TrpE feedback mutants reveal roadblocks and conduits toward increasing secondary metabolism in Aspergillus fumigatus

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\textbf{A B S T R A C T}

Small peptides formed from non-ribosomal peptide synthetases (NRPS) are bioactive molecules produced by many fungi including the genus Aspergillus. A subset of NRPS utilizes tryptophan and its precursor, the non-proteinogenic amino acid anthranilate, in synthesis of various metabolites such as Aspergillus fumigatus fumiquinazolines (Fqs) produced by the \textit{fmq} gene cluster. The \textit{A. fumigatus} genome contains two putative anthranilate synthases – a key enzyme in conversion of anthranilic acid to tryptophan – one beside the \textit{fmq} cluster and one in a region of co-linearity with other \textit{Aspergillus} spp. Only the gene found in the co-linear region, \textit{trpE}, was involved in tryptophan biosynthesis. We found that site-specific mutations of the TrpE feedback domain resulted in significantly increased production of anthranilate, tryptophan, \textit{p}-aminobenzoate and fumiquinazolines FqF and FqC. Supplementation with tryptophan restored metabolism to near wild type levels in the feedback mutants and suggested that synthesis of the tryptophan degradation product kynurenic acid could negatively impact Fq synthesis. The second putative anthranilate synthase gene next to the \textit{fmq} cluster was termed \textit{icsA} for its considerable identity to isochorismate synthases in bacteria. Although \textit{icsA} had no impact on \textit{A. fumigatus} \textit{Fq} production, deletion and over-expression of \textit{icsA} increased and decreased respectively aromatic amino acid levels suggesting that \textit{icsA} can draw from the cellular chorismate pool.

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1. Introduction

The Kingdom Fungi constitutes an unparalleled genomic resource of natural product (also called secondary metabolite) pathways with many fungi containing up to 70 secondary metabolite gene clusters in their genome (Cacho et al., 2014). Although some natural products – both harmful and beneficial – are produced in copious quantities under laboratory growth conditions, most are produced either in small quantities or not at all. Numerous efforts have recently been aimed at inducing production of these lowly expressed metabolites, primarily through over-expression of the secondary metabolite cluster genes in endogenous and/or exogenous host systems (Anyagoo and Mortensen, 2015).

Not all over expression efforts have resulted in success. Many reasons could account for these failures, ranging from toxicity of the products to the host system, unknown post-transcriptional regulations, requirement of other genes not in the cluster, and lack of sufficient primary metabolite pools. All natural products originate from various primary metabolites. Polyketides are derived from acyl coenzyme A (CoA) and malonyl-CoA units, terpenes from isoprene units and non-ribosomal peptides from proteinogenic and non-proteinogenic amino acids (Keller et al., 2005).

Non-ribosomal peptides are synthesized by non-ribosomal peptide synthetases (NRPS), which consist minimally of A (adenylating) and PCP (peptide carrier protein), C (condensation) and releasing domains. The A domain determines which amino acid is incorporated into the growing chain. For example, \textit{Aspergillus fumigatus} \textit{FmqA} is a three A domain NRPS incorporating anthranilate, \textit{L}-tryptophan and \textit{L}-alanine into the precursor peptide fumiquinazolone F (FqF) (Ames et al., 2010). FqF is further processed by additional enzymes to the end metabolite FqC (Fig. 1). As a considerable subset of fungal NRPS utilize both anthranilate and \textit{L}-tryptophan in production of valuable alkaloid peptides (Ames and Walsh, 2010), we became interested in the potential contribution of modulating tryptophan biosynthesis to increase...
production of alkaloid peptides using fumiquinazolines (Fqs) as the targeted metabolites.

Fig. 2 presents the known and predicted A. fumigatus enzymes involved in aromatic amino acid metabolism. Chorismate is a common intermediate for the aromatic amino acids anthranilate, L-tryptophan, L-phenylalanine and L-tyrosine in yeast (Braus, 1991). L-tryptophan is derived from chorismate by five enzymatic steps encoded by four genes in A. fumigatus (Fig. 2 and Table 1). Anthranilate synthase (AS) [EC 4.1.3.27] catalyzes the production of anthranilate from chorismate in the L-tryptophan biosynthesis branch. ASs have been characterized in bacteria (Bae et al., 1989; Ito et al., 1969; Palmer, G.C. et al., 2013), in plants (Morino et al., 2005; Saika et al., 2012; Tozawa et al., 2001) and in Saccharomyces cerevisiae (Braus, 1991; Graf et al., 1993). In yeast, AS consists of two subunits (AAS-I and AAS-II) that are necessary to yield anthranilate from chorismate by addition of an amino group from L-glutamine (Graf et al., 1993). AAS-I is responsible for chorismate binding, and AAS-II is a glutamine amidotransferase that catalyzes the transfer of the amino group (Graf et al., 1993). A tryptophan feedback loop specified by amino acids in the AAS-I subunit (Graf et al., 1993) makes AS the rate-limiting enzyme in L-tryptophan synthesis. Feedback inhibition resistant forms of this enzyme – allowing for increased tryptophan pools – have been associated with increases in several plant natural products including the alkaloid lochericine in transgenic hairy root cultures of the periwinkle Catharanthus roseus (Hughes et al., 2004) and indole alkaloids in rice (Dubouzet et al., 2013). In Aspergillus spp., the AAS-II encoding gene, trpE, was first characterized in Aspergillus nidulans in 1977 (Käfer, 1977) but, until now, trpE encoding AAs-I has only been defined by predicted enzymatic activity.

Here, we show that AAS-I feedback mutants in A. fumigatus enhance Qp production. The A. fumigatus genome contains two putative AAS-I proteins, one beside the fmq cluster and one in a region of co-linearity with other Aspergillus spp. Over-expression of the fmq-cluster-associated gene (icsA/Afu6g12110) could not complement the tryptophan auxotrophy of the trpE (Afu6g12580) deletion mutant, and had no significant difference on Qp production. However, over-expression of icsA resulted in significantly decreased anthranilate, L-tryptophan, L-phenylalanine, and L-tyrosine levels, suggesting that IcsA metabolizes chorismate into a yet unknown product in A. fumigatus. Phylogenetic analysis showed it to be closely related to bacterial isochorismate synthases. The second gene, trpE (Afu6g12580), encoded the canonical AAS-I required for L-tryptophan production. Creation of synthetic feedback resistant alleles of TrpE resulted in strains that produced approximately 1.5-fold more FqF and FqC, and displayed a 120+ fold increase in intracellular levels of anthranilate and up to 3+ fold more tryptophan than the control strain on minimal medium. Supplementation with L-tryptophan eliminated any gain in Fq production and in fact lowered production in the single site feedback mutant, potentially through increases in the L-tryptophan degradation product L-kynurenine.

2. Material and methods

2.1. Strains and medium

Strains used or created in this study are listed in Table 2. The genetic background of the primary strain used in this study is A. fumigatus AF293 (Osherov et al., 2001). All strains were maintained as glycerol stocks at −80 °C, and activated on solid glucose minimal media (GMM) at 37 °C (Shimizu and Keller, 2001). Growth media was supplemented with 1.26 g/L uridine and 0.56 g/L uracil for pyrG auxotrophs, 1 g/L L-arginine for argB auxotrophs, and 1 g/L L-tryptophan for trpE auxotrophs.

2.2. Genetic manipulations

Fungal DNA extraction, gel electrophoresis, restriction enzyme digestion, Southern blotting, hybridization and probe preparation were performed according to standard methods (Sambrook and Russell, 2001). For DNA isolation, A. fumigatus strains were grown for 24 h at 37 °C in steady state liquid GMM, supplemented as needed for auxotrophs. DNA isolation was performed as described by Sambrook and Russell (2001). Gene deletion mutants in this study were constructed by targeted integration of the deletion cassette through transformation (Lim et al., 2012b; Szewczyk et al., 2006). The deletion cassettes were constructed using a double-joint fusion PCR (DJ-PCR) approach (Lim et al., 2012b; Szewczyk et al., 2006). A. fumigatus protoplast generation and transformation were carried out as previously described (Lim et al., 2012b; Szewczyk et al., 2006). The plasmids used in this work are listed in Table S1 and all primers are listed in Table S2.
An *A. fumigatus trpE* (Afu6g12580) disruption cassette consisted of the following: 1 kb DNA fragment upstream of the *trpE* start codon (primers DAf6g12580F1 and DAf6g12580R1), a 2.7 kb selectable auxotrophic marker, *A. fumigatus argB* (Afu6g09830), cloned from plasmid pJMP4 (Palmer, J.M. et al., 2013) (primers GF A. Fumi argB F and GF A. Fumi argB R), and 1 kb DNA fragment downstream of the *trpE* stop codon (primers DAf6g12580F2 and DAf6g12580R2). The deletion construct was transformed into AF293.6 (pyrG1, argB1). Transformants were screened for arginine prototroph in media supplemented with L-tryptophan for *trpE* auxotrophs, uridine and uracil for pyrG auxotrophs. Transformants obtained were verified by PCR using primers PM-F6g12580F and PM-F6g12580R, and Southern analysis using a *trpE* probe SB1 (primers DAf6g12580F1 and DAf6g12580R2) (Fig. S1). TPMW6.2 (*AtrpE*, *pyrG1*) was chosen for further experiments.
In order to complement the ΔtrpE mutant with a wild-type and feedback insensitive trpE copies, the plasmids pPMW1 (trpE\(^{\text{C}}\)) and pPMW2 (trpE\(^{\text{S77L}}\)) and pPMW3 (trpE\(^{\text{S66R,S77L}}\)) were constructed using in vivo yeast recombination (Yin et al., 2013b) of 4 linear DNA fragments consisting of (1) the gpdA(p) promoter obtained by PCR from plasmid pMP9.1 (Lim et al., 2012a) (with primers PM-yAup-gpdA F and PM-yAup-gpdA R), (2) a AspgDNA2 shuttle plasmid pYH-yA-riboA (Pal et al., 2012), (3) the first half of wild-type or a Ser66Arg-mutated trpE gene (obtained by PCR from A. fumigatus wild-type genomic DNA with primers PM-Afu6gR2, respectively (Table S2). The selection marker, cassette. To construct the ΔicsA gene (obtained by PCR AF293.1 (pyrG1; argB1 Xue et al. (2004)) using the primer pair ParapyrGF/ParapyrGR from parasiticus pyrG and A. fumigatus genomic DNA (Yin et al., 2013b) to release Avr II and Sac II sites of pJMP9.1 (Lim et al., 2012). The resulting plasmids pPMW4 (OE::trpE\(^{\text{C}}\)), pPMW5 (OE::trpE\(^{\text{S77L}}\)), pPMW6 (OE::trpE\(^{\text{S66R,S77L}}\)) and the parent plasmid pMP9.1 were transformed into the auxotrophic ΔtrpE mutant TPMW6.2 (ΔtrpE, pyrG1) to yield mutants TPMW8.9 (trpE\(^{\text{C}}\)), TPMW9.5 (trpE\(^{\text{S77L}}\)), TPMW10.9 (trpE\(^{\text{S66R,S77L}}\)) and TPMW12.4 (ΔtrpE), respectively. These mutants were selected for tryptophan and pyrimidine prototrophy in media supplemented with l-tryptophan for trpE auxotrophs, in case site- in vivo yeast recombination (Yin et al., 2013b) of 4 linear DNA fragments consisting of (1) the gpdA(p) promoter obtained by PCR from plasmid pMP9.1 (Lim et al., 2012a) (with primers PM-yAup-gpdA F and PM-yAup-gpdA R), (2) a AspgDNA2 shuttle plasmid pYH-yA-riboA (Pal et al., 2012), (3) the first half of wild-type or a Ser66Arg-mutated trpE gene (obtained by PCR from A. fumigatus wild-type genomic DNA with primers PM-Afu6gR2, respectively (Table S2). The selection marker, cassette. To construct the ΔicsA gene (obtained by PCR AF293.1 (pyrG1; argB1 Xue et al. (2004)) using the primer pair ParapyrGF/ParapyrGR from parasiticus pyrG and A. fumigatus genomic DNA (Yin et al., 2013b) to release Avr II and Sac II sites of pJMP9.1 (Lim et al., 2012). The resulting plasmids pPMW4 (OE::trpE\(^{\text{C}}\)), pPMW5 (OE::trpE\(^{\text{S77L}}\)), pPMW6 (OE::trpE\(^{\text{S66R,S77L}}\)) and the parent plasmid pMP9.1 were transformed into the auxotrophic ΔtrpE mutant TPMW6.2 (ΔtrpE, pyrG1) to yield mutants TPMW8.9 (trpE\(^{\text{C}}\)), TPMW9.5 (trpE\(^{\text{S77L}}\)), TPMW10.9 (trpE\(^{\text{S66R,S77L}}\)) and TPMW12.4 (ΔtrpE), respectively. These mutants were selected for tryptophan and pyrimidine prototrophy in media supplemented with l-tryptophan for trpE auxotrophs, in case site- in vivo yeast recombination (Yin et al., 2013b) of 4 linear DNA fragments consisting of (1) the gpdA(p) promoter obtained by PCR from plasmid pMP9.1 (Lim et al., 2012a) (with primers PM-yAup-gpdA F and PM-yAup-gpdA R), (2) a AspgDNA2 shuttle plasmid pYH-yA-riboA (Pal et al., 2012), (3) the first half of wild-type or a Ser66Arg-mutated trpE gene (obtained by PCR from A. fumigatus wild-type genomic DNA with primers PM-Afu6gR2, respectively (Table S2). The selection marker, cassette. To construct the ΔicsA gene (obtained by PCR AF293.1 (pyrG1 and TPMW6.2 (ΔtrpE, pyrG1) using a pyrG marker cassette. To construct the icsA deletion cassette, two 1 kb fragments flanking the ORF were amplified from AF293 genomic DNA using the primer pair DAfu6gF1/DAfu6gR1 and DAfu6gF2/DAfu6gR2, respectively (Table S2). The selection marker, Aspergillus parasiticus pyrG, was PCR amplified from plasmid pJW24 (Calvo et al., 2004) using the primer pair ParapyrGF/ParapyrGR.
To construct an icsA over-expression strain at its locus, its native promoter was replaced with the constitutive promoter, gpdA(p), amplified from plasmid pJM9.1 using the primer pair OEpyrGppD/F/OEpyrGppDR. The two 1 kb fragments for the icsA over-expression cassette were amplified from A. fumigatus genomic DNA using the primer pair DAfu6gF1/OEAFfu6gR1 and OEAFfu6gF2/DAfu6gR2, respectively (Table S2). Transformants were selected for pyrimidine prototrophy in media without any supplements and amended with l-tryptophan in case TPMW6.2 was used as its native promoter was replaced with the constitutive promoter, (OE::icsA). Over-expression of icsA was verified via Northern analysis for TPMW1.70 compared to the complemented parental strain TJW55.2, and the icsA deletion strain TPMW1.13 (Fig. S4).

2.3. Phylogenetic analysis

For phylogenetic analysis, reviewed and curated sequences from the Swiss-Prot database (www.uniprot.org) of proteins containing a chorismate binding domain (isochorismate synthase, anthranilate synthase and p-aminobenzoate synthase) were retrieved and aligned using ClustalW (MegAlign, DNASTar, Madison, WI, USA) together with the protein sequences of Afu6g121110/OEsA, Afu6g04820/PabaA, and Afu6g12580/TrpE (www.aspergillus.org) (Cerqueira et al., 2014). From the alignment, the chorismate binding domains were extracted based on the conserved canonical binding domain from the Conserved Domain Database (CDD) (www.ncbi.nlm.nih.gov). Phylogenetic analysis of the chorismate binding domains was performed using MAFFT (http://mafft.cbrc.jp/alignment/software/) (Katoh et al., 2002) and (http://www.microbesonline.org/fasttree/) (Price et al., 2009). Results were visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree/) collapsing branches with bootstrap values below 70% support value. For phylogenetic analysis of the indoleamine 2,3-dioxygenase (ido) proteins, sequences from Aspergillus and Fusarium species were obtained from the National Center for Biotechnology Information (NCBI) by performing a protein blast search using the S. cerevisiae Bna2p sequence as query. Sequences were processed and visualized including Ido sequences previously analyzed by Yuasa and Ball (2011, 2012, 2013) as described for the chorismate binding domain proteins above.

2.4. Physiology experiments

Colony diameters of strains were measured after 3 days of growth at 37 °C on solidified GMM and GMM supplemented with 5 mM l-tryptophan, respectively. Strains were point-inoculated onto the media at 10^6 conidia total (in 5 µL). Conidial production studies were performed on solid GMM and tryptophan plates. For each plate, a 10 µL top layer of cool but molten agar that contained 10³ spores of each strain, respectively, was added. Strains were cultured at 37 °C for 24 h. A core of 1.5 cm diameter was removed from the plates and homogenized in 2 mL 0.01% Tween 80 to release the spores. Spores were counted on a hemocytometer. Three replicates were used for each assay and statistical significance was calculated with analysis of variance (ANOVA) using Prism 6 software (GraphPad).

2.5. Primary metabolites extraction and analysis

A. fumigatus strains were inoculated into 50 mL of liquid GMM and GMM supplemented with 5 mM l-tryptophan, respectively, at 10³ conidia per mL and cultured at 25 °C and 250 rpm for 84 h in triplicates. Fusarial tissue was collected by rapid filtration using miracloth and immediately frozen in liquid nitrogen. About 0.1 g of fungal tissue was transferred to a 15 mL conical vial containing 3 mL extraction solvent (2/2/1 (v:v:v) acetonitrile/methanol/water) cooled on dry ice. After homogenization and centrifugation, 2 mL of supernatant was filtered using a 0.45 µm PTFE Mini-UniPrep filter (Agilent). The supernatant of these hyphal metabolites were used for metabolite analysis. For metabolite measurement, samples were dried under N₂ and resuspended in LC–MS grade water (Sigma–Aldrich). Samples were analyzed using an HPLC–MS system consisting of a Dionex UPLC® coupled by electro-spray ionization (ESI; negative mode) to a hybrid quadrupole–high-resolution mass spectrometer (Q Exactive orbitrap, Thermo Scientific) operated in full scan mode. Liquid chromatography separation was achieved using an ACQUITY UPLC® BEH C18 (2.1 × 100 mm column, 1.7 µm particle size, Waters). Solvent A was 97:3 water:methanol with 10 mM tributylamine and 10 mM acetic acid, pH 8.2; solvent B was methanol. The gradient was: 0 min, 5% B; 1.5 min, 5% B, 11.5 min, 95% B; 12.5 min, 95% B; 13 min, 5% B; 14.5 min, 5% B. Autosampler and column temperatures were 4 °C and 25 °C, respectively. Metabolite peaks were identified by their exact mass and matching retention time to those of pure standards (Sigma–Aldrich).

2.6. RNA extraction and northern analysis

For northern expression analysis of trpE and icsA in A. fumigatus wild-type AF293 at different time point, wild-type strain AF293 was grown in liquid glucose minimal media (GMM) (replacing nitrate with 20 mM glutamine as nitrogen source) at 37 °C and 250 rpm for 24 h. Then mycelia were collected, transferred into solid GMM and grown in duplicates for the indicated time at 37 °C, To verify over-expression of icsA via northern blot, TPMW1.13 (ΔicsA), TPMW1.70 (OE::icsA) and the complemented strain TJW55.2 were grown in liquid GMM at 37 °C and 250 rpm for 24 h. For northern analysis, strains TPMW8.9 (trpEc), TPMW9.5 (trpEcST77), TPMW10.9 (trpEcS66R,ST77) and the complemented control strain TJW55.2 were inoculated into 50 mL of liquid GMM containing 20 mM glutamine as nitrogen source as indicated in the text according to Schrettl et al. (2008). Strains were grown for 24 h at 37 °C, 250 rpm with an initial spore concentration of 10⁶ conidia per mL. Mycelia were harvested by filtration through Miracloth (Calbiochem) and 2 g of mycelia were transferred into 50 mL of liquid GMM and GMM supplemented with 5 mM l-tryptophan, respectively. Strains were grown in duplicates for 1 h at 29 °C and 250 rpm. Mycelia were harvested by filtration through Miracloth. Total RNA was extracted with Trizol reagent (Invitrogen) from freeze-dried mycelia, according to the manufacturer’s protocol. Northern analysis was performed as described by Sambrook and Russell (2001). Northern analysis for trpE used trpE probe SB1 (primers DAfu6g12580F1 and DAfu6g12580R2), for icsA used icsA probe SB3 (primer pair DAfu6gF1/DAfu6gR2). Other probes for northern analysis were constructed using primers listed in Table S2 and labeled with dCTP α³²P.

2.7. Secondary metabolite extraction and profiling

Secondary metabolites of strains were extracted after 6 days of growth at 29 °C on solidified GMM and GMM supplemented with 5 mM l-tryptophan, respectively. Strains were point-inoculated onto the media at 10³ conidia total (in 5 µL). Agar cores (1.5 cm in diameter) were prepared in triplicate for each strain cultured. Three cores from each plate were extracted with 2.5 mL of ethyl acetate. The solvent was evaporated and suspended in 500 µL 19.5/79.5/1 (v/v/v) acetonitrile/water/formic acid. Subsequently, the samples were filtered using a 0.45 µm PTFE Mini-UniPrep filter.
vial (Agilent) and 50 μL of the filtrate analyzed by high-performance liquid chromatography (HPLC) (Perkin Elmer) coupled to a photo diode array (PDA) as described by Wiemann et al. (2014). Fq quantification was performed as described by Ames and Walsh (2010).

3. Results

3.1. The A. fumigatus genome contains a canonical anthranilate synthase and a putative isochorismate synthase

When characterizing the fnq cluster (Ames et al., 2010; Lim et al., 2014), a putative anthranilate synthase subunit I (AAS-I), Afu6g12110, was found 8.3 kb downstream of the fnq gene cluster (Fig. 1A). Considering the requirement for both L-tryptophan and anthranilate for Fq synthesis (Fig. 1B), it seemed reasonable that Afu6g12110 could be involved in synthesis of both amino acids. Further analysis of the genome, however, revealed a canonical AAS-I, Afu6g12580, which was present in region of co-linearity in all sequenced Aspergillus species. In contrast genes displaying significant homology to Afu6g12110 were only detected in a subset of Aspergillus species located at dispersed genomic locations (http://www.aspergillusgenome.org). A comparison of the two proteins indicated they shared 30% identity (E value = 6 × 10⁻23), primarily along the conserved chorismate binding domain. As a first step toward determining the function of the two potential anthranilate synthases in A. fumigatus, a phylogenetic tree was created. Our results show that Afu6g12580 is closely related to characterized AAS-I of fungi including A. nidulans, Neurospora crassa, and S. cerevisiae (Fig. 3) and thus was named TrpE following terminology used in A. nidulans. By contrast, the protein sequence of Afu6g12110 was most closely related to bacterial isochorismate synthases (Fig. 3) and named lcsA for isochorismate synthase. Notably lcsA lacked the feedback inhibition domain found in TrpE and all known AAS-I (Fig. 4A) but contained a chorismate binding domain also found in PabaA (Afu6g04820). Under culture conditions with nitrate as sole nitrogen source, trpE but not lcsA was strongly expressed (Fig. 4B).

To determine if these proteins were required for tryptophan biosynthesis, we deleted and over-expressed both genes in the A. fumigatus AF293 genetic background. Southern analysis and Northern analysis of transformants for each gene identified correct biosynthesis, we deleted and over-expressed both genes in the (Table 2). The ΔtrpE mutant could not grow unless supplemented with L-tryptophan. Complementation of ΔtrpE with a trpE allele restored wild-type-like growth (Fig. 5A). In contrast neither the ΔlcsA nor the OE::lcsA strains exhibited a phenotypic difference to the respective control strains on any media examined in this study (Figs. 5A and S5). Furthermore, the OE::lcsA allele could not complement the tryptophan auxotrophy of ΔtrpE (Fig. 5A) thus suggesting that lcsA played no major role in tryptophan or anthranilate biosynthesis.

3.2. TrpE feedback mutants exhibit increased production of anthranilate and fumiquinazolines FqF and FqC and lcsA mutants alter amino acid pools

To create feedback insensitive mutants in A. fumigatus, we first aligned the TrpE sequence with known AAS-I proteins and readily located the amino acids involved in feedback inhibition (Fig. 5B). Two mutant alleles were created, one was designed to change serine residue 77 to leucine (S77L), and the other was designed to additionally change serine residue 66 to arginine (S66R). These residues were chosen as they have been shown essential for tryptophan feedback inhibition in yeast (Graf et al., 1993) and some plants (Kanno et al., 2005; Saika et al., 2012). Transformation of the ΔtrpE strain with a wild-type and these two mutant trpE alleles, respectively, yielded three strains TPMW8.9 (trpE⁺), TPMW9.5 (trpE⁶⁶R) and TPMW10.9 (trpE⁷⁷L) for comparison of aromatic acid metabolism and Fq synthesis (Fig. S2).

Although there were no significant differences in growth or sporulation among the three strains on either GMM or GMM supplemented with 5 mM L-tryptophan (Fig. 5C), we observed a large intracellular buildup of anthranilate (ca. 120 fold vs. control strain trpE⁺) in the feedback mutant trpE⁶⁶R grown on GMM medium (Fig. 6A). The substantial increase in intracellular anthranilate was consistent with the loss of tryptophan feedback in this enzyme, which resulted in the increased biosynthesis of the related intermediates. In agreement with this, we also observed increased tryptophan levels (4-fold) in the trpE⁶⁶R mutant. Surprisingly, we also found that p-aminobenzoate (7-fold) and to a small extent phenylalanine (1.4-fold) were increased in this mutant. The trpE⁷⁷L-trpE⁶⁶R feedback mutant displayed smaller but still significant increased levels of anthranilate (ca. 13-fold) and p-aminobenzoate (5.5-fold). Examination of Fq production in these three strains showed that FqC and FqF production was significantly increased by 1.3–1.8-fold in both feedback mutants grown on GMM medium (Fig. 6A).

We then assessed the metabolome of the lcsA mutants. Although there was no significant impact of either mutant on Fq production, deletion of lcsA resulted in a significant increase in phenylalanine and tyrosine levels (as well as alanine) whereas OE::lcsA exhibited significantly decreased anthranilate, L-tryptophan, L-phenylalanine, and L-tyrosine levels (Fig. 6B). Together this data suggested that lcsA metabolizes chorismate into a yet unknown metabolite thereby making the common substrate less available for TrpE and AroC, respectively (Fig. 2).

To assess if the TrpE feedback mutants could be pushed to further increase Fq production, media was supplemented with L-tryptophan. We found this treatment primarily restored metabolite levels of the feedback mutants to those of wild type. There were no differences between wild type and the trpE⁷⁷L mutant (Fig. 6C). The trpE⁶⁶R-lcsA mutant still accumulated significantly more anthranilate, p-aminobenzoate and phenylalanine than wild type but at much less fold than in GMM medium. Furthermore, in contrast to results on GMM, this mutant produced less FqC and FqF than wild type (Fig. 6C). Finally, two tryptophan degradation products, L-kynurenine and indolepyruvate were detected in all three strains grown on tryptophan medium. The single site mutant produced significantly more L-kynurenine than either the wild type or the double site mutant, possibly suggesting that increases in L-kynurenine production could be related to decreases in Fq production (Fig. 6C).

3.3. Transcriptional profiling of tryptophan metabolism genes

To help interpret the changes in primary and secondary metabolite synthesis on GMM and GMM supplemented with L-tryptophan, we next examined a transcriptional profile of L-tryptophan metabolic genes in both GMM and GMM medium supplemented with L-tryptophan for the two feedback mutants (trpE⁷⁷L and trpE⁶⁶R⁷⁷L) and complemented control trpE⁺ (Fig. 7). Genes were identified through blast analysis using characterized genes from tryptophan metabolic enzymes in S. cerevisiae and KEGG L-tryptophan metabolism (http://www.genome.jp/)
Fig. 3. Maximum likelihood phylogenetic analysis of chorismate-binding domains extracted from 99 characterized protein sequences. Monophyletic leaves of relevant proteins are boxed in gray. *A. fumigatus* proteins are indicated in bold.
(kegg/pathway.html) to identify putative genes and encoded proteins illustrated in Fig. 2 and Table 1.

Chorismate is a common precursor for metabolic pathways leading to the formation of \( \alpha \)-tryptophan, \( p \)-amino benzoate, salicylate, and \( \alpha \)-phenylalanine/\( \alpha \)-tyrosine in microbes and plants (Braus, 1991; Maeda and Dudareva, 2012; Tzin and Galili, 2010). Four putative enzymes, encoded by \( pabaA \), \( aroc \), \( icsA \), and \( trpE/trpC \) (Fig. 2, Table 1), theoretically could compete for and utilize chorismate as a substrate to catalyze the committed step of the respective pathways to produce \( p \)-amino benzoate, \( \alpha \)-phenylalanine/\( \alpha \)-tyrosine, isochorismate, and \( \alpha \)-tryptophan in \( A. fumigatus \) (Fig. 2). Although we did not detect isochorismate in our analysis, over-expression of \( icsA \) decreased anthranilate, \( \alpha \)-tryptophan, \( \alpha \)-phenylalanine, and \( \alpha \)-tyrosine levels suggesting that \( IcsA \) can reduce the cellular chorismate pool (Fig. 6B). Furthermore, our metabolic data (Fig. 6) supported the activities of the other enzymes and suggested that...
pabaA and aroC may be up-regulated in the feedback-domain site mutants grown on GMM medium. However, this was not borne out at the transcriptional level as there were no differences in pabaA or aroC expression between the wild type and feedback mutants in either GMM or L-tryptophan supplemented medium (Fig. 7). In fact, there were no differences in gene expression between these three strains for any of the genes assessed. Tryptophan supplementation, however, did impact gene expression. Two putative L-tryptophan degradation genes, aroH and idoB were highly up-regulated in all three strains (Fig. 7). aroH (Afu2g13630) encodes a putative aromatic aminotransferase involved in transamination of L-tryptophan to generate indolepyruvate and idoB (Afu4g09830) encodes a putative indoleamine 2,3-dioxygenase which could be involved in dioxygenation of L-tryptophan as the first step to produce L-kynurenine. This matched well with production of these two products by A. fumigatus grown on L-tryptophan supplemented medium (Fig. 6B). Two other genes, Afu3g14250 and Afu7g02010, also encode putative indoleamine 2,3-dioxygenases with Afu7g02010 weakly expressed under these conditions. Based on the phylogenetic analysis of indoleamine 2,3-dioxygenases (Fig. S6), we name these genes idoA (Afu3g14250), idoB (Afu4g09830), and idoC (Afu7g02010).

4. Discussion

A. fumigatus is an opportunistic human pathogen renown for its secondary metabolites that are thought to contribute to disease development (Bok et al., 2005). The fumiquinazolines are signature metabolites of A. fumigatus and comprise a family of cytotoxic peptidyl alkaloids, which have received considerable interest due to their complex biochemistry, antitumor properties and cellular localization (Ames et al., 2010; Han et al., 2007; Lim et al., 2014).
but until now, nothing is known of the effect of primary metabolism on their biosynthesis. Here our efforts focused on the effect of manipulating tryptophan biosynthesis on Fq production.

Anthraniolate is a non-proteinogenic aryl α-amino acid generated from enzymatic amination of chorismate by anthranilate synthases (ASSs), and serves as the framework for the indole ring of the amino acid l-tryptophan (Amer and Walsh, 2010). Along with l-tryptophan, anthranilate is incorporated into several fungal peptide and amino acid L-tryptophan (Ames and Walsh, 2010). Along with L-tryptophan, anthranilate is incorporated into several fungal peptidyl alkaloids including Fq, the benzodiazepine dione, ardeemin and asperlicin (Amer and Walsh, 2010; Walsh et al., 2013). AS consists of two subunits, anthranilate synthase subunit I (AAS-I) termed TrpE as described here, and AAS-II termed TrpC (Käfer, 1977), respectively in Aspergillus species. We found trpE was present in a region of co-linearity in all sequenced Aspergillus spp. while a second protein, IcsA, encoded by a gene near the fmq cluster and with considerable homology to TrpE was more closely related to bacterial isochorismate synthases and lacked the tryptophan feedback domain found in TrpE and all known AAS-I (Figs. 3 and 4A). The ΔtrpE mutant required exogenous l-tryptophan for growth and could not be rescued by OE::icsA. However, over-expression of icsA decreased anthranilate, l-tryptophan, l-phenylalanine, and l-tyrosine levels suggesting that IcsA can draw from the cellular chorismate pool (Fig. 6B). A protein alignment of the A. fumigatus IcsA and TrpE with characterized bacterial ASS-I and isochorismate synthases revealed that important residues that changed enzymatic activity of the Salmonella typhimurium ASS-I from producing anthranilate to isochorismate (Plach et al., 2015) are identical to the A. fumigatus IcsA, suggesting that it is likely responsible for isochorismate formation although we did not identify this metabolite in our study. Identification of the putative IcsA metabolic product will be a future task.

The bacterium Pseudomonas aeruginosa possesses two ASs, TrpEG and PhnAB (Palmer, G.C. et al., 2013). Although only the former and not the latter is required for l-tryptophan biosynthesis in P. aeruginosa, over-expression of phnAB can rescue a trpEG deletion. A third protein harboring a chorismate binding domain is bifunctional PchA in P. aeruginosa (Fig. 3) and was shown to be involved in isochorismate and subsequently salicylic acid production, that is incorporated into the bacterium’s siderophores, mediating iron uptake (Serino et al., 1997). Based on the chorismate binding domains, IcsA from A. fumigatus is closely related to Mbtl from Mycobacterium tuberculosis (Fig. 3), that is also responsible for salicylic acid formation (Harrison et al., 2006). E. coli harbors two enzymes, EntC and MenE, that are responsible for isochorismate production (Buss et al., 2001), that also group with IcsA in our analysis (Fig. 3). Bioinformatic analysis shows some but not all Aspargillus contain putative IcsA homologs, all missing the feedback domain and often located near secondary metabolite clusters. Although fungal siderophores do not contain salicylic acid or isochorismate (Haas, 2014), A. fumigatus was shown to produce at least one other amino acid-derived small molecule with iron binding abilities (Wiemann et al., 2014; Yin et al., 2013a). What the role, if any, IcsA-like proteins play in fungal physiology has yet to be elucidated.

The importance of the feedback regulation of AS in the control of metabolic flow in l-tryptophan production and secondary metabolites has been demonstrated in many plants expressing feedback-resistant AS genes (Dubouzet et al., 2013; Hong et al., 2006; Hughes et al., 2004; Ishihara et al., 2006; Li and Last, 1996; Tozawa et al., 2001). Here, inactivation of the putative feedback domain in TrpE of A. fumigatus also had a significant effect on aromatic amino acid metabolism and Fq production with the single mutation strain trpE577L consistently having a greater impact on metabolism than the double site mutant. It was only this mutant which showed a significant increase in l-tryptophan pools, similar to reports of increased l-tryptophan pools in feedback mutants in plants (Hughes et al., 2004; Ishihara et al., 2006; Li and Last, 1996; Tozawa et al., 2001). This same strain also showed modest but significant increases in one of the two other aromatic amino acids, l-phenylalanine (Fig. 6A). As Fqs contain l- alanine, l-tryptophan, and anthranilate, the decreased l- alanine was consistent with the increased Fqs. However, both feedback mutants showed large increases in anthranilate and p-aminobenzoate that possibly diverted precursor pools from fumiquinazolines as reflected by the modest increases in FqE and FqC when grown on minimal medium (Fig. 6A). p-aminobenzoate is synthesized by PabaA and PabaB (Table 2 and Fig. 1) in Aspergillus spp. and strains with inactivation of either gene require p-aminobenzoate for growth (Brown et al., 2000; Mellado et al., 2015; Tang et al., 1994). Possibly less efficient versions of these enzymes could reduce p-aminobenzoate accumulation and enhance Fq synthesis. Studies in E. coli and Arabidopsis thaliana have shown that the p-aminobenzoate synthase complex is not inhibited by tryptophan, but by 2-fluorochorismate and methotrexate, respectively (Bulloch et al., 2004; Parsons et al., 2002; Sahr et al., 2006). Whether or not these or analogous inhibitors could direct metabolite flux toward Fq biosynthesis in A. fumigatus could be explored in future studies.

An interesting finding was that supplementation with tryptophan restored metabolite levels in the TrpE feedback mutants to those near the control strain. This suggests that there is an additional regulatory control mechanism(s) for tryptophan synthesis other than anthranilate synthase that is controlled differentially by externally added tryptophan and tryptophan produced by A. fumigatus itself. Intracellular amino acid pools are stored in vacuoles (Sekito et al., 2008) and their accessibility will differ from supplementation from external sources. Our data allude to different intra- and extracellular amino tryptophan sensing mechanisms in A. fumigatus, supporting a hypothesis of distinct nitrogen sensing mechanisms in other filamentous fungi (Wagner et al., 2013; Wiemann and Tüzynski, 2013). Our expression profiling data suggests that several tryptophan degradation pathways could be important in this regard (Fig. 7). The decrease of FqE and FqC in the TrpE single feedback mutant grown on l-tryptophan amended medium may be reflective of not only differential regulatory mechanisms and diversion of chorismate to p-aminobenzoate and l-phenylalanine but also to the increase in the degradation product l-kyurenine (Fig. 6C).

l-Kynurenine is produced by indoleamine 2,3-dioxygenase (Ido) activity. Whereas S. cerevisiae has a single Ido gene (BNA2) (Panozzo et al., 2002), A. fumigatus, like many other filamentous Ascomycetes, possesses three putative Ido genes, idoA (Afu3g14250), idoB (Afu4g09830) and idoC (Afu7g02010) (Fig. 2 and Table 1) in its genome (Yuasa and Ball, 2013). One of them, idoB, was highly induced and a second, idoC, slightly induced by external l-tryptophan (Fig. 7). Enzymatic studies of Aspergillus oryzae Ido enzymes suggest two of the three enzymes, IdoX and IdoJ, may participate in tryptophan degradation (Yuasa and Ball, 2011). The Idos showed higher activity and lower K_m values (higher affinity for substrates), similar to the enzymatic properties of Bna2p in S. cerevisiae (Yuasa and Ball, 2011; 2012). However, idoA, the homolog of A. oryzae idox, was not induced by tryptophan under our conditions (Fig. 7). Instead idoB, the homolog of A. oryzae idox was most highly induced by l-tryptophan. The third Ido in A. oryzae, IdoY, had a closer relationship to bacterial Idos than fungal Idos (Fig. S6) and showed very high K_m values (low affinity for substrates) and low catalytic efficiencies for l-tryptophan (Yuasa and Ball, 2012). idoC, the homolog of idoy, was slightly induced by l-tryptophan. Our data suggests that A. fumigatus idoB is mostly likely to contribute to l-kyurenine production.

I-Tryptophan can also be metabolized via the indolepyruvate pathway. AroH (termed Aro8 in S. cerevisiae (Iraqui et al., 1998)) is a key enzyme in the transamination of I-tryptophan to generate indolepyruvate and aroH was highly induced by I-tryptophan feeding. Similar to S. cerevisiae, AroH could also participate in L-phenylalanine and L-tyrosine synthesis in A. fumigatus (Fig. 2) and therefore could direct metabolite pools to L-phenylalanine and L-tyrosine as well as toward I-tryptophan degradation. Thus it is likely that both AroH and IdoB activities could divert pathways from fumiquinazoline synthesis, a hypothesis we will explore in future studies. 

In summary, we have genetically characterized two putative A. fumigatus chorismate binding proteins, TrpE and IcsA. TrpE was required for I-tryptophan production and its deletion results in tryptophan auxotroph, and over-expression of the fmq-cluster-associated gene (icsA/Atfu6g12110) could not complement the tryptophan auxotroph of deletion trpe mutant or significantly change the Fq production. However, deletion and over-expression of icsA resulted in significantly changed anthranilate, L-tryptophan, L-phenylalanine, and L-tyrosine levels, suggesting that IcsA metabolizes chorismate into a yet unknown product in A. fumigatus. Synthetetic feedback resistant alleles of TrpE had a significant effect in the synthesis of the sideroaphore mycobaclin, reveals it to be a salicylate synthase. J. Bacteriol. 188, 6081–6091.


