

Keratinophilic fungi: Their role in nature and degradation of keratinic substrates

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Summary

Keratinophilic fungi are natural colonizers of keratinic substrates. Some are keratinolytic and play an important ecological role in decomposing α -keratins, the insoluble fibrous proteins. Because of the tight packing of their polypeptide chains in α -helix structures and their linkage by disulphide bridges, they are poorly biodegradable. Two main forms of attack have been identified: surface erosion and radial penetration. In surface erosion, the sequence of degradation proceeds as the level of keratinisation (the cystine crosslinks) of the components of the keratinic matrix increases. In radial penetration, on the other hand, specialized hyphae can penetrate like a drill through the matrix, irrespective of the degree of keratinisation. This may illustrate how the growth can change direction and how secretory activity may concentrate at the tips of the penetrating hyphae.

Key words

Keratinophilic fungi, Role in nature, Keratin decomposition

After insects, fungi are the second largest group of organisms [1]. Their unusually wide morphological diversity is matched by a singular behavioural diversity, while the many life strategies they have evolved explain their enormous importance in evolution, the ecosystem, human progress, and most of the processes that take place on Gaia i. e. the Earth considered as a whole, the atmosphere, the oceans, biota and lithosphere [1].

Diversity is also a feature of fungal nutrition. Nine main categories, representing a series of continua and based on nutritional mode and ecological behaviour, have been devised from various combinations of saprotrophy, necrotrophy and biotrophy [2]. Moreover, many fungi do not confine themselves to a single mode but display varying degrees of flexibility in response to changes in their environment [2].

The ability of fungi to adapt quickly to such changes primarily stems from the ease with which they acquire and store genetic information through special hyphal fusion mechanisms that result in the coexistence of different nuclear types, known as heterokaryosis. Differential gene expression rather than selection of nuclei appropriate for a particular way of life seems the mechanism of choice [2]. The direction of nutritional evolution may therefore be assumed to be determined by the options imposed on a fungus by its environment, including, where appropriate the narrowing of specialism within a single mode [2].

The characteristics of the genetic stock and its close interaction with the environment appear, therefore, to be features of great importance in the phylogenetic and ontogenetic evolution of fungi. They also provide a theoretical explanation for aspects of the biology of those fungi which are endowed with an affinity for keratinic substrates.

Keratinophilic and keratinolytic fungi

The cellular and extracellular structural compounds of microorganisms, plants and animals are hardly ever found in the pure state in natural situations, but in association with other more or less complex molecules. During degradation by decomposing organisms, these molecules are used fairly quickly. Structural compounds, on other hand, become available after a long interval, although for many pathogens and primary colonizers they are potential sources of carbon, and possible nitrogen and sulphur [2].

in vivo, keratin molecules are organized with various other proteins and cementing substances in more or less keratin-rich structures. A keratinic substrate, such as a hair fragment, placed in the soil will be colonized by many microorganisms, including fungi, which collaborate in using the various components differentially and progressively, according to their complexity and availability, until it is completely mineralized.

Burying of keratinic baits, especially human and animal hairs, is a routine way of isolating the so-called "keratinophilic fungi", which, in fact, are usually endowed with a limited competitive activity and cannot be isolated by ordinary soil plating and soil-dilution plating. The "Tokava" hair-baiting method, an acronym of Toma, Karling and Vanbreuseghem who introduced it [3], has since been modified by Orr [4] by adding antibiotics to the soil-moistening water to restrict bacterial colonization. Numerous fungi, including many Ascomycetes, can be isolated from the baits after a certain incubation period. But which of them are keratinophilic? The term itself is ambiguous, since it has been used to indicate both the der-

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matophytes (regarded as keratinophilic par excellence) and other colonizers, which are often selected arbitrarily from those more closely related to the dermatophytes in the teleomorph or microconidic and arthroconidic phase, while their true role during colonization is rarely examined in depth.

According to Griffin [5] and De Vries [6], some fungi that colonize these baits are not able to attack keratin, but merely use the products of its partial demolition by other fungi, the protoplasmic residues of the keratinic matrices or substances naturally present on their surface. Griffin [5] showed that aqueous extracts of intact human and animal hairs contained appreciable quantities of uric acid, urea, ammonia, pentoses, glycogen, phenols and amino acids i. e. compounds that are probably enough in themselves to support the growth of some fungi. Griffin [5] and De Vries [6] observed a fungal succession on hair in soil. The primary colonizers, in their opinion, were chytrids, followed at first by *Fusarium*, *Penicillium* and *Mucor* species, capable of using intercellular substances which are very easy to digest, and later by *Chaetomium*, *Gliocladium* and *Humicola* species, which are able to break down more resistant substances. The final group were typical keratinophilic hyphomycetes, such as *Trichophyton* and *Microsporum* species. In nature, however, the substrate groupings of decomposer soil fungi are not so rigid, but often occur in an overlapping sequence [7].

It therefore seems necessary to draw a clear distinction between the roles of these colonizers. The only possibility, although this does not reproduce the natural situation exactly, mainly because no influence is exerted by other organisms, is to use supplementary *in vitro* keratinolysis tests according to Ajello & Georg [8] or English [9] for each species isolated. For the reasons mentioned above, the simple growth of a fungus on keratin residues, often visible with the naked eye, is not a sufficient demonstration of keratinolytic activity. Morphological examination at various resolution levels is the only way of determining the real capacity of each species through the demonstration of lytic spaces and specialised fungal organs in which keratin is present. Biochemical examinations do not provide equally reliable information, since the production of extracellular proteases [10] and the presence of Lowry-positive peptides [11] are not enough to show that keratin has been degraded. It is, in fact, uncertain whether peptides are actually released from keratin or other keratin-associated proteins [10, 12]. Experiments with purified dermatophytic proteases on native keratin have shown that the weight lost after their repeated action is no greater than the expected content of non-keratinic compounds [11]. This is probably because breaking the disulphide bridges is the first step in keratin demolition and this could not be done by proteases alone [13-17]. The mechanisms involved, however, are still unclear, though sulphitolysis, which denatures keratin non-enzymatically seems one of the most likely [15,17-19].

Little-known papers by Polish workers [20,21] propose that the term keratinolytic be reserved for colonizers that are really capable of attacking and demolishing keratin, whereas those that accompany them and only use the more readily degradable substances should be called keratinophilic.

I have adopted this distinction in my own work. More recently, however, my thoughts have returned to the etymological meaning of the word keratinophilic, which is derived from Greek and simply means "endowed with an affinity for keratinic substrates". Keratinophilic fungi, therefore, can be said to include all natural colonizers of

these substrates, whereas keratinolytic fungi are only those that have been shown to attack keratin itself in some way.

The fact remains, however, that the most active keratinolytic fungi are dermatophytes and their correlates, especially *Microsporum*, *Trichophyton*, *Aphanoascus*, *Chrysosporium*, *Geomyces*, *Gymnoascus*, *Malbranchea* and *Myceliophthora* species, though forms of attack have equally been reported for some species of *Alternaria*, *Beauveria*, *Cladosporium*, *Mucor*, *Paecilomyces*, *Penicillium* and *Scopulariopsis* [22-25].

It should also be borne in mind that keratinolysis, like many other fungal biochemical activities, does not seem either a constant or a species-specific character [22,23,25]. Both active and nonactive isolates actually occur within a given species in the same environmental conditions. Variations may also be observed in the manner and intensity in which each isolate attacks the substrate and differentiates specialised structures for this purpose. Dermatophytes themselves are no exception in this respect [26,27].

When referring to species, therefore, and not to precise isolates whose activities can be fairly easily checked *in vitro*, it is always advisable to speak about "potentially keratinolytic species". This is one of the aspects of the biodiversity of fungi that emerges when their populations are investigated.

The keratinolytic fungi in the cycle of matter

Heraclitus concept of becoming, taken up by other Greek philosophers including Aristotle (becoming always takes place between opposites), and by positivists and other schools of thought in modern times, is both precisely applied and experimentally confirmed in biology. Nothing in nature is immobile. Everything flows, becomes, is transformed. It is the secret of life, whose survival on Earth depends solely on two factors [28]:

- a continuous supply of energy from an external source: the sun;

- the cycle of matter i. e. the cyclical passage of matter from its inorganic to its organic form and vice versa (this is Aristotle's becoming between opposites).

The first phase of this cycle is mainly achieved through photosynthesis: the leading actors are the green plants, which with the aid of the sun's energy produce organic matter that is then transformed and passed through the food chain to animals. The second phase occurs through respiration: the leading actors are all the organisms, but especially those that form the microflora of the soil. In the soil, which is a particularly important habitat for saprotrophic organisms, organic substances from animals and plants are reconverted to an inorganic form and so made available once again for plants.

It follows from this that no natural compound accumulates in the Earth's crust: sooner or later, depending on the complexity of its molecule, it is mineralised by some microorganism or group of microorganisms in the immense chemical laboratory called the soil. Even the keratins. These are insoluble fibrous proteins derived from the ectoderm and are poorly biodegradable. There are two kinds of keratins [29]:

- α -keratins: these contain most of the common amino acids, but they are primarily rich in cystine residues and, therefore, disulphide bridges: rigid, brittle forms in horns and nails contain up to 22% cystine; soft, flexible forms in the skin, and in hair and wool contain between

10 and 14%;

- β -keratins: these lack both cystine and cysteine, but are rich in amino acids with short side chains, especially glycine, alanine and serine. They are found in the fibres of spiders and silkworms, in scales and in the claws and beaks of reptiles and birds.

Only the α -keratins constitute an ecological problem. Their resistance to degradation by microbes is the result of the tight packing of their polypeptide chains in α -helix structures and their linkage by disulphide bridges. Few organisms are capable of demolishing them: perhaps a few actinomycetes, some *Bacillus* strains and the thermophilic *Fervidobacterium pennavorans* [13,17,30-32]; some animals, such as the larvae of wool, feather and fur moths, birds of prey and the Mallophagi [33,34], perhaps directly or commensally with microorganisms in their intestinal microflora; fungi, especially the dermatophytes and some soil species belonging to the Ascomycetes, the mitosporic fungi and Mucorales, and in very wet damp soils and aquatic environments, some Chytridiales, Saprolegniaceae and Leptolegniaceae [32]. Many of the data in the literature, however, require confirmation because of the confusion between keratinophilia and keratinolysis, and the use of unsatisfactory methods to demonstrate the latter.

The ecological role of fungi in the demolition of keratinic remains is undoubtedly of prime importance, even if their activity is hard to quantify. Many estimates have been made of the amount of lignified cellulose synthesised by primary producers through photosynthesis and then restored to the atmosphere in the form of carbon dioxide, and through the activity of fungi that decompose this complex and refractory polymer. Woody plants, in fact, probably contain about 80% of the organic carbon in biological material [35]. The activity of the fungi in this context is fundamental, both for replenishing the supply of carbon dioxide and other inorganic compounds and for the important task of removing nature's debris and garbage. Without this activity, the world would soon be submerged by plant residues, and this would probably exclude most living organisms from their natural habitat.

On the other hand, I have never found estimates for the input of organic resources on the Earth on the part of consumers and particularly their more complex, refractory and persistent polymers like chitin and keratin. These are certainly less abundant organic resources than those directly produced by phototrophs, but they are no less important for that [2].

In natural environments, therefore, keratinolytic fungi are involved in recycling the carbon, nitrogen and sulphur in α -keratins. Their presence and distribution seem to depend largely on the amount of keratinic material available due either to man or to domestic animals, synanthropic and wild animals [22,23,36], especially where human and animal populations exert strong selective pressure on the environment. Other ecological and environmental factors, such as pH, temperature or altitude, seem to be less important because these fungi show a wide tolerance toward them [37-40].

The α -keratins: from polypeptide chains to keratinized tissues

The main unit of the α -keratins is protofibril, a structure with four polypeptide chains in two pairs that wind around each other to form dextrose α -helices [41]. These also wind around each other to form a sinistrorse superhelix that is rendered stable by hydrogen bridges

within each chain running parallel to its major axis, and by disulphide bridges formed by cystine residues between adjacent chains [29,41]. Protofibrils join to form microfibrils. In hairs, wool and nails, microfibrils turn in spirals to form macrofibrils [41,42]. Protofibrils and microfibrils are still linked by disulphide bridges, whose number determines both their rigidity [41] and their resistance to degradation by microbes.

Hairs and nails are easily collected. They therefore provide the keratinic substrates most frequently used as baits and in keratinolysis assays. Their anatomical and histochemical features illustrate the patterns and dynamics of their demolition. Since much of the literature on these subjects lies outside the field of interest for mycologists, I will briefly summarise the main points.

Hairs have a complex, heterogeneous structure. The distribution and organisation of keratin's several parts are equally variable [43-50].

The cuticle forms a very efficient defence against injury from the environment. Its cells contain large amounts of amorphous keratin, especially in the thicker outer layer (layer A) and an inner layer, both of which have a high cystine content. The exocuticle is also rich in cystine, though its keratin is irregularly distributed. In the endocuticle, on the other hand, there is very little keratin but a prevalence of cytoplasmic residues (mitochondria, nucleus and endoplasmic reticulum).

Apart from a thin, irregularly keratinized sheet just below their membrane the cortical cells are not layered. They display a prevalence of fibrillar keratin. Microfibrils combine to form bundles or macrofibrils that are tightly packed in the whole of the cellular volume and lie parallel to the long axis of the hair. Each microfibril and macrofibril is surrounded by an amorphous matrix. There are marked biochemical differences between these structures. Microfibrils are composed of high molecular weight keratin and little cystine, whereas their matrix contains low molecular weight keratin and is very rich in cystine. The macrofibrillar matrix contains very little keratin, and its chemical composition and properties are similar to those of the endocuticle. Medullary cells are not always present in the inner portion of the hair shaft. They seem to be devoid of cystine, but contain relatively large amounts of glutamic acid [44].

The nail plate consists of a dorsal, an intermediate and a ventral layer that differ in thickness and in the compactness and type of bond between their cells [42,51]. In general, however, a nail is more uniform than a hair and may be cytologically and ontogenetically compared to its cortex. Analysis of keratin nail shows that hairs and nails contain practically the same fractions [42], while ultrastructural and ultrahistochemical studies have revealed that micro and macrofibrils are organized like in the hair cortex and that there is a layer with many disulphide bridges on the periphery of every nail cell, particularly in the intermediate layer [42].

Degradation of keratinic substrates

Investigation of the ability of fungi to decompose keratin has been mainly biochemical and morphological. Biochemical studies of keratinolysis by fungi and bacteria have mostly characterized the enzymes secreted, firstly because they may be important as determinants of virulence in the case of pathogenic fungi [10], and secondly because they are applied biotechnologically in the disposal of refuse and the production of animal foods, fertilisers, glue and rare amino acids from poultry farm and tannery wastes [17,52]. Most workers have attributed the

degradation of keratinic substrates to the production of specific and mostly extracellular proteolytic enzymes called keratinases, whose secretion appears to be induced by the presence of keratin in the substrate [53-57]. However, those secreted by *T. rubrum*, which appear to be responsible for keratinolysis, also seem to be expressed constitutively even in the stationary phase [58].

Extracellular keratinases are produced by bacteria [17,59] and fungi [54,55,57,60-66]. Inhibitor profiles suggest that they are all serine proteinases. Their molecular weights generally range from 30 to 50 kDa; many are active at pH 7 to 8, others at acid values [67-70], and others, such as those produced by *Streptomyces pactum* [17], *Bacillus licheniformis* [59] and *Fervidobacterium pennavorans* [52], at a slightly basic pH. An intracellular keratinase with similar characteristics is produced by *T. gallinae* [71]. There appears to be some antigenic relationship between the dermatophytic keratinases [12].

However, some authors have observed that, although the keratinases of fungi and bacteria are much more active than nonspecific proteases such as trypsin and papain in terms of their speed and effect on keratinic matrices, their activity is limited, so much so that even purified dermatophytic [55,57,61,63,72] and bacterial [17,52] preparations are unable to dissolve native keratin. As already stated, the breaking of disulphide bridges, probably by sulphitolysis, is thought to be the initial step in demolition, and this renders keratin more accessible to proteolytic enzymes. This could explain the absence of extracellular keratinases in the early degradation stages [65].

At present, therefore, real demonstrations of keratinolysis are provided by *in vitro* morphological investigations. The first light microscope observations were those of Vanbreuseghem [73], Barlow & Chattaway [74] and De Vries [6]. Of fundamental importance, however, was the research of English, who provided extremely detailed descriptions of hyphal changes during hair colonization by dermatophytes [75,76] and other keratinolytic fungi [9,77,78], which had been isolated from the substrate by digestion.

Morphological expressions of keratinolysis on hair, the fungal structures involved and their relation with the matrix, and the sequence of events have been examined in depth in light microscopy and SEM studies [22-25]. Two main forms of attack, namely surface erosion and radial penetration, have been identified, and an attempt at reconstructing their respective invasion patterns has been made. In surface erosion, there is a gradual destruction of a hair from the outside inwards by hyphae, which work their way under the cuticular scales, lift up the cuticle and then digest the scales, starting from the inner side. The hyphae engaged in this activity may either retain their normal appearance or dilate to form short branches and give rise to the palm of the hand structures [75]. In the cortex, these hyphae develop still further and some may lead to the formation of flattened branched fronds (English's eroding mycelium). These changed hyphae, together with those that retain their normal appearance, form a variously extensive mantle around the hair that is responsible for uniform surface erosion. The palm of the hand structures and the eroding mycelium can cause erosion pockets by means of large cells or a more or less compact pseudoparenchyma which slowly form cavities in the cortex. These vary in size, but are a little larger than the hyphae that produced them. The hyphae may develop further and invade other areas of the cortex via the spaces between the cells.

In radial penetration, there is a random attack by variously specialised hyphae that penetrate the hair at

right angles to its surface. These are English's boring hyphae and perforating organs [75,76], wider boring hyphae, which are structures intermediate in diameter, and swollen boring hyphae, which are similar to the boring hyphae when they penetrate the outer cortex but are dilated in balloon-like formations when they reach what are probably less compact regions of the hair. The lysis areas around these forms of penetration are considerably larger than the hyphae that produce them. Both forms of attack usually coexist. *Myceliophthora vellerea* and *Chrysosporium tropicum* isolates, however, display surface erosion only, despite the remarkable efficiency of their demolition of the hair [25].

Further information is supplied by transmission electron microscopy. Pathogenic and nonpathogenic dermatophytes have received the most attention in a wealth of papers [79-86] that mainly illustrate the enzymatic side of demolition. Particular mention may be made of Kunert & Krajci's investigation of *in vitro* degradation of keratin in human hair invaded by the dermatophyte *Microsporum gypseum* [83]. By following in the tracks of Mercer & Verma [79], they showed that the sequence of degradation of the individual hair components corresponded to their degree of keratinization i. e. their cystine content. In the cuticle, penetration of the hyphae below the scales was followed by invasion of the nonkeratinized cell membranes and the endocuticle with its many cytoplasmic residues. The exocuticle, the thin sheet adjacent to the inner face of the scales and especially the outer layer A were the most resistant. In the cortex, the cell membranes and cytoplasmic residues were again the first to be digested. These were followed by the intermacrofibrillar matrix, the thin sheet and the microfibrils. The most resistant part was the intermacrofibrillar matrix.

The same conclusions were reached [24,87] in the studies of two nondermatophytic keratinolytic fungi, *Chrysosporium tropicum* and *Scopulariopsis brevicaulis*. These showed, however, that the sequence described earlier [83] is characteristic of surface erosion, but that the sequence is not followed in radial penetration, where the boring hyphae start on the outside from appressorium-like formations and pass through the layers of the hair irrespective of their degree of keratinisation (Figures 1-3). This may illustrate their ability to concentrate their secretory activity at their tips and thus act like a drill. The images showed, however, that the enzymes spread laterally after this drilling and gradually digest the hair components in the sequence described. The boring hyphae spread in this way and may also branch out to increase the lysis area.

The perforating organs of the dermatophytes, which were not found in *C. tropicum* or *S. brevicaulis*, may have the same features as the boring hyphae, though this has never been demonstrated. There are other aspects of the dermatophytes which have never been made clear or described in detail, such as the initial stages of their attack on the cuticle and the origin of their perforating organs. It is not clear, for example, whether they are already differentiated on the outside of the scales, like the boring hyphae, or only when they reach the cortex.

Despite these gaps in our knowledge, strong analogies have been demonstrated of the way in which hairs are attacked by the dermatophytes and common soil fungi, such as *C. tropicum* and *S. brevicaulis*.

Nails have been employed as the growth substrate for keratinolytic fungi much less frequently. There are some data for *Scopulariopsis*, particularly *S. brevicaulis* [24,88], which is commonly observed in onychomycosis [89], sometimes as a primary invader but more often as

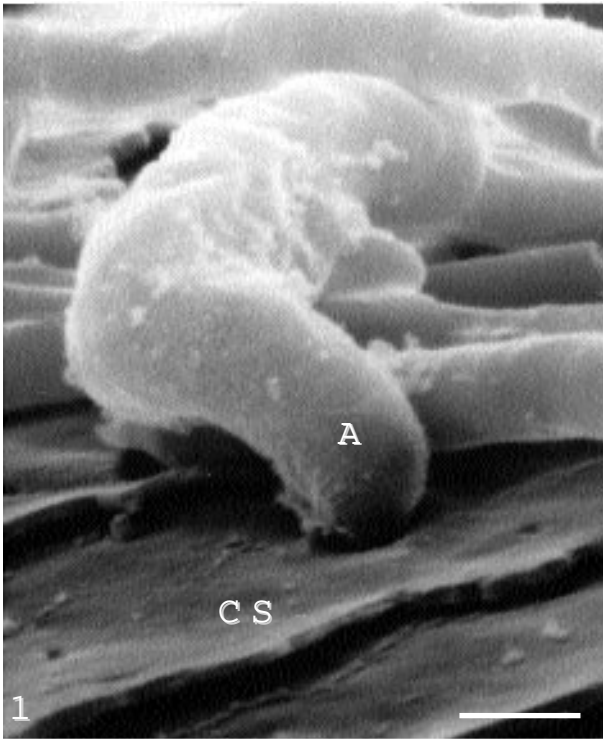


Figure 1. Development of boring hyphae in human hair. SEM of an appressorium-like formation (A), that is the starting point of a boring hypha, on the surface of a cuticular scale (CS). Bar= 2 μ m. From Fusconi & Filipello Marchisio [87].

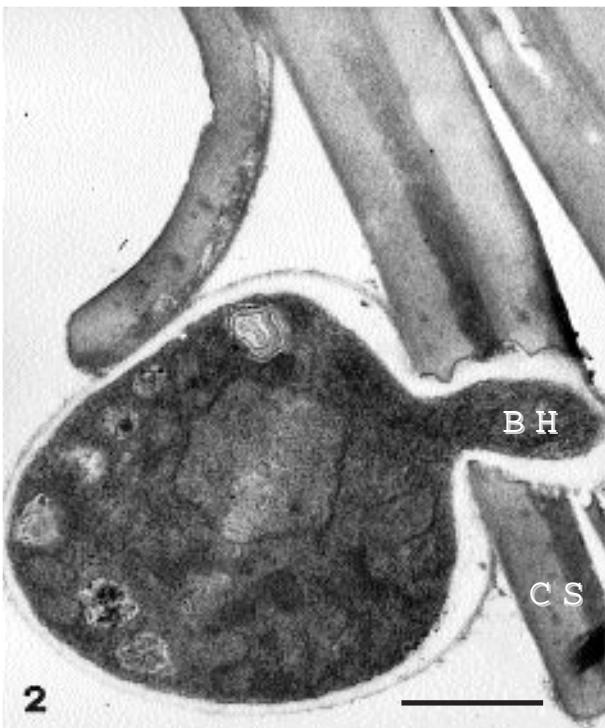


Figure 2. Development of boring hyphae in human hair. TEM of longitudinal section of a young boring hypha (BH) that penetrates to a depth of two cuticular scales (CS). Note the festooned lysis area. Bar= 1 μ m. From Fusconi & Filipello Marchisio [87].

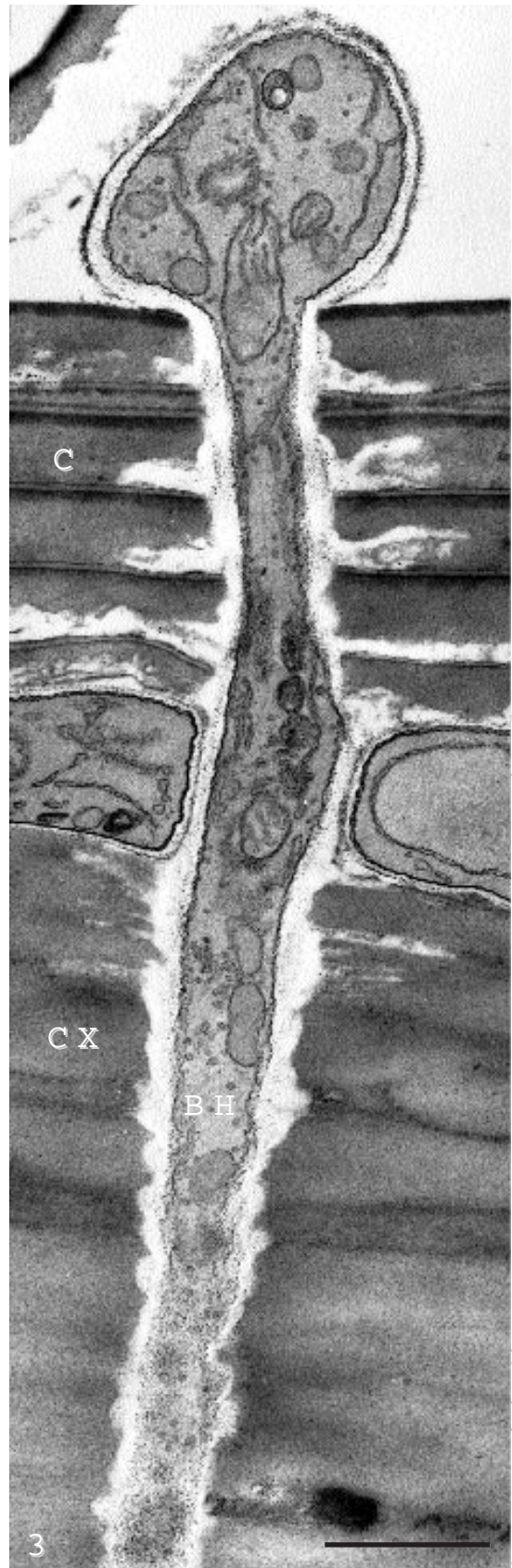


Figure 3. Development of boring hyphae in human hair. TEM of longitudinal section of a boring hypha (BH) that crosses the whole cuticle (C) and part of the cortex (CX). Bar= 1 μ m. From Fusconi & Filipello Marchisio [87].

secondary to dermatophytosis or injury [90,91]. It demolishes nails in much the same way as the hair cortex and follows the usual sequence determined by the cystine content [24]. The few boring hyphae observed also behaved as in the hair [24].

Is there an evolutionary pathway from the soil keratinolytics to human and animal pathogens?

The host's epithelium is the main barrier to passive fungal invasion [12]. Its penetration is apparently the outcome of enzymatic degradation of its surface macromolecules [12], including the keratins [30,58]. The keratinolytic activity of an organism can therefore be taken as a putative virulence factor [10].

If the ability of a fungus to demolish α -keratins *in vitro* is of real significance in predicting its ability to infect *in vivo*, then all the soil keratinolytics are potential pathogens. For the dermatophytes, one can imagine an evolution via numerous intermediate conditions from primitive soil saprotrophs to more specialised species acting solely as parasites and able to recognise only one type of host [30]. Similarly each soil keratinolytic species could therefore evolve and acquire the ability to invade keratinized human and animal tissues [30]. Many keratinolytic saprotrophs have often been isolated from human and animal skin, fur and feathers, but it is not always possible to show whether they are transient or occasional guests or

whether they are responsible for lesions [30]. In the laboratory they are often regarded simply as contaminants [30]. Their pathogenetic role is therefore an open question. There is, however, a growing body of evidence that both geophilic dermatophytes and other soil keratinolytics may be pathogens. Sufficient confirmation of this can be found by consulting the Atlas of Clinical Fungi edited by de Hoog & Guarro [89].

This exciting interpretation is corroborated in the Introduction, which mentions the close interaction between fungi and the environment that seems to allow the fungi to select the nutritional options and behaviour patterns best suited to their survival in a given environment. The only cloud on this horizon, however, is the fact that very few studies have investigated the *in vivo* significance of keratinolytic activity on the penetration of host barriers. This means that the importance of keratinolysis in dermatophyte invasion of the epidermis still has to be established [12]. In addition, a number of other factors dependent on the fungus, the host and the environment are probably required for initiating and developing infection [92]. Any understanding of pathogenesis must take account of the total *in vivo* growth conditions, including the exposure of cells to specific products expressed by the host at various stages of tissue invasion and colonisation by the pathogen. The expression of putative virulence genes under defined growth conditions may not reflect the true nature of the pathogen's response *in vivo* [10].

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