Determination of keratin degradation by fungi using keratin azure

J. A. SCOTT*,† & W. A. UNTEREINER‡
**Department of Public Health Sciences/Gage Occupational and Environmental Health Unit, University of Toronto, Toronto, †Gamma-Dynacare Medical Laboratories, Brampton and ‡Department of Botany, Brandon University, Brandon, Canada*

Azure dye-impregnated sheep's wool keratin (keratin azure) was incorporated in a high pH medium and overlaid on a keratin-free basal medium. The release and diffusion of the azure dye into the lower layer indicated production of keratinase. Fifty-eight fungal taxa, including 49 members of the Arthrodermataceae, Gymnoascaceae and Onygenaceae (Order Onygenales), were assessed for keratin degradation using this method. The results were comparable to measures of keratin utilization reported in studies using tests based on the perforation or erosion of human hair *in vitro*.

Keywords hair perforation, keratin azure, keratin degradation, keratinolytic, keratinophilic, Onygenales

Introduction

Keratins are the largest and most complex family of cytoskeletal intermediate filament proteins of animal cells, particularly epithelia [1]. The durability of keratins is a direct consequence of their complex architecture. Keratin molecules form parallel, intertwined heterodimers consisting of one each of acidic Type I keratins and basic or neutral Type II keratins. Antiparallel couplets of heterodimers comprise protofilaments, which pair to form protofibrils. Each filament of keratin, in turn, consists of four bundled protofibrils. This complex tertiary and quaternary structure is richly stabilized by disulphide bonds, a construction endowing keratin with a durability and resilience that is matched by few other polypeptides.

The enzymatic ability of fungi to decompose keratin has long been interpreted as a key innovation in the evolution of animal dermatopathogenicity. Evidence in support of this idea derives from two observations: (i) keratins are common, extremely resistant animal polypeptides; and (ii) fungi capable of the enzymatic degradation of these polymers are restricted largely to a single lineage of filamentous fungi, the ascomycete Order Onygenales, which also contains a disproportionate number of pathogens.

Keratin utilization has been reported in a wide variety of organisms including non-filamentous and filamentous bacteria [2,3], helminths [4], water moulds [5] and filamentous fungi [6,7]. The refractory nature of keratins and the specialization of microbial enzyme systems responsible for their degradation led Currah [8] to hypothesize that specialized keratinases evolved only once in the filamentous fungi.

In addition to keratin, keratinaceous materials such as skin, hair, nails, hoofs and horns contain a large proportion of non-keratin protein. The term 'keratinolytic' is used for fungi exhibiting the enzymatic ability to attack and utilize keratin. However, relatively little consideration has been given to the distinction between keratin utilization and simple occurrence on keratinaceous material nourished by constituents other than keratin. In acknowledgement of this distinction, fungi merely inhabiting keratinaceous substrates but lacking manifest keratinolytic activity have sometimes been termed 'keratinophilic' [9].

Interest in keratin degradation as a pathogenicity factor and as a taxonomic determinant has led to the development of a number of methods for its assessment. Most of these techniques require direct microscopic examination of fungally colonized human hairs in order to detect erosion or fungal penetrating bodies, also known as perforating organs [10,11]. Formation of

Received 26 July 2002; Accepted 16 May 2003
Correspondence: J. A. Scott, Department of Public Health Sciences/Gage Occupational and Environmental Health Unit, University of Toronto, 223 College Street, Toronto, ON, Canada M5T 1R4.

© 2004 ISHAM
DOI: 10.1080/13693780310001644680

the latter structures distinguishes a specialized subset of keratinophilic fungi, particularly the agents of animal dermatophytoses, and therefore has become an important differential character at the species level in the dermatophytic genera *Microsporum* and *Trichophyton* [6,12].

Our method for assessing keratin utilization is based on procedures for the assessment of cellulolytic activity [13] and chitin degradation [14], whereby fungi are grown on an azure-dye impregnated substrate and their degradative abilities assessed via dye released during the degradation process. In contrast to hair-based methods, our technique requires as little as seven days and is read by gross examination of culture tubes instead of by microscopy.

Materials and methods

A basal medium (BM) was prepared in a final volume of 1 l that contained 15 g Bacto Agar (Difco, Detroit, MI, USA) and 100 ml of each of the following solutions (prepared individually in a total volume of 1 l): (i) major salts stock solution (5.0 g KCl, 5.0 g $MgSO_4 \cdot 7H_2O$, 0.01 g $CaCl_2 \cdot 2H_2O$); (ii) buffer stock solution (14.2 g NaH_2PO_4 adjusted to pH 9.0 with a concentrated solution of KH_2PO_4); (ii) micronutrients stock solution (40 g $NaH_2PO_4 \cdot H_2O$, 20 g $FeCl_3 \cdot 6H_2O$ and 1 ml each of a solution containing 1000 mg/l $MnCl \cdot 6H_2O$, 1000 mg/l $ZnSO_4 \cdot 7H_2O$, 100 mg/l $Na_2MoO_4 \cdot 2H_2O$, 250 mg/l $CuSO_4 \cdot 5H_2O$). The pH was adjusted to 9.0 by the addition of a solution of concentrated NaOH. This formulation is a modification of the medium described by Ahmad and Malloch [15] and it differs in using a phosphate buffer rather than Tris, and in lacking the disodium salt of EDTA, thiamine chloride, biotin and vitamin B_{12}. These modifications allowed us to eliminate extraneous sources of organic carbon and nitrogen that might interfere with the keratin utilization assay.

Approximately 15 ml BM was dispensed into 25-ml screw-capped, French square bottles and autoclaved at 15 p.s.i. for 15 min. The bottles were cooled in an upright position. Finely chopped keratin azure (Sigma, St Louis MO, USA) was suspended to a concentration of 4 mg/ml in BM and autoclaved. Finally, 1 ml of this overlay medium was dispensed aseptically into each bottle.

Keratin azure tubes were inoculated with a single 4-mm disk cut with a cork borer from actively growing cultures on BM amended with 1.0 g/l glucose, 0.5 g/l Bacto Peptone and 0.5 g/l Bacto Yeast Extract (Difco). Keratin azure tubes were incubated at 21°C in darkness and examined at 1, 2, 4 and 6 weeks following inoculation. All test fungi were assessed in triplicate. Degradation of keratin was inferred from the release of azure dye into the uncolored, lower layer of BM. Assays were scored by direct visual examination in artificial daylight by comparison of an inoculated tube with an uninoculated reference tube (see Fig. 1). Dye release and growth were scored at 1, 2, 4 and 6 weeks. Test strains used in this study are listed in Table 1.

Results

Dye release results for 1 and 4 weeks are shown in Table 2. With a few exceptions, our results corresponded well to established reports documenting keratin degradation assessed by hair perforation. Our results confirmed that the ability to degrade keratin is a variable character within the families of the Onygenales (Table 2).

Most strains tested on keratin azure medium showed a rapid response (e.g. dye release at 7 days); however, a few isolates of known keratinolytic species showed negative keratin degradation at 4 weeks and only produced weak dye release at 6 weeks (e.g. *Trichophyton rubrum*, data not shown). Density of growth did not appear to be well correlated with the degree of dye

Fig. 1 Keratin azure test tubes following 14 days incubation. Left, *Aspergillus niger* (ATCC 9642) showing negative reaction; right, *Microsporum canis* (Gamma-Dynacare Medical Laboratories GDML 9-7140) showing positive reaction indicated by azure dye release into basal medium.

Table 1 Isolates used in this study and substrate sources

Species	Substrate and locality	Source*
Arthrodermataceae		
Arthroderma curreyi Berkeley	not known	CBS 138.26
A. gypseum (Nannizzi) Weitzman *et al.*	not known	ATCC 22925 T† (mt+)‡
A. incurvatus (Stockdale) Weitzman *et al*.	*ex* skin, *H. sapiens*, UK	CBS 174.64 T
A. otae (Hasegawa and Usui) McGinnis *et al*.	*ex* ringworm of *Felis domesticus* (cat), Japan	ATCC 28328 T (mt−)
A. quadrifidum Dawson and Gentles	not known	ATCC 22954 T (mt+)
A. silverae Currah *et al*.	*ex* dung of *Alopex lagopus* (arctic fox), Svålbard	UAMH 6715 T
Chrysosporium vallenarense van Oorschot and Piontelli	*ex* dung of *A. lagopus*, Svålbard	UAMH 6914
Ctenomyces serratus Eidam	*ex* soil, Australia	CBS 187.61 NT§
Epidermophyton floccosum (Harz) Langeron and Milochevitch	*ex Homo sapiens* (human), the Netherlands	CBS 553.84
Microsporum canis Bodin	scraping and hair *ex* male *H. sapiens*, Canada	UAMH 2338
M. cookei Ajello	*ex H. sapiens*, Canada	OMH H1-10
M. persicolor (Sabouraud) Guiart and Grigorakis	*ex H. sapiens*, Canada	WUC 399
Trichophyton krajdenii Kane *et al.*	*ex* skin lesion of *H. sapiens*, Canada	UAMH 3244 T
T. mentagrophytes (Robin) Blanchard	not known	UAMH 6256
T. mentagrophytes ('red' variant)	*ex H. sapiens*, Canada	OMH 607678
T. mentagrophytes (granular variant)	*ex H. sapiens*, Canada	OMH 646544
T. mentagrophytes (velvety variant)	*ex H. sapiens*, Canada	OMH 566803
T. raubitschekii Kane *et al.*	*ex H.* sapiens, Canada	OMH 6-1286
T. rubrum (Castellani) Sabouraud	*ex* feet of *H. sapiens*, Canada	UAMH 2129
T. simii (Pinoy) Stockdale *et al.*	*ex H. sapiens*, Canada	OMH 1585214
Gymnoasceae		
Arachniotus ruber (van Tieghem) Schroeter	*ex* soil, UK	CBS 352.90 NT
Arachnomyces minimus Malloch and Cain	*ex* decayed wood, Canada	CBS 324.70 T
Gymnascella aurantiaca Peck	*ex* soil, Russia	ATCC 22394 T
Gymnoascoideus petalosporus Orr *et al.*	*ex* skin lesion of *H. sapiens*, India	ATCC 34351 T
Gymnoascus reessii Baranetsky	*ex* soil, USA	CBS 410.72
Onygenaceae		
Amauroascus aureus (Eidam) von Arx	decayed wood, Japan	ATCC 18654 NT
A. mutatus (Quelet) Rammeloo	*ex* soil, USA	ATCC 22395
A. niger Schroeter	*ex* soil, USA	ATCC 22339 NT
A. purpureus Ito and Nakagiri	*ex* soil, Japan	IFO 32622 T
Aphanoascus fulvescens (Cooke) Apinis	*ex* dung of *Ursus* sp. (bear), Canada	CBS 111.58
A. mephitalis (Malloch and Cain) Cano and Guarro	carnivore dung, Canada	ATCC 22144 T
A. terreum (Randhawa and Sandhu) Apinis	*ex* soil, India	ATCC 16413 T
Apinisia graminicola La Touche	decomposing grass clippings, UK	CBS 721.68 T
Ascocalvatia alveolata Malloch and Cain	carnivore dung, Canada	ATCC 22147 T
Auxarthron californiense Orr and Kuehn	*ex* dung of *Neotoma* sp. (packrat), USA	ATCC 15600 T
A. zuffianum (Morini) Orr and Kuehn	*ex* lung of *Cynomys ludovicianus* (prairie dog), USA	CBS 219.58 NT
Chrysosporium keratinophilum D. Frey *ex* Carmichael	*ex* soil, New Zealand	CBS 392.67 T
C. tropicum Carmichael	*ex* woollen overcoat, Solomon Islands	MUCL 10068
Nannizziopsis vriesii (Apinis) Currah	*ex* skin and lungs of *Ameiva* sp. (lizard), The Netherlands	ATCC 22444 T
Neogymnomyces demonbreunii (Ajello and Cheng) Orr	*ex* soil, USA	ATCC 18394 NT
Onygena equina (Wildenow) Persoon	hoof of *Bos taurus* (cow), Germany	ATCC 22731
Polytolypa hystricis Scott and Malloch	dung of *Erethizon dorsatum* (American porcupine), Canada	UAMH 7299 T
Renispora flavissima Sigler *et al.*	*ex* bat guano and soil, USA	ATCC 38503 T (mt+)
Shanorella spirotricha Benjamin	feathers of a dead bird, USA	ATCC 12594 T
Spiromastix grisea Currah and Locquin-Linard	dung of *Canis aureus* (jackal), Algeria	UAMH 6836
S. tentaculatum Guarro *et al.*	*ex* soil, Somalia	UAMH 7098 T
S. warcupii Kuehn and Orr	*ex* soil, Australia	ATCC 14964 T
S. warcupii Kuehn and Orr	*ex* soil, Burundi	UAMH 7099
Uncinocarpus reesii Sigler and Orr	feathers, Australia	ATCC 34533 T (mt−)
Trichocomaceae		
Aspergillus alliaceus Thom and Church	culture contaminant, Canada	MUCL 42693
A. niger van Tieghem	wireless set [radio], locality unknown	MUCL 19001
Byssochlamys nivea Westling	not known	CBS 100.11 T

Table 1 (*Continued*)

Species	Substrate and locality	Source*
Eurotium herbariorum (Wiggers *ex* Fr.) Link	unpainted board, USA	ATCC 16469 NT
Petromyces alliaceus Malloch and Cain	*ex* soil, Australia	ATCC 16891 T
Trichocoma paradoxa Junghuhn	substrate unknown, Japan	CBS 247.57
Positive and negative control isolates		
Chaetomium globosum Kunze *ex* Fr.	leaf of *Triticum aestivum* L., Belgium	MUCL 28850
Pochonia chlamydosporia (Goddard) Zare and W. Gams	*ex* soil, Guinea	CBS 594.66
Schizophyllum commune Fr.	not known	CBSC 15-6275B

*Cultures are deposited in the following collections: ATCC, American Type Culture Collection, Manassas, VA, USA; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CBSC, Carolina Biological Supply Company, Burlington, NC, USA; IFO, Institute for Fermentation, Osaka, Japan; MUCL, Mycothèque de l'Université Catholique de Louvain, Louvain-la-Neuve, Belgium; OMH, Ontario Ministry of Health, Toronto, ON, Canada; UAMH, University of Alberta Microfungus Collection and Herbarium, Edmonton, AB, Canada; WUC, Culture collection of W.A. Untereiner, Brandon University, Brandon MB, Canada. †Strain derived from the type specimen. ‡Mating type. §Strain derived from the neotype specimen.

release; for example, *Amauroascus purpureus* caused dye release in the absence of visible growth. Thus, the visual assessment of mycelial production was not considered to be informative for the scoring of this test.

As is evident in Table 2, a positive keratin azure test at 28 days for members of the Arthrodermataceae was generally predictive of the ability to form hair perforating bodies. We tested twenty members of this family, all of which are known from keratinaceous substrata, and 14 of which have been reported to digest hair by formation of perforating bodies or erosion. In general, members of the genus *Microsporum* with positive hair perforation tended to yield a positive keratin azure result after 7 days incubation, whereas *Trichophyton* spp. with this ability were slower to release azure dye, with most becoming positive between days 7 and 28 of incubation. Although most of our results are in agreement with published reports of keratinophilic activity in the Arthrodermataceae, there are a few notable exceptions. Most remarkably, *Trichophyton krajdenii* (= the nodular variant of *T. mentagrophytes* ss. lat.) is known to perforate hair but was negative in our assay [16]. Two other species, *Chrysosporium vallenarense* and *Ctenomyces serratus*, are reported to decompose hair [11,17] but dye release was not observed in our test for either of these taxa.

None of the members of the Gymnoascaceae we tested have been investigated systematically for *in-vitro* hair degradation either by perforation or erosion, although Currah [8] collectively described this group as non-keratinolytic based on unpublished records. All taxa of Gymnoascaceae we examined were negative for keratin degradation in our assay.

In total, 24 members of Onygenaceae (*sensu* Currah [8]) were tested. Thirteen percent of taxa tested clearly demonstrated dye release at 7 days. This proportion increased to 39% after 28 days incubation. Two additional taxa, *Onygena equina* and *Uncinocarpus reesii*, demonstrated dye release only after 42 days (data not shown). With several exceptions, our results agreed with Currah's interpretation of this family as exhibiting keratinolytic activity. Currah [8] reported the genus *Amauroascus*, including *A. aureus*, to be keratinolytic. We did not observe keratin degradation in this taxon using our assay, a finding that is unusual given the common occurrence of the species on keratinaceous substrata. However, the strain used in our study was derived from the neotype of this taxon, which originated from decaying wood and which may be poorly representative of the currently accepted species concept. *Neogymnomyces demonbreunii* was reported to be keratinolytic by Currah [8] but did not show dye release in our assay. Currah [8] additionally listed the genus *Auxarthron* as keratinolytic, presumably on the basis of hair perforation studies; however, our data did not indicate keratin degradative ability in *A. californiense* or *A. zuffianum*. Like *Amauroascus aureus*, the strains of *Auxarthron* we used were derived from atypical habitats (e.g. dung of packrat and lung of prairie dog, respectively), and therefore may be uncharacteristic for these taxa.

Apart from the Onygenales, a very different fungus that released azure dye in our assay was *Chaetomium globosum* (Chaetomiaceae, Sordariales). This species was reported by Domsch and colleagues [18] to attack wool keratin and other keratinaceous substrates to a limited extent by means of boring hyphae.

Discussion

Azure-based culture media assays have been employed to study fungal utilization of numerous complex

Table 2 Results of keratin degradation tests, occurrence on keratinous substrata and ability to decompose hair

Species	Occurrence on keratinaceous substrates (y/n)	Mode of hair decomposition	Dye release in keratin azure test (+/–)		
			7 days	28 days	Growth
Arthrodermataceae					
Arthroderma curreyi	y	er [23,45]; bh [23]	–	+	sparse
A. gypseum	y	pb [10,23]	+	+	good
A. incurvatus	y	pb [6]	+	+	good
A. otae (anamorph *M. canis*)	y	pb [6,10,46]	–	–	sparse
A. quadrifidum	y	pb [6,46]	–	–	sparse
A. silverae	y	pb [17]	–	–	moderate to good
Chrysosporium vallenarense	y	pb after 60 days [11]	–	–	good
Ctenomyces serratus	y	+[35]; er [11]	–	–	sparse
Epidermophyton floccosum	y	neg [6,46]	–	–	sparse
Microsporum canis	y	pb [6,10,46]	+	+	sparse
M. cookei	y	pb [6,46,47]	+	+	sparse
M. persicolor	y	pb [6,47,48]	–	+	sparse
Trichophyton krajdenii	y	pb [6,16,46,47]	–	–	sparse
T. mentagrophytes	y	pb [6,10,46,47]	+	+	moderate
T. mentagrophytes var. red	y	pb [10,46,47]	–	+	sparse
T. mentagrophytes var. granular	y	pb [6,10,46,47]	–	+	good
T. mentagrophytes var. velvety	y	pb [6,10,46,47]	–	+	good
T. raubitschekii	y	neg [46,49]	–	–	sparse
T. rubrum	y	neg [6,10,46,47]	–	–	sparse
T. simii	y	pb [6,46]	–	+	good
Gymnoascaceae					
Arachniotus ruber	y	NT	–	–	moderate to good
Arachnomyces minimus	y	NT	–	–	sparse
Gymnascella aurantiaca	n	NT	–	–	sparse
Gymnoascoideus petalosporus	y	NT	–	–	sparse
Gymnoascus reessii	n	NT	–	–	sparse
Onygenaceae					
Amauroascus aureus	n	+[8]	–	–	moderate
A. mutatus	n	+[8]	–	+	moderate to good
A. niger	n	+[8]	–	+	sparse to moderate
A. purpureus	n	NT	–	+	no visible growth
Aphanoascus fulvescens	y	+[50,51]; pb [45]	+	+	sparse
A. terreum	y	+[8,35]	+	+	good
A. mephitalis	y	NT	+	+	sparse
Apinisia graminicola	n	NT	–	–	sparse
Ascocalvatia alveolata	y	+[8]	–	+	sparse
Auxarthron californiense	n	+[8]	–	–	moderate to good
A. zuffianum	n	+[8]	–	–	sparse
Chrysosporium keratinophilium	y	er/bh [23]; pb [45,52]	–	–	sparse
C. tropicum	y	er/bh [23,53]; pb [45,52]	–	–	moderate to good
Nannizziopsis vriesii	y	+[35]	–	+	sparse to moderate
Neogymnomyces demonbreunii	y	+[8]	–	–	sparse
Onygena equina	y	+[8]	–	–	sparse
Polytolypa hystricis	y	neg [42]	–	–	moderate
Renispora flavissma	n	+[35,54]	–	+F	good
Shanorella spirotricha	y	+[8]	–	+	good
Spiromastix grisea	y	neg [42]	–	–	moderate
S. tentaculatum	n	neg [42]	–	–	moderate
S. warcupii	n	neg [42]	–	–	sparse to moderate
Uncinocarpus reesii	y	er [45]; pb [55]	–	–	good
Trichocomaceae					
Aspergillus alliaceus	n	NT	–	–	sparse
A. niger	n	NT	–	–	moderate
Byssochlamys nivea	n	NT	–	–	sparse
Eurotium herbariorum	n	NT	–	–	no visible growth
Petromyces alliaceus	n	NT	–	–	good

Table 2 (*Continued*)

Species	Occurrence on keratinaceous substrates (y/n)	Mode of hair decomposition	Dye release in keratin azure test (+/–)		
			7 days	28 days	Growth
Trichocoma paradoxa	n	NT	–	–	no visible growth
Keratinase positive and negative isolates					
Chaetomium globosum	y	bh [56]	–	–*	moderate
Pochonia chlamydosporia	n	+[18]; er/bh [23]	–	+F	sparse to moderate
Schizophyllum commune	n	NT	–	–	sparse

*, Some release of dye but without appreciable clearing of the upper layer; –, negative; +,digests hair by unspecified process(es); bh, produces boring hyphae; er, surface erosion; F, faint; good, mycelium well-developed, evident without the use of a dissecting microscopy; moderate, mycelium moderately well-developed; ng, no growth or no mycelium evident; NT, not tested; pb, produces perforating bodies; sparse, mycelium hardly evident.

organic carbon sources such as cellulose [13,19,20], chitin [14] and lignin [13]. Apodaca and McKerrow [21] employed dye release from keratin azure, measured by spectrophotometric assay, as a measure of keratinase activity in liquid culture. Keratin azure-containing media have also been used to assess keratinase production by the nematode *Strongyloides* [4].

The most widely used method for assessing hair decomposition was described by Ajello and Georg [10], modified from Vanbreuseghem [22]. Short segments of human hair are sterilized by autoclaving, combined with sterile distilled water amended with a few drops of sterile 10% yeast extract as a starter carbohydrate. They are then inoculated with the test fungus. Following one or more weeks of incubation at 25°C, hairs are examined microscopically for signs of deterioration such as erosion, perforating bodies or boring hyphae. The production of perforating bodies is always accompanied by some degree of surface erosion, whereas boring hyphae may be produced in the absence of surface erosion [23].

Reports vary on the extent to which hair type, donor age and sex influence susceptibility to fungal attack. Some authors [24,25] have stressed the importance of these factors in the accurate evaluation of hair decomposition, while others have dismissed these variables as unimportant [26,27]. The disparity of such reports suggests that hair-based evaluation of keratin degradation may be subject to considerable inconsistency. A degree of standardization, however, is often achieved. Despite the overall lack of definitive consensus in this area, evaluators have generally recognized that children's hair tends to become perforated more rapidly than does adult hair [26]. For this reason, many authors have recommended the use of prepubertal blonde scalp hair for keratinolysis test procedures [28–31]. The appropriateness of this recommendation is probably ascribable to a combination of factors. First, melanin pigments are known to inhibit the formation of perforation organs or mask their presence [24]. Second, sebum secretion increases dramatically at puberty, and at the same time there is a shift in the ratio of wax esters to cholesterol esters in sebum as well as an overall increase in free fatty acids [32], which are known to possess mild antifungal properties [33]. Third, prepubertal hair is less likely than adult hair to have been subjected to cosmetic chemical alteration involving materials such as dyes and the chemicals used in introducing permanent waves. Some authors [30,34] have included a preparatory organic solvent defatting procedure to eliminate potentially fungitoxic waxes from hairs to be used in the perforation test. The extent to which such measures truly allow valid interlaboratory replication has not been tested.

Another area of inconsistency in hair-based methods is found in the criteria used in microscopic evaluation of colonized hairs and in the subsequent interpretation of whether or not keratinolysis has occurred. Carmichael [11] interpreted hair degradation by characterizing the ability of test fungi to erode or perforate sterilized human hair that had been sprinkled on glucose-salts agar. A similar method for assaying keratinolytic activity was later used by Van Oorschot [35] in her revision of *Chrysosporium* and allied genera, but she did not distinguish between perforation and erosive deterioration. Presently, no standard substrate characteristics, incubation conditions or assessment criteria have been widely adopted. As a consequence, literature reports of keratinolysis in some species are contradictory. For example, Marchisio *et al*. [31] reported surface erosion of hairs by *Chrysosporium carmichaelii* using blonde prepubertal hair whereas Bahuguna and Kushwaha [7] reported no morphological changes when the same species was inoculated onto blonde and black hair from donors of unspecified age. It must be added that the taxonomic difficulty of identifying some Onygenalean fungi, especially *Chry-*

sosporium species, may also contribute to some variability in keratinolysis results, particularly in studies not using standard reference strains or those in which novel isolates have not been deposited in culture collections for verification by other investigators.

Our keratin azure tube test has two main advantages over hair-based methods for assessing keratin degradative ability: (i) it is readily standardized and therefore less subjective than microscopic evaluation of hairs, and (ii) assessment is accomplished by direct visual evaluation of culture tubes rather than microscopy.

In developing our test, we used a basal medium intended to favor keratinase activity. The medium composition was based on optimal conditions for production and functionality of known keratinases. Specifically, our medium was buffered to a high pH [36,37] and it lacked starter sugars. The latter factor was in recognition of the observation that proteolytic activity is frequently suppressed by glucose [21,38,39]. Our recent trials have suggested that the use of carbohydrate-free Czapek's–Dox medium [40] as a basal medium for our keratin utilization assay provides results comparable to those obtained with BM (data not shown).

The recognition of keratinolytic ability as indicated by erosion or fungal penetrating bodies has been interpreted as an important phylogenetic character in the taxonomy of the Onygenales [8,41]. Scott *et al.* [42] noted that procedural and stochastic variability in hair decomposition made this character unreliable as a family level-determinant for the separation of the Gymnoascaceae (non-keratinolytic) from the Onygenaceae (keratinolytic) [8]. Indeed, recent evidence has suggested that the Onygenaceae *sensu* Currah [8] is polyphyletic [43,44]. Thus, the comparative re-examination of keratinophily in the framework of molecular-based phylogeny may clarify the systematic utility of this character and may provide useful insight into the role of keratin utilization in the evolution of this group of fungi. At the same time, the results of the present study make it clear that the keratin azure tube assay may provide a useful species-specific character for the laboratory identification of members of the Onygenaceae.

Acknowledgements

Financial support was provided in the form of an AW Mellon Postdoctoral Fellowship in Plant Systematics and a NSERC operating grant to WAU. We thank David Malloch, John Rippon and Lynne Sigler for insightful discussions and suggestions for the improvement of the pre-submission draft of this paper. We are indebted to the late Julius Kane for sharing his wisdom and vibrant enthusiasm for dermatophytic fungi, inspiring us to this and other work on keratinophilic fungi. We thank Richard Summerbell, the MUCL Culture Collection (Université Catholique de Louvain, Belgium) and Gamma-Dynacare Medical Laboratories (Brampton, Ontario) for supplying a number of the cultures used in this study.

References

1 Latkowski JM, Freedberg IM. Epidermal cell kinetics, epidermal differentiation, and keratinization. In: Freedberg IM, Eisen AZ, Wolff K, *et al* (eds). *Fitzpatrick's Dermatology in General Medicine*. New York: McGraw-Hill, 1999: 133–144.
2 Zaghoul TI, Al-Bahra M, Al-Azmeh H. Isolation, identification and keratinolytic activity of several feather-degrading bacterial isolates. *Appl Biochem Biotechnol* 1998; **70–72**: 207–213.
3 Morihara K, Oka T, Tsuzuki H. Multiple proteolytic enzymes of *Streptomyces fradiae*: production, isolation, and preliminary characterization. *Biochim Biophys Acta* 1967; **139**: 382–397.
4 Tadeusz M. A metalloproteinase secreted by the infective larvae of *Strongyloides papillosus*. *Acta Parasitol* 1999; **44**: 193–198.
5 Seymour RL, Johnson TW. Saprolegniaceae: A keratinophilic *Aphanomyces* from soil. *Mycologia* 1973; **65**: 1312–1318.
6 Padhye AA, Young CN, Ajello L. Hair perforation as a diagnostic criterion in the identification of *Epidermophyton, Microsporum* and *Trichophyton* species. *Pan American Scientific Publications* 1980; **396**: 115–120.
7 Bahuguna S, Kushwaha RKS. Hair perforation by keratinophilic fungi. *Mycoses* 1989; **32**: 340–343.
8 Currah RS. Taxonomy of the Onygenales: Arthrodermataceae, Gymnoascaceae, Myxotrichaceae and Onygenaceae. *Mycotaxon* 1985; **24**: 1–216.
9 Marchisio VF. Keratinolytic and keratinophilic fungi of children's sandpits in the city of Turin. *Mycopathologia* 1986; **94**: 163–172.
10 Ajello L, Georg LK. In vitro cultures for differentiating between atypical isolates of *Trichophyton mentagrophytes* and *T. rubrum*. *Mycopathol Mycol Appl* 1957; **8**: 3–17.
11 Carmichael JW. *Chrysosporium* and some aleuriosporic hyphomycetes. *Canad J Bot* 1962; **40**: 1137–1174.
12 Kane J, Summerbell RC, Sigler L, Krajden S, Land G. *Laboratory Handbook of Dermatophytes*. Belmont, CA: Star Publishing, 1997.
13 Thorn RG. The use of cellulose azure as a crude assay of both cellulolytic and lignolytic abilities of wood-inhabiting fungi. *Proc Japan Acad Series B Phys Biol Sci* 1993; **69**: 29–34.
14 Untereiner WA, Malloch D. Patterns of substrate utilization in species of *Capronia* and allied black yeasts: ecological and taxonomic implications. *Mycologia* 1999; **91**: 417–427.
15 Ahmad I, Malloch D. An evaluation of carbon and nitrogen assimilatory patterns for taxonomic differentiation of *Penicillium* species. *Mycologia* 1999; **91**: 1031–1044.
16 Kane J, Scott JA, Summerbell RC, Diena BB. *Trichophyton krajdenii* sp. nov.: an anthropophilic dermatophyte. *Mycotaxon* 1992; **45**: 307–316.
17 Currah RS, Abbot SP, Sigler L. *Arthroderma silverae* sp. nov. and *Chrysosporium vallenarense*, keratinophilic species from antarctic and montane habitats. *Mycol Res* 1996; **100**: 195–198.
18 Domsch KH, Anderson TH, Gams W. *Compendium of Soil Fungi*. London: Academic Press, 1980.

19 Cresswell MA, Attwell RW, Dempsey MJ. Detection of cellulolytic actinomycetes using cellulose azure. *J Microbiol Meth* 1988; **8**: 299–302.
20 Smith RE. Rapid tube test for detecting fungal cellulase production. *Appl Environ Microbiol* 1977; **33**: 980–981.
21 Apodaca G, McKerrow JH. Expression of proteolytic activity by cultures of *Trichophyton rubrum*. *J Med Vet Mycol* 1990; **28**: 159–171.
22 Vanbreuseghem R. La culture des dermatophytes in vitro sur les cheveux isolés. *Ann Parasitol* 1949; **24**: 559–573.
23 Ali-Shtayeh MS, Jamous RMF. Keratinophilic fungi and related dermatophytes in polluted soil and water habitats. In: Kushwaha RKS, Guarro J (eds). *Biology of Dermatophytes and Other Keratinophilic Fungi*. Bilbao: Revista Iberoamericana de Micología, 2000: 51–59.
24 Lu YC. A new method for the study of hair digestion by dermatophytes. *Mycopathol Mycol Appl* 1961; **17**: 225–235.
25 Rai MK, Qureshi S. Screening of different keratin baits for isolation of keratinophilic fungi. *Mycoses* 1994; **37**: 295–298.
26 Salkin IF, Hollick GE, Hurd NJ, Kemna ME. Evaluation of human hair sources for the in vitro hair perforation test. *J Clin Microbiol* 1985; **22**: 1048–1049.
27 Vanbreuseghem R. Technique biologique pour l'isolement des dermatophytes du sol. *Ann Soc Belg Méd Trop* 1952; **32**: 173–178.
28 Guarro J, Figueras MJ, Cano J. Degradación de pelo humano in vitro por *Trichophyton mentagrophytes*. *Microbiologia SEM* 1988; **4**: 29–37.
29 Kane J, Summerbell RC, Sigler L, Krajden S, Land G. Appendix – Media and Methods. In: Kane J, Summerbell RC, Sigler L, Krajden S, Land G (eds). *Laboratory Handbook of Dermatophytes*. Belmont, CA: Star, 1997: 330–331.
30 Kunert J. Keratin decomposition by dermatophytes. 1. Sulfite production as a possible way of substrate denaturation. *Z Allg Mikrobiol* 1973; **13**: 489–498.
31 Marchisio VF, Fusconi A, Rigo S. Keratinolysis and its morphological expression in hair digestion by airborne fungi. *Mycopathologia* 1994; **127**: 103–115.
32 Downing DT, Stewart ME, Strauss JS. Lipids of the epidermis and the sebaceous glands. In: Freedberg IM, Eisen AZ, Wolff K, *et al* (eds). *Fitzpatrick's Dermatology in General Medicine*. New York: McGraw-Hill, 1999: 144–155.
33 Kunert J. Physiology of keratinophilic fungi. In: Kushwaha RKS, Guarro J (eds). *Biology of Dermatophytes and Other Keratinophilic Fungi*. Bilbao: Revista Iberoamericana de Micología, 2000: 77–85.
34 Page RM. Observations on keratin digestion by *Microsporum gypseum*. *Mycologia* 1950; **42**: 591–602.
35 Van Oorschot CAN. A revision of *Chrysosporium* and allied genera. *Stud Mycol* 1980; **20**: 1–89.
36 Anon. *The Merck Index*, 12th edn. 1996.
37 Hibino T. Purification and characterization of keratin hydrolase in psoriatic epidermis: application of keratin-agarose plate and keratin-polyacrylamide enzymography methods. *Anal Biochem* 1985; **147**: 342–352.
38 Apodaca G, McKerrow JH. Regulation of *Trichophyton rubrum* proteolytic activity. *Infect Immun* 1989; **57**: 3081–3090.
39 Brasch J, Martins B-S, Christophers E. Enzyme release by *Trichophyton rubrum* depends on nutritional conditions. *Mycoses* 1991; **34**: 365–368.
40 Malloch D. *Moulds: Their Isolation, Cultivation and Identification*. Toronto: University of Toronto Press, 1981.
41 Chabasse D. Phénomènes d'adaptation parasitaire des champignons kératinophiles telluriques et conséquences en pathologie humaine et animale. *Crypt Mycol* 1997; **18**: 71–79.
42 Scott JA, Malloch D, Gloer JB. *Polytolypa*, an undescribed genus in the Onygenales. *Mycologia* 1993; **85**: 503–508.
43 Sugiyama M, Mikawa T. Phylogenetic analysis of the non-pathogenic genus *Spiromastix* (Onygenaceae) and related onygenalean taxa based on large subunit ribosomal DNA sequences. *Mycosci* 2001; **42**: 413–421.
44 Untereiner WA, Scott JA, Naveau FA, Currah RS, Bachewich J. Phylogeny of *Ajellomyces*, *Polytolypa* and *Spiromastix* (Onygenaceae) inferred from rDNA sequence and non-molecular data. *Stud Mycol* 2002; **47**: 25–35.
45 Sigler L. *Chrysosporium* and molds resembling dermatophytes. In: Kane J, Summerbell RC, Sigler L, Krajden S, Land G (eds). *Laboratory Handbook of Dermatophytes*. Belmont, CA: Star, 1997: 261–331.
46 Summerbell RC, Kane J. The genera *Trichophyton* and *Epidermophyton*. In: Kane J, Summerbell RC, Sigler L, Krajden S, Land G (eds). *Laboratory Handbook of Dermatophytes*. Belmont, CA: Star, 1997: 131–191.
47 Rippon JW. *Medical Mycology: the Pathogenic Fungi and the Pathogenic Actinomycetes*, 3rd edn. Philadelphia: Saunders, 1988.
48 Land G. The genus *Microsporum*. In: Kane J, Summerbell RC, Sigler L, Krajden S, Land G (eds). *Laboratory Handbook of Dermatophytes*. Belmont, CA: Star, 1997: 193–211.
49 Kane J, Salkin IF, Weitzman I, Smitka C. *Trichophyton raubitschekii*, sp. nov. *Mycotaxon* 1981; **13**: 259–266.
50 Cano J, Guarro J. The genus *Aphanoascus*. *Mycol Res* 1990; **94**: 355–377.
51 Cano J, Guarro J, Figueras MJ. Study of the invasion of human hair in vitro by *Aphanoascus* spp. *Mycoses* 1991; **34**: 145–152.
52 Kushwaha RKS. The genus *Chrysosporium*, its physiology and biotechnological potential. In: Kushwaha RKS, Guarro J (eds). *Biology of Dermatophytes and Other Keratinophilic Fungi*. Bilbao: Revista Iberoamericana de Micología, 2000: 66–76.
53 Marchisio VF. Keratinophilic fungi: Their role in nature and degradation of keratinic substrates. In: Kushwaha RKS, Guarro J (eds). *Biology of Dermatophytes and Other Keratinophilic Fungi*. Bilbao: Revista Iberoamericana de Micología, 2000: 86–92.
54 Sigler L, Gaur PK, Lichtwardt RW, Carmichael JW. *Renispora flavissima*, a new gymnoascaceous fungus with tuberculate *Chrysosporium* conidia. *Mycotaxon* 1979; **10**: 133–141.
55 Sigler L, Carmichael JW. Taxonomy of *Malbranchea* and some other hyphomycetes with arthroconidia. *Mycotaxon* 1976; **4**: 349–488.
56 English MP. The saprophytic growth of non-keratinophilic fungi on keratinized substrata, and a comparison with keratinophilic fungi. *Trans Brit Mycol Soc* 1965; **48**: 219–235.