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Selenate sensitivity of a *laeA* mutant is restored by overexpression of the bZIP protein MetR in *Aspergillus fumigatus*

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Abstract

LaeA is a conserved global regulator of secondary metabolism and development in filamentous fungi. Examination of *Aspergillus fumigatus* transcriptome data of *laeA* deletion mutants have been fruitful in identifying genes and molecules contributing to the *laeA* mutant phenotype. One of the genes significantly down regulated in *A. fumigatus laeA* is *metR*, encoding a bZIP DNA binding protein required for sulfur and methionine metabolism in fungi. LaeA and MetR deletion mutants exhibit several similarities including down regulation of sulfur assimilation and methionine metabolism genes and ability to grow on the toxic sulfur analog, sodium selenate. However, unlike *metR*, *laeA* strains are able to grow on sulfur, sulfite, and cysteine. To examine if any parameter of the *laeA* phenotype is due to decreased *metR* expression, an over-expression allele (*OE::metR*) was placed in a *laeA* background. The *OE::metR* allele could not significantly restore expression of MetR regulated genes in *laeA* but did restore sensitivity to sodium selenate. In *A. nidulans* a second bZIP protein, MetZ, also regulates sulfur and methionine metabolism genes. However, addition of an *OE::metZ* construct to the *A. fumigatus laeA OE::metR* strain still was unable to rescue the *laeA* phenotype to wildtype with regards gliotoxin synthesis and virulence in a zebrafish aspergillosis model.

Keywords

Aspergillus fumigatus; sulfur metabolism; transcription factors; selenate

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Introduction

The filamentous fungus *Aspergillus fumigatus* is an opportunistic human pathogen frequently associated with diseases including allergic bronchopulmonary aspergillosis, mycotoxicosis, and invasive aspergillosis (IA), this latter disease with mortality rates up to 90% (Abad et al., 2010). Similar to other pathogenic microorganisms, *A. fumigatus* must obtain nutrients from its host. Indeed, it is becoming evident that blocking nutrient acquisition could be a key to preventing or ameliorating microbial pathogenesis (Abu Kwaik and Bumann, 2013). In its non-pathogenic state, *A. fumigatus* is a saprophyte and widespread in nature, obtaining nutrients from dead and decaying organic matter. Because of its saprophytic life cycle and thermo-tolerance, it is able to survive in variable environmental conditions and successfully compete for nutrients (Fleck et al., 2011).

Metabolic attributes of fungi play a significant role in pathogenicity. Recently, several studies have linked primary metabolism to the pathogenicity of fungi, including but not limited to nitrogen and carbon metabolism and availability of essential micronutrients like zinc, copper and iron (reviewed in (Boral et al., 2017)). For example, *A. fumigatus* is known to produce several siderophores including ferricrocin, ferrichrome, ferrichrome C, and N',N'',N-triacetylfusarinine C (TAF) out of which ferricrocin and TAF were found to be most abundant. These metabolites are required for iron scavenging for the fungus and deletion of *sidA*, encoding l-ornithine N 5-monooxygenase which is the first committed step in siderophore synthesis, generated a strain unable to grow either *in vitro* or *in vivo* in multiple vertebrate models of IA including murine and zebrafish models (Hissen et al., 2005; Knox et al., 2014; Schrettl et al., 2004). Copper homeostasis is also important in *A. fumigatus* virulence as it was shown that AceA and MacA (Mac1), transcription factors required for Cu mediated defense responses, are essential for pathogenesis in neutropenic models of IA depending on fungal strain (Cai et al., 2017; Wiemann et al., 2017). These are but a few examples where nutrient acquisition has been associated with the pathogenicity and overall survival of the fungus.

The ability of the fungus to adapt to its environment – be it as a saprophyte or pathogen – is multifactorial ranging from genes and molecules involved in primary metabolism to secondary metabolism. LaeA is one of the major virulence factors in *A. fumigatus* which was discovered in 2004 as a global regulator of secondary metabolism in *Aspergillus* spp. (Bok and Keller, 2004). LaeA is part of a conserved transcriptional complex in ascomycete fungi involved in orchestrating development with metabolism (Amaike and Keller, 2009; Bok and Keller, 2004; Jain and Keller, 2013; Lopez-Berges et al., 2013; Seiboth et al., 2012). Loss of this gene results in decreased virulence in all pathogenic fungi examined to date (Lopez-Berges et al., 2013; Wiemann et al., 2010; Wu et al., 2012) including *A. fumigatus* where *laeA* deletants were found aberrant in spore production, invasive growth and secondary metabolism (Ben-Ami et al., 2009; Bok et al., 2005; Dagenais and Keller, 2009; Sugui et al., 2007). The pleiotropic nature of LaeA was revealed from transcriptional microarray studies of *laeA* deletants where numerous genes and molecules involved in virulence and nutrient acquisition were mis-regulated compared to wild-type including some genes involved in the aforementioned siderophore and copper homeostasis pathways (Perrin et al., 2007). Among these genes were several LaeA regulated transcription factors

potentially contributing to the fitness and virulence defects of the *laeA* strain. For example, *nosA*, encoding a Zn(II)(2)Cys(6) transcription factor important in sexual development in *A. nidulans* (Vienken and Fischer, 2006) and significantly up-regulated in *A. fumigatus laeA*, was found to contribute to a germination defect of *laeA* mutants (Soukup et al., 2012).

Another class of transcription factors (TFs) are the basic leucine zipper (bZIP) domain TFs, a conserved family of TFs regulating diverse metabolic and stress response pathways in eukaryotes (Piłsyk et al., 2015; Wee et al., 2017). Filamentous fungi typically contain well over a dozen of these TFs (Kong et al., 2015). bZIPs typically form homo or hetero-dimers and bind to the promotor regions to regulate the transcription of their target genes. Several fungal bZIPs have been characterized and implicated in multiple phenomena including remediation of secondary metabolism (Sekonyela R et al., 2013), virulence (Amich et al., 2013; Kong et al., 2015; Schrettl et al., 2010), and the oxidative stress response (Wee et al., 2017). A connection between bZIP proteins and *laeA* have been found in both *A. flavus* and *A. nidulans*. In the former, the bZIP nitrogen regulatory gene *meaB* is overexpressed in the *A. flavus laeA* mutant and creation of synthetic *meaB* overexpression strains were found to mimic the *OE::laeA* phenotype (Amaike et al., 2013; Shaaban et al., 2010). The *A. nidulans* bZIP protein RsmA was identified in a multicopy-suppressor screen to find genes that could remediate *laeA* loss. Overexpression of *rsmA* was able to restore production of sterigmatocystin via transcriptional activation of sterigmatocystin biosynthetic genes (Shaaban et al., 2010).

In *A. fumigatus*, the bZIP MetR, which positively affects the transcriptional activation of sulfur assimilation and methionine anabolic genes in *Aspergillus* spp., is required for virulence (Amich et al., 2013; Natorff et al., 2003). MetR mutants are easily detected not only by inability to grow without added methionine to the medium but also to the resistance of *metR* mutants to selenate, a toxin taken up by sulfate permeases (which are not expressed in *metR* mutants, (Arst, 1968; Brzywczy et al., 2011). The regulation of sulfur metabolism by MetR might be more complex than originally thought as another TF (named MetZ) containing a bZIP domain similar to MetR was identified and described in *A. nidulans* (Piłsyk et al., 2015). *metZ* expression was not only dependent on MetR but it was also found to be under the regulation of sulfur metabolite repression (SMR) system unlike MetR itself. The SMR system allows for tight regulation of transcription of sulfur assimilation genes by selectively activating the TFs depending on sulfur status of the cell. Thus it was deduced that MetZ seems to be controlling the sulfur assimilation genes by fine tuning their expression (Piłsyk et al., 2015).

Because a previous transcriptional profiling study showed that both *metZ*, *metR* and many MetR regulated genes showed lower expression levels in *laeA* when compared to wild-type (Perrin et al., 2007), here we investigated if either MetR or MetZ contributed to the *laeA* phenotype in *A. fumigatus*. We hypothesized that downregulation of *metR* in particular could contribute to some of the phenotypes associated with *laeA* loss, including resistance to selenate, down regulation of sulfur/methionine assimilation/anabolic genes and reduced virulence (reviewed in (Jain and Keller, 2013). To address this question, we overexpressed either *metR* alone or in combination with *metZ* in the *laeA* background. Although the overexpression strains gained a wild type sensitivity to selenate, there was little increase in

MetR regulated gene expression and no restoration of wild type virulence in a zebrafish model of invasive aspergillosis.

Materials and Methods

Fungal strains and culture conditions

The strains and plasmids used in this study are listed in Table 1. All strains were grown at either 37°C or 25°C with appropriate supplements (2 mM methionine, 1.26 g/L uridine, and 0.56 g/L uracil). The strains were stored as glycerol stocks at –80°C.

Physiological studies

10³ conidia from each strain were point-inoculated (5 µl) onto solid glucose minimal media (GMM) (Shimizu and Keller, 2001) in three biological replicates and incubated at 25°C for 12 days and at 37°C for 5 days. Two mM methionine was used as a supplement for all strains when *metR* was included in an experiment. Radial growth was assayed by measuring the diameter of point-inoculated colonies after 5 days and 12 days incubation at 37°C and 25°C, respectively. Conidia were counted from agar cores taken from the center of the point-inoculated cultures.

Sulfur and methionine utilization

Dilution plating was performed with the strains on MM-S (minimal media minus sulfur) with different sulfur sources. Different sulfur sources included sodium sulfate (2 mM), sodium sulfite (2 mM), L-methionine (5 mM), L-methionine (5 mM) + sodium selenate (0.5 mM and 1 mM), and cysteine (2 mM). Glucose minimal media (GMM) and MM-S were used as a control. Dilution plating was done from 10⁵ spores to 10⁰ spores in 1 µl. The plates were incubated at 37°C for 48 hours.

Northern analysis

The strains were analyzed for gene expression using standard Northern blotting procedure. 10⁷ fungal spores/ml were grown in liquid minimal media for either 24 hours at 37°C or 60 hours at 25°C shaking at 250 r.p.m. Total RNA was extracted using Isol-RNA Lysis Reagent (5 Prime) as described previously (Sambrook J, 2001). Approximately 20 µg of total RNA was used for blotting. Blots were hybridized with DNA fragments generated by PCR of wild-type (WT) gDNA with gene specific primers (Table 2).

Semi-quantitative RT-PCR analysis

Semi-qRT-PCR analysis was performed using 10 µg RNA which was digested with DNase I (NEB Cat# M0303L) to remove any contaminating genomic DNA. cDNA synthesis reactions were performed using the Bio-Rad iScript cDNA Synthesis Kit (Cat# 170-8891) according to manufacturer's protocols. 50 ng of cDNA was used per reaction to amplify specific fragments using gene specific primers (Table 2).

Quantitative RT-PCR analysis

qRT-PCR analysis was performed using 10 µg RNA which was digested with DNase I (NEB Cat# M0303L) to remove any contaminating genomic DNA. cDNA synthesis reactions were performed using the Bio-Rad iScript cDNA Synthesis Kit (Cat# 170-8891) according to manufacturer's protocols. 50 ng of cDNA was used per reaction to amplify using gene specific primers (Table 2). qRT-PCR was performed using a Sensi FAST SYBR® No-ROX Kit (Bioline USA Inc., Taunton, MA, USA) on a CFX96 real-time PCR system (Bio-Rad). Relative quantification data were calculated for each gene using the threshold cycle (CT) method with actin as an internal control and normalization to the wild type. Statistical significance was measured using analysis of variance (ANOVA) in GraphPad Prism v6, and comparisons to the wild type were done using Dunnett's test.

Transformation and genetic manipulation

Fungal transformation was done as previously described (Palmer et al., 2008). The construct for the overexpression of *metR* was made using double-joint PCR (Szewczyk et al., 2006; Yu et al., 2004). Briefly, approximately 1.5-kb 5' flanking region was PCR amplified from gDNA of AF293 and fused to the *A. parasiticus pyrG::gpdA (P)* from plasmid pJMP9 and the *metR* open reading frame (primers are listed in Table 2). The *laeA* gene was disrupted with *A. fumigatus argB* in the AF293.6 (*pyrG1, argB1*) background to create TJMP19.2 (*laeA, pyrG1*). Over expression of *metR* was achieved by transformation of the construct into AF293.1 (*pyrG1*) and TJMP19.2 (*laeA, pyrG1*), which created TRSR22.1 (*OE::metR*) and TRSR22.3 (*laeA, OE::metR*) respectively. These strains were confirmed by Southern and northern blot (Fig S-1A–B and Fig S-2A). Deletion of *metR* was achieved by using double joint PCR (Szewczyk et al., 2006; Yu et al., 2004). Approximately 1 Kb of the 5' and 3' flanking region was PCR amplified from gDNA of AF293 and fused to *A. parasiticus pyrG* gene from pJMP7. The deletion cassette was transformed into AF293.1 (*pyrG1*) to generate TSJA1.1 (*metR*) which was confirmed by Southern and northern blotting (Fig S-1C and Fig S-2B). The construct for overexpression of both *metR* and *metZ* was assembled using homologous recombination in yeast from an adapted protocol (van Leeuwen et al., 2015). Briefly, *metR* ORF including terminator regions, *metZ* ORF including terminator regions, and *niiA niaD* divergent promoter were amplified from Af293 gDNA and fused with *A. parasiticus pyrG* amplified from pJMP9. The above fragments contained 20–35 bp overlapping bases to the correct flanking fragments for *in vivo* yeast recombination to create pSJA1. Plasmids were confirmed by restriction digests and sequencing. The overexpression construct was then transformed into a wild type background Af293.1 (*pyrG1*) generating TSJA2.1 (*OE::metRmetZ*) and a *laeA* background TJMP19.2 (*laeA, pyrG1*) generating TSJA3.1 (*OE::metRmetZ; laeA*). All strains were confirmed by PCR, Southern, and northern blotting. Both *OE::metRmetZ* and *OE::metRmetZ; laeA* were always grown on minimal media with nitrate as nitrogen source to induce over-expression including when grown for northern analysis.

Gliotoxin Analysis

Strains were assessed for gliotoxin production. Briefly, 5×10^6 conidia per ml were inoculated into 125ml flasks containing 50ml liquid minimal media. Strains were grown at

25°C for 3 days shaking at 250rpm. Three-day old cultures were harvested, mycelia was filtered and 5 ml of culture supernatant was extracted with 5ml of organic solvent (ethyl acetate). The top layer of the ethyl acetate was then taken off and instantly dried in a speed-vac. Dried extracts were re-suspended in 50% acetonitrile, filtered through 0.2µ filters, and analyzed on HPLC. The gliotoxin peak was identified by comparing with purified standard bought from Sigma (Cat#: G9893).

Pathogenicity

Virulence was assessed in a vertebrate model of invasive aspergillosis using larval zebrafish as previously described (Knox et al., 2014) with slight modifications. Based on previous studies showing a requirement for functional neutrophils to observe a virulence attenuation for the *A. fumigatus laeA* strain (Knox et al., 2014), all infections were performed in macrophage-deficient *irf8*^{-/-} larvae in which neutrophils retain function (Shiau et al., 2015). Briefly, conidial stocks in PBS at a concentration of 1.5×10^8 cells/mL were mixed with 1% phenol red to obtain a final injection concentration of 1.0×10^8 . Spore suspensions were microinjected at a volume of 3 nL/larvae and localized to the hindbrain ventricle of anesthetized 48 hours post fertilization larvae. Each infected larva was housed individually in a single well of a 96-well plate in ~100 µL of E3-MB and stored at 28.5 °C for the duration of each 7-day survival assay. Mortality was determined by lack of detectable heartbeat. Statistical analysis for survival assays were performed by pooling 3 independent experimental replicates and generating *P* values via Cox proportional hazards regression analysis.

Results

LaeA regulates expression of sulfur assimilation and methionine metabolism genes

In 2007, Perrin *et al.* conducted a genome wide transcriptional microarray study with an *A. fumigatus laeA* mutant compared to wild-type. This study revealed that *metR* and *metZ* were downregulated (2 fold and 1.5-fold respectively) in the *laeA* mutant. MetR and MetZ are bZIP transcription factors regulating the transcription of genes involved in sulfur assimilation, methionine and cysteine metabolism in *Aspergillus* spp. (Amich et al., 2013; Natorff et al., 2003). Further analysis of the microarray data for the genes associated with sulfur assimilation and methionine metabolism indicated that many of the genes in the pathway were significantly down-regulated in *laeA* as compared to wild-type (Fig 1 and Table 3) (Perrin et al., 2007).

Overexpression of *metR* does not restore expression of sulfur assimilation genes in *laeA*

To determine if decreased expression of *metR* was responsible for the decreased transcription of the sulfur/methionine pathway genes in *laeA*, two over-expression (OE) strains were created through standard transformation processes (*OE::metR* and *OE::metR; laeA*) (Fig S-1A–B and Fig S-2A). Gene expression was assessed using quantitative RT-PCR in all the strains as compared to wild-type and *laeA* controls. The decrease in expression of these genes, including *metR* itself, was largely confirmed in the *laeA* strain (Fig 2). However, the *OE::metR* allele had little impact on these genes in either

the wild-type or *laeA* background, suggesting a need for additional transcriptional machinery for MetR function.

MetR overexpression does not restore wild type sporulation to *laeA*

Although we did not see an increase in expression in the sulfur/methionine pathway genes in the *OE::metR* strains, we considered it possible that this allele might impact other aspects of the *laeA* phenotype. Thus *OE::metR* and *OE::metR; laeA* strains were assessed for any ability to restore *laeA* phenotypes to wild type growth, in particular colony growth and sporulation. The *OE::metR; laeA* was unable to restore wild type characteristics in any of these parameters as shown in Fig 3a–c (radial growth) and Fig 3d–e (spore production). Radial growth and sporulation of *OE::metR* was comparable to wild-type (Fig 3a–e).

Over-expressing *metR* in *laeA* background restores sensitivity to the toxic sulfur analog selenate

Sodium selenate is a toxic analog of sulfate that has been used to identify *Aspergillus nidulans* sulfur assimilation mutants including mutations in sulfate permeases (Sul1 and Sul2), ATP sulfurylase and PAPS reductase (Arst, 1968; Natorff et al., 2003). The most distinct characteristics of *metR* and sulfur assimilation mutants are their inability to grow on several sulfur sources and resistance to sodium selenate. As shown in Fig 4a, *laeA* exhibited wild type growth on medium supplemented with sulfite, sulfate, methionine and cysteine but exhibited a *metR* phenotype of enhanced growth on sodium selenate supplemented medium compared to wild type. *OE::metR* in a *laeA* background, on the other hand, restored sensitivity to selenate as the double mutant grew much slower than *laeA* as indicated by its decreased radial growth (Fig 4a). This was confirmed by point inoculation on agar plates and quantifying the radial growth (Fig 4b–d).

Addition of a MetZ overexpression construct does not restore a wild type developmental or gliotoxin phenotype to *laeA*

Considering that a second bZIP transcription factor MetZ works in possible coordination with MetR in *A. nidulans*, we considered that over-expressing the *A. fumigatus* homolog of MetZ could be important in restoration of some of the *laeA* phenotypes. Thus we generated two over-expression strains, *OE::metRmetZ*, and *OE::metRmetZ laeA* (Fig S-3A–B and Fig S-4). These strains were assessed for colony growth and sporulation. The *OE::metRmetZ laeA* was unable to restore wild type characteristics in any of these parameters as shown in Fig 5a (radial growth), Fig 5b (spore production). Radial growth and sporulation of *OE::metRmetZ* was comparable to wild-type (Fig 5a–e). However the *OE::metRmetZ; laeA* strain was sensitive to selenate, similar to that of *OE::metR; laeA* and wild-type (Fig 6a–c).

LaeA is a known positive regulator of the toxin gliotoxin (Bok and Keller, 2004). Thus we were also interested to determine any impact of *OE::metR* and *OE::metRmetZ*, on gliotoxin synthesis, especially due to the known interplay of gliotoxin metabolism and the methyl/methionine cycle (Owens et al., 2015). As shown in supplementary Fig S-6, gliotoxin synthesis remained depressed in all strains harboring a *laeA* allele.

Overexpression of *metR* and *metZ* in the *laeA* background does not impact the virulence attenuation of *laeA* in immunosuppressed zebrafish larvae

With *LaeA* being a known virulence factor, we next investigated whether reconstitution of *metR* and *metZ* expression in a *laeA* background would restore virulence. While *LaeA* is a known pathogenicity factor in several models of invasive aspergillosis, the mechanism(s) underlying *LaeA*'s role in pathogenicity remain largely unknown. To this end, we utilized the larval zebrafish as a vertebrate model of invasive aspergillosis to test if *MetR* or *MetZ* had a measurable impact on *LaeA*-mediated pathogenicity. Importantly, our data (Fig 7) recapitulate the previously described virulence attenuation for a *laeA* strain of *A. fumigatus* compared to an isogenic control in macrophage-deficient larvae (Knox et al., 2014). Overexpression of *metR* and *metZ* in a *laeA* background, however, failed to produce any significant change in virulence. Additionally, overexpression of *metR* and *metZ* alone was insufficient to alter the virulence in reference to an isogenic control (Fig 7).

Discussion

LaeA, a nuclear protein affecting global gene expression, is a conserved protein in filamentous fungi and of significant interest due its requirement for morphological and chemical development (Bok and Keller, 2004; Lopez-Berges et al., 2013; Seiboth et al., 2012; Zhang et al., 2016), biofuel enzyme activity (Li et al., 2016; Seiboth et al., 2012) and involvement in virulence of plant, fungal and human pathogenic fungi (Amaike and Keller, 2009; Bi et al., 2013; Karimi Aghchegh et al., 2013; Kumar et al., 2017; Lopez-Berges et al., 2013). Microarray analysis of *laeA* mutants show *LaeA* to regulate at least 10 percent of the genome (Fekete et al., 2014; Perrin et al., 2007; Wang et al., 2017). Our interest in *metR*, encoding a bZIP transcription factor known to regulate sulfur assimilation genes in *Aspergillus nidulans* and *A. fumigatus*, developed because the transcript level of this gene was significantly reduced in a *laeA* strain (Perrin et al., 2007). Because several of the known phenotypes of the *laeA* strain have been attributed to or corrected by mis-regulation of specific TFs (e.g. gliotoxin reduction due to suppression of *gliZ*, (Bok and Keller, 2004) or germination and radial growth aberrations associated with over expression of *nosA*, (Soukup et al., 2012), we thought it possible that some *laeA* traits could be a consequence of altered *metR* expression.

As might be expected for a strain with weak *metR* expression, many *MetR* regulated genes are down regulated in the *laeA* mutant (Fig 1 and 2), similar to reports of *metR* mutants (Amich et al., 2013; Brzywczy et al., 2011). Another similarity between *laeA* and *metR* was their relative resistance to the toxic metabolite selenate (Fig 4 and 6). Previous reports indicate that selenate is taken up by cells through sulfur regulated permeases and is incorporated into sulfur containing compounds (including amino acids). Due to this replacement of S with Se, resulting proteins become non-functional and the fungus starves to death. Enzyme activity associated with Se toxicity include PAPS reductase and ATP sulfurylase. In 1968, Arst reported that a double sulfur uptake/ATP sulfurylase mutant (the first two steps of the sulfur assimilation pathway, Fig. 1) was able to tolerate roughly fifty times more selenate than a single mutant of PAPS reductase (Arst, 1968; Natorff et al., 2003; Tweedie and Segel, 1970). We speculated that *laeA* resistance to selenate might stem from

the fact that ATP sulfurlyase (*AFU3g06530*) and PAPS reductase (*AFU3g06540*) are downregulated in this mutant, hence selenate is not taken up by the cells as efficiently as in wild-type. Although OE:*metR* in a *laeA* background restored the sensitivity to selenate, the expression of ATP sulfurlyase gene (*AFU3g06530*) PAPS reductase (*AFU3g06540*) were not significantly increased in the OE::*metR*; *laeA* double mutant which might suggest other enzymes or permeases involved in selenate transport or possibly post-transcriptional regulation of these enzymes. Moreover, due to broad transcriptional reprogramming that MetR is involved in the cell, we cannot rule out the possibility of non-specific effects of its over-expression.

What makes it particularly more complex is that the transcription factors related to sulfur metabolism are controlled by ubiquitin ligases belonging to SCF family (Sieko et al., 2014). In *A. nidulans*, the activity of MetR is dependent on the sulfur status of the cell and controlled by ubiquitin ligases encoded by the *scon* genes which have homologs in *A. fumigatus* although not very well characterized. In *Schizosaccharomyces pombe* Zip1 (MetR) is phosphorylated, allowing it to interact with SCF-Pof1 leading to its degradation (Harrison et al., 2005) and in budding yeast SCF-Met30 complex plays a role in degradation of Met4 (MetZ) (Rouillon et al., 2000). Similarly, it is thought that MetR is repressed under high cellular methionine/cysteine levels consequently repressing the MetR regulated genes (Piłsyk et al., 2015). An assessment of the cellular levels of sulfur/sulfur-containing amino acids present in the *A. fumigatus* mutants (*laeA* and OE::*metR*; *laeA*) could possibly point towards a mechanism for selenate resistance. Moreover, *A. fumigatus* sulfur acquisition could be different from *A. nidulans* since some sulfate transporters identified in *A. nidulans* seems to be absent in *A. fumigatus* like the *astA* gene (alternative sulfate transporter) (Piłsyk et al., 2007). Furthermore, considering the pleiotropic effects of *LaeA*, we speculate that the transcriptional remodeling in OE::*metR*; *laeA* would be different from OE::*metR* and could also contribute to the different phenotypes.

Because no other wild-type physiological or developmental characteristics were recovered OE:*metR*; *laeA* double mutant, including gene expression of sulfur metabolism genes, we considered that MetR function might require presence of MetZ due to the report that MetZ – only found in the Eurotiales fungal taxon - works in co-ordination with MetR to regulate sulfur and methionine assimilation in *A. nidulans* (Piłsyk et al., 2015). However, overexpression of both bZIPs MetR and MetZ in the *laeA* strain still did not restore gene expression of the downregulated genes of sulfur metabolism pathway in a *laeA* background as shown in Fig S-5 for select genes. This may not be surprising after considering the qRT-PCR results (Fig 2) where we found that *metZ* was significantly downregulated in the OE::*metR* strains in addition to *laeA* strains. This result points, once again, to the complexity of the transcriptional regulation of sulfur metabolism. We speculate that in *A. fumigatus*, *metR* and *metZ* may not necessarily work in synergy consequently overexpressing both bZIPs at the same time may not help in restoring expression of sulfur metabolism genes. However, sensitivity to selenate was again restored, which might solely be due to MetR since OE::*metR* was equally sensitive to selenate despite reduction in *metZ* gene expression.

As mentioned earlier, *laeA* strains also exhibit reduced ability to produce a gliotoxin, an epipolythiodioxopiperazine alkaloid characterized by a unique disulfide bridge (Bok and Keller, 2004). Our results suggest that gliotoxin production was not restored by overexpressing either MetR or MetZ. This may be explained by the fact that although MetR is required for utilization of sulfur sources except methionine (Amich et al., 2013), the biosynthetic gene cluster of gliotoxin on the other hand may be expressed independently of these bZIPs and requires the expression of other TFs like GliZ (Bok and Keller, 2004).

LaeA is also a well-established virulence determinant in plant and animal ascomycete fungal pathogens (reviewed in (Jain and Keller, 2013), yet ascribing the specific pathway(s) regulated by the pleiotropic *LaeA* to virulence attenuations has remained difficult. Therefore, we hypothesized that overexpression of *metR* (also a virulence factor, (Amich et al., 2013) and *metZ* in a *laeA* background might restore virulence to wild-type levels in a larval zebrafish model of invasive aspergillosis. As described previously, there is a host immune-specific virulence defect for *laeA* exclusively within macrophage-deficient, but not neutrophil-deficient, zebrafish larvae (Knox et al., 2014). From this, we evaluated the role of MetR and MetZ to remediate virulence attenuation in a *laeA* strain of *A. fumigatus* using *irf8*^{-/-} animals (Shiau et al., 2015). Consistent with most of our other data in which overexpression of *metR* and *metZ* fails to restore most of the sulfur- and methionine-related phenotypes of the *laeA* strain, we observed no amelioration of virulence attenuation in the *laeA;OE::metRmetZ* strain compared to *laeA* (Fig 7). Taken together, in light of the metabolic flexibility and redundancy of *A. fumigatus* pathogenicity (Amich and Krappmann, 2012), restoration of full virulence in a *laeA* strain of *A. fumigatus* will likely not be accomplished through the reinstallation of a single gene or pathway.

This study was motivated from previous reports where synthetic expression of transcription factors regulated by *LaeA* were able to restore some of the associated *laeA* phenotypes (Amaike et al., 2013; Soukup et al., 2012). Here, we report here that despite an overlap in positive regulation of sulfur/methionine assimilation pathway genes by *LaeA* and MetR, overexpression of *metR* was unable to rescue the aberrancies associated with *laeA* strains except for selenate tolerance. Addition of an overexpression *metZ* allele – postulated to work in concert with MetR in *A. nidulans* – displayed an equivalent phenotype as the *OE::metR laeA* strain thus also failing to restore sulfur/methionine gene expression. Although restoration of selenate sensitivity in the over-expression strains makes a convincing argument for upregulation of sulfur uptake pathway by MetR, it was difficult to call to a specific mechanism at this point.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. Sulfur metabolism genes are downregulated in *laeA* deletion mutants.
2. *laeA* mutants are resistant to selenate (toxic analog of sulfate).
3. Over-expression of *metR* and *metZ* restores selenate sensitivity in *laeA* mutants.
4. Over-expression of *metR* and *metZ* does not restore other *laeA* abberancies.

Figure 1. Sulfur/methionine assimilation pathway in yeast with their homologs in *A. fumigatus* (reformatted from (Thomas and Surdin-Kerjan, 1997))

The genes shown in red are significantly down-regulated in *laeA* compared to wild-type as analyzed in Perrin et al., 2007.

Figure 2. qRT-PCR analysis of select genes in *A. fumigatus* strains

Relative gene expression of each gene in OE::metR, $\Delta laeA$, and OE::metR $\Delta laeA$ *A. fumigatus* strains is shown. metR and Act1 (Afu6g04740) serve as a control. Expression of each gene was normalized to Actin (Act1) and relative expression was calculated by comparing to wild type. Asterisks in the figure indicate statistical significance (*, **, ***, **** indicating p-value <0.05, 0.01, 0.001, 0.0001 respectively). Labels on top indicate gene annotations in *Aspergillus nidulans*, labels in parenthesis indicate yeast homologs.

Figure 3. Radial growth and sporulation of *A. fumigatus* strains

A. 10^3 conidia from wild-type, OE::metR, *laeA*, and OE::metR *laeA* strains were point inoculated on glucose minimal media and incubated at both 25°C and 37°C for 12 days and 5 days respectively. Pictures of top of the plates are shown. **B and C.** Radial growth was quantified and plotted using GraphPad Prism software. Levels not connected by the same letter are significantly different ($p < 0.05$) according to a 1 way ANOVA test. **D and E.** Spores from each strain were counted using a haemocytometer. Means $\pm$ standard deviations are indicated for three replicates of each strain. Levels not connected by same letter are significantly different ($P > 0.05$) according to a 1 way ANOVA test.

Figure 4. Sulfur and methionine utilization of *A. fumigatus* strains

A. Dilution plating was performed with wild-type, OE::metR, *laeA*, OE::metR *laeA* and *metR* strains on MM-S (minimal media minus sulfur) with different sulfur sources. Different sulfur sources included sodium sulfate (2mM), sodium sulfite (2mM), L-methionine (5mM), L-methionine (5mM) + sodium selenate (0.5mM and 1mM), and cysteine (2mM). Glucose minimal media (GMM) and MM-S were used as a control. Dilution plating was done from 10^5 spores to 10^0 spores in $1\mu\text{l}$. The plates were incubated at 37°C for 48 hours. **B.** Strains were grown on glucose minimal media and MM-S with 5mM L-methionine and 0.5mM sodium selenate at 37°C for 3 days. **C and D.** Radial growth was quantified by measuring diameters and plotting them using GraphPad Prism software. Means $\pm$ standard deviations are indicated for three replicates of each strain. Levels not connected by same letter are significantly different ($P > 0.05$) according to a 1 way ANOVA test.

Figure 5. Average radial growth of *A. fumigatus* strains

A. 10^3 conidia from wild-type, OE::metRmetZ, *laeA*, and OE::metRmetZ *laeA* strains were point inoculated on glucose minimal media and incubated at both 25°C and 37°C for 12 days and 5 days respectively. Pictures of top of the plates are shown. **B and C.** Radial growth was quantified and plotted using GraphPad Prism software. Levels not connected by the same letter are significantly different ($p < 0.05$) according to a 1 way ANOVA test. **D and E.** Spores were counted using a haemocytometer. Means $\pm$ standard deviations are indicated for three replicates of each strain. Levels not connected by same letter are significantly different ($P > 0.05$) according to a 1 way ANOVA test.

Figure 6. Selenate sensitivity assay of *A. fumigatus* strains

A. Wild-type, OE::metRmetZ, *laeA*, and OE::metRmetZ *laeA* strains were grown on glucose minimal media and MM-S with 5mM L-methionine and 0.5mM sodium selenate at 37°C for 3 days. **B and C.** Radial growth was quantified using ImageJ (area) and plotting using GraphPad Prism software. Means ± standard deviations are indicated for three replicates of each strain. Levels not connected by same letter are significantly different ($P > 0.05$) according to a 1 way ANOVA test.

Figure 7. Virulence in a vertebrate model of invasive aspergillosis

Infections were performed using macrophage-deficient *irf8*^{-/-} zebrafish larvae at 2 days post fertilization and individual larvae followed for survival through 7 days post infection (dpi). Survival data shown on a Kaplan-Meier plot from three pooled independent experimental replicates. Statistical analysis was performed using Cox proportional-hazard regression analysis.