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Metabolomics of *Aspergillus fumigatus*

JENS C. FRISVAD, CHRISTIAN RANK, KRISTIAN F. NIELSEN & THOMAS O. LARSEN
Center for Microbial Biotechnology, DTU Biosys, Technical University of Denmark, Kgs. Lyngby, Denmark

Aspergillus fumigatus is the most important species in *Aspergillus* causing infective lung diseases. This species has been reported to produce a large number of extrolites, including secondary metabolites, acids, and proteins such as hydrophobins and extracellular enzymes. At least 226 potentially bioactive secondary metabolites have been reported from *A. fumigatus* that can be ordered into 24 biosynthetic families. Of these families we have detected representatives from the following families of secondary metabolites: fumigatins, fumigaclavines, fumiquinazolines, trypacidin and monomethylsulochrin, fumagillins, gliotoxins, pseurotins, chloroanthraquinones, fumitremorgins, verruculogen, helvolic acids, and pyripyropenes by HPLC with diode array detection and mass spectrometric detection. There is still doubt whether *A. fumigatus* can produce tryptoquivalins, but all isolates produce the related fumiquinazolines. We also tentatively detected sphingofungins in *A. fumigatus* Af293 and in an isolate of *A. lentulus*. The sphingofungins may have a similar role as the toxic fumonisins, found in *A. niger*. A further number of mycotoxins, including ochratoxin A, and other secondary metabolites have been reported from *A. fumigatus*, but in those cases either the fungus or its metabolite appear to be misidentified.

Keywords Metabolomics, *Aspergillus* section *Fumigati*, extrolites, sphingofungin

Introduction

Aspergillus fumigatus is the dominating species causing fungal lung diseases in humans and animals [1–6]. Other species in *Aspergillus* section *Fumigati* and its teleomorphic (sexual) state *Neosartorya* are also able to cause aspergillosis, however. These species include *A. lentulus* [7], *N. pseudofischeri* [8,9] and *N. udagawae* [10]. While *Neosartorya* species produce both a sexual state with ascospores and an asexual state with conidiospores, the *Aspergillus* species only produce conidiospores. Several new species have recently been described in section *Fumigati* and *Neosartorya* [11–14], and an overview of the 23 species of *Neosartorya* and 10 species in *Aspergillus* section *Fumigati* is provided by Samson *et al*. [14]. It is well known that isolates of *A. fumigatus* are able to produce many extrolites [14–17], but of high importance is gliotoxin, that has been found in lungs or other infected tissues. Gliotoxin was found after experimental aspergillosis [18], and has also been found naturally occurring in turkey lungs infected with *A. fumigatus* [19]. Gliotoxin has also been found in a bovine udder infected with *A. fumigatus* [20] and in human tissues [21]. Gliotoxin may not be the only mycotoxin involved in mycosis [22] as several other extrolites have been reported from *A. fumigatus* [1,15,16,23–26]. The antibiotic fumigacin (which was later shown to be a mixture of helvolic acid and gliotoxin) has also been found in human and animal pulmonary tissues [27–30].

Two isolates of *A. fumigatus* have been full-genome sequenced and arrays are being developed for this species [31–33]. The closely related species *Neosartorya fischeri* is also being full-genome sequenced and this species and another closely related species *A. lentulus* and several new species we have described, can be compared with, and used as controls, for *A. fumigatus*, as their extrolite profiles are also known [11]. There are still extrolites that have not been identified in *A. fumigatus* and allied species [34–36], but a large

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Correspondence: Jens C. Frisvad, Center for Microbial Biotechnology, DTU Biosys, Technical University of Denmark, Building 221, Soltofts Plads, DK-2800 Kgs. Lyngby, Denmark. Tel: +45 45 25 26 26; E-mail: jcf@bio.dtu.dk

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number of extrolites are well characterized. Many extrolites from *A. fumigatus* are associated with the conidiospores, including gliotoxin, trypacidin, verruculogen and fumigaclavine A [37–41] and are thus likely to have effects in the initial lung infection process. The global regulator gene *laeA* appear to have an effect on conidial morphology, including associated extrolites such as hydrophobins and other extrolites [42–44].

It is the purpose of this paper to list, update and revise the profile of extrolites associated with *A. fumigatus* and to analyse 40 strains of *A. fumigatus* to examine the chemo-consistency in this species.

Materials and methods

Many isolates of *Aspergillus* section *Fumigati* were examined for extrolite profiles using HPLC and MS methods, and few isolates of *A. fumigatus* and *A. lentulus* were specifically screened for sphingofungins. *A. fumigatus* isolates from different sources were emphasized (Table 1). All isolates were inoculated on Czapek yeast autolysate (CYA) agar, yeast extract sucrose (YES) agar, malt extract autolysate (MEA) agar, Oat meal (OAT) agar at 25°C and on CYA at 37°C (see Samson *et al*. [45] for formulae). Secondary metabolites were extracted from CYA and YES agar after 7 days of growth in darkness, using the extraction solvent ethyl acetate/dichloromethane/methanol (3:2:1, v/v/v) with 1% (v/v) formic acid.

HPLC with diode array detection and high resolution mass spectrometric detection (HPLC-DAD-HRMS), was performed on an Agilent 1100 system with a Luna C18 II column (Phenomenex, Torrance, CA) and equipped with a photo diode array detector (DAD), and coupled to a LCT orthogonal time-of-flight MS (Waters-Micromass, Manchester, UK), with a Z-spray ESI source and a LockSpray probe [46]. Furthermore all isolates were analyzed by HPLC-DAD using the method of Frisvad and Thrane [47,48] as modified by Smedsgaard [49].

Samples were analyzed in positive and negative electrospray interphase (ESI^+ and ESI^-) using a water-acetonitrile linear gradient system starting from 15% acetonitrile which was increased to 100% in 20 min and holding 100% for 5 min [50]. In both ESI^+ and ESI^- two scan functions (1 s each) were used: the first with a potential difference of 14 V between the skimmers scanning m/z 100 to 900; the second with 40 V between the skimmers scanning m/z 100–2000.

Data analysis was performed as described previously [50], peaks were matched against an internal reference standard database ($\sim$730 compounds), the 33557 compounds in Antibase 2007 [51], previous data from our group [17], and the review data in this paper.

Table 1 Isolates of *Aspergillus fumigatus* examined

Isolate	Source
CBS 542.75 = ATCC 26606	Man, USA
CBS 143.89	Man, France
CBS 144.89 = CEA10*	Man, France
CBS 145.89	Man, France
CBS 146.89	Man, France
CBS 147.89	Man, France
IBT Stend1	Man, hospital Sundby, Denmark
IBT Stend2	Man, hospital Hvidovre, Denmark
Af293*	Man, United Kingdom
CBS 133.61^T = ATCC 1022	Chicken lung, Connecticut, USA
IBT 16904	Saltern,Slovenia
IBT 16902	Saltern, Slovenia
IBT 16903	Saltern, Slovenia
IBT 16901	Saltern, Slovenia
IBT 24004	Saltern, Slovenia
ATCC 32722	Soil, Canada
NRRL 1979 = IBT 15720	Soil, USA
CBS 113.26 = ATCC 1028	Soil, Germany
CBS 132.54 = QM 6b	Soil
CBS 457.75 = WB 5452	Soil, India
CBS 151.89	Stone, Germany
CBS 152.89	Stone, Germany
NRRL 5587	?
IBT D47i	Soil, Indonsia
CCRC 32120	Soil, Taiwan
IBT 25732	Soil under banana tree, Kenya
IMI 376380 = IBT 23720	Unknown (reported ochratoxin A producer)
CBS 545.65 = ATCC 16913	Unknown
WB 5033 = IBT 22612	Unknown (white conidia)
IBT 23737	Unknown, Denmark
IBT 14904 = ATCC 32722	Unknown, USA
CBS 192.65 = IHEM 4392	Feed, Netherlands
CBS 148.89	Maize, France
CBS 149.89	Maize, France
CBS 150.89	Beetroot, France
IBT 21997 = A195	Feed, Spain (reported ochratoxin A producer)
IBT 22023	Silage, Germany
IBT PerHag	Silage, Sweden
IBT 22234	Ex tea factory, Uganda
IBT 21711	Food, Italy

*Full genome sequenced strains.

Results

The profile of extrolites produced by *A. fumigatus* has to be collected from several literature sources in

conjunction with actual metabolic profiling of a series of isolates of *A. fumigatus*. The data obtained here were verified by comparison with authentic standards, similar UV and high resolution mass spectra and literature data. We examined the isolates of *A. fumigatus* for all main secondary metabolites that have been reported in the literature, often reported from one isolate only. In this way we confirmed that isolates of *A. fumigatus* produced some secondary metabolites consistently, others by approximately half of the isolates, and some reported secondary metabolites were apparently not produced by *A. fumigatus* (Table 3). It is well known that the production of extrolites is depending on the growth medium and environmental factors [52–54], but on the media Czapek yeast autolysate (CYA) agar and yeast extract sucrose (YES) agar a large number of these representatives of the 24 families of extrolites are detectable using HPLC with diode array detection [24]. The extrolites most consistently produced were fumiquinazolines A/B, C/D and F/G (100%), gliotoxin (38%), fumigaclavine C (100%), fumitremorgins (100%), fumagillin (100%), helvolic acid (98%), pseurotin A (100%), fumigatins (35%), chloroanthraquinones (70%), melanins (100%, only verified, however, by observing that the bluegreen pigment is produced by all the isolates), and pyripyropenes (48%) (Table 3). The growth conditions and the incubation time chosen may not have been optimal for production of all extrolites by *A. fumigatus*.

(1) Epoxysuccinic acid and difructosedianhydride (tricarboxylic acid cycle)

Epoxysuccinic acid seems to be the major organic acid produced by *A. fumigatus* [55,56], whereas production of citric acid appears to be weak [57]. The role of epoxysuccinic acid in the life cycle of *A. fumigatus* is unknown. Atypical carbohydrates, such as difructose dianhydride may also be produced by *A. fumigatus* [58].

We did not examine any of the cultures for these two metabolites, as the detection method was not adequate for these particular metabolites.

(2) Fumigatins (polyketides)

The fumigatins and spinulosins consist of at least 21 polyketide extrolites (Table 1) and have been thoroughly studied concerning their biosynthesis [59–69]. Fumigatin and spinulosin are reported to be antibiotically active against several gram-negative and gram-positive bacteria [23,28] and fumigatin was cited to be toxic against experimental animals by Austwick [1], while Cole and Cox [23] claimed that vertebrate toxicity was unknown. However, later it was shown that fumiquinones A and B, in the same biosynthetic family, are toxic to other kinds of animals (nematodes) [70]. Antibacterial and antinematodal activity of the fumigatins and spinulosins can maybe explain their prevalence in soil isolates of *A. fumigatus*.

We found fumigatin in several isolates (35%), but it was most common in soil-borne *A. fumigatus* (Table 3). However, one isolate from a patient produced fumigatin.

(3) Trypacidins (polyketides, nitrogen in one extrolite)

Trypacidin [71–73] and monomethylsulochrin [15,71,73] were isolated and their structure elucidated by Turner [71] and Balan *et al*. [73]. Trypacidin is antiprotozoal and also an antimicrobial antibiotic [37,74]. Two other related extrolites, asperfumin, and the nitrogen containing asperfumoid has also been detected in *A. fumigatus* [15]. Trypacidin and monomethylsulochrin has been found in all isolates examined of *A. fumigatus* [11].

Most isolates (75%) examined by us produced trypacidin and monomethylsulochrin (Table 3). Isolates producing these metabolites also produced the chloroanthraquinones (Table 3).

(4) Chloro-anthraquinones or –anthrones (polyketides)

Emodin, physcion [15], 2-chloro-emodin, 2-chloro-citreorosein, 2-chloro-1,3,8-trihydroxy-6-methyl-9-anthrone, and 2-chloro-1,3,8-trihydroxy-6-hydroxymethyl-9-anthrone have been reported from *A. fumigatus* [75]. These polyketides have not been reported to have a role in the infection process.

We found UV spectrum evidence for production of several of the anthraquinone metabolites in many strains of *A. fumigatus* (Table 3).

(5) Melanins (polyketides)

Aspergillus fumigatus is able to produce polyketide derived melanins via a heptaketide shortening from YWA1 to 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN), which is the basis for production of 1,8-dihydroxynaphthalene, the pentaketide compound that will polymerize to melanin [76–78], giving *A. fumigatus* the green conidium colour. Melanin has been mentioned as one of several potential virulence factors in *A. fumigatus* [79,80].

As all isolated had blue-green conidia, they probably have the ability to produce this 1,8-dihydroxynaphthalene derived polymer.

(6) Sphingofungins and fumifungin (polyketides + alanine)

The sphingofungins A-D from *A. fumigatus* ATCC 20857 (from soil in Uruguay) are antifungal agents [81,82] and are potent and specific inhibitors of serine palmitoyl transferase, an enzyme essential in the the biosynthesis of sphingolipids. *Paecilomyces variotii* produces sphingofungins E and F [83]. Fumifungin [84] was isolated from what was probably *A. viridinutans*, as the fungus also produced viriditoxin, but sphingofungins may also be produced by *A. fumigatus sensu stricto*. These metabolites share a similar backbone to the carcinogenic mycotoxins the fumonisins produced by *Fusarium* species and an *Aspergillus* species, *A. niger* [85] and may thus be potential inhibitors of human nerve cells. Fumonisins have been shown to cause pulmonary edema in pigs [86] and down-regulates basal IL-8 expression in pig intestine [87] and therefore sphingofungins may be likely candidates to be involved in the lung infection process, also in humans.

We examined two isolates from section *Fumigati* for production of sphingofungins: the full genome sequenced *A. fumigatus* Af293 and *A. lentulus* IBT 27201. HPLC-MS data strongly indicated that both species are able to produce these compounds (Fig. 2). A viriditoxin producer that also produced fumifungin [84] was probably not *A. fumigatus* or *A. lentulus*, as none of these species are able to produce viriditoxin. The fumifungin producing strain could have been *A. viridinutans*, *Neosartorya aurata*, or *N. denticulata* as these three species produces viriditoxin [14].

(7) Pseurotins (mixed biosynthetic origin: polyketide + phenylalanine)

The pseurotins were first isolated from *Pseudeurotium ovalis* (pseurotins A, B, C, D and E) [88–91], but were later isolated from *A. fumigatus* (pseurotin A, 8-O-demethylpseurotin, pseurotin F1 and F2 and synerazol) [92,93]. These compounds are chitin synthase inhibitors, but only the epoxy-pseurotin, synerazol, has antifungal activity. It is not known whether the pseurotins have biological activities of relevance for the lung infection process. The closely related compound azaspirene has been isolated from a *Neosartorya* species [94].

We found that pseurotin A was produced by all 40 strains examined of *A. fumigatus*, but some additional pseurotins, as identified based on UV-VIS spectra, were often produced at the same time.

(8) Ergosterols (triterpenes)

Apart from ergosterol, produced by all fungi, *A. fumigatus* has been reported to produce ergosterolpalmitat, ergosterolperoxide [95], ergosta-4,6,8(14),22. -tetraen-3-one, ergosta-4,22-diene-3β-ol, 5α,8α-epidioxy-ergosta-6,22-diene-3β-ol [15]. Ergosterolperoxide has some antiviral properties [96].

We found ergosterol in all 40 isolates of *A. fumigatus* examined, but did not screen for the other ergosterol derived compounds.

(9) Helvolic acids (triterpenes)

Helvolic acid [15,97–101] is an antibiotic that is active against both gram-positive and gram-negative bacteria. Other products in the biosynthetic family, such as helvolinic acid and 7-desacetoxyhelvolic acid have been isolated from *Sarocladium oryzae* [102,103], but not yet from *A. fumigatus*. The fusidic acids may also be closely related, but has not been found in *A. fumigatus* [[104], pp. 264–265]. The reported toxicity of helvolic acid may be due to contamination with gliotoxin [1,23].

Helvolic acid was produced by nearly all strains (98%) we examined of *A. fumigatus* (Table 3).

(10) Fumagillins (sesquiterpenes)

Trans-fumagillin was isolated from *A. fumigatus* by Eble and Hanson [105], and its structure elucidated by Tarbbell [106,107] and McCorkindale and Sime [108]. Further extrolites in this biosynthetic family have been isolated later, inclusive fumagiringillin [109], demethoxyfumagillol [110], Sch528647 [111], RK-95113 [112] and closely related metabolites [113]. Ovalicin [114,115], FR-111142 [116], or FR65814 [117] may also be produced, but have been reported from other fungi. The fumitoxins, toxic to both animals and plants [118–122] were never structure elucidated, but based on the chemical data presented, they appear to be members of the fumagillin biosynthetic family. β-transbergamoten is a precursor of fumagillin [123].

We detected fumagillin in all strains of *A. fumigatus* examined (Table 3).

(11) Fatty acids (fatty acids) and hexahydroxyprenyls (polyterpenes)

A fumigatus has been reported to produce a series of hydroxypolyprenols [124], ubiquinones, phthioic acid and other lipids [125–127]. The role of these metabolites in the infection process is unknown, but other lipids (oxylipins) have been shown to be involved in virulence [128].

We did not screen for these lipids in the 40 extracts of *A. fumigatus*.

(12) Siderochromes (N-hydroxyornithine with either three glycines or one glycine and two serins)

Fusigen [129], ferricrocin and N′,N″,N‴-triacetylfusarinine C [130,132] are important iron-chelating extrolites from *A. fumigatus*, that may play a significant role in the infection process in animals [133,134].

As the production requires special substrates depleted for iron, we did not examine the cultures for the siderophores in our screening process.

(13) Gliotoxins (phenylalanine, m-tyrosine, methionine)

Gliotoxin was isolated from a strain of *A. fumigatus* by Johnson *et al*. [135] and Menzel *et al*. [98] and later structure elucidated [136]. Fumigacin [27,29] has been found in animal and human tissue, but fumigacin was later found to be a mixture of gliotoxin and helvolic acid. Gliotoxin has been claimed to be involved in diseases caused by *A. fumigatus* [40,137,138]. The less toxic bisdethiobis(methylthio)gliotoxin has also been reported from *A. fumigatus* [139,140] as has gliotoxin G, the tetrasulphide analogue of gliotoxin [139]. Other gliotoxins, including gliotoxin monoacetate [141,142], and gliotoxin E and G [143,144] may also be extrolites of *A. fumigatus*, but have been isolated from *Trichoderma virens* and *Penicillium lilacinoechinulatum* (Frisvad JC and Thrane U, unpublished).

Gliotoxin is best produced on media with low C/N ratio, so the media used here for screening of *A. fumigatus* extrolites were not optimal for its expression. When tested on such media [17] all isolates of *A. fumigatus* seems to be able to produce gliotoxin.

(14) Fumigaclavins (tryptophane and terpene unit (dimethylallyl))

Agroclavine, festuclavine, elymoclavine, chanoclavine I, fumigaclavine A, B, and C [15,145–149] are produced by *A. fumigatus*. The biosynthetic genes for the ergot alkaloids in *A. fumigatus* have been studied by Coyle and Panaccione [150]; Li and Unsöld [151]; Unsöld and Li [152], Stack *et al*. [153].

All 40 isolates examined of *A. fumigatus* produced fumigaclavine C (Table 3), the end-product in the biosynthetic family.

(15) Fumitremorgins, verruculogen, tryprostatins, cyclotrypostatins and spirotryprostatins (tryptophane, proline and terpene (dimethylallyl) groups)

Brevianamide F [154,155] is a conceived precursor of the diverse biosynthetic family of fumitremorgins, including verruculogen [24,156–159], cyclotrypostatins [160], tryprostatins [161–163], spirotryprostatins [164,165], fumitremorgin A & B [140,166,172], fumitremorgin C [15,146,159,173], TR-2 [174], TR-3 = 12,13-dihydroxyfumitremorgin C and demethoxyfumitremorgin C [162,163], 12,13-dihydrofumitremorgin C [159,175], and 15-acetoxyverruculogen [23]. In all, this extrolite family consists of 20 known members. Tryprostatin A is an inhibitor of microtubule assembly [176], and in general the fumitremorgins are cell cycle inhibitors and tremorgenic mycotoxins [23].

We found that the fumitremorgins (A, B, C), TR-2 and verruculogen were produced by all isolates of *A. fumigatus* examined (Table 3), but the full genome sequenced Af293 [31] only produce brevianamide F [17,177].

(16) Simple diketopioperazines (two amino acids)

Alanyl-leucyl and alanyl-isoleucyl, prolyl-phenylalanyl, prolyl-glycyl, prolyl-prolyl, prolyl-valyl, 4-hydroxyprolyl-leucyl, 4-hydroxyprolyl-phenylalanyl, and prolyl-leucyl diketopirazines, all consisting of L-amino acids, have all been reported from *A. fumigatus* [15,178,179]. Several of those are antibiotically active [15].

We did not detect any of those simple diketopiperazines in *A. fumigatus*.

(17) Pyripyropenes (meroterpenoids and nicotinic acid)

Pyripyropenes A-R have been reported from *A. fumigatus* [180–187]. The pyripyropenes have the ability to inhibit acyl-CoA:cholesterol acyltransferase and may thus play a role in the infection process.

We found that approximately half of the isolates of *A. fumigatus* produced pyripyropenes (48%, Table 3). We did not detect those metabolites in Af293, but the other full genome sequence strain of *A. fumigatus* (CBS 144.89 = CEA 10) did produced pyripyropenes.

(18) Fumiquinazolines (anthranilic acid, tryptophane, valine)

Fumiquinazolines A-E [140,188,189] were reported from marine isolates of *A. fumigatus*. These quinazolins have been reported to be moderately cytotoxic.

We found that the fumiquinazolines were consistently produced by all 40 isolates of *A. fumigatus* examined (Table 3).

(19) Tryptoquivalines (anthranilic acid, tryptophane, valine, terpene unit (dimethylallyl))

The tryptoquivalines and tryptoquivalones were originally isolated from *A. clavatus* [190,191] but tryptoquivaline A and E to N has been reported from *A. fumigatus* [192–195]. It was later shown that *A. fumigatiaffinis* is a very efficient producer of (some of) these tryptoquivalines [11,14]. However, tryptoquivaline J was isolated from a strain of *A. fumigatus* by Afiyatullov [159], so it is possible that also *A. fumigatus* can produce at least some of these extrolites.

The tryptoquivalins were not detected in our analyses of 40 isolates of *A. fumigatus*. Earlier reports of tryptoquivalins [25] from *A. fumigatus* were apparently based on the fact that the UV-VIS spectra of the tryptoquivalins and the fumiquinazolines are quite similar. HPLC-HR-MS analysis showed that the major compounds with such UV spectra were all fumiquinazolines.

(20) N-(2-cis(4-hydroxyphenyl)ethenyl)-formamide

N-(2-cis(4-hydroxyphenyl)ethenyl)-formamide is a platelet aggregation inhibitor that was isolated from a strain identified as *A. fumigatus* [196].

We were not able to detect this extrolite in any of 40 extracts of *A. fumigatus*.

(21) Restrictocins (polypeptides)

Restrictocin, mitogillin and 'asp F1', small basic proteins, are cytotoxins that cleave ribosomal RNA [197–199]. The culture originally examined (ATCC 34475 = NRRL 2869) was first identified as *A. restrictus*, but later reidentified as *A. fumigatus* [200]. A leader sequence in the gene coding for these proteins protects the producer strains from suicide [201], and these proteins have also been identified as major allergens from the conidia, mycelium and culture filtrate of *A. fumigatus* [198]. 'Asp F1' was first found in urine of patients that suffered from invasive aspergillosis [197], so these compounds may be of significance in *A. fumigatus* mediated aspergillosis.

We did not screen *A. fumigatus* for any proteins in this study.

(22) Volatile extrolites (including sesquiterpenes, alcohols and ketones)

Sesquiterpenes provisionally detected from from *A. fumigatus* include 10(14)-(−)-aromadendrene, bicycloelemene, bicyclooctane-2-one, camphene, α-cadinene, 2-carene, caryophyllene, α & β-curcumene, cyclooctene, dihydroedulane I, β-elemol, α-farnesene, *trans*-β-farnesene, (−)-fenchol, germacrene A & B, italicene, a-longipenene, megastigma-4,6(E),8(Z)-triene, p-mentha-6,8-dien-2-ol, 2-methyl-2-bornane, 2-methylenebornanene, a-muurolene, neo-allo-ocimene, and β-phellandrene (39). Other volatile metabolites reported include 2-acetyl-5-methylfuran, anisole, 3-cycloheptane-1-one, 2,3-dimethylbutanoic acid methyl ester, 2,5-dimethylfuran, 4,4-dimethylpentenoic acid methyl ester, dodecane, 4-ethylbutan-4-olide, 2-ethylfuran, 2-ethyl-5-methylfuran, 1-ethyl-2-methylbenzene, furaneol, 3-hexanone, isopropylfuran, 1-methoxy-3-methylbenzene, 2-methylbutanoic acid and its methyl ester, 4-methyl-2-(3-methyl-3-butenyl)furan, 3-methyl-1-heptene, 6-methyl-2-heptanone, 2-methyl-2,4-hexadiene, 2-methylphenole, 1-(3-methylphenyl)-ethanone, 3-octanone, 1,3,6-octatriene, styrene, 3,5,7-trimethyl-2E,4E,6E,8E-decatetraene, 2,3,5-trimethylfuran (39). The role of these volatiles in the infection process of *A. fumigatus*, if any, is unknown.

(23) Primary metabolites

The vitamin riboflavin has also been found in *A. fumigatus* [202,203], and so has several other primary widespread primary metabolites.

(24) Biotransformations

A. fumigatus is also capable of converting some plant metabolites for example melitolic acid to 4-hydroxycoumarin and o-coumaric acid to dicoumarol [204] and phenylacetic acid to 2,6-dihydroxyphenylacetic acid [205]. It is also not known whether this ability to bioconvert metabolites play a role in the animal infection process.

(25) Proteins

Apart from the restrictocins, *A. fumigatus* also produce hydrophobins and several extracellular enzymes and these do play a role in the fungal infection process [16,206].

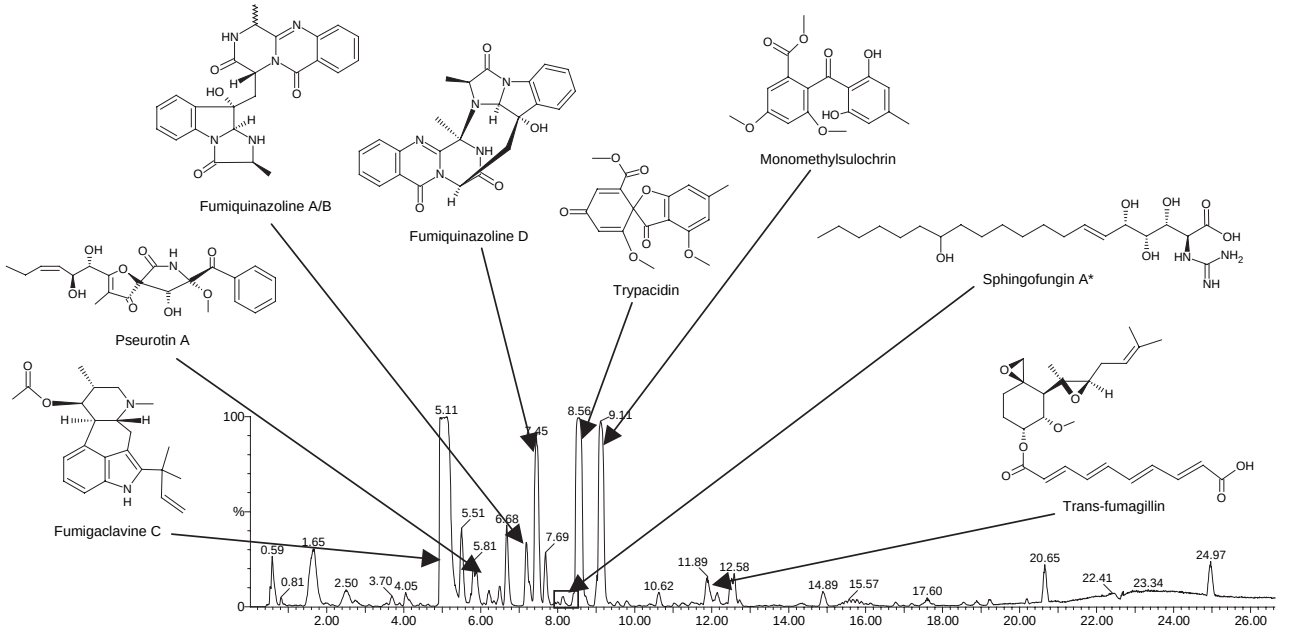

Fig. 1 LC-MS BPI (base peak ion) ESI (electrospray ionization)+ trace of raw extract of *Aspergillus fumigatus* Af293 on CYA agar, depicting major and typical extrolites from the species. Box indicates window for the sphingofungins in this isolate. Sphingofungin A is one of four possible compounds with almost identical masses to be present, based on mass traces.

(26) Extrolites erroneously reported from Aspergillus fumigatus

Aspergillus fumigatus has been claimed to produce a large number of mycotoxins and other extrolites, including ochratoxin A [207–210], indications of aflatoxin [211], cyclopiazonic acid [212], kojic acid [213,214], sterigmatocystin [215] and fumifungin+viriditoxin [84]. The isolates producing these mycotoxins and other biologically active extrolites appear to be misidentified. For example cyclopiazonic acid is produced by *A. lentulus* and isolates of the latter species can have been mistaken for *A. fumigatus* [17]. The isolate reported to produce fumifungin also produced viriditoxin [84], and the latter is a typical metabolite produced by *A. viridinutans*, another member of *Aspergillus* subgenus *Fumigati* section *Fumigati* [14,216–218]. In the case of ochratoxin A, sterigmatocystin and aflatoxin, probably the mycotoxin itself was misidentified.

Molecules that may be artefacts, such as GERI-BP002-A [219] that is a sterol biosynthesis inhibitor, have been reported as extrolites of *A. fumigatus*. This compound may or may not be a real secondary metabolite.

Expansolide, antafumicin A & B, and cytochalasin E were all reported from a strain claimed to be *A. fumigatus* [220]. These extrolites have only been found in *A. clavatus* [221], so it is highly probable that the reported producing strain represented the latter species.

Ruakuric acid has been isolated from a strain of *A. fumigatus* growing in conjunction with a coral lichen in hot sulfurous springs, New Zealand [222]. We have not been able to examine this culture and we have not yet detected compunds with the characteristics of ruakuric acid from any strain of *A. fumigatus sensu stricto*.

Aurasperone C was reported from *A. fumigatus* by Mitchell *et al*. [36], but this metabolite is a common metabolite in *Aspergillus* section *Nigri* [223] and we have not been able to detect it in any strain of *A. fumigatus* in this study.

Fumigatonin was also reported from *A. fumigatus* [224], but the isolation of the chemically related novofumigatonin from *A. novofumigatus* [35] indicates that fumigatonin is produced only by *A. novofumigatus*.

None of the isolates of *A. fumigatus* examined here (Table 1) produced aflatoxins, antafumicins, cyclopiazonic acid, cytochalasin E, expansolides, kojic acid, ochratoxins, sterigmatocystin, or viriditoxin.

(27) Extrolites of A. fumigatus *Af293*

Af293, the full genome sequenced strain of *A. fumigatus* [31] produced, fumigaclavines, fumiquinazolines, trypacidin and mono-methylsulochrin, fumagillins,

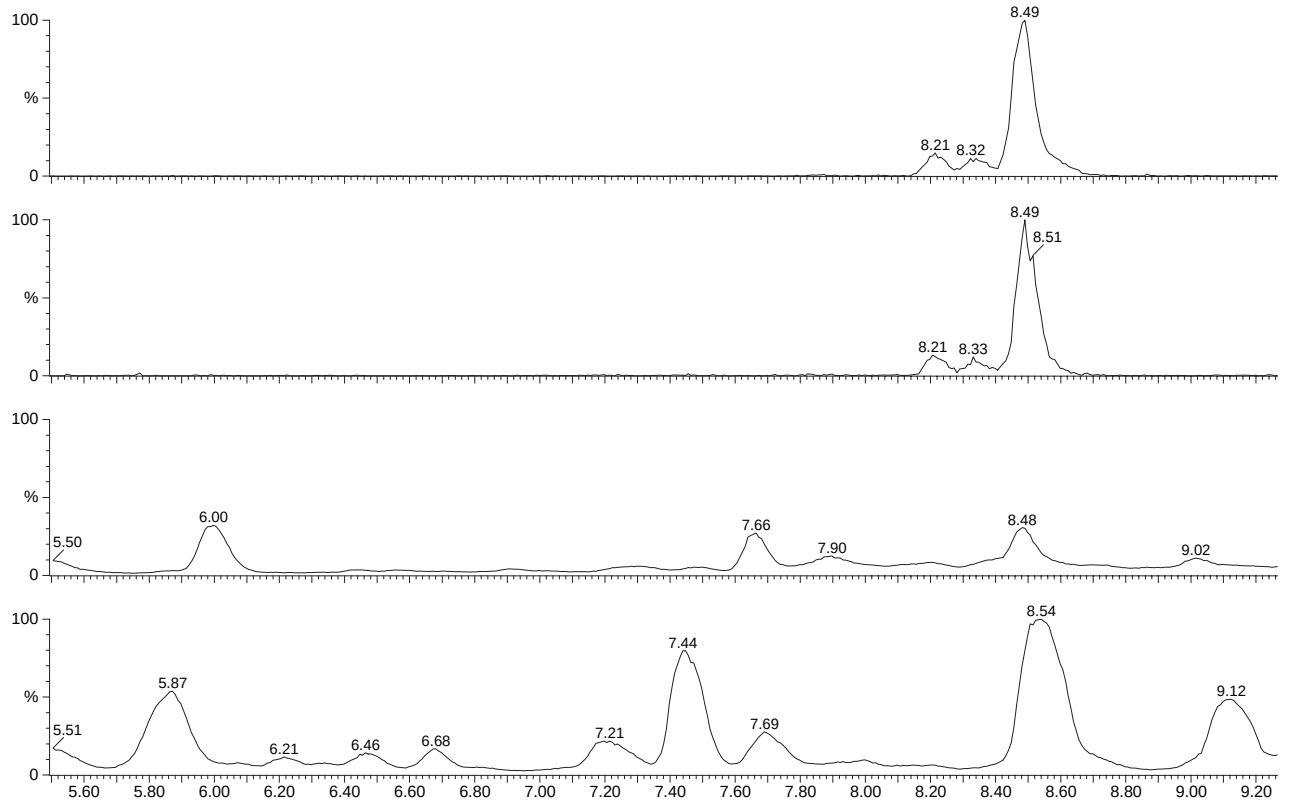

Fig. 2 Comparison of *A. lentulus* and *A. fumigatus* production of sphingofungins. Though very different in general extrolite profile, both of these isolates produce the same profile of what appears to be sphingofungins. From the bottom: Af293 TIC (total ion count) ESI+ (CYA agar); IBT 27201 *Aspergillus lentulus* TIC ESI+ (CYA agar); 432.30±0.05 Da mass trace for Af293 and IBT 27201 respectively (this mass fits both sphingofungin A ($C_{21}H_{41}N_3O_6$) and sphingofungin C, D and fumifungin (the latter three: $C_{22}H_{41}NO_7$).

gliotoxins, pseurotins, chloroanthraquinones, fumitremorgins, verruculogen, helvolic acids and sphingofungins (Fig. 1). The presence of sphingofungins A, C, D, or fumifungin (or all of those) was indicated by HPLC-MS analysis (Fig. 2). The formulae of these extrolites and the other secondary metabolites produced by *A. fumigatus* are shown in Fig. 1 and Fig. 3.

Discussion

We have been able to detect most of the major mycotoxins and other extrolites known from *A. fumigatus* in clinical strains, including the full genome sequenced Af293 and CBS 144.89 (=CEA10). These two strains produced gliotoxin, helvolic acid, fumagillin, fumigaclavine C, brevianamide F, fumiquinazolines and pseurotin A. Af293 and CBS 144.89 differed in that Af293 produced trypacidin, mono-methylsulochrin and some chloroanthraquinones, whereas CBS 144.89 produced a series of fumitremorgins (TR-2, fumitremorgin A, B, C and verruculogen). We also detected what we tentatively identified as sphingofungins in Af293, but have not screened other *A. fumigatus* strains for these extrolites yet. The other clinical strains also produced most of the well known secondary metabolites of *A. fumigatus* (Table 3), but 50% of the clinical strains did not produce fumigatin and pyripyropenes. However other investigators have found these extrolites in a lower proportion of the strains examined for example from silage [225] or from saw mills [226]. This may be because some of the strains were *A. lentulus* [17] or other strains similar to *A. fumigatus* [14]. Furthermore our results show that on the media CYA and YES only few strains show small peaks of gliotoxin, while they are produced in much higher amounts on media such as yeast extract agar (YE) [17]. Therefore the frequency of gliotoxin (38%) in *A. fumigatus* may be much higher in reality. On the other hand, the media CYA and YES are very good media for production of most other secondary metabolites from *A. fumigatus*, and we recommend to use those media in screening for all other secondary metabolites than gliotoxin.

The number of biosynthetic families of secondary metabolites reported to be produced by *A. fumigatus*

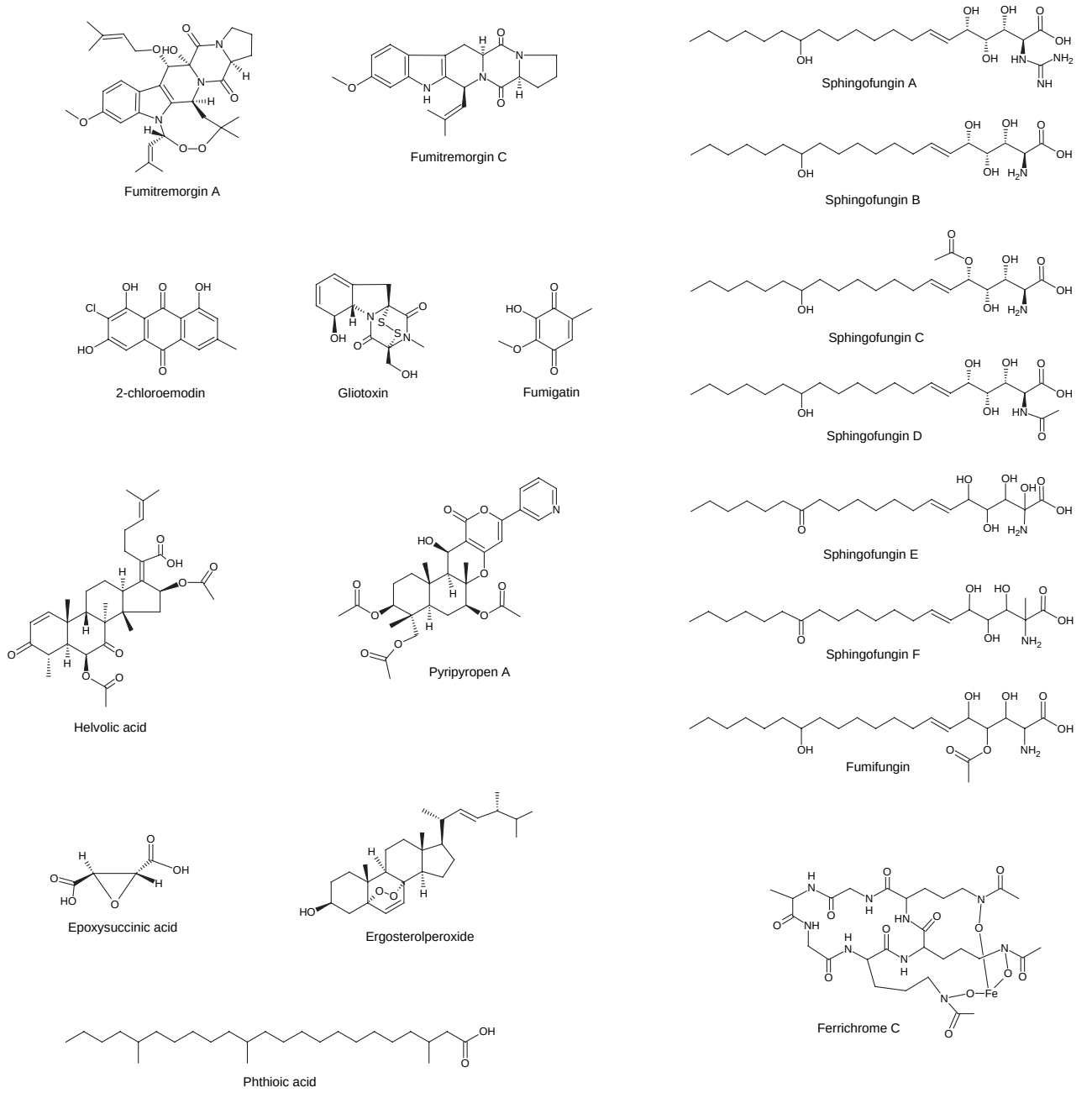

Fig. 3 Collection of single extrolites presenting the different compound classes, in addition to those in Fig. 2, produced by *Aspergillus fumigatus.*

(24) is impressive and so is the number of individual extrolites [226] (Table 2). We have not screened for all these 226 metabolites, but have concentrated on the most toxic or otherwise bioactive major metabolites from each biosynthetic family. It is still an open question how many of these secondary metabolites are involved in the infection process of lungs, but there is evidence that at least gliotoxin is involved [21,40,43, 137,138,227].

Nierman *et al*. [31] found by bioinformatic analysis of the full genome of *A. fumigatus* that 14 gene clusters indicated that they coded for non ribosomal peptide synthases (NRPS). Examination of the chemical phenotype (exometabolome) of *A. fumigatus* shows that gliotoxins, fumigaclavines, fumitremorgins, fumiquinazolins, siderochromes, diketopiperazines, restrictocins, N-(2-cis(4-hydroxyphenyl)ethenyl-formamide, and possibly tryptoquivalines, in addition to amino acids that

Table 2 Extrolites produced by isolates of *Aspergillus fumigatus*

Extrolite family	Number of members	Individual members
1. Epoxysuccinic acid	1	Epoxysuccinic acid
2. Fumigatins	21	Fumigatin
		Fumigatin oxide
		Fumigatin chlorohydrins
		Fumigatol
		Spinulosin
		Spinulosin hydrate
		Spinulosin quinol-hydrat
		Dihydrospinulosin quino
		Phyllostine
		Orsellinic acid
		Orcinol
		m-cresol
		3,4-dihydroxytoluquinone
		4-hydroxy-3-methoxytoluquinone
		3-hydroxytoluquinone
		3,6-dihydroxytoluquinone
		3-hydroxy-4-methoxy-
		Toluquinone 1,6-epoxide
		4-carboxy-5,5′-dihydroxy-3,3′-dimethyldiphenyl
		Fumiquinone A
		Fumiquinone B
3. Trypacidins	6	Trypacidin
		Bisdechlorogeodin
		Monomethylsulochrin
		Sulochrin-2′-methylether
		Asperfumin
		Asperfumoid
4. Anthraquinones and anthrones	5	Emodin
		Physcion
		2-chloroemodin
		2-chloro-1,3,8-trihydroxy-
		6-methylanthrone
		2-chloro-1,3,8-trihydroxy-
		6-hydroxymethylanthrone
5. Melanins	8	YWA1
		1,3,6,8-THN
		Flaviolin
		Scytalone
		1,3,8-THN
		2-HJ
		Vermelone
		1,8-DHN
6. Sphingofungins	7	Sphingofungin A-D
		Sphingofungins E-F?
		Fumifungin
7. Pseurotins	11	Pseurotin A-E
		8-O-demethylpseurotin A
		Synerazol
		RK-95113
		Azaspirene?
		FD-839
		Pseurotin F2
8 Sterols	7	Ergosterol
		Ergosterolperoxide
		Ergosterolpalmitat
		24-methylenophenol
		Ergosta-4,6,8(14),22-tetraen-3-one
		Ergosta-4,22-diene-3β-ol 5α,8α-Epidioxy-ergosta-
		6,22-diene-3β-ol
9. Helvolic acid	3	Helvolic acid
		Helvolinic acid
		7-desacetoxyhelvolic acid
10. Fumagillins	13	Fumagillin
		Fumitoxins A-D
		Fumagillol

Table 2 (*Continued*)

Extrolite family	Number of members	Individual members
		5-Demethoxyfumagillol
		Fumagiringillin
		FR-111142
		Sch528647
		RK-95113
		Ovalicin?
		β-*trans*-bergamotene
11. Hexahydropolyprenyls	5	Hexahydropolyprenyls A-E
11a. Phthioic acid	2	Phthioic acid
		Teichoic acid
11b. Hydroxyphenyls	1	bis(2-hydroxy-3-tert. butyl-5-Methylphenyl)methane (= GERI-BP002-A)
12. Siderochromes	2	Fusigen
		Ferrichrome
13. Gliotoxins	5	Gliotoxin
		Gliotoxin E
		Gliotoxin G
		S-methylgliotoxin
		bisdethiobis(methylthio)-Gliotoxin
14. Fumigaclavins	7	Agroclavine
		Elymoclavin
		Chanoclavine I
		Festuclavin
		Fumigaclavine A-C
15. Fumitremorgins	20	Brevianamide F
		Fumitremorgin A-C
		Verruculogen
		15-acetoxyverruculogen
		Demethoxyfumitremorgin C
		12,13-dihydroxyfumitremorgin C (= TR-3)
		12,13-dihydrofumitremorgin C
		TR-2
		Cyclotryprostatin A-D
		Dehydrotryprostatin
		Tryprostatin A & B
		Spirotryprostatin A & B
		'Compound 6′
16 Simple diketopiperazines	9	Alanyl-leucyl and alanyl-isoleucyl, prolyl-phenylalanyl, prolyl-glycyl, prolyl-prolyl, prolyl-valyl, 4-hydroxyprolyl-leucyl, 4-hydroxyprolyl-phenylalanyl, and prolyl-leucyl diketopirazines
17. Pyripyropenes	19	Pyripyropene A-R
		GERI-BP001-A
18. Fumiquinazolines	7	Fumiquinazoline A-G
19. Tryptoquivalins	11	Tryptoquivaline?
		Nortryptoquivaline?
		Tryptoquivaline E-N?
20. N-(2-cis(4-hydroxyphenyl)ethenyl-formamide	1	
21. Restrictocins	3	Restrictocin
		Mitogillin
		Asp F1
22. Volatile extrolites	25+28	
Sum:		
24 Biosynthetic families	226	secondary metabolites

involve the addition of the polyketides: sphingofungins, pseurotins, trypacidins, pyripyropenes are all NRPS dependent. This could account for in all, 13 biosynthetic families that involve amino acids. However, many of the secondary metabolites of *A. fumigatus* are of mixed biosynthetic origin. Concerning terpene involvement helvolic acids, ergosterolperoxide, fumagillin, hexahydropolyprenols are pure terpene secondary metabolites, but the fumigaclavines, fumitremorgins and tryptoquivalines have added dimethylallyl groups, so at least 7 gene clusters should contain genes coding for terpene biosynthesis. Nierman *et al.* [31] found that there were 7

Table 3 Consistency in production of extrolites by *Aspergillus fumigatus* from different sources as evaluated using HPLC and isolates grown on CYA and YES agar for one week at 25°C

Isolate	Source	Secondary metabolite family[£]										
		2	3	4	9	10	13	14	15	18	7	17
CBS 542.75	man, USA	−	−	−	+	+	+	+	+	+	+	+
CBS 143.89	man, F	−	−	−	+	+	−	+	+	+	+	+
CBS 144.89	man, F	−	−	−	+	+	+	+	+	+	+	+
CBS 145.89	man, F	−	−	+	+	+	−	+	+	+	+	−
CBS 146.89	man, F	−	−	−	+	+	+	+	+	+	+	+
CBS 147.89	man, F	−	−	−	+	+	−	+	+	+	+	+
IBT Stend1	man, DK	−	+	+	+	+	−	+	+	+	+	−
IBT Stend2	man, DK	+	+	+	+	+	−	+	+	+	+	−
Af293	man, UK	−	+	+	+	+	+	+	+	+	+	−
CBS 133.61[T]	Chicken lung	−	+		+	−	+	+	+	+	+	−
IBT 16904	Saltern,SL	+	+	+	+	+	−	+	+	+	+	+
IBT 16902	Saltern, SL	+	+	+	+	+	+	+	+	+	+	+
IBT 16903	Saltern, SL	+	+	+	+		−	+	+	+	+	+
IBT 16901	Saltern, SL	+	+	+	+	+	−	+	+	+	+	+
IBT 24004	Saltern, SL	−	+	+	+	+	−	+	+	+	−	+
ATCC 32722	Soil, CAN	+	+	+	+	+	−	+	+	+	+	−
NRRL 1979	Soil, USA	+	+	+	+	+	+*	+	+	+	+	+
CBS 113.26	Soil, D	−	+	+	+	+	−	+	+	+	+	+
CBS 132.54	Soil?	−	−	−	−	+	−	+	+	+	+	+
CBS 457.75	Soil, IND	−	−	−	+	+	+	+	+	+	+	−
CBS 151.89	Stone, D	+	+	+	+	+	−	+	+	+	+	+
CBS 152.89	stone, D	+	+	+	+	+	−	+	+	+	+	+
NRRL 5587	?	−	−	−	+	+	−	+	+	+	+	−
IBT D47I	soil, INDO	−	+	+	+	+	−	+	+	+	+	−
CCRC 32120	soil, TAI	−	+	+	+	+	−	+	+	+	+	−
IBT 25732	soil, K	−	+	+	+	+	−	+	+	+	+	−
IMI 376380	?	−	+	+	+	−	−	+	+	+	+	+
CBS 545.65	?	−	+	−	+	−	−	+	−	+	+	−
WB 5033	?	−	+	+	+	+	+	+	+	+	+	−
IBT 23737	?, DK	+	+	+	+	+	−	+	+	+	+	−
ATCC 32722	?, USA	+	+	+	+	+	+	+	+	+	+	−
CBS 192.65	Feed, NL	+	+	+	+	+	+	+	+	+	+	+
CBS 148.89	Maize, F	−	+	+	+	+	+	+	+	+	+	+
CBS 149.89	Maize, F	−	−	−	+	+	+	+	+	+	+	+
CBS 150.89	Beetroot, F	−	+	+	+	+	−	+	+	+	+	+
IBT 21997	feed, E	−	+	+	+	+	−	+	+	+	+	−
IBT 22023	silage, D	−	+	+	+	+	+	+	+	+	+	−
IBT PerHag	Silage, S	−	+	+	+	+	+	+	+	+	+	−
IBT 22234	Tea factory, U	+	+	+	+	+	−	+	+	+	+	−
IBT	food, I	+	+	−	+	+	−	+	+	+	+	−

[£] Metabolite family nr (frequency of extrolite production out of 40 strains).
*Gliotoxin detected originally.
2. Fumigatin (35%).
3. Trypacidin (75%).
4. Chloro-anthraquinones or -anthrones (70%).
9. Helvolic acid (98%).
10. Fumagillins (93%).
13. Gliotoxin (>38%).
14. Fumigaclavines (100%).
15. Fumitremorgins (98%).
18. Fumiquinazolines (100%).
7. Pseurotins (100%).
17. Pyripyropenes (48%).

gene clusters accounting for dimethylallyl tryptophane synthases and here we can account for three of these. However pure terpene secondary metabolite clusters were not mentioned. It has been shown that the fumonisins are not only depending on the fumonisin gene cluster, but also on regulating genes on other chromosomes and environmental factors [228,229]. Finally polyketide synthases (PKS) include fumigatin, trypacidins, chloroanthraquinones, melanins, sphingofungins, pyripyropenes and pseurotins, accounting for at least 7 PKS gene clusters, only half of the 14 PKS gene clusters listed by Nierman *et al*. [31]. However, the PKS and NRPS gene clusters may be differently organized and thus pure PKS gene clusters and mixed PKS-NRPS clusters may be difficult to detect using bioinformatic methods. The gene clusters for gliotoxin, fumitremorgins, fumigaclavines and siderophores have been provisionally detected [153,177,227,230].

The production of sphingofungins by *A. fumigatus* has been reported previously in *A. fumigatus* ATCC 20857 [81,82], and the closely related sphingofungin called fumifungin was isolated from a fungus identified as *A. fumigatus* Y-83,0405 [84]. We have not been able to get those cultures, but the latter appear to be *A. viridinutans* rather than *A. fumigatus*, as it also produced viriditoxin. The detection of compounds with the mass around 432.3 strongly indicates that *A. fumigatus* Af 293 and *A. lentulus* IBT 27201 produce these sphingofungins, but as sphingofungin A, C, D or fumifungin almost have the same mass, it would be necessary to isolate, purify and characterize these compounds using NMR to be sure they are actually sphingofungins. It is intriguing that the mass trace for *A. fumigatus* for these compounds is almost identical to that of *A. lentulus*, indicating a common biosynthesis. Since there are few secondary metabolites in common between *A. fumigatus* and *A. lentulus* [17], it is tempting to speculate that the sphingofungins might be involed in the lung infection process. All 10 species of *Aspergillus* and 23 species of *Neosartorya* should be analyzed for sphingofungins to see if all or only the pathogenic species produce them. The production of sphingofungins was consistent on YES and CYA agar only with variations in amounts.

We have shown that strains of *A. fumigatus* produce the same profile of secondary metabolites, but that all metabolites are not necessarily expressed phenotypically in any given strain. The new possibilities of bioinformatic search based on full genome sequenced strains will show whether genes for fumigatin and for pyripyropenes are present in a strain like Af293, where we as yet have never detected these secondary metabolites. If the geneclusters are present, they may be silent or defective in the strains that do not produce them. However, many strains consistently produce a series of different secondary metabolites, the most consistent being fumigaclavines, fumitremorgins, fumiquinazolins, pseurotins, helvolic acid and fumagillin. The polyketides are less consistently expressed, but maybe other growth media or stimuli will induce their production.

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