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Dysregulated Gliotoxin Biosynthesis Attenuates the Production of Unrelated Biosynthetic Gene Cluster-Encoded Metabolites in *Aspergillus fumigatus*

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Running head: Biosynthetic Gene Cluster interactions.

Dysregulated Gliotoxin Biosynthesis Attenuates the Production of Unrelated Biosynthetic Gene Cluster-Encoded Metabolites in *Aspergillus fumigatus*.

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Abstract

Gliotoxin is an epipolythiodioxopiperazine (ETP) class toxin, contains a disulfide bridge that mediates its toxic effects via redox cycling and is produced by the opportunistic fungal pathogen Aspergillus fumigatus. The gliotoxin bis-thiomethyltransferase, GtmA, attenuates gliotoxin biosynthesis in A. fumigatus by conversion of dithiol gliotoxin to bis-thiomethylgliotoxin (BmGT).

Here we show that disruption of dithiol gliotoxin bis-thiomethylation functionality in A. fumigatus results in significant remodelling of the A. fumigatus secondary metabolome upon extended culture. RP-HPLC and LC-MS/MS analysis revealed the reduced production of a plethora of unrelated biosynthetic gene cluster-encoded metabolites, including pseurotin A, fumagillin, fumitremorgin C and tryprostatin B, occurs in A. fumigatus ΔgtmA upon extended incubation. Parallel quantitative proteomic analysis of A. fumigatus wild-type and ΔgtmA during extended culture revealed cognate abundance alteration of proteins encoded by relevant biosynthetic gene clusters, allied to multiple alterations in hypoxia-related proteins. The data presented herein reveal a previously concealed functionality of GtmA in facilitating the biosynthesis of other BGC-encoded metabolites produced by A. fumigatus.
Introduction

Ascomycetes constitute the largest phylum of the fungal kingdom and produce an incredible array of natural products. Although many of these compounds are known as medicinal therapeutics or industrial chemicals, several natural products are potent toxins which pose substantial threats to human food supplies and health (Schueffler and Anke 2014). The opportunistic fungus Aspergillus fumigatus produces a wealth of these potent natural product toxins which are encoded by multigene biosynthetic gene clusters (BGCs) (Bignell et al. 2016).

Gliotoxin has been shown to be a virulence attribute in invasive aspergillosis due to its cytotoxic, genotoxic and apoptosis-stimulating properties. It is produced through a sequential series of enzymatic steps, which are predominantly encoded by the gli BGC (Dolan et al. 2015). Gliotoxin biosynthesis is also influenced by GtmA (TmtA in Scharf et al. (2014)), a thiol-directed methyltransferase encoded outside the gli BGC, which specifically bis-thiomethylates both thiols of dithiol gliotoxin to form bis-thiomethylgliotoxin (BmGT), resulting in the attenuation of gliotoxin formation (Dolan et al. 2014). Lines between defined BGCs have been blurred by elegant work describing the existence of intertwined biosynthetic gene clusters which are involved in the formation of more than one chemical product (Wiemann et al. 2013). For example, A. fumigatus encodes a “supercluster” in the subtelomeric region of chromosome 8, in which the genes responsible for the production of two natural products, pseurotin and fumagillin, are physically intertwined (Wiemann et al. 2013). Like gliotoxin, these other natural products also have potent toxicities. For example, pseurotin A is an immunosuppressive spirocyclic that has been shown to have interesting biological activities including the ability to induce the cellular differentiation of PC12 neuronal cells (Komagata et al. 1996), monoamine oxidase inhibitory activity (Maebayashi et al. 1985) and chitin synthase inhibitory activity (Wenke et al. 1993), highlighting many potential applications of this metabolite. Fumagillin has antibiotic and antifungal activity. It was also found to exhibit anti-cancer properties and anti-angiogenic activity as a inhibitor of the of the human type 2 methionine aminopeptidase (MetAP2) (Sin et al. 1997; Hou et al. 2009).
Despite the fact that BGCs often possess a pathway-specific transcription factor, the production of several otherwise unconnected natural products has been shown to be regulated by global regulators of secondary metabolism such as LaeA. This functionally enigmatic regulator has been shown to regulate the production of several *A. fumigatus* secondary metabolites (SMs) including gliotoxin, fumagillin, pseurotin A and helvolic acid (Perrin *et al.* 2007). LaeA has also been shown to counteract the establishment of heterochromatin marks, thus activating SM production, inferring that LaeA regulates BGC-encoded metabolism by modifying chromatin structure (Nützmann *et al.* 2011). This suggests that fungal SM is controlled by a rigorous hierarchy of regulatory mechanisms.

Understanding the diversity of regulatory strategies controlling the expression of these pathways is therefore critical if their biosynthetic potential is to be explored for new drug leads. No single medium under standardized growth conditions can secure expression of the full potential for producing the secondary metabolome of a fungal culture. However, there are many strategies to enable fungal cultures to produce even more BGC-encoded metabolites; for instance by modifying the media constituents, adding other microorganisms, using light or using longer incubation periods, alternate temperatures or deploying low or high pH (Ochi & Hosaka 2013).

The filamentous fungus *Aspergillus terreus* produces the ETP acetylaranotin (Guo *et al.* 2013). Similar to gliotoxin, this metabolite and its derivatives have been shown to display an array of interesting bioactivities including the induction of apoptosis in cancer cell lines and antifungal activity (Guo *et al.* 2013; Choi *et al.* 2011; Suzuki *et al.* 2000; Li *et al.* 2016). Three novel bis(methylthio)dioxopiperazine derivatives of the epipolythiodioxopiperazine (ETP) emestrin were produced by *Podospora australis* following static incubation of cultures at 23 °C for 14 days (Li *et al.* 2016). Like *P. australis*, *A. fumigatus* produces many thiomethylated forms of gliotoxin upon incubation for two weeks at 25 °C in the dark (Forseth *et al.* 2011). Extending the incubation duration of *A. terreus* cultures from 6 d to 42 d resulted in the production of four thiomethylated
forms of acetylaranolotin, which were undetectable at earlier incubation time points. One of these bioactive SMs (bisdethiobis(methylsulfanyl)apoaranotin) exhibited growth inhibitory properties against *Mycobacterium tuberculosis* H37Ra with an MIC value of 1.56 μg/ml, thus highlighting the potential of extended incubation to develop exciting natural product derivatives (Haritakun *et al.* 2012). As the generation of these thiomethylated forms of acetylaranolotin was likely dependent on an *A. terreus* GtmA homolog, this research prompted us to explore the effect of extended culture incubation on *A. fumigatus* wild-type and ΔgtmA. Moreover, despite significant work describing the production and isolation of these ETP derivatives (Dolan *et al.* 2014; Scharf *et al.* 2014; Liang *et al.* 2014), the downstream effects of removing this ETP bis-thiomethylation functionality and concomitant perpetuation of gliotoxin biosynthesis, have not been explored to date in extended cultures.

**Materials and Methods**

**RP-HPLC and LC-MS detection of natural products from *A. fumigatus* culture supernatants**

*A. fumigatus* wild-type, ΔgtmA and gtmA<sup>e</sup> strains (Dolan *et al.* 2014) were grown (10<sup>8</sup> conidia/ml) in quadruplicate (Czapek-Dox broth, 200 rpm, 3 d; then static, 25 d 37 °C). Culture supernatants and ethyl acetate organic extracts (100 μl) were analysed by RP-HPLC with UV detection (Agilent 1200 system), using a C18 RP-HPLC column (Agilent Zorbax Eclipse XDB-C18 Semi-Preparative; 5 μm particle size; 4.6 x 250 mm) at a flow rate of 2 ml/min (Figure 1). A mobile phase of water and acetonitrile with 0.1 % (v/v) trifluoroacetic acid, was used under various gradient conditions.

For LC-MS analysis, organic extracts were diluted 1/10 in 0.1 % (v/v) formic acid and spin filtered prior to LC-MS analysis (Agilent Ion Trap 6340). Gliotoxin (purity: 98%) and BmGT (purity: 99%) standards were obtained from Sigma-Aldrich and Enzo Life Sciences, respectively. Fumagillin, pseurotin A, tryprostatin B and fumitremorgin C were identified based on published *m/z* ratios, retention times and fragmentation patterns as described previously (O’Keeffe *et al.* 2014). All data
were analysed using built-in GraphPad prism version 5.01 functions, as specified. The level of significance was set at \( p < 0.05 (*) \), \( p < 0.001 (**) \), and \( p < 0.0001 (***) \), unless otherwise stated.

**Comparative quantitative proteomic analysis of *A. fumigatus* wild-type and mutant strains**

As shown in Figure 1, Mycelia were then harvested and snap frozen in liquid N\(_2\). No significant differences in biomass were noted for these strains (Supplementary Figure 1 and 2). Mycelial lysates were prepared in lysis buffer (100 mM Tris-HCl, 50 mM NaCl, 20 mM EDTA, 10% (v/v) glycerol, 1 mM PMSF, 1 \( \mu \)g/ml pepstatin A, pH 7.5) with grinding, sonication and clarified using centrifugation. The resultant protein lysates were precipitated using trichloroacetic acid/acetone and resuspended in 100 mM Tris-HCl, 6 M urea, 2 M thiourea, pH 8.0. After dithiothreitol reduction and iodoacetamide-mediated alkylation, sequencing grade trypsin combined with ProteaseMax surfactant was added. All peptide mixtures were analysed via a Thermo Fisher Q-Exactive mass spectrometer coupled to a Dionex RSLCnano. LC gradients ran from 4-35 % B over 2 h, and data was collected using a Top15 method for MS/MS scans. Comparative proteome abundance and data analysis was performed using MaxQuant software (Version 1.3.0.5), with Andromeda used for database searching and Perseus used to organise the data (Version 1.4.1.3) (Cox & Mann 2008).

**Results and Discussion**

**Late-stage culture metabolomics reveals that dysregulated gliotoxin biosynthesis due to gtmA absence influences the biosynthesis of other secondary metabolites**

Comparative RP-HPLC analysis of culture supernatants at 3 d showed that the overall SM profile was not altered significantly by the deletion of \( gtmA \) (Figure 2A), except that gliotoxin production was significantly increased and \( \text{bis-} \)-thiomyethylgliotoxin (BmGT) production was abolished as described previously (Dolan *et al.* 2014). However, when the culture incubation time was increased by 25 d, a decrease in several compounds was specifically detected in *A. fumigatus* \( \Delta gtmA \) by RP-HPLC DAD (at 254 nm and 351 nm) (Figure 2B,C). The production of these compounds was restored in the complemented strain (\( gtmA^+ \)). These compounds were fraction-collected, analysed by
LC-MS and identities were elucidated based on known m/z values and fragmentation patterns. Pseurotin A (p = 0.0001) and fumagillin (p = 0.0005) were produced at significantly decreased concentrations in \textit{A. fumigatus} \textit{Δgta} (Figure 2D). Furthermore, LC-MS/MS analysis of ethylacetate extracted culture supernatants also uncovered that the detected concentration of tryprostatin B (p = 0.0005) and fumitremorgin C (p = 0.0074) were significantly lower in the \textit{Δgta} strain (Figure 2D). This suggested that Gta activity may influence the production of other SM through the production of BmGT or, alternatively, by augmenting gli-cluster activity.

In order to uncover if the absence of BmGT was directly responsible for the metabolite alterations, the extended culture experiment was repeated and exogenous BmGT was added (10 μg/ml final) to \textit{A. fumigatus} \textit{Δgta} prior to static incubation for 25 days (n = 4). Methanol was added to the control cultures. Exogenously added BmGT did not result in the restoration of pseurotin A or fumagillin production in \textit{A. fumigatus} \textit{Δgta} to wild-type levels, suggesting that BmGT does not directly facilitate the production of these metabolites (Figure 2E).

Label-Free Quantitative (LFQ) proteomics of late-stage cultures reveals that the absence of gta expression and resulting dysregulated gliotoxin biosynthesis leads to widespread proteomic alterations.

The metabolomic analysis demonstrated that \textit{A. fumigatus} \textit{Δgta} has an altered SM profile in comparison with that of the wild-type or \textit{gta} cultured under identical conditions. Comparative label-free quantitative (LFQ) proteomic analysis was carried out to further elucidate the involvement of Gta activity in this metabolite profile alteration. A total of 1468 proteins were detected in \textit{A. fumigatus} wild-type vs. \textit{Δgta} analysis (Figure 3). Two proteins were uniquely detected in \textit{A. fumigatus} \textit{Δgta} and 87 proteins were significantly more abundant in this mutant. Proteins (n = 35) were not detected in \textit{A. fumigatus} \textit{Δgta} and 136 proteins were significantly less abundant in this strain compared to wild-type. This dataset was cross referenced with the \textit{gta} vs. \textit{Δgta} dataset in order to confirm which of these changes were directly due to the absence of \textit{gta}.

Based on this enrichment, 27 proteins were not detected in \textit{A. fumigatus} \textit{Δgta} and 93 proteins
were significantly less abundant in this strain. Two proteins were uniquely detected in *A. fumigatus ΔgtmA* under this condition and 71 proteins were significantly more abundant in this mutant (Table S1 and S2).

Four proteins encoded by the *gli* cluster were shown to be significantly more abundant (log2-fold) in *ΔgtmA* compared to the wild-type. The MFS gliotoxin efflux transporter GliA (AFUA_6G09710; 1.82652), the membrane dipeptidase GliJ (AFUA_6G09650; 1.66652), a predicted O-methyltransferase GliM (AFUA_6G09680; 1.62724) and the glutathione S-transferase GliG (AFUA_6G09690; 1.40425) were increased in abundance in ΔgtmA (Table S1). Additionally, a putative short chain dehydrogenase (AFUA_4G08710; 1.37478) and a ThiJ/PfpI family protein (AFUA_5G01430; 1.93416), which were previously shown to be induced by gliotoxin exposure were more abundant in this condition. RmtA (AFUA_1G06190; 1.06), a putative arginine methyltransferase previously shown to act as a global regulator in *A. flavus*, mediating broad effects on secondary metabolism and development in this organism (Satterlee *et al.* 2016), also exhibited significantly elevated abundance. However, its role in *A. fumigatus* has yet to be elucidated.

Co-incident with the reduced levels of selected SMs, several proteins for which cognate transcripts have previously been shown to be induced by hypoxia, or by exposure to neutrophils, were increased in abundance in *A. fumigatus ΔgtmA*. Two proteins which are repressed by gliotoxin exposure and two which are repressed by hypoxia (Vödisch *et al.* 2011) were significantly decreased in abundance in ΔgtmA. This may be a response to the sustained gliotoxin production in *A. fumigatus ΔgtmA* as the abundance of these proteins is returned to wild-type levels in the complemented strain. Proteins which had been shown previously to be induced by hypoxia exposure were significantly more abundant in *A. fumigatus ΔgtmA* compared to the wild-type (Vödisch *et al.* 2011). These include a putative transaldolase (AFUA_5G09230; 1.58072), a putative glyceraldehyde 3-phosphate dehydrogenase (AFUA_5G01030; 1.34405), an argininosuccinate lyase (AFUA_3G07790; 1.34137), a 6-phosphogluconate dehydrogenase (AFUA_6G08050; 1.31184), a putative mevalonate kinase (AFUA_4G07780; 1.07826), and an
essential 1, 3-beta-glucanosyltransferase (AFUA_2G05340; 1.0025). Additionally, glutathione synthase (AFUA_5G06610; 1.67077), which is an ortholog of the *Saccharomyces cerevisiae* GSH2 glutathione biosynthetic protein, known to be induced by oxidative stress in this organism (Sugiyama et al. 2000), was more abundant. Additionally, proteins formerly shown to be induced by neutrophil exposure were also more abundant in ΔgtmA (Sugui et al. 2008). These included a putative carbon-nitrogen family hydrolase (AFUA_5G02350; 1.567), a putative myo-inositol-phosphate synthase (AFUA_2G01010; 1.21826) and an aldehyde reductase (AKR1) (AFUA_6G10260; 1.09358). Mannitol 2-dehydrogenase which has a predicted role in mannitol metabolism (AFUA_4G14450; 1.52766) was also more abundant. Mannitol is an important virulence determinant of pathogenic fungi. Its high antioxidant capacity aids in suppressing the reactive oxygen species mediated attacks from neutrophils (Wyatt et al. 2014). This suggests that the sustained expression of the *gli*-cluster in long-term cultures may be translated as an oxidative challenge to *A. fumigatus*.

Six transporter proteins were found to be significantly more abundant in ΔgtmA. These were an ABC transporter Cdr1B (AFUA_1G14330; 2.4345), a putative MFS monocarboxylate transporter (AFUA_3G03320; 2.29482) (located in an uncharacterised SM cluster (Lind et al. 2016), the ABC multidrug transporter Mdr1 (AFUA_5G06070; 2.19999), a putative plasma membrane H⁺ ATPase Pma1 (AFUA_3G07640; 1.3888), the low affinity plasma membrane zinc transporter ZrfB (AFUA_2G03860; 1.30673), which is induced by zinc depletion and the amino acid permease Gap1 (AFUA_7G04290; 1.0342). Pma1 was upregulated during conidial germination and in response to amphotericin B and downregulated by caspofungin treatment (Gautam et al. 2008; Cagas et al. 2011). Overexpression of the Cdr1B transporter was reported to be responsible for azole resistance in a clinical setting (Fraczek et al. 2013). The Zn²⁻-Cys₆ transcription factor AtrR was recently shown to be responsible for regulating cdr1B expression in *A. fumigatus* (Hagiwara et al. 2017).
Aminoacyl-tRNA synthetases are central enzymes in translation which provide the charged tRNAs needed for protein synthesis (Guo et al. 2010). Tyrosyl-tRNA synthetase (AFUA_5G10640; 2.85558), Seryl-tRNA synthetase (AFUA_5G05490; 1.22428), Putative valyl-tRNA synthetase (AFUA_8G04800; 1.22314), Isoleucyl-tRNA synthetase (AFUA_1G13710; 1.10632) and a putative Lysyl-tRNA synthetase (AFUA_6G07640; 1.05645) were significantly more abundant in *A. fumigatus ΔgtmA* compared to the wild-type. Several cell wall-related proteins were increased in abundance in *A. fumigatus ΔgtmA*. These included the GPI-anchored cell wall beta-1,3-endoglucanase Bgt2 (AFUA_3G00270; 1.35218) and three beta(1-3)glucanosyltransferases which belong to the 7-member GEL family (Fontaine et al. 2003; Gastebois et al. 2010); Gel5 (AFUA_8G02130; 1.23988), Gel3 (AFUA_2G12850; 1.00391), and Gel4 (AFUA_2G05340; 1.0025; previously shown to be increased in hypoxia). GltA, a UDP-galactopyranose mutase (AFUA_3G12690; 1.3312), was also significantly more abundant. *A. fumigatus ΔgltA* is devoid of galactofuranose and displays attenuated virulence in a low-dose mouse model of invasive aspergillosis (Schmalhorst et al. 2008). Interestingly, the small monomeric GTPase RasA (AFUA_5G11230; 1.65165) was also significantly more abundant in ΔgtmA. A ΔrasA mutant demonstrated a phenotype of cell wall instability and slow germination (Fortwendel et al. 2008). Higher levels of RasA abundance may be linked to the cell wall remodelling described above.

As mentioned above, *A. fumigatus ΔgtmA* 28 d cultures showed a significant decrease in the production of pseurotin A and fumagillin compared to the wild-type strain. Complementary LFQ proteomic analysis revealed a significant decrease in abundance of several proteins encoded by the chromosome 8 supercluster (AFUA_8G00100-00720) (Wiemann et al. 2013), in agreement with the metabolomic analysis which revealed the decreased production of pseurotin A and fumagillin (Figure 4). A total of 15 proteins from this cluster were detected as less abundant in ΔgtmA. Interestingly, 19 of the 98 proteins detected as significantly decreased in abundance in ΔgtmA are encoded on Chromosome 8, whereas 2 of the 38 proteins which were detected as increased in abundance in ΔgtmA were similarly located. O’Keeffe et al. (2014) demonstrated that an intact
gliotoxin self-protection mechanism, mediated by GliT, is essential to regulate the biosynthesis of apparently unrelated metabolites such as pseurotin A, fumagillin and fumitremorgins. Herein, we have shown that when *A. fumigatus* cultures are incubated for extended duration, the absence of GtmA, resulting in dysregulated gliotoxin production, also has downstream effects on the biosynthesis of apparently unrelated natural products produced by this organism. A total of 17 proteins from the supercluster (Wiemann et al. 2013) were detected as less abundant in ΔgtmA. Interestingly, 23 of the 136 proteins detected as significantly decreased in abundance in ΔgtmA are encoded on Chromosome 8. This is in comparison to 2 of the 72 proteins detected as increased in abundance in ΔgtmA being encoded on Chromosome 8. Several proteins encoded by the supercluster were either undetectable or significantly decreased in abundance in *A. fumigatus* ΔgtmA (Table 3; Table S2). Four proteins involved in the synthesis of fumitremorgins were significantly decreased in abundance in ΔgtmA. FtmPT1 (AFUA_8G00210; not detected) and FtmPT2 (AFUA_8G00250; not detected), two prenyltransferases involved in fumitremorgin B biosynthesis, FtmD, an O-methyltransferase involved in fumitremorgin B synthesis (AFUA_8G00200; not detected), and FtmF, an alpha-ketoglutarate-dependent dioxygenase which catalyses the conversion of fumitremorgin B to verruculogen (AFUA_8G00230; -4.32466). Six proteins associated with fumagillin biosynthesis were not detected or significantly less abundant. These were the fumagillin polyketide synthase (Fma-PKS) (AFUA_8G00370; not detected), the fumagillin phytanoyl-CoA-oxidase FmaF (AFUA_8G00480; -4.17772), FmaD, the fumagillin O-methyltransferase (AFUA_8G00390; -3.85167), a hypoxia induced protein; encoded in the fma gene cluster (AFUA_8G00430; -3.24847), FmaC, a putative fumagillin alpha/beta hydrolase (AFUA_8G00380; -3.16375) and a putative O-methyltransferase; encoded in the fma gene cluster (AFUA_8G00400; -2.85247). Four proteins involved in pseurotin A biosynthesis were significantly less abundant. PsoB, PsoD, PsoC, a putative pseurotin A methyltransferase (AFUA_8G00550; -3.28917), PsoA, the pseurotin A non-ribosomal peptide synthetase (AFUA_8G00540; -1.60039), a pseurotin A dual-functional mono- oxygenase/methyltransferase PsoE (AFUA_8G00560; not
detected) and PsoF, a putative pseurotin A dual methyltransferase/monoxygenase (AFUA_8G00440; -2.74114) (Table 3; Table S2). A glutathione S-transferase (AFUA_4G14380; -2.96915) encoded within the helvolic acid cluster (AFUA_4G14380–4850) (Mitsuguchi et al. 2009) was also decreased in abundance in A. fumigatus ΔgtmA.

Two proteins shown previously to be repressed by gliotoxin exposure (Carberry et al. 2012) were shown to be significantly decreased in abundance in ΔgtmA: a mitochondrial peroxiredoxin (AFUA_4G08580; -1.96889) with a predicted role in cell redox homeostasis regulation and a putative alcohol dehydrogenase (AFUA_7G01000; -1.17862) involved in ethanol metabolism. A putative thioredoxin (AFUA_8G01090; not detected) and an M repeat protein (AFUA_6G08660; -1.99673), which were shown to be hypoxia-repressed were also decreased in expression (Vödisch et al. 2011) (Table S2). The thiol methyltransferase GtmA (Dolan et al. 2014 & 2017) was uniquely detected in the wild-type condition and abundance was restored in the complemented strain. Several cell wall associated proteins were also not detected or decreased in abundance in A. fumigatus ΔgtmA mutant in comparison with the wild-type. These included the conidial hydrophobin RodA (AFUA_5G09580; not detected), a putative glycoprophosphatidylinositol (GPI)-anchored cell wall protein MP-2 (AFUA_2G05150; not detected) and a putative phiA family cell wall protein (AFUA_3G03060; -1.5461).

Several mitochondrial-associated proteins were not detected or less abundant in ΔgtmA. These included an uncharacterized protein (AFUA_1G13195; not detected) with orthologs involved in cristae formation and integral to mitochondrial inner membrane, a putative iron-sulfur cluster biosynthesis protein extrinsic to mitochondrial inner membrane (AFUA_3G06492, not detected), a putative mitochondrial intermembrane space translocase subunit (AFUA_1G04470; 1.97737), a putative mitochondrial peroxiredoxin (AFUA_4G08580; -1.96889) with a predicted role in cell redox homeostasis regulation, a putative prohibitin (AFUA_2G09090; -1.5947) with orthologs involved in mitochondrion inheritance, a putative mitochondrial 2-oxodicarboxylate carrier protein
(AFUA_1G09660; -1.56908), a putative adenylate kinase with mitochondrial intermembrane space localization (AFUA_1G07530; -1.4474), a putative outer mitochondrial membrane protein porin (AFUA_4G06910; -1.31711), a putative mitochondrial genome maintenance protein Mgm101 (AFUA_2G09560; 1.26767), a mitochondrial glycerol-3-phosphate dehydrogenase (AFUA_1G08810; -1.19813) and a putative mitochondrial processing peptidase alpha subunit with a role in protein processing involved in protein targeting to mitochondrion (AFUA_1G11870; -1.1768). Interestingly, several hypoxia-induced proteins were either not detected or decreased in abundance in A. fumigatus ΔgtnA (Blatzer et al. 2011). These included a gamma-glutamyltranspeptidase (AFUA_4G13580, not detected); SrbA-regulated during hypoxia, Putative flavohemoprotein (AFUA_4G03410; -1.42304), Putative outer mitochondrial membrane protein porin (AFUA_4G06910; -1.31711), Ubiquinol-cytochrome c reductase iron-sulphur subunit precursor (AFUA_5G10610; -1.19224) with a predicted role in oxidative phosphorylation, an aspartic acid endopeptidase (AFUA_3G11400; -1.18445) and a thiamine biosynthesis protein (AFUA_5G02470; -1.11766).

Gliotoxin can act as a sporulation signal for A. nidulans development under mildly reducing conditions through NapA oxidative stress regulation, as reflected by green conidial pigmentation (Zheng et al. 2015). Although no phenotypic differences were noted for ΔgtnA, it is conceivable that the absence of gliotoxin bis-thiometylation can alter the normal course of fungal development by sustaining the gli-cluster activation signal. It is speculated that this may then lead to downstream redox stress signalling, cell wall remodelling and the modification of secondary metabolite profiles.

Conclusions

Overall these data show that the loss of GtnA-mediated thiometylation upon extended culture duration has downstream effects on seemingly unrelated BGC-encoded metabolites in A. fumigatus. The exact mechanism of this effect is unclear, however, it is likely that the sustained gli-cluster activation in the absence of GtnA artificially prioritises the expression of this cluster,
resulting in extensive downstream proteomic remodelling, which occurs at the expense of other
BGCs in this organism. We also explored the possibility that BmGT itself acts as a signal to sustain
the expression of the unrelated BGC-encoded metabolites, which decrease in its absence. Adding a
high concentration (10 μg/mL) of BmGT to the cultures did not complement the phenotype,
suggesting that BmGT itself does not sustain the expression of these unrelated clusters. It’s
important to note that although *A. fumigatus* exposure to GT results in a rapid intracellular
accumulation due to the redox properties of this metabolite (Bernardo *et al.* 2003), BmGT does not
have this capability due to the absence of the characteristic disulfide bridge. This means that we
cannot completely rule out that the intracellular accumulation of BmGT is responsible this
phenotype. However, as shown in our earlier study, exposure of *A. fumigatus* to 5 μg/ml BmGT
results in a distinct proteomic response, increasing the GliT and GtmA protein abundance 2 fold
(Dolan *et al.* 2014). This would suggest that despite its inability to accumulate intracellularly to the
same extent as GT, exogenously added BmGT can act as a signalling molecule in *A. fumigatus*
when applied exogenously.

Although several master regulators have been shown to orchestrate secondary metabolism in
fungi, this work highlights the importance of the BGC-encoded metabolites themselves as important
signals in rewiring SM production. Despite our extensive functional and mechanistic insight into
how these BGC-encoded metabolites are synthesised, the precise mechanism of how intracellular
signals orchestrate temporal control over SM production have yet to be understood. Further work
will focus on the precise nature of how exactly these competing metabolic signals are integrated,
resulting in the expression of a ‘typical’ secondary metabolome of *A. fumigatus* and other
pathogenic fungi.

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**Figure 1.** Flow diagram describing the experimental setup. Wild-type, ΔgtmA and gtmAC were grown for 3 d shaking at 37°C. Cultures where then incubated at 37 °C, static for a further 25 d. Culture supernatants were analysed by RP-HPLC at 3 d, Samples were analysed by RP-HPLC, LC-MS/MS and quantitative proteomics at 28 d.

**Figure 2.** A. RP-HPLC analysis of *A. fumigatus* wild-type, ΔgtmA and gtmAC culture supernatants at 72 h. No alteration of the secondary metabolite profile was altered, except the absence of BmGT and increase in GT due to the absence of gtmA. B/C. RP-HPLC analysis of *A. fumigatus* WT, ΔgtmA and gtmAC culture supernatants at 28 d. Major alterations in the SM profile are evident, notably at 254 nm and 351 nm. The abundance of the compounds pseurotin A and fumagillin were significantly reduced in the ΔgtmA strain. D. LC-MS/MS analysis of organically extracted culture supernatants of *A. fumigatus* wild-type, ΔgtmA and gtmAC culture supernatants at 28 d. Pseurotin A, fumagillin, tryprostatin B and fumitremorgin C were detected at significantly reduced levels in ΔgtmA. E. Exogenous addition of methanol or BmGT (10 μg/ml) to ΔgtmA (quadruplicate) prior to static incubation for 25 d did not result in the restoration of pseurotin A or fumagillin production levels to that of the wild-type strain. Bars represent pseurotin A or fumagillin RP-HPLC intensity (mAU) at 254 nm.

**Figure 3.** Venn-diagram illustrating the proteins with altered abundance in *A. fumigatus* wild-type when compared to ΔgtmA. Heat map depicting hierarchal clustered expression data of the 260 proteins of differential abundance in ΔgtmA compared to the wild-type.

**Figure 4.** Absence of GtmA during long-term incubation of *A. fumigatus* results in the increased expression of the gli-cluster, widespread proteomic alterations and the decreased abundance of BGC-encoded enzymes located on chromosome 8. This in turn results in the reduced production of the respective compounds.
Table 1: Top 10 proteins with increased abundance in A. fumigatus ΔgtmA compared to wild-type following extended culture. Data sorted by fold change, in descending order.

Table 2: Top 10 proteins with decreased abundance in A. fumigatus ΔgtmA compared to wild-type following extended culture. Data sorted by fold change, in descending order.

Table 3: Proteins encoded by the intertwined secondary metabolite supercluster on chromosome 8 of A. fumigatus (AFUA_8G00100-00720) with decreased abundance in A. fumigatus ΔgtmA compared to wild-type and gtmaΔ grown for 28 days in Czapec-Dox media. Data sorted by fold change, in descending order.

Supplementary Figure 1: Image of A. fumigatus wild-type and ΔgtmA long term incubation cultures immediately prior to harvesting.

Supplementary Figure 2: Calculated mycelial dry weight from snap frozen, lyophilised mycelia for A. fumigatus wild-type, ΔgtmA and gtmaΔ following long term incubation.

References


https://doi.org/10.2520/myco1975.1985.22_33


https://doi.org/10.1073/pnas.1103523108


https://doi.org/10.1007/s00253-012-4551-9


https://doi.org/10.1128/EC.00109-08


https://doi.org/10.1021/pr1012812


Table 1: Top 10 proteins with increased abundance in *A. fumigatus ΔgtmA* compared to wild-type following extended culture. Data sorted by fold change, in descending order.

<table>
<thead>
<tr>
<th>Protein Description</th>
<th>Log₂(Fold Increase)</th>
<th>Peptides</th>
<th>Seq coverage [%]</th>
<th>Protein IDs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldo-keto reductase. In uncharacterised secondary metabolite cluster.</td>
<td>2.85624</td>
<td>9</td>
<td>36.2</td>
<td>AFUA_2G01410</td>
</tr>
<tr>
<td>Tyrosyl-tRNA synthetase, cytoplasm, nucleus localization</td>
<td>2.85558</td>
<td>10</td>
<td>34.5</td>
<td>AFUA_5G10640</td>
</tr>
<tr>
<td>ABC transporter; Cdr1B, mutation causes increased azole sensitivity</td>
<td>2.4345</td>
<td>18</td>
<td>16.2</td>
<td>AFUA_1G14330</td>
</tr>
<tr>
<td>MFS monocarboxylate transporter, putative</td>
<td>2.29482</td>
<td>2</td>
<td>4.5</td>
<td>AFUA_3G03320</td>
</tr>
<tr>
<td>ABC multidrug transporter Mdr1</td>
<td>2.19999</td>
<td>25</td>
<td>25</td>
<td>AFUA_5G06070</td>
</tr>
<tr>
<td>Aminotransferase family protein, putative</td>
<td>2.09468</td>
<td>12</td>
<td>38</td>
<td>AFUA_2G13295</td>
</tr>
<tr>
<td>Glycerate dehydrogenase</td>
<td>2.01153</td>
<td>7</td>
<td>28</td>
<td>AFUA_1G13630</td>
</tr>
<tr>
<td>ThiI/PfpI family protein; abundant in conidia</td>
<td>1.93416</td>
<td>9</td>
<td>55.1</td>
<td>AFUA_5G01430</td>
</tr>
<tr>
<td>Glutamyl-tRNA(Gln) amidotransferase, subunit A</td>
<td>1.93045</td>
<td>12</td>
<td>34.5</td>
<td>AFUB_092380</td>
</tr>
<tr>
<td>MFS gliotoxin efflux transporter GliA, encoded in the gliotoxin biosynthetic gene cluster</td>
<td>1.82652</td>
<td>4</td>
<td>9.2</td>
<td>AFUA_6G09710</td>
</tr>
</tbody>
</table>
Table 2: Top 10 proteins with decreased abundance in *A. fumigatus ΔgtaM* compared to wild-type following extended culture. Data sorted by fold change, in descending order.

<table>
<thead>
<tr>
<th>Protein Description</th>
<th>Log₂(Fold Decrease)</th>
<th>Peptides</th>
<th>Seq coverage [%]</th>
<th>Protein IDs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-heme Fe(II) and alpha-ketoglutarate-dependent dioxygenase; catalyses the conversion of fumitremorgin B to verruculogen</td>
<td>-4.32466</td>
<td>21</td>
<td>87.3</td>
<td>AFUA_8G00230</td>
</tr>
<tr>
<td>Phytanoyl-CoA dioxygenase family protein</td>
<td>-4.17772</td>
<td>12</td>
<td>46.8</td>
<td>AFUA_8G00480</td>
</tr>
<tr>
<td>O-methyltransferase, putative</td>
<td>-3.85167</td>
<td>11</td>
<td>70.6</td>
<td>AFUA_8G00390</td>
</tr>
<tr>
<td>IgE-binding protein</td>
<td>-3.76683</td>
<td>3</td>
<td>34.5</td>
<td>AFUA_6G00430</td>
</tr>
<tr>
<td>Putative methyl transferase; member of the pseurotin A gene cluster; conidia-enriched protein; hypoxia induced protein</td>
<td>-3.28917</td>
<td>28</td>
<td>79.7</td>
<td>AFUA_8G00550</td>
</tr>
<tr>
<td>Cysteine-rich secreted protein</td>
<td>-3.27599</td>
<td>15</td>
<td>63.3</td>
<td>AFUA_7G01060</td>
</tr>
<tr>
<td>Conserved hypothetical protein, hypoxia induced protein</td>
<td>-3.24847</td>
<td>12</td>
<td>84.7</td>
<td>AFUA_7G01060</td>
</tr>
<tr>
<td>DltD N-terminal domain protein (BF Unique)</td>
<td>-3.16375</td>
<td>15</td>
<td>78.4</td>
<td>AFUA_8G00380</td>
</tr>
<tr>
<td>Glutathione S-transferase, putative</td>
<td>-2.96915</td>
<td>11</td>
<td>41</td>
<td>AFUA_4G14380</td>
</tr>
<tr>
<td>Putative secreted 1,4-beta-D-glucan glucanhydrolase</td>
<td>-2.90284</td>
<td>27</td>
<td>53.3</td>
<td>AFUA_7G06140</td>
</tr>
</tbody>
</table>
Table 3: Proteins encoded by the intertwined secondary metabolite supercluster on chromosome 8 of *A. fumigatus* (AFUA_8G00100-00720) with decreased abundance in *A. fumigatus ΔgtmA* compared to wild-type grown for 28 days in Czapek-Dox media. Data sorted by fold change, in descending order.

<table>
<thead>
<tr>
<th>Protein Description</th>
<th>Log₂ (Fold Decrease)</th>
<th>Peptides</th>
<th>Seq coverage [%]</th>
<th>Protein IDs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Putative prenyltransferase involved in fumitremorgin B biosynthesis</td>
<td>Absent</td>
<td>16</td>
<td>39.8</td>
<td>AFUA_8G00250</td>
</tr>
<tr>
<td>Polyketide synthase (PKS), encoded in the fma (fumagillin) secondary metabolite gene cluster; required for fumagillin biosynthesis</td>
<td>Absent</td>
<td>27</td>
<td>15.4</td>
<td>AFUA_8G00370</td>
</tr>
<tr>
<td>Protein of unknown function</td>
<td>Absent</td>
<td>8</td>
<td>18.2</td>
<td>AFUA_8G00630</td>
</tr>
<tr>
<td>Translation elongation factor eEF-1, gamma subunit, putative</td>
<td>Absent</td>
<td>9</td>
<td>36.7</td>
<td>AFUA_8G00580</td>
</tr>
<tr>
<td>Putative brevianamide F prenyltransferase, predicted to convert brevianamide F to tryprostatin B; involved in the biosynthesis of fumitremorgins</td>
<td>Absent</td>
<td>13</td>
<td>32.3</td>
<td>AFUA_8G00210</td>
</tr>
<tr>
<td>Putative O-methyltransferase with a predicted role in fumitremorgin B synthesis</td>
<td>Absent</td>
<td>22</td>
<td>68.9</td>
<td>AFUA_8G00200</td>
</tr>
<tr>
<td>Non-heme Fe(II) and alpha-ketoglutarate-dependent dioxygenase; catalyses the conversion of fumitremorgin B to verruculogen</td>
<td>-4.32466</td>
<td>21</td>
<td>87.3</td>
<td>AFUA_8G00230</td>
</tr>
<tr>
<td>Putative iron-dependent oxygenase; encoded in the fma (fumagillin) secondary metabolite gene cluster</td>
<td>-4.17772</td>
<td>12</td>
<td>46.8</td>
<td>AFUA_8G00480</td>
</tr>
<tr>
<td>Predicted O-methyltransferase; encoded in the fma (fumagillin) secondary metabolite gene cluster</td>
<td>-3.85167</td>
<td>11</td>
<td>70.6</td>
<td>AFUA_8G00390</td>
</tr>
<tr>
<td>Putative methyl transferase; member of the pseudotin A gene cluster; conidia-enriched protein; hypoxia induced protein</td>
<td>-3.28917</td>
<td>28</td>
<td>79.7</td>
<td>AFUA_8G00550</td>
</tr>
<tr>
<td>Hypoxia induced protein; encoded in the fma (fumagillin) secondary metabolite</td>
<td>-3.24847</td>
<td>12</td>
<td>84.7</td>
<td>AFUA_8G00430</td>
</tr>
<tr>
<td>Gene Cluster</td>
<td>Score</td>
<td>Fold Change</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>----------------------------------------------------------------------------</td>
<td>--------</td>
<td>-------------</td>
<td>-----------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Putative alpha/beta hydrolase; encoded in the fma (fumagillin) secondary</td>
<td>-3.16375</td>
<td>15</td>
<td>metabolite gene cluster</td>
<td></td>
</tr>
<tr>
<td>Protein of unknown function; encoded in the fma (fumagillin) secondary</td>
<td>-2.85247</td>
<td>10</td>
<td>metabolite gene cluster</td>
<td></td>
</tr>
<tr>
<td>Baeyer-Villiger monooxygenase (BVMO); hypoxia induced protein; encoded in</td>
<td>-2.74114</td>
<td>41</td>
<td>the fma (fumagillin) secondary metabolite gene cluster</td>
<td></td>
</tr>
<tr>
<td>Non-ribosomal peptide synthetase (NRPS); PKS/NRPS hybrid; multidomain</td>
<td>-1.60039</td>
<td>105</td>
<td>protein; required for pseurotin A production; transcript induced by voriconazole; induced by hypoxia and in infected mouse lungs</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1

_A. fumigatus_ Shaking Culture

- WT
- ΔgtmA
- gtmAC

200 rpm, 72 h; 37 °C.

_A. fumigatus_ Static Culture

- WT
- ΔgtmA
- gtmAC

Static, 25 d; 37 °C.

RP-HPLC Analysis

Quantitative Proteomics

RP-HPLC / LC-MS Analysis
Figure 2

B. Wild-Type

C. Wild-Type

Type

tmA

gtmA\textsuperscript{C}

PseudoA
Bis(methyl)gliotoxin
Gliotoxin

PseudoA
Bis(methyl)gliotoxin
Gliotoxin

PseudoA

Fumagill
Figure 3

Wild-Type  \( \Delta gtmA \)

\[ \begin{align*}
\uparrow & 171 \quad \text{Proteins Increased in Abundance} \\
\downarrow & 1208 \quad \text{Shared Proteins} \\
\uparrow & 89 \quad \text{Proteins Increased in Abundance}
\end{align*} \]
Figure 4

Fumagillin, Pseudothil and Fumitremorgin Biosynthesis

GliA

2SAM

GtiM

Dithiol Gliotoxin

GliT

O2

Gliotoxin Biosynthesis

Gliotoxin

Bis(methyl)gliotoxin