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Secondary metabolite arsenal of an opportunistic pathogenic fungus

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Aspergillus fumigatus is a versatile fungus able to successfully exploit diverse environments from mammalian lungs to agricultural waste products. Among its many fitness attributes are dozens of genetic loci containing biosynthetic gene clusters (BGCs) producing bioactive small molecules (often referred to as secondary metabolites or natural products) that provide growth advantages to the fungus dependent on environment. Here we summarize the current knowledge of these BGCs—18 of which can be named to product—their expression profiles *in vivo*, and which BGCs may enhance virulence of this opportunistic human pathogen. Furthermore, we find extensive evidence for the presence of many of these BGCs, or similar BGCs, in distantly related genera including the emerging pathogen *Pseudogymnoascus destructans*, the causative agent of white-nose syndrome in bats, and suggest such BGCs may be predictive of pathogenic potential in other fungi.

This article is part of the themed issue 'Tackling emerging fungal threats to animal health, food security and ecosystem resilience'.

1. Introduction

Of the many informative biological stories uncovered from sequences of thousands of fungal genomes, one garnering extreme interest is the discovery of large numbers of secondary-metabolite (SM) encoding biosynthetic gene clusters (BGCs) in specific fungal taxa. Much of the interest is driven through a quest to find novel pharmaceuticals as many fungal SMs display valuable bioactivities, presumably evolved in their protective role against both biotic and abiotic stressors [1]. Furthermore, a subset of pathogenic fungi produces SMs that increase their virulence attributes on hosts. These SM-producing fungi include plant, insect and vertebrate pathogens.

An understanding that fungal SMs could adversely impact humans and other vertebrates can be traced back to the 1960s where the SM aflatoxin, produced by the seed pathogen *Aspergillus flavus*, resulted in deaths of thousands of poultry in England [2]. Toxic fungal SMs found in food and feed products are collectively known as mycotoxins. Owing to the harmful effects of these metabolites, worldwide efforts have resulted in the identification and characterization of the most common mycotoxin BGCs including not only aflatoxin (and the closely related sterigmatocystin BGC) but also fumonisin, trichothecene, ochratoxin, zearalenone, citrinin, ergot alkaloid, cyclopiazonic acid and patulin BGCs (reviewed in [3]). Although touted for their toxic, mutagenic, teratogenic or carcinogenic impacts on vertebrates, emerging data are now suggesting that these mycotoxins—and other BGC metabolites for that matter—serve as fitness factors for the producing fungi. For example, emerging data suggest that aflatoxin may act as an endogenous redox signal protecting the fungus from

oxidative stress [4]. The connection of oxidative stress with BGC activation is, in fact, so common in fungi [5–7] that several reviews address the protective nature of fungal SMs and their possible use as oxidative treatments in human disorders [8,9].

Oxidative stress protection is but one property of a subset of SMs. Demonstrated bioactivities of other SMs range between metal acquisition, UV protection and antimicrobial activity (often via targeting of specific critical enzymes and hence potentially damaging to host tissues but useful in pharmaceutical settings). Considering that fungi can contain dozens of BGCs within their genomes, the potential of BGC metabolites to contribute to disease development is considerable. It is not yet possible to survey the total secondary metabolome of a single fungus to accurately predict how SMs could drive pathogenic ability, as characterization of the entire set of BGCs—including knowledge of structure and bioactivity of the SMs, BGC expression during pathogenesis and possible synergism of the multiple SMs—is needed for such a charge. Yet we can start to speculate on what BGCs might signal pathogenic potential from studies of the opportunistic human pathogen *Aspergillus fumigatus*.

Owing to its importance as a serious pathogen of immunocompromised patients, efforts have focused on characterizing the SM arsenal of this fungus with a view to determining any role of these compounds in virulence. These efforts have led to, arguably, *A. fumigatus* having the greatest number of characterized BGCs of any fungus studied with the possible exception of the genetic model *Aspergillus nidulans* [10]. Initial genome sequence predicted a total of 22 BGCs [11], but additional bioinformatic analysis now suggests over 30 BGCs are present in the genome of *A. fumigatus* [12]. The DHN-melanin cluster, first characterized in 1999 [13], has garnered considerable attention as a virulence factor in this organism [14].The second BGC to be characterized in *A. fumigatus* produced gliotoxin, which, like DHN-melanin, impacts virulence [15–18]. Following a reoccurring theme in studies of the roles of SM in fungal biology, both DHN-melanin and gliotoxin exhibit bioprotective properties well beyond a role as simply a virulence factor. While not directly examined in *A. fumigatus*, melanins in other fungi provide protection from UV radiation [19] and gliotoxin provides redox homeostasis properties to the fungus [20].

Since identification of the gliotoxin BGC, a successive wave of studies revealed the endocrocin [21], fumigaclavine [22], fumagillin [23], fumiquinazoline [24], fumisoquin [25], fumitremorgin [26,27], helvolic acid [28], hexadehydroastechrome [29], neosartoricin (fumicycline) [30], pseurotin [23], pyripyropene [31] and trypacidin [32] BGCs in this species. The two siderophore BGCs are not presented as such, but can be pieced together from several studies to yield a two-gene ferricrocin cluster composed of SidI and SidC [33,34] and a six-gene fusarinine C cluster (composed of SidJ, SidF, SidH, SidD, SitT and MirD [34–38]). Additional BGCs can be inferred from orthology to known clusters in other fungi including the conserved nidulanin A BGC described in *A. nidulans* [39], a fusarielin-like cluster described in *Fusarium graminearum* [40], and a fumonisin-like cluster (fumonisin is synthesized by many *Fusarium* spp.) probably producing sphingofungin.

Although there are several algorithms designed to identify fungal BGCs, including anti-SMASH [41], SMURF [42] and MIDDAS-M [43], these programs err on the side of over prediction, inability to discern superclusters and in missing non-canonical clusters (e.g. those lacking typical SM synthetases such as polyketide synthases (PKSs), non-ribosomal peptide synthetases and terpene cyclases). Therefore, it is not possible at this moment to give a precise number of total BGCs for any SM-rich fungus including *A. fumigatus*. With this in mind, here we consider only 26 *A. fumigatus* BGCs that either from previous characterization or from strong bioinformatic support, are likely to produce a predictable SM (electronic supplementary material, table S1), examine their expression in a murine model of infection and expand on the conservation of these BGCs in other fungi.

2. Results and discussion

(a) Twenty-six clusters

In addition to the 18 clusters that have been characterized either in *A. fumigatus* or other fungi, eight additional loci strongly support production of predictable SMs (all sequence data used, via MultiGeneBlast (MGB) analysis, to identify clusters in other fungi are presented as electronic supplementary material, Data S1). Focus on these 26 does not preclude the existence of other BGCs in *A. fumigatus*.

BGC 1. This five-gene cluster encodes a probably highly reduced polyketide as determined by the enzymatic domains present in the PKS. While not found in other sequenced *Aspergillus* species, the arrangement and gene number is conserved in the wheat pathogen *Phaeosphaeria nodorum* and the pneumocandin-producing fungus *Glarea lozoyensis*.

BGC 2 (*Nidulanin A*). This cluster produces the cyclic tetrapeptide nidulanin A, found in all *Aspergillus* and *Penicillium* species. Originally characterized in *A. nidulans*, currently there is no known property or function for this metabolite [39]. However, the non-ribosomal synthetase (NRPS) generating nidulanin is also involved in synthesis of fungisporin [44], which has been shown to have antibacterial properties [45].

BGC 3 (*Ferricrocin*). This two-gene cluster contains *sidI* and *sidC* that encode proteins required for synthesis of the siderophore ferricrocin in *A. fumigatus*. The pairing of *sidI* and *sidC* genes is common across Ascomycete genera [33,34]. Ferricrocin, along with fusarinine C, is critical for *A. fumigatus* virulence [46].

BGC 4. This six-gene cluster is likely to produce a polyketide with similarities to fusarielins, produced by *Fusarium* and *Aspergillus* spp. [47,48] and variations (4–6 genes) of this cluster are also found in *Colletotrichum fioriniae*, *Pestalotiopsis fici* and some *Metarhizium* spp. Fusarielins exhibit antibacterial, antifungal, anticancer, antiangiogenic and antiproliferative properties [49]. Existence of a fusarielin-like cluster in *A. fumigatus* was first noted in a study characterizing the *F. graminearum* fusarielin BGC [40].

BGC 5 (*DHN-melanin*). A well-characterized cluster producing the polyketide DHN-melanin giving the characteristic greyish green coloration of *A. fumigatus* conidia. A near identical cluster is found in closely related *Aspergillus* spp. including *Aspergillus clavatus* and *Neosartorya fischeri*. As mentioned above, numerous studies have described the importance of this metabolite in virulence in *A. fumigatus* [50]. In fact, a repeating theme in fungal biology is the requirement of spore melanins for virulence in numerous pathogenic fungi [51].

BGC 6 (*Fumigaclavine*). Fumigaclavines are bioactive alkaloids with numerous bioactivities ranging from antibacterial [52] to vasorelaxant [53]. Fumigaclavines are considered mycotoxins and purified compounds have a toxic impact on mammalian cells [54]. However, a role for these compounds in pathogenesis has not yet been shown in *A. fumigatus* [55]. The fumigaclavine-producing eleven-gene cluster—or a significant part of it—is also present in members of the dermatophytic genera *Arthroderma* and *Trichophyton*, the insect-pathogenic genus *Metarhizium* and a miscellaneous group of fungi including *Byssochlamys spectabilis*, *G. lozoyensis* and *Claviceps purpurea*.

BGC 7. This cluster encodes another uncharacterized highly reduced polyketide that is present in some aspergilli including the opportunistic pathogen *Aspergillus terreus* and some *Penicillium* spp.

BGC 8 (*Fusarinine C*). This second siderophore six-gene cluster is conserved in many fungi [56]. Similar clusters of four and five genes are also present in many species and may represent divergence or the absence of the missing genes.

BGC 9 (*Hexadehydroastechrome*). This eight-gene BGC, or variations of it, is present sporadically in *Aspergillus* spp. including *A. terreus* [57], the dermatophytic genera *Arthroderma* and *Trichophyton*, the plant pathogenic *Fusarium oxysporum* and *Fusarium avenaceum*, and partially in the blackleg pathogen *Leptosphaeria maculans*. Over-production of this metabolite enhances virulence in a murine model of *A. fumigatus* infection [29], and its activity is associated with iron homeostasis and alterations in siderophore synthesis [58].

BGC 10. This cluster contains an NRPS and NRPS-like gene suggesting that the metabolite will be a small peptide. A subsection of this cluster is present in the closely related *N. fischeri* but with little homology in other fungi.

BGC 11. This uncharacterized eight-gene BGC contains several homologues to the *Fusarium* fumonisin BGC [59] and thus is predicted to produce the similarly structured polyketide sphingofungin [60]. Fumonisin is a potent mycotoxin impacting ceramide synthase activity [61]. Sphingofungins act as antifungal compounds by targeting serine palmitoyltransferase [62]. This BGC is largely conserved in *Arthroderma*, *Trichophyton* and *Metarhizium* spp.

BGC 12. The presence of conserved enzymatic domains in the NRPS in this uncharacterized cluster suggests it will encode a small peptide 2–3 amino acids in size. This cluster is found in *N. fischeri* and a subset of the genes in a few other *Aspergillus* spp.

BGC 13. (*Endocrocin*). The polyketide endocrocin is another spore pigment with antimigratory effects on neutrophils [21]. Endocrocin, trypacidin and neosartoricin all belong to a class of related non-reduced polyketides with aromatic ring structures [63]. Variations of all these clusters are common in many fungi, with such clusters often encoding a spore pigment or toxin. The fungus *Pseudogymnoascus destructans*, causing the white-nose syndrome of bats, contains at least one cluster with considerable similarity to several genes in these non-reduced polyketide BGCs [63].

BGC 14 (*Trypacidin*). Another spore pigment, this compound is produced by a 13-gene BGC [32,64]. The endocrocin and trypacidin BGCs show redundancy as both synthesize the spore pigment endocrocin [32]. Trypacidin is known and named for its antiprotozoal (e.g. *Trypanosoma cruzi*) activity [65]. Trypacidin is toxic to lung cells [66].

BGC 15 (*Helvolic acid*). Following on trypacidin, helvolic acid also exhibits antiprotozoal activity [67] and is produced by several fungal spp. in addition to *A. fumigatus*, including several plant and insect pathogens and many endophytes [28,68,69]. Along with gliotoxin and fumagillin, helvolic acid exhibits cilioinhibitory activity [70] and is a potent antimicrobial [71].

BGC 16. An uncharacterized NRPS-like cluster conserved in closely related *Aspergillus* spp. including *N. fischeri* and, partially, *A. clavatus*. The composition of genes in this predicted BGC is less supportive of a *bona fide* cluster than in the cases of all other uncharacterized BGCs here presented.

BGC 17. This BGC contains the largest NRPS gene in the *A. fumigatus* genome with 6 adenylation and 10 condensation domains, suggestive of a 6–10 residue peptide. This BGC appears limited to *A. fumigatus* and *N. fischeri*. Although the metabolite produced by this BGC has not been characterized, disruption of the NRPS results in a hypervirulent strain as assessed in insect and murine models. The authors suggest that BGC17 product may be structural in nature [72].

BGC 18 (*Fumisoquin*). This newly described BGC [25] is also found in *Penicillium solitum*, *F. graminearum* and *F. pseudograminearum*.

BGC 19. An uncharacterized NRPS cluster immediately adjacent to the gliotoxin BGC. As with BGC 17, this cluster appears limited to *A. fumigatus* and *N. fischeri*.

BGC 20 (*Gliotoxin*). Variations of this 13-gene BGC are conserved in numerous fungi including may plant pathogens [15]. Gliotoxin, belonging to a class of natural products known as epipolythiopiperazines, contributes to the virulence of *A. fumigatus* and a similar epipolythiopiperazine, sirodesmin, is a potent phytotoxin [73].

BGC 21 (*Fumiquinazoline*). Various fumiquniazolines are reported to be produced by *Aspergillus*, *Penicillium* and *Acremonium* spp. [74] with one report of antibacterial properties of fumiquinazoline F [75]. This BGC, expressed in spores of *A. fumigatus* [24], is also found in the saprophyte *Pseudogymnoascus pannorum*.

BGC 22 (*Pyripyropene A*). Pyripyropenes are produced by several *Aspergillus* and *Penicillium* spp. [76]. MGB locates the BGC to these genera and the nematode endoparasite, *Hirsutella minnesotensis* where it is likely a virulence factor, surmising from the several patents claiming its use as a nematicide [77]. Pyripyropene is of considerable interest as a potent and selective acyl-coenyzmeA::acholesterol acyltransferase 2 (ACAT2) inhibitor [78].

BGC 23 (*Neosartoricin*). This polyketide cluster is conserved in many species, again including the dermatophytic genera *Arthroderma* and *Trichophyton* and insect pathogen *Metarhizium*. The orchid mycorrhizal species *Oidiodendron maius* also appears to contain this or a similar BGC.

BGC 24 (*Fumitremorgin*). Similar to the pyripyropene BGC, the fumitremorgin gene cluster is not only present in *A. fumigatus* and *N. fischeri* but also *H. minnesotensis*. Studies have identified antifungal and insecticidal properties of this and related metabolites [79].

BGC 25 (*Fumagillin and pseurotin*). The genes for these two SMs are intertwined in a supercluster [26]. This BGC is conserved in part in *Metarhizium* spp., *Scedosporium apiospermum*, *P. solitum* and *Colletotrichum sublineola*. Fumagillin is a potent inhibitor of methionine aminopeptidases, is considered a possible therapeutic targeting angiogenesis due to

this property [80] and is currently used to treat microsporidian fungal infections [81]. Several biological activities have been attributed to pseurotin including antibacterial properties [52] and inhibition of IgE production [82].

BGC 26. This uncharacterized BGC is likely to produce a polyketide-terpene hybrid, possibly with some similarity to the *Aspergillus flavus* toxin aflatrem [83], and is found in *N. fischeri*.

How might these BGC metabolites affect virulence of *A. fumigatus* or other pathogenic fungi? Gene-deletion studies from six of the BGCs (3, 5, 8, 9, 17 and 20) have shown impacts on virulence in murine models of invasive aspergillosis. Another eight BGC (6, 13, 14, 15, 21, 22, 24 and 25) produce metabolites that have toxic effects on various organisms ranging from bacteria to mammals, which may indicate contributions of these metabolites to virulence, perhaps in a synergistic manner. One unexpected finding from our MGB analysis was the conservation of many of these clusters in other pathogenic fungi; in particular, the presence of many of the *A. fumigatus* BGCs in the insect-pathogenic genus *Metarhizium*, the dermatophytic genera *Arthroderma* and *Trichophyton*, but also in several plant-pathogenic species. Supporting a case for a role of these BGC metabolites in virulence, the products (pyripyropene and fumitremorgin) of two clusters conserved in the nematode parasite *H. minnesotensis* show insecticidal properties.

Although relatively low in BGC numbers (approx. one dozen), it is interesting to note that the emerging bat pathogen *P. destructans* contains a BGC with similarities to the endocrocin BGC. Endocrocin, as noted above, is an *A. fumigatus* spore metabolite that inhibits neutrophil migration. Because studies have documented an aberrant bat immune response to infections by *P. destructans* (formerly *Geomyces destructans* [84–86]), it is intriguing to speculate that some *P. destructans* SMs might be playing a role in these responses.

(b) Expression studies

Our previous transcriptome analyses revealed that simultaneous contributions of multiple *A. fumigatus* SMs are likely during colonization of the mammalian host niche [87] whereby comparative analysis of *A. fumigatus* gene expression in laboratory culture versus the lungs of neutropenic mice during initiation of growth revealed coordinate upregulation of BGCs 5, 8, 7, 12, 20, 24 and 25 during infection of the mammalian lung. Despite the technical complexity of the experimentation, the boundaries of BGCs were clearly delineated, adding weight to the relevance of their clustered groupings to transcriptional regulation. In this study, we took an alternative view, asking whether genes of predicted BGCs were (i) expressed and (ii) prioritized over other metabolic gene functions during morphogenesis and colonization of the host environment. We used quantile normalized transcript abundance (calculated per gene as a function of all other expressed genes in the analysis) as a proxy measure of overall transcript abundance, thereby assessing gene expression levels for all BGC genes at 4 and 14 h post-infection (figure 1). Of the total cohort of $n = 226$ genes (electronic supplementary material, table S1), detectable expression of $n = 171$ (76%) was achievable (figure 1) and of these, 80 genes (47%) were expressed at significantly higher levels than the population median. Concordant with our previous analyses [87] and those assessing pathogenicity of SM-deficient null mutants [46,50,54,55,66,70,82], greater than or equal to 50% of detectable transcripts arising from BGCs 3, 5, 6, 13, 14, 15 and 25 exhibited abundances, which significantly exceeded the median value of that of the sampled gene population at one or both time-points of the analysis. A similar picture was observed for the majority of other BGCs, with the notable exceptions of BGCs 2, 9, 18, 24, 15, 11 and 26, which might be of limited relevance to murine infection or, alternatively, have relevance to time points of infection occurring later than those we studied here (as, notably, overexpression of the BGC 9 product increases virulence in the murine model of IA [29]). Concordant with roles for metal ion homeostasis in mammalian pathogenicity, genes of the siderophore BGCs 3 and 8 are highly expressed. Afu1g17200 of BCG3, known to encode an enzyme required for synthesis of conidial siderophores and germination of *A. fumigatus* spores in iron-depleted environments [36] was preferentially expressed at 4 h but not 14 h post-infection, as were many genes of the uncharacterized BGC 10 (figure 1). The relevance of BGCs 5 and 8 (directing biosynthesis of DHN-melanin and fusarinine C, respectively) has been conclusively demonstrated in neutropenic hosts via analysis of null mutants [36,50]. Although DHN-melanin is often regarded as a critical spore-associated metabolite we found heightened transcript abundance of several key biosynthetic genes at both the 4 h and 14 h time points, while the relevance of fusarinine C appears to escalate with time. Genes of the gliotoxin BGC 20 were predominantly upregulated, although, as we have previously discussed and noted [87], gliotoxin is not a virulence factor in neutropenic hosts. It is likely that the stresses encountered in the host environment provoke the expression of a bespoke 'blend' of SM production, the net effect of which is to undermine host defenses thus creating a hospitable niche. The remaining critical question to be addressed is the impact of synergistic SM activities upon host physiology and innate immunity.

3. Conclusion

While products of BGCs are not the only factors that contribute to pathogenicity of any fungus, certain specific SMs are known virulence factors and many can modify host responses to pathogenic fungi. These metabolites have largely been understudied with regard to their impact on pathogenesis, in part, due to the immense work required to identify and characterize a complete secondary metabolome. Some BGCs encode metabolites that are fitness factors that enable a fungus to obtain nutrients and cofactors (e.g. metal acquiring metabolites) or protect from stress (e.g. oxidative, UV, other microbes) in any environment, and do not easily predict/indicate degree of virulence or pathogenicity. The complex expression of specific BGCs within a single species is also likely to be a factor impacting pathogenic abilities yet we are at a point where we cannot confidently predict which BGC couplings are synergistic but only speculate on this possibility. Nevertheless, the identification of conserved BGCs—especially those that have been demonstrated to exhibit fitness or bioactive properties—in more than one fungus lends credibility to a view that such BGCs may constitute part of the arsenal for both persistent and emergent fungal pathogens.

4. Material and methods

The MGB was used to detect homologous clusters across distantly related fungal species [88]. MGB architecture searches

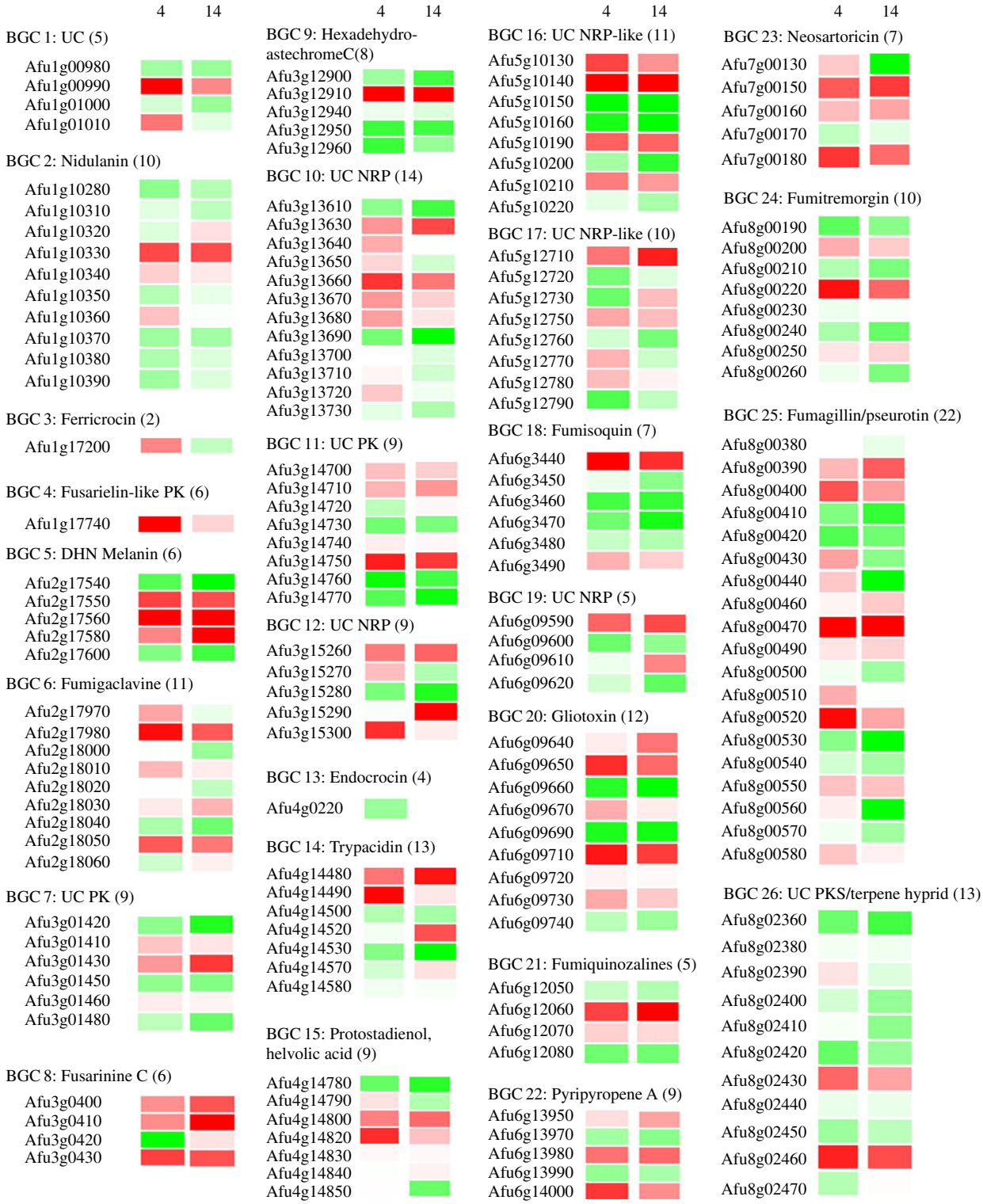

Figure 1. Expression of BGC genes during colonization of the mammalian lung. Transcript abundances of BGC genes at 4 h ($n = 6$ technical replicates) and 14 h ($n = 4$ technical replicates) post-infection of the neutropenic murine host (mean value of transcript abundance from a pooled sample of $n = 7$ mice per time-point). Quantile normalized transcript abundance, per gene, per time-point, calculated as a function of all expressed genes in the analysis, was used as a proxy measure of standing in the overall ranking of transcript abundance. Transcript abundance, at each time-point, relative to all expressed *A. fumigatus* genes in the analysis is represented as greater (red) or lower (green) than the population median. Numbers in parentheses indicate the number of genes per cluster predicted by MultiGeneBlast.

were carried out with the per cent identity threshold set to 25%, the synteny weight set to 0 and the maximum intergenic distance set to 110% of the span of the corresponding *A. fumigatus* cluster with a minimum bound of 25 kb. Other parameters were set to default. The input for each search was a multiFASTA file of the amino acid sequences of the proteins predicted to be encoded by the genes in the region of an *A. fumigatus* predicted cluster or hypothetical cluster variant, erring on the side of over-inclusion to detect cluster boundaries. A custom fungal database, previously described, was used for these searches [63].

(a) Murine infections

A previously published method for examination of fungal gene expression in neutropenic mice was used [87]. Briefly, intraperitoneal injections of cyclophosphamide (150 mg kg^{-1},

Endoxana, Asta Medica) were conducted on days −3 and −1, with a single subcutaneous dose of hydrocortisone acetate (112.5 mg kg^{-1}, Hydrocortistab, Sovereign Medical) on day −1. *Aspergillus fumigatus* spores from clinical isolate Af293 were prepared and intranasally administered to mice as described previously [87]. For each of the 4 and 14 h time-points seven mice were infected and then humanely sacrificed. Bronchoalveolar lavage (BAL) was performed using three to four aliquots of 37° sterile saline per mouse. BAL fluids (BALFs) were snap frozen in liquid nitrogen.

(b) *Aspergillus fumigatus* RNA extraction, amplification and hybridization

The BALF samples were centrifuged at 14 000 r.p.m. for 5 min and the pellet was washed with 500 µl ice-cold water. BALFs were pooled, suspended in 450 µl ME-RLC buffer (Qiagen) and ground in liquid nitrogen with a pestle and mortar. RNA was then extracted using RNeasy® Kit (Qiagen). Quality of RNA was checked by Nanodrop ND-1000 Spectrophotometer (Nanodrop, Wilmington, DE, USA). RNA with an A260/280 and an A260/230 ratio > 1.9 was used for the experiments.

Fungal RNA was doubly amplified prior [87] to array hybridization. A 70-mer oligotide glass slide DNA microarray platform was used (Pathogen Functional Genomic Resource Centre, JCVI, Rockville) and doubly amplified RNA derived from 4 and 14 h time points was hybridized, respectively, in technical duplicate or triplicate. Washed slides were scanned and data captured using GenePix 4000b (Axon Instruments, Foster City, CA, USA) semiconfocal microarray scanner and associated GenePix Pro 3.0 software. Images were saved as .TIF files and TIGR Spotfinder [89] was used to quantitate fluorescent intensities. Poor-quality spots were identified manually and flagged. After Spotfinder analysis, results were exported as TIGR software-compatible .MEV files following local background subtraction.

(c) Microarray normalization

A single-channel approach was applied using quantile normalization to extract, from dual channel co-hybridizaton data [90,91], a comparative, ranked view of Af293 transcript abundance. From .MEV files, fluorescence intensity values were captured on a gene-by-gene basis, corresponding to either (with duplicate spots) $n = 6$ technical replicates for the 4 h time point or (with duplicate spots) $n = 4$ technical replicates for the 14 h time point. Values were quantile normalized across all replicates [92] using GeneSpring v. 11.0.5 (Agilent Technologies). Following normalization, spot intensity values were averaged between technical replicates at each time point, producing a single measurement of transcript abundance per gene at each time point. Quantile-normalized transcript abundance, per gene, per time point, calculated as a function of all expressed genes in the analysis, was used as a proxy measure of standing in overall ranking of transcript abundance.

Ethics. Murine infections were performed in accordance with local and international ethical governance, under UK Home Office Project Licence PPL/70/6487, in dedicated facilities at Imperial College London.

Data accessibility. Raw and quantile normalized data have been uploaded to the Gene Expression Omnibus http://www.ncbi.nlm.nih.gov/geo/ under the title 'Secondary metabolite arsenal of an opportunistic pathogenic fungus'. Accession number pending. Fasta format sequence data used to perform MGB analysis have been uploaded as the electronic supplementary material, Data S1.

Authors' contributions. K.T. carried out the MultiGeneBlast data analysis; T.C.C. performed the murine experimentation and devised the analytical methodology for transcriptome analyses, E.B. and N.P.K. conceived of the study, coordinated the study and helped draft the manuscript. All authors contributed to writing of the manuscript and gave final approval for publication.

Competing interests. We have no competing interests.

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