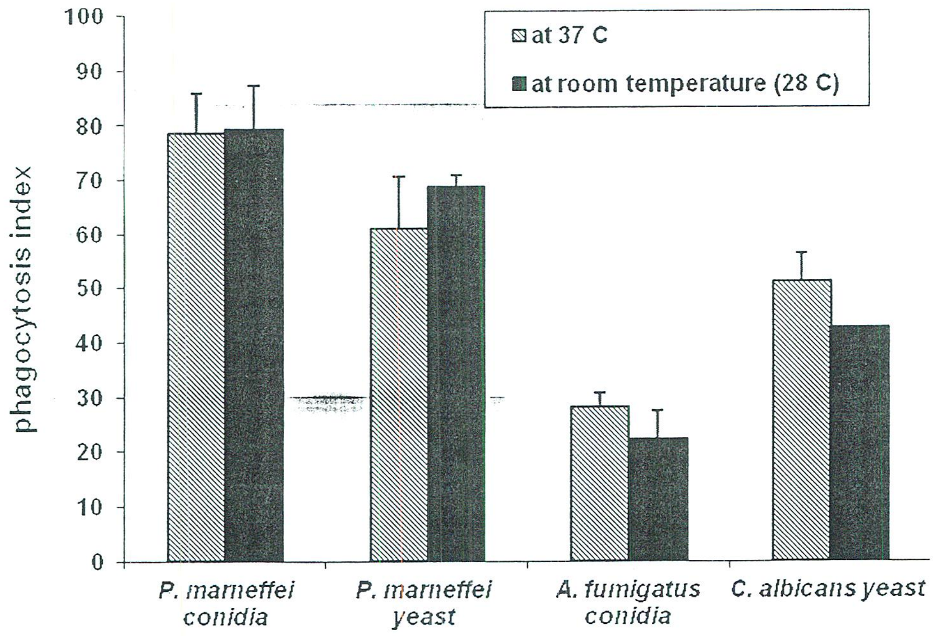



**Fig. 2. Phagocytosis of *P. marneffei*, *A. fumigatus* and *C. albicans* cells by *A. castellanii* ATCC 30324 at different temperature. Bars represent the phagocytic index by amoebae either at 37 °C (hatched bars), or at room temperature (28 °C) (solid bars), and each bar denotes one standard deviation. The phagocytiosis index was determined by counting the total number of *A. castellanii* with internalized conidia per 100 *A. castellanii* cells.**

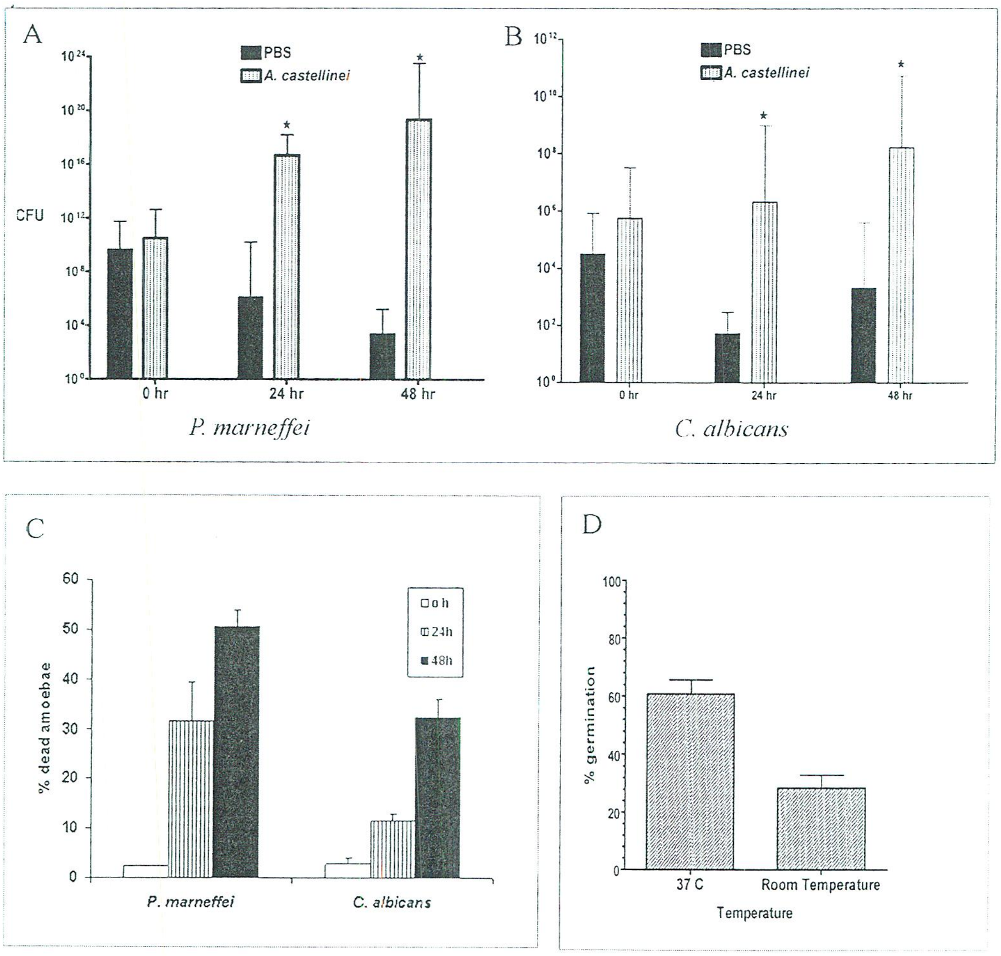



**Fig. 3. Fungal cell counts after incubation with or without amoebae in PBS at room temperature (28°C). Bars represent CFU at different times: solid bars denote CFU at 0 h and hatched bars denote CFU at 48 h. The error bars each represent one standard deviation. There are significant differences ($P \leq 0.05$) in *P. marneffei* (A) or *C. albicans* (B) incubated with amoebae and in PBS at 24 and 48 h. (C) The percentage of dead amoebae (*A. castellanii*) after incubation with *P. marneffei* and *C. albicans* at different time points, 0, 24 and 48 h. Amoeba cell viability was interfered by the ability of the cell to exclude trypan blue dye. (D) The germ tube formation of *C. albicans* within *A. castellanii* after incubation at 37°C and 28°C (room temperature) for 2 hours. Data are the means of triplicate wells, and the standard errors are represented by the error bars.**

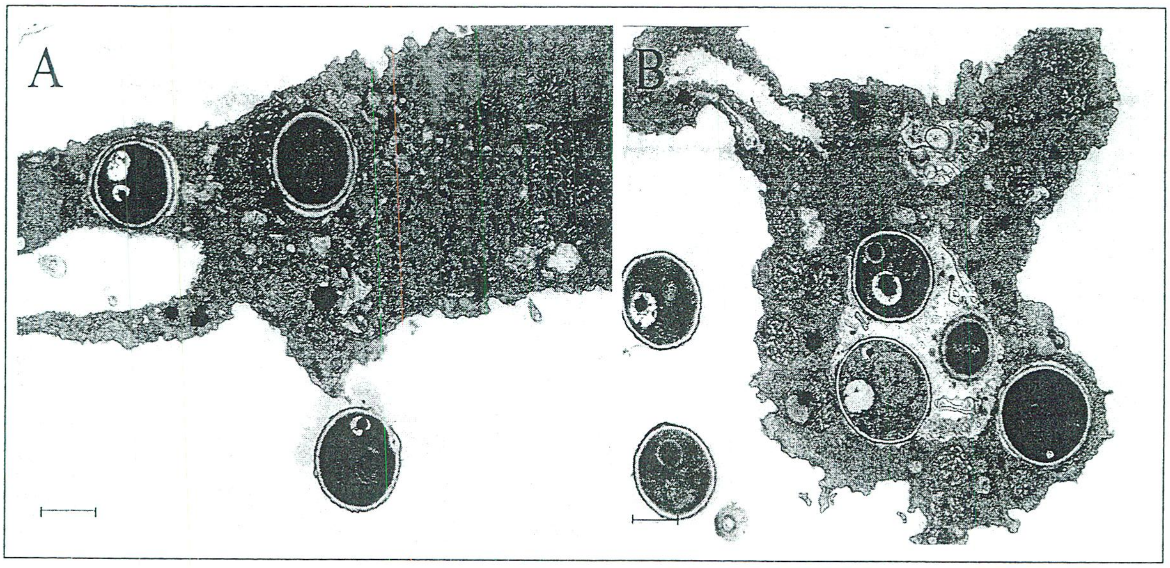



**Fig. 4. Transmission electron micrographs of *P. marneffei* interacted with *A. castellanii* after incubation at room temperature (28°C) for 30 min (A) and 2 h (B). The scale bars represented 1$\mu$m.**

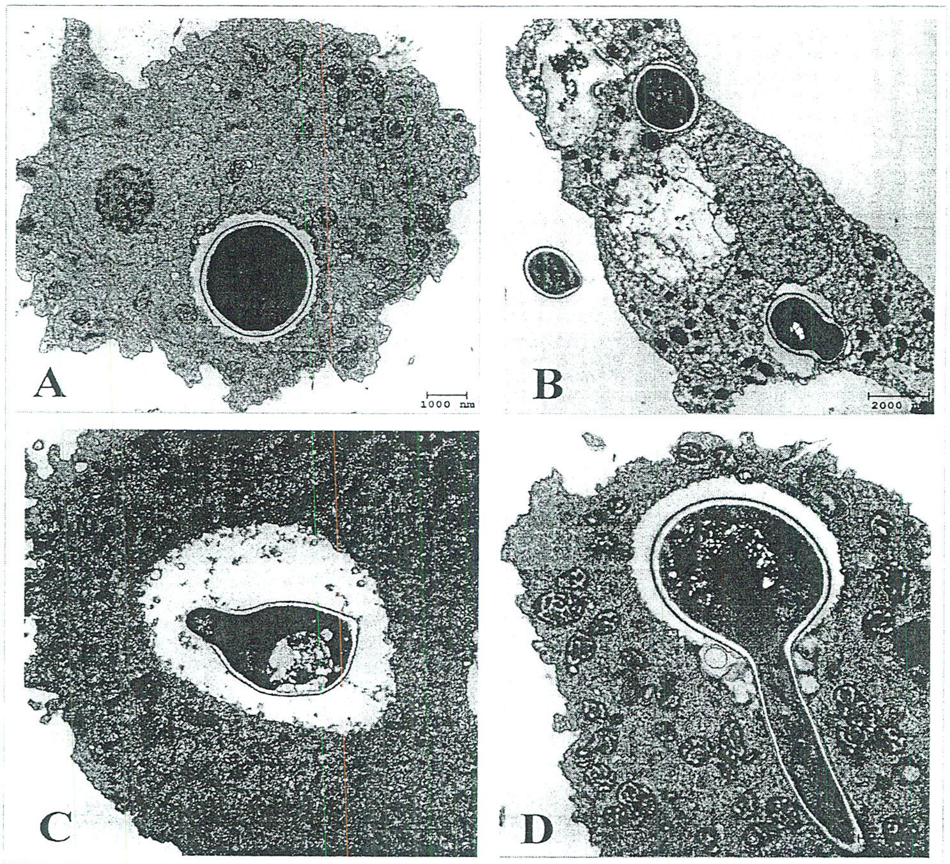



**Fig. 5.** Transmission electron micrographs of *C. albicans* within *A. castellanii* at 2 h after incubation at 37°C. Yeast cells in a membrane–bound vacuole surrounding the fungal cell 2 h post incubation (A and B). *C. albicans* started producing germ tube in amoeba (C and D). The scale bars shown in panel A was 1 µm while B, C and D were 2 µm.

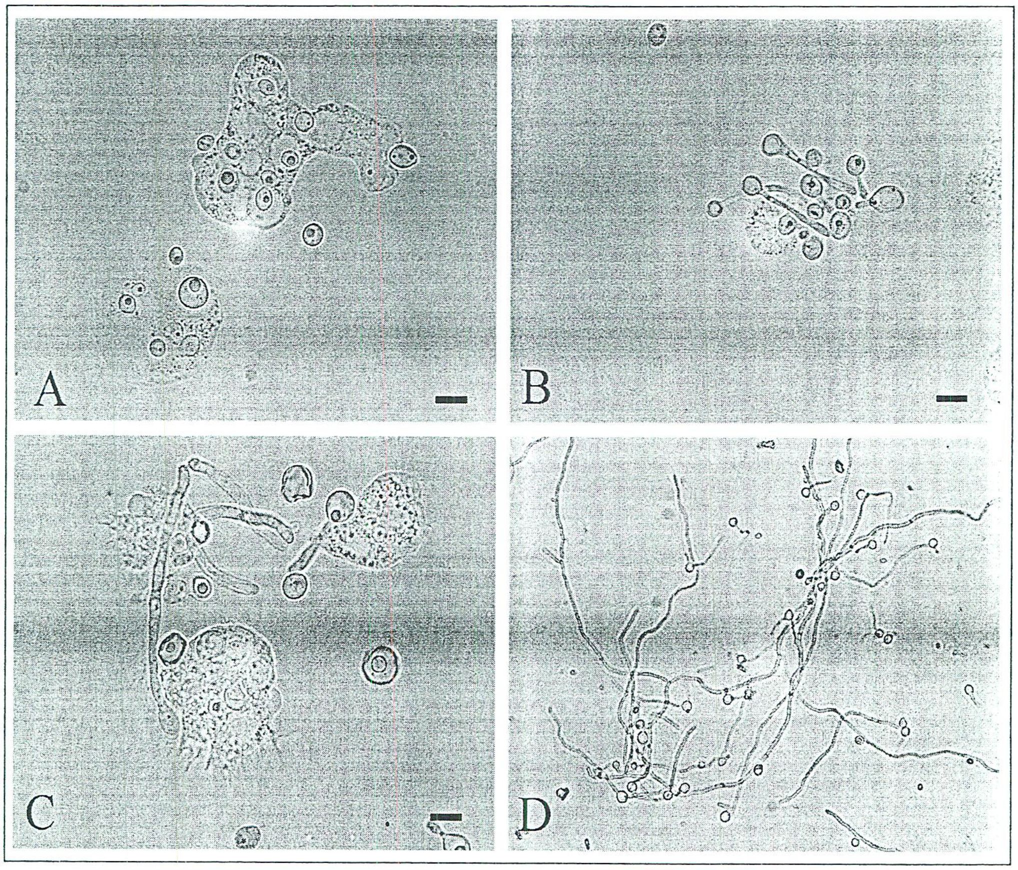



Fig. 6. Transformation of *C. albicans* from yeast forms to hyphal or filament forms at 37°C. Micrograph illustrating an internalized *C. albicans* yeast cells surrounded by a membrane-bound vacuole after 30 min cocultures with *A. castellanii* (A). Germ tubes were produced after 2 h incubation with amoebae (B) and 24 h (C). Micrograph depicting morphology changes of yeast cells after 48 h of incubation with amoebae illustrating hyphal forms of *C. albicans* (D). Bars represent 5 μm.

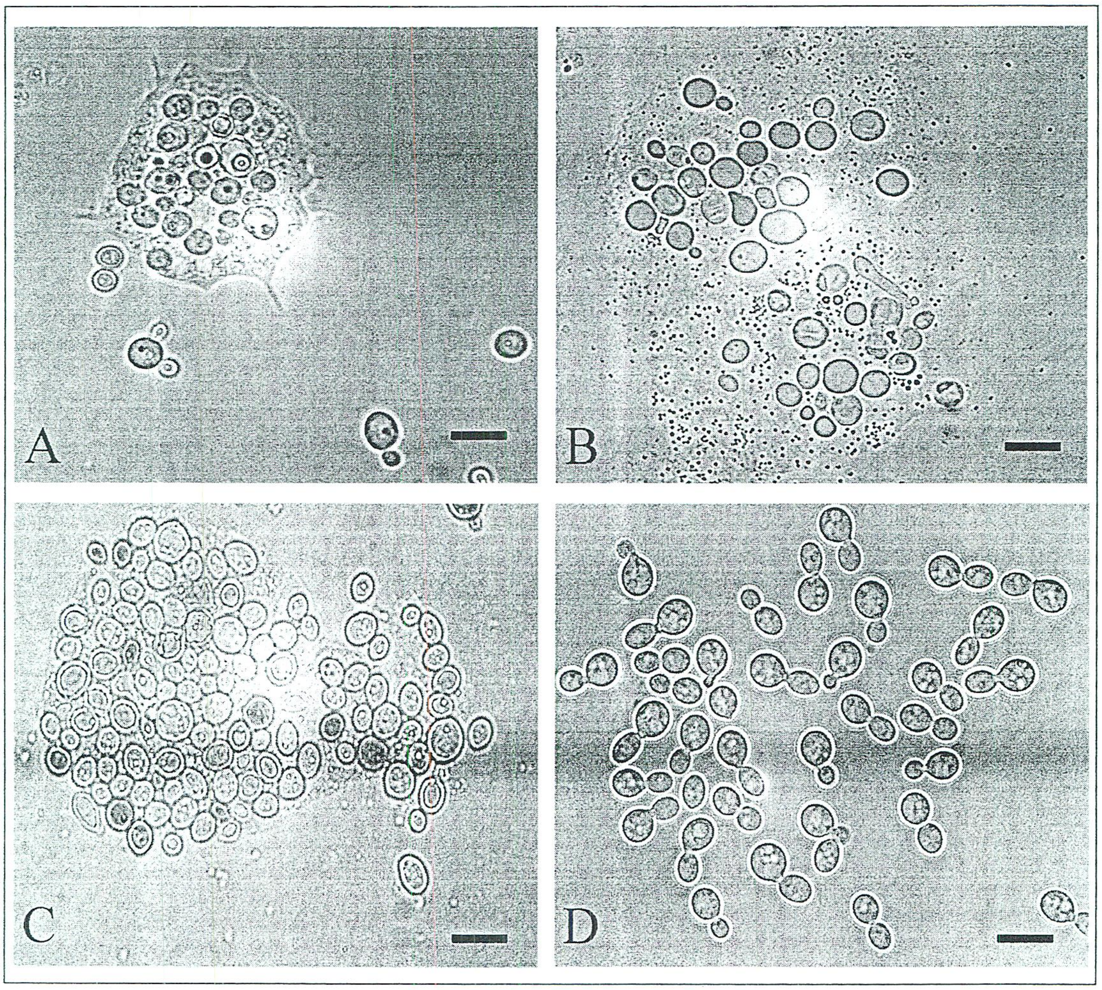
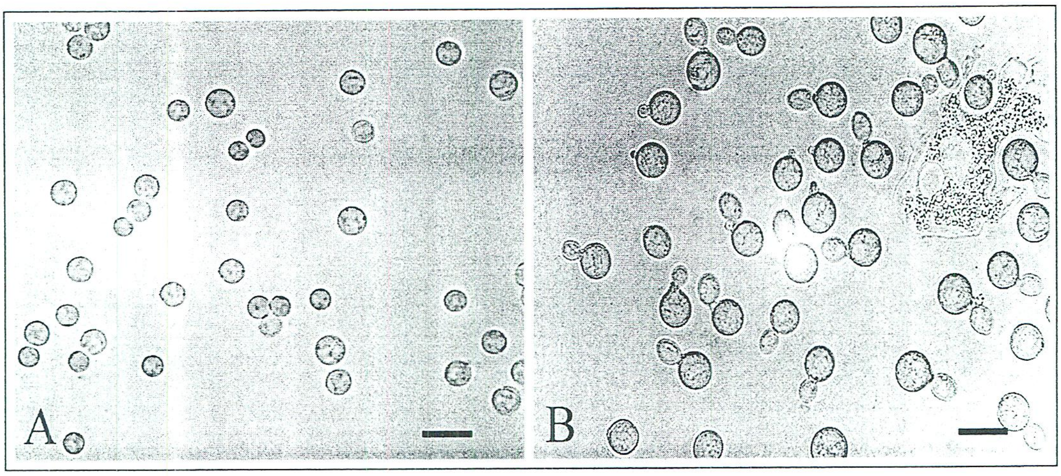



**Fig. 7. Interaction of *C. albicans* with amoebae at room temperature (28°C). The amoebae phagocytosed *Candida* yeast cells after 30 min (A) and 2 h (B) of incubation. Micrograph depicting yeast cells of *C. albicans* in proximity to an amoeba cell after 24 h (C) and 48 h (D) of incubation. Bars represent 5 μm.**

Supporting data




**Supporting information 1. Interaction of *C. albicans* with *A. castellanii* at room temperature (28 °C) after 48 h of incubation in PBS (A) and with *A. castellanii* (B). Bars represent 5µm.**