Low nutrient *Eucalyptus* wood chip agar: a semi-quantitative medium for assessing melanin production by *Cryptococcus gattii*

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Abstract

Cryptococcus gattii is commonly associated with *Eucalyptus* spp. and is able to cause potentially serious disease in immunocompromised humans. As the virulence factors of many fungi are thought to have evolved in response to environmental pressures, it is important to understand their expression in association with environmental factors. We report here that melanisation, a key virulence factor that aids survival of *C. gattii* in its environment, is induced in culture by propagating the fungus on a modified agar supplemented with *Eucalyptus* wood chips. Furthermore, this medium provides a semi-quantitative assessment of melanin production that differs with major *C. gattii* genotypes. Melanin production on the *Eucalyptus* agar was enhanced when the concentration of peptone and glucose were limited, suggesting that melanisation may be induced in *C. gattii* as a response to nutrient stress.

Key words: *C. gattii*, cryptococcosis, melanisation, *Eucalyptus*, wood agar.

Introduction

Cryptococcosis is a mycotic infection caused by the basidiomycetous yeasts *Cryptococcus gattii* and *Cryptococcus neoformans* that can develop from a mild disease into a severe life-threatening state. The fungus is acquired through the inhalation of airborne propagules, which deposit in the alveoli of the lung and if not cleared can cause a pulmonary infection. From the lung, *Cryptococcus* can spread hematogenously to extra-pulmonary tissues to infect the brain, causing meningitis and meningoencephalitis (Lin & Heitman, 2006). Cryptococcal meningoencephalitis is fatal if not treated, especially if the host is immunocompromised, and HIV-AIDS patients are particularly at risk (Casadevall, 1999).

C. neoformans and *C. gattii* are normally saprobic fungi that are acquired from environmental reservoirs. The major reservoir of *C. neoformans* appears to be pigeon guano, whereas *C. gattii* is commonly isolated from decaying wood in hollows of *Eucalyptus* trees, making it a pathogen of particular importance in Australia (Ellis & Pfeiffer, 1990). *Eucalyptus camaldulensis,* a species of red gum widely distributed in mainland Australia is the most reliable source for *C. gattii* isolation, although sporadic isolation has been reported from additional *Eucalyptus* species, including *E. tereticornis, E. rudis* (flooded gum), *E. gomphocephala* (tuart) and *E. blakelyi* (Blakely's red gum) (Krockenberger *et al.* 2002; Vilcins *et al.* 2002). There have also been reports of *C. gattii* isolation in Canada, Brazil and India from tree species other than the *Eucalyptus*, expanding its identified geographical and ecological range (Lazéra *et al.* 1998; MacDougall *et al.* 2007; Randhawa *et al.* 2008, Refojo *et al.* 2009).

C. gattii and *C. neoformans* have key virulence factors that increase their degree of pathogenicity. One of these is the ability to produce melanin (Gomez & Nosanchuk, 2003). Melanin production is used to differentiate *C. neoformans* and *C. gattii* from most other environmental yeast species, and is indicated by a brown to black pigment on agar containing diphenolic compounds (Kwon-Chung *et al.* 1992). During infection, melanin protects *Cryptococcus* cells by reacting with and neutralizing the free radicals produced by macrophages (Jacobson & Tinnell, 1993). Additionally, melanin provides structural support for the cell, prevents T-cell response and cytokine secretion by the host, reduces antibody-mediated phagocytosis and protects cells against some microbicides and antifungals (Casadevall *et al.* 2000; Doering *et al.* 1999; van Duin *et al.* 2002; Ikeda *et al.* 2003).

The production of melanin is attributed to the polymerisation of polyphenols that are produced when phenol oxidase catalyses the conversion of phenolic compounds to quinines (Casadevall *et al.* 2000). *Cryptococcus* lacks tyrosinase, which is required for the endogenous production of dihydroxyphenols (Salas *et al.* 1996), and the fungus must therefore acquire diphenolic compounds from the environment, which are subsequently converted into melanin intermediates (Buchanan & Murphy 1998).

As melanin is an important virulence factor, quantifying melanin levels can be useful for understanding the virulence of different *Cryptococcus* strains and species. Bird seed agar is routinely used to induce melanin production, and results in small, heavily pigmented colonies. However, as *Eucalyptus* wood is considered to be a primary natural substrate for *C. gattii,* the ability to convert this substrate to melanin may be important in producing virulent infectious propagules in the natural environment. This study was carried out to determine the optimal *Eucalyptus* medium to quantify melanin production among different *C. gattii* strains. We show here that a semi-quantitative assessment of melanin production can be made on a *Eucalyptus* wood-chip agar that is supplemented with a limited level of nutrients.

Materials and methods

C. gattii strains

A total of 33 isolates of *C. gattii* were used in this study and were sourced from infected humans and animals (Table 1). Isolates belonged to the three major molecular genotypes of *C. gattii* (VGI, VGII and VGIII; Meyer *et al.*, 1999). Mating type

Table 1 *Cryptococcus* isolates used in this study.

Isolate name	Molecular and mating type	Clinical Source	WA	Melanin production		
				Tone	Intensity	Rating[1]
PNG9	VGI-MATα	Human	Papua New Guinea	F	7	9
PNG14	VGI-MATα	Human	Papua New Guinea	F	7	9
PNG20	VGI-MATα	Human	Papua New Guinea	F	8	10
V7	VGI-MATα	Animal (cat)	The Oaks, NSW	E	6	8
V20	VGI-MATα	Animal (dog)	Camden, NSW	D	2	2
Q00	VGI-MATα	Animal (quokka)	Western Australia	F	8	10
Q98	VGI-MATα	Animal (cockatoo)	Western Australia	E	8	9
V3	VGI-MATα	Animal (cat)	Hornsby Hts, NSW	F	8	10
NT-9	VGI-MATα	Human	Northern Territory	E	6	8
NT-10	VGI-MATα	Human	Northern Territory	F	5	8
PNG19	VGI-MAT**a**	Human	Papua New Guinea	E	8	9
PNG27	VGI-MAT**a**	Human	Papua New Guinea	F	8	10
V12	VGI-MAT**a**	Animal (dog)	Camden, NSW	E	6	8
NT-2	VGI-MAT**a**	Human	Northern Territory	E	6	8
NT-4	VGI-MAT**a**	Human	Northern Territory	F	7	9
V9	VGI-MATα	Animal (cat)	Coogee, NSW	E	7	8
V4	VGI-MATα	Animal (cat)	Chiswick, NSW	E	7	8
V6	VGI-MATα	Animal (cat)	Marrickville, NSW	F	6	8
V26	VGI-MATα	Animal (cat)	St Andrews, NSW	F	6	8
NT-13	VGI-MATα	Human	Northern Territory	E	6	7
RDH-2	VGI-MATα	Human	Northern Territory	E	5	8
MK1231N	VGI-MATα	Animal (koala)	Western Australia	F	7	8
MK1219N	VGI-MATα	Animal (koala)	Caversham, WA	E	8	9
MK1031	VGI-MATα	Animal (Maltese dog)	Western Australia	E	7	8
MK1168	VGI-MATα	Animal (bulldog)	Western Australia	F	7	9
McBride	VGI-MATα	Animal (cat)	Sydney, NSW	F	4	6
R265	VGI-MATα	Human	Vancouver Island	F	5	7
MK914	VGI-MATα	Animal	Vancouver Island	F	5	7
PNG30	VGI-MATα	Human	Papua New Guinea	E	4	5
PNG34	VGI-MATα	Human	Papua New Guinea	D	3	3
VPB 66270	VGI-MATα	Animal (cat)	Western Australia	D	5	4
MK970b	*C. neoformans*	Animal (koala)	Perth, WA	D	4	4
MK1212N	*C. neoformans*	Animal (koala)	Perth, WA	F	7	9

[1]Melanin rating (lowest: 1 – highest: 10) based on scales of tone (A–F), which measures the amount of black present in the colour, and intensity (1–8) which measures level of colour saturation.

(MAT**a** or MATα) was determined by PCR using established techniques (Halliday *et al.* 1999).

Agar media

Bird seed agar

Fifty grams of *Guizotia abyssinica* (niger) seed was ground to a fine powder in a electric blender, mixed with 1 L MilliQ water and boiled for 30 min. Seed fragments were removed by passing the mix through filter paper, the volume was adjusted to 1 L and 1 g each of glucose, potassium dihydrogen orthophosphate and creatinine were added and dissolved. The pH was adjusted to 5.5, 15 g agar was added and the solution was autoclaved (110˚C; 20 min). After cooling to ~ 50˚C the agar was dispensed into 90 mm Petri dishes.

Agar supplemented with E. camaldulensis wood

Plain agar with *E. camaldulensis* woodchips—This agar is based on the media by Ren *et al.* (2006) but uses *E. camaldulensis* woodchips in place of wood from other tree species. Twenty grams of *E. camaldulensis* wood chips (obtained from hollows in *E. camaldulensis* trees growing in the Murray Darling Basin, NSW) - were ground in a coffee grinder to fragments of < 1 cm, autoclaved at 110°C for 30 min and mixed with 1 L of autoclaved 2% agar solution. The media was poured into 90 mm Petri dishes.

Sabourauds Dextrose Agar (SDA) supplemented with *E. camaldulensis* woodchips—SDA media was made using 10 g peptone, 40 g glucose, 15 g agar and 1 litre MilliQ water. Twenty grams of sterile *E. camaldulensis* wood chips prepared as above were combined with the SDA mixture and dispensed into 90 mm Petri dishes.

Low Nutrient Sabourauds Dextrose Agar (LN-SDA) supplemented with *E. camaldulensis* woodchips—LN-SDA used 5 g peptone, 20 g glucose, 28 g agar and 1 L MilliQ water. Twenty grams of sterile *E. camaldulensis* wood chips were prepared and added as above

Non-*Eucalyptus* LN-SDA agar—As a control, plates containing LN-SDA without the *E. camaldulensis* woodchips were prepared.

C. gattii culture and melanin production

Yeast cell concentrations were standardised by emulsifying a single *C. gattii* colony in 1 mL of sterile saline, measuring the optical density (OD) at 520 nm and adjusting the concentration to 10^8 cells/mL. A 5 µL (5 x 10^5 cells/mL) volume of the standardised yeast cell suspension was pipetted onto the bird seed or *Eucalyptus* wood agar and allowed to dry. Plates were

incubated at 30°C for 13 days. All strains were plated in triplicate.

Assessment of melanin production

Following growth, colonies were assessed for hue, intensity and tone according to the Methuen Handbook of Colour (Kornerup & Wanscher 1978). Hue is the attribute of colour that determines its name in common speech, such as red, yellow or blue. Each colour can vary in intensity, from essentially colourless to an intense, bright shade. Tone measures the amount of black present in a colour. As melanin is a dark brown-black pigment, both intensity and tone are expected to increase with increased levels of melanin production. The Methuen Handbook of Colour grades intensity from 1 to 8, where 8 is the most intense, and tone is ranked A–F, which range from a basic hue to an increasingly deep tone. Based on these values, each strain was then assigned an overall score from 1–10, which reflected the extent of black-brown pigmentation, and thus the level of melanin produced. Statistical difference between groups was assessed using the Student's T-test.

Proof that the brown pigment was melanin and not other pigmented compounds was done by extracting the pigment from ~ 300 mg wet weight of melanized cells using hot acid (6 M HCl; 100˚C) for 30 min. Non-melanin pigments are extracted into the acid by this method, whereas melanins are acid-stable (Chaskes *et al.* 2008).

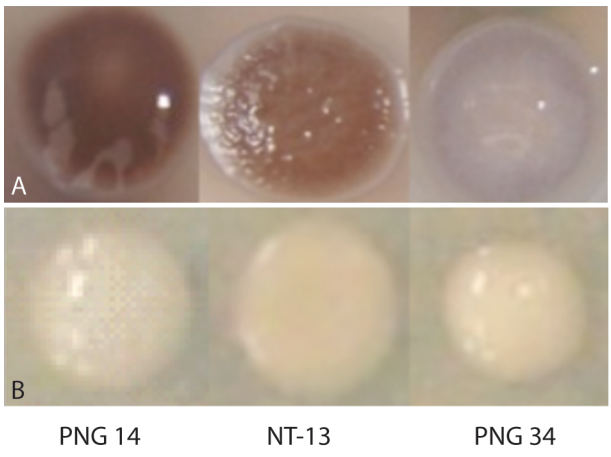

Fig. 1 A. *C. gattii* strains grew to form large colonies with varying levels of pigmentation on low-nutrient SDA supplemented with *Eucalyptus* wood chips (LN-SDA). **B.** The same colonies grown on SDA only are shown for comparison. All colonies were incubated for 13 days at 30˚C.

Results

All *C. gattii* strains grew well on SDA, forming large colonies at 7 days. On bird seed agar, the strains grew slowly and developed pigmentation after 48 hours. These results indicated that the strains could be induced to produce melanin and were from viable stock cultures.

Isolates grown on the plain agar supplemented with *Eucalyptus* woodchips only did not grow to form colonies but did show development of pigmentation after 13 days of incubation at 30°C. Conversely, strains on SDA supplemented with *Eucalyptus* wood chips grew to form large colonies but did not pigment. *C. gattii* strains that were grown on the LN-SDA containing *Eucalyptus* woodchips formed prominent colonies that pigmented intensely and to varying degrees (Fig. 1). The pigments were confirmed to be melanin as they were not extracted by hot acid. Melanin formation was reproducible across the three replicate plates, and was scored with respect to hue, tone and intensity to give an overall melanin score (Table 1). There was significantly less melanin produced in isolates belonging to molecular type VGIII compared to VGI (p = 0.019) and VGII (p = 0.016). There was no significant difference in melanin levels between VGI and VGII (p = 0.098).

Discussion

Melanisation was demonstrated by all strains of *C. gattii* when grown on media containing *Eucalyptus* wood chips with limited levels of peptone and glucose. *Eucalyptus* wood contains high levels of phenols (Amen-Chen *et al.*, 1997), which are required for melanisation by *C. gattii,* and simple sugars are likely to be low, indicating that this medium may approximate conditions for *C. gattii* growth in the environment. Interestingly, the level of melanisation varied with the molecular genotype of the isolates, with VGIII isolates producing significantly less melanin that VG I or VGII strains. The VGI genotype has a well-established ecological association with *E. camaldulensis* (Ellis & Pfeiffer 1990; Saul *et al.*, 2008b), and VGII isolates have been found on some *Eucalyptus* species (Saul *et al.* 2008a). In contrast, VGIII has never been found in *Eucalyptus* samples and has instead been isolated from almond and other tree species (Meyer *et al.* 2003). Ren *et al.* (2006) found the source of wood to be important in inducing melanin by *C. gattii*, and it would be interesting to see if VGIII isolates could produce more melanin on different wood-based agars.

As well as a role in virulence, melanin is important in the survival of fungi in the environment and is produced by a large number of different fungal species. Melanin protects cells against UV, solar and gamma radiation, predation by amoebae, excreted hydrolytic enzymes, extremes of heat and cold and the toxic effects of heavy metals (reviewed in Nosanchuk & Casadevall 2006). As *C. gattii* is an environmental organism, melanin production is likely to be important during dispersal and colonisation of new environmental hosts. The current study suggests *C. gattii* growing in association with *Eucalyptus* trees is likely to be heavily melanized and therefore capable of enhanced survival in the environment.

Melanisation appears to be enhanced by physiological stress. Studies on the black yeast *Trimmatostroma* found melanin levels to increase with increasing levels of saline stress (Kogej *et al.* 2006). Melanisation by *Cryptococcus* on birdseed agar is reported to be more reliable when glucose is limited (Hazen & Howell 2003), and in the current study, melanisation was dramatically increased when the levels of glucose and peptone supplied in the media were halved. These results indicate that melanisation by *C. gattii* may be a response to nutrient stress. Future studies will further characterise the melanisation properties of *C. gattii* strains in conditions of low environmental nutrients.

We have demonstrated that incorporation of *Eucalyptus* wood into the culture medium of *C. gattii* provides an improved agar for studying melanin production, a key virulence factor of strains of this important human pathogen. *Eucalyptus* agar provides a

rapid, semi-quantitative method of assessing the extent of melanin production in different *C. gattii* strains.

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References

Amen-Chen C, Pakdel H, Roy C 1997. Separation of phenols from *Eucalyptus* wood tar. *Biomass and Bioenergy* 25–27.

Buchanan KL, Murphy JW 1998. What makes *Cryptococcus neoformans* a pathogen? *Emerging Infectious Diseases* **4**, 71–83.

Casadevall A, Perfect JR 1999. *Cryptococcus neoformans* ASM Press, Washington, DC. 541 pp.

Casadevall A, Rosas AL, Nosanchuk JD 2000 Melanin and virulence in *Cryptococcus neoformans*. *Current Opinion in Microbiology* **3**, 354–358.

Chaskes S, Frases S, Cammer M, Gerfen G, Casadevall A 2008. Growth and pigment production on D-Tryptophan medium by *Cryptococcus gattii*, *Cryptococcus neoformans,* and *Candida albicans*. *Journal of Clinical Microbiology* **46**, 255–264.

Doering TL, Nosanchuk JD, Roberts WK, Casadevall A 1999. Melanin as a potential cryptococcal defence against microbicidal proteins. *Medical Mycology*. **37**, 175–181.

Ellis DH, Pfeiffer TJ 1990. Natural habitat of *Cryptococcus neoformans* var. *gattii*. *Journal of Clinical Microbiology*. **28**, 1642–1644.

Gomez BL, Nosanchuk JD 2003. Melanin and fungi. *Current Opinion in Infectious Diseases* **16**, 91–96.

Halliday CL, Bui T, Krockenberger M, Malik R, Ellis DH, Carter DA 1999. Presence of alpha and **a** mating types in environmental and clinical collections of *Cryptococcus neoformans* var. *gattii* strains from Australia. *Journal of Clinical Microbiology* **37**, 2920–2926.

Hazen KC, Howell SA 2003. *Candida, Cryptococcus* and other yeasts of medical importance. In: *Manual of Clinical Microbiology* (eds. Murray PR, Baron EJ, Jorgensen JH, Pfaller MA, Yolken RH), pp. 1693–1711. ASM Press, Washington, DC.

Ikeda R, Sugita T, Jacobson ES, Shinoda T 2003. Effects of melanin upon susceptibility of *Cryptococcus* to antifungals. *Microbiology & Immunology* **47**, 271–277.

Jacobson ES, Tinnell SB 1993. Antioxidant function of fungal melanin. *Journal of Bacteriology* **175**, 7102–7104.

Kogej T, Gorbushina AA, Gunde-Cimerman N 2006. Hypersaline conditions induce changes in cell-wall melanization and colony structure in a halophilic and a xerophilic black yeast species of the genus *Trimmatostroma*. *Mycological Research* **110**, 713–724.

Kornerup A, Wanscher JH 1978. *Methuen Handbook of Colour* E. Methuen, London.

Krockenberger MB, Canfield PJ, Malik R 2002. *Cryptococcus neoformans* in the koala (*Phascolarctos cinereus*): Colonization by *C. n.* var. *gattii* and investigation of environmental sources. *Medical Mycology* **40**, 263–272.

Kwon-Chung KJ, Wickes BL, Stockman L, Roberts GD, Ellis D, Howard DH 1992. Virulence, serotype, and molecular characteristics of environmental strains of *Cryptococcus neoformans* var. *gattii*. *Infection & Immunity*. **60**, 1869–1874.

Lazéra MS, Cavalcanti MAS, Trilles L, Nishikawa MM, Wanke B 1998. *Cryptococcus neoformans* var. *gattii*: Evidence for a natural habitat related to decaying wood in a pottery tree hollow. *Medical Mycology* **36**, 119–122.

Lin X, Heitman J 2006. The biology of the *Cryptococcus neoformans* species complex. *Annual Review of Microbiology* **60**, 69–105.

MacDougall L, Kidd SE, Galanis E, Mak S, Leslie M, Cieslak P, Kronstad JW, Morshed MG, Bartlett KH 2007. Spread of *Cryptococcus gattii* in British Columbia, Canada, and detection in the Pacific Northwest, USA. *Emerging Infectious Diseases* **13**, 42–50.

Meyer W, Castañeda A, Jackson S, Huynh M, Castañeda E & the International *Cryptococcus* Study Group 2003. Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates. *Emerging Infectious Diseases* **9**, 189–195.

Meyer W, Marszewska K, Amirmostofian M, Igreja RP, Hardtke C, Methling K, Viviani MA, Chindamporn A, Sukroongreung S, John MA, Ellis DH, Sorrell TC 1999. Molecular typing of global isolates of *Cryptococcus neoformans* var. *neoformans* by polymerase chain reaction fingerprinting and randomly amplified polymorphic DNA-a pilot study to standardize techniques on which to base a detailed epidemiological survey. *Electrophoresis.* **20**, 1790–1799.

Nosanchuk JD, Casadevall A 2006. Impact of melanin on microbial virulence and clinical resistance to antimicrobial compounds. *Antimicrob. Agents Chemother.* **50**, 3519–3528.

Randhawa HS, Kowshik T, Chowdhary A, Preeti Sinha K, Khan ZU, Sun S, Xu J 2008. The expanding host tree species spectrum of *Cryptococcus gattii* and *Cryptococcus neoformans* and their isolations from surrounding soil in India. *Medical Mycology* **46**, 823–833.

Ren P, Springer DJ, Behr MJ, Samsonoff WA, Chaturvedi S, Chaturvedi V 2006. Transcription factor STE12α has distinct roles in morphogenesis, virulence, and ecological fitness of the primary pathogenic yeast *Cryptococcus gattii*. *Eukaryotic Cell* **5**, 1065–1080.

Refojo N, Perrotta D, Brudny M, Abrantes R, Hevia AI, Davel G 2009. Isolation of *Cryptococcus neoformans* and *Cryptococcus gattii* from trunk hollows of living trees in Buenos Aires City, Argentina. *Medical Mycology* **47**, 177–184.

Salas SD, Bennett JE, Kwon-Chung KJ, Perfect JR, Williamson PR 1996. Effect of the laccase gene CNLAC1, on virulence of *Cryptococcus neoformans*. *Journal of Experimental Medicine* **184**, 377–386.

Saul N, Ellis DH, Currie B, Malik R, Carter D, Krockenberger M 2008a. Environmental isolation and molecular diversity of environmental and clinical isolates of *Cryptococcus gattii* VGII in Arnhem Land, an area of high endemic disease in Australia. In: *7th International Conference on Cryptococcus and Cryptococcosis*, Nagasaki, Japan.

Saul N, Krockenberger M, Carter D 2008b. Evidence of recombination in mixed mating type and α-only populations of *Cryptococcus gattii* sourced from single *Eucalyptus* tree hollows. *Eukaryotic Cell* **7**, 727–734.

van Duin D, Casadevall A, Nosanchuk JD 2002. Melanization of *Cryptococcus neoformans* and *Histoplasma capsulatum* reduces their susceptibilities to amphotericin B and caspofungin. *Antimicrobial Agents & Chemotherapy* **46**, 3394–3400.

Vilcins I, Krockenberger M, Agus H, Carter D 2002. Environmental sampling for *Cryptococcus neoformans* var. *gattii* from the Blue Mountains National Park, Sydney, Australia. *Medical Mycology* **40**, 53–60.