Curr Genet (2003) 44: 211–215
DOI 10.1007/s00294-003-0434-z

RESEARCH ARTICLE

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4′-Phosphopantetheinyl transferase-encoding *npgA* is essential for siderophore biosynthesis in *Aspergillus nidulans*

Received: 18 April 2003 / Revised: 27 June 2003 / Accepted: 15 July 2003 / Published online: 24 September 2003

Abstract *Aspergillus nidulans* produces two major siderophores: it excretes triacetylfusarinine C to capture iron and contains ferricrocin as an intracellular iron-storage compound. Siderophore biosynthesis involves the enzymatic activity of nonribosomal peptide synthetases (NRPS). NRPS contain 4′-phosphopantetheine as an essential prosthetic group, which is attached by 4′-phosphopantetheinyl transferases. *A. nidulans* appears to possess at least one gene, *npgA*, encoding such an enzyme. Using a strain carrying a temperature-sensitive allele, *cfwA2*, we showed that NpgA is essential for biosynthesis of both the peptide bond-containing ferricrocin and the ester bond-containing triacetylfusarinene C. The *cfwA2* strain was found to be iron-starved at the restrictive temperature during iron-replete conditions, consistent with the siderophore system being the major iron-uptake system—as we recently demonstrated. Northern analysis indicated that, in contrast to other genes which are involved in siderophore biosynthesis and uptake, expression of *npgA* is not controlled by the GATA-transcription factor SreA. It was shown previously that NpgA is required for biosynthesis of penicillin, pigment, and potentially lysine via the α-aminoadipate pathway. Supplementation with lysine plus triacetylfusarinine C restored normal growth of the *cfwA2* strain at the restrictive temperature, suggesting that the growth defect of the mutant is mainly due to impaired biosynthesis of siderophores and lysine.

Keywords Iron · Siderophore · Lysine · Phosphopantetheinyl transferase

Communicated by U. Kück

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Introduction

Virtually all organisms require iron for their growth, because this metal is used in many different types of cofactors, e.g. heme moieties and iron-sulfur clusters. Despite the fact that iron is the fourth most abundant element in the earth′s crust, the amount of bioavailable iron is very limited, because this metal is most commonly found as an insoluble ferric oxyhydroxide. Thus, microorganisms need specialized iron-mobilization systems (Guerinot 1994; Leong and Winkelmann 1998). In order to solubilize environmental iron, most microorganisms synthesize and excrete siderophores—low molecular weight, Fe(III)-specific chelators—in iron-depleted conditions. Subsequently, cells recover the iron from the ferric-siderophore complexes via specific uptake mechanisms (Winkelmann 2001; Haas 2003). Furthermore, most fungi possess intracellular siderophores as an iron-storage compound (Matzanke 1994). Siderophores have often been suggested to function as virulence factors, because the acquisition of iron is a key step in the infection process of any pathogen, since this metal is tightly sequestered by high-affinity iron-binding proteins in mammalian hosts (Weinberg 1999). Siderophore biosynthesis and uptake also represent possible targets for antifungal chemotherapy, because the underlying biochemical pathways are absent in human cells.

Various *Aspergillus* species are important pathogens of immunocompromised hosts, causing pneumonia and invasive disseminated diseases with high mortality (Latge 1999). In contrast to *A. fumigatus*, which may be regarded as the most important airborne pathogenic fungus, *A. nidulans* is a much rarer cause of human disease but represents a filamentous model fungus (Brookman and Denning 2000). *A. nidulans* produces two major siderophores: it excretes triacetylfusarinine C (TAFC) and contains intracellular ferricrocin (FC) (Charlang et al. 1981; Oberegger et al. 2001). Subsequent to chelating Fe(III), the siderophore-Fe(III) complex is taken up by MirB, a transporter belonging to the major

facilitator protein superfamily (Haas et al. 2003). After uptake, TAFC is cleaved by an esterase, the cleavage products are excreted, and Fe(III) is transferred to FC (Oberegger et al. 2001; Eisendle et al. 2003). TAFC is a cyclic "tripeptide" consisting of three N^2-acetyl-N^5-*cis*-anhydromevalonyl-N^5-hydroxyornithine residues linked by ester bonds; and FC is a cyclic "hexapeptide" with the structure Gly-Ser-Gly-(N^5-acetyl-N^5-hydroxyornithine)$_3$ (Winkelmann 1993). Based on biochemical and genetic studies of several microorganisms, biosynthesis of FC and TAFC is assumed to proceed according to the enzymatic steps depicted in Fig. 1 (Plattner and Diekmann 1994). Recently, we found that siderophore biosynthesis is essential for viability of *A. nidulans* (Eisendle et al. 2003). In this study, two genes involved in siderophore biosynthesis are characterized: *sidA* encoding L-ornithine N^5-monooxygenase, the first committed step in siderophore biosynthesis, and *sidC*, which encodes a nonribosomal peptide synthetase (NRPS) involved in synthesis of ferricrocin. NRPSs are large multifunctional enzymes with a modular construction able to assemble compounds from a remarkable range of proteinogenic and nonproteinogenic precursors (Kleinkauf and Von Dohren 1996; Weber and Marahiel 2001). Each module contains an adenylation domain, a condensation domain, and a peptidyl carrier domain. Like the acyl carrier domains in fatty acid and polyketide synthases, the peptidyl carrier domain requires attachment of a 4′-phosphopantetheine (Ppant) group by a dedicated PPTase. It was anticipated that most organisms employing more than one Ppant-dependent pathway also have more than one 4′-phosphopantetheinyl transferase (PPTase; Walsh et al. 1997). In contrast, it was shown that *Pseudomonas aeruginosa* possesses merely a single PPTase, which is required for both fatty acid and siderophore synthesis (Finking et al. 2002). Primarily, it was suggested that *Neurospora crassa*, *A. fumigatus*, and *A. nidulans* possess only a single PPTase (Keszenman-Pereyra et al. 2003). The encoding *A. nidulans* gene, *npgA*, was isolated by complementation of a null-pigment mutant (Han and Han 1993; Chung et al. 1996; Kim et al. 2001). Subsequently, *npgA* was found to complement a *Saccharomyces cerevisiae* strain defective in *lys5*, which encodes a PPTase required for activation of the α-aminoadipate semialdehyde reductase *Lys2* (Ehmann et al. 1999; Mootz et al. 2002). *Lys2* and consequently *Lys5* are essential for lysine biosynthesis in this yeast. Utilizing a strain harboring a temperature-sensitive allele of *npgA*, *cfwA2* (Aguirre et al. 1993), it was shown that NpgA is also required for synthesis of penicillin, which involves a NRPS step (Keszenman-Pereyra et al. 2003). Recently, a second putative PPTase-encoding gene was identified in the genome sequences of various fungi, including *A. nidulans*. However, its function remains unclear so far (D. Keszenman-Pereyra and G. Turner, personal communication).

Here, we show that NpgA is essential for siderophore biosynthesis and that the growth defect of the *cfwA2* strain is mainly due to impairment of the biosynthesis of siderophores and lysine.

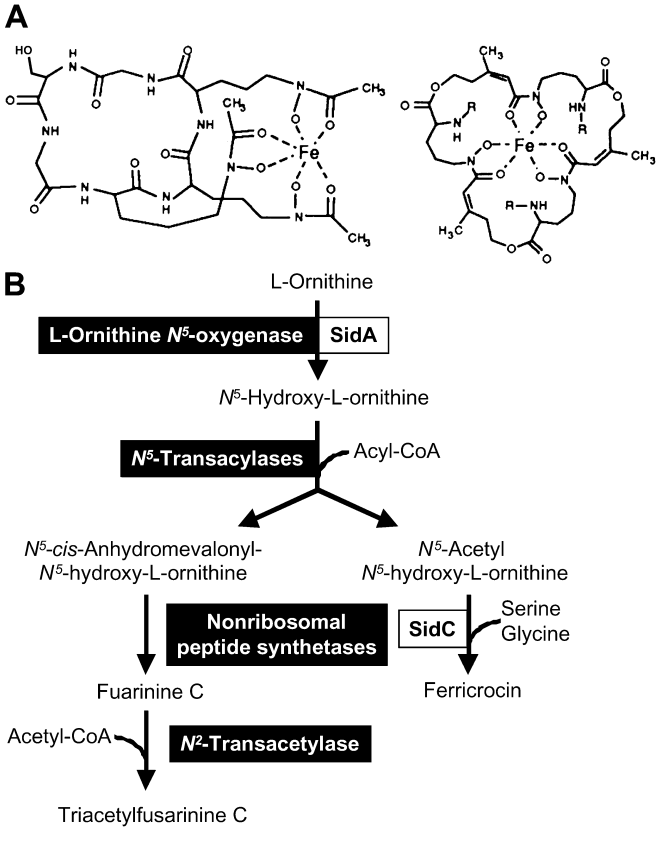

Fig. 1A, B Siderophores of *Aspergillus nidulans*. **A** Chemical structure of ferricrocin (FC) and triacetylfusarinine C (TAFC), adapted from Winkelmann (1993) with permission of the publisher. **B** Biosynthetic pathway for FC and TAFC, according to Plattner and Diekmann (1994)

Materials and methods

Strains, vectors, growth media, and general molecular techniques

The *A. nidulans* strains WGTRAN (*argB2::argB bgA0, biA1*), SRKO1 (*sreA::argB bgA0, biA1*), and AJC12:36 (*pabaA1, yA2, cfwA2*) are designated in the text as *wt*, ΔsreA, and *cfwA2*, respectively. Construction of WGTRAN and SRKO1 was described by Haas et al. (1999) and Haas et al. (2003); and *cfwA2* was isolated by Aguirre et al. (1993).

Fungal strains were grown at 25 °C, 37 °C, or 42 °C, as indicated, in *Aspergillus* minimal medium (AMM) according to Pontecorvo et al. (1953) containing 1% glucose as carbon source, 20 mM glutamine as nitrogen source, 10 μM $FeSO_4$, 20 μg/l biotin, and 4 mg *p*-aminobenzoic acid/l. For low-iron media (−Fe-AMM), iron was omitted. Standard molecular techniques were performed as described by Sambrook et al. (1989).

Identification, quantification, and purification of siderophores

TAFC and FC were isolated from *A. nidulans* as described by Oberegger et al. (2001). Crude identification of extracellular siderophore production was performed using the chrome azurol S liquid assay (Payne 1994). Characterization and quantification of extracellular and cellular siderophores were performed by

reversed-phase HPLC chromatography according to Konetschny-Rapp et al. (1988), as described by Oberegger et al. (2001). Extracellular siderophore production was normalized to the dry weight of the mycelia; and the intracellular siderophore content was normalized to the protein content of the cellular extract.

Northern analysis

Generally, 15 μg of total RNA were electrophoresed on 1.2% agarose-2.2 M formaldehyde gels and blotted onto Hybond N membranes (Amersham). The hybridization probes used in this study were generated by PCR, using oligonucleotides 5′-AGCC CGGTGTGAAAAGAG-3′ and 5′-AACAGGAGGAGGATTG CGCC-3′ for *mirA*, 5′-ACACCCGCCCTCTAACCG-3′ and 5′-CA CACCCCAGTCGCACAG-3′ for *npgA*, and 5′-CGGTGATGAG GCACAGT-3′ and 5′-CGGACGTCGACATCACA-3′ for γ-actin-encoding *acnA* (Fidel et al. 1988).

Results and discussion

NpgA function is essential for biosynthesis of both ferricrocin and triacetylfusarinine C

An *A. nidulans* strain harboring the temperature-sensitive *npgA* allele *cfwA2* did not grow at the restrictive temperature of 42 °C using liquid or solid AMM. Therefore, *cfwA2* was grown at 37 °C, a temperature at which *cfwA2* displayed about 30% of the *wt* growth rate. In order to analyze the influence of NpgA on siderophore biosynthesis, *cfwA2* and *wt* were grown for 24 h during iron-depleted conditions. Subsequently, the production of extracellular and intracellular siderophores was quantified as described by Oberegger et al. (2001). At the permissive temperature of 25 °C, *cfwA2* showed about 90% of the intracellular and extracellular siderophore production of *wt*. In contrast, at 37 °C, the production of TAFC and FC by *cfwA2* decreased to 3% and 2%, compared with *wt* (Fig. 2). These data demonstrate that NpgA is essential for the biosynthesis of both siderophores, ferricrocin and TAFC. As shown in Fig. 1, FC synthesis involves the NRPS SidC—which requires activation by a Ppant. In contrast to FC, the modified ornithine residues of TAFC are linked by ester bonds and the enzymatic steps involved are unclear so far. On the one hand, the requirement of NpgA for TAFC synthesis is therefore surprising. On the other hand, it has been shown that some NRPS can also form ester bonds—an example is *Escherichia coli* EntF, which is involved in the synthesis of the catecholate-type siderophore enterobactin (Crosa and Walsh 2002). Deficiency in the TAFC biosynthesis of *cfwA2* at the restrictive temperature suggests that TAFC synthesis involves a Ppant-dependent enzymatic step—most likely an NRPS.

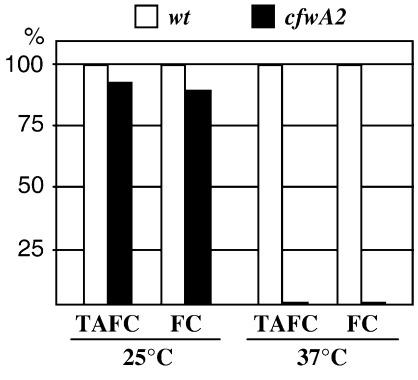

Fig. 2 Reversed-phase HPLC analysis of intracellular and extracellular siderophore production of *A. nidulans wt* and *cfwA2* at 37 °C and 25 °C. The fungal strains were grown for 24 h in liquid low-iron *Aspergillus* minimal medium (AMM); and the production of extracellular (TAFC) and intracellular (FC) siderophore was analyzed, as described in the Materials and methods. *Columns* represent the siderophore production normalized to *wt* at the respective temperature. Samples were prepared in triplicate, and SD did not exceed 10%

cfwA2 is iron-starved during iron-replete conditions

In *A. nidulans*, the siderophore system is the major iron-uptake system (Eisendle et al. 2003). Impairment of siderophore biosynthesis, e.g. deletion of the siderophore-biosynthesis gene *sidA* causes iron starvation during iron-replete conditions. Expression of genes involved in siderophore biosynthesis and uptake is repressed by iron; and this control is mediated in part by the transcriptional repressor SREA (Haas et al. 1999; Oberegger et al. 2001, 2002a). Northern analysis of *mirA*, which encodes a siderophore transporter upregulated under iron depletion (Oberegger et al. 2001; Haas et al. 2003), displayed that, in contrast to *wt*, *cfwA2* is iron-starved during iron-replete conditions at 37 °C (Fig. 3A). Therefore, with respect to iron homeostasis, *cfwA2* grown at 37 °C shows a similar phenotype to that of strains with defects in siderophore-biosynthesis genes.

Northern analysis indicated that, in contrast to *mirA* and numerous other genes which are involved in siderophore biosynthesis and uptake (Oberegger et al. 2002a), expression of *npgA* is only slightly regulated by iron availability. *npgA* transcript levels are about 2-fold upregulated during iron-depleted conditions, compared with iron-replete conditions (Fig. 3B). ΔsreA displays the same *npgA* expression pattern as *wt*, indicating this regulation is SreA-independent (Fig. 3B). In this respect, it is interesting to note that we previously showed that an iron-regulatory mechanism exists in *A. nidulans* which does not involve SreA (Oberegger et al. 2002b).

The growth defect of *cfwA2* is due to impaired biosynthesis of siderophores and lysine

Like *S. cerevisiae*, *A. nidulans* synthesizes lysine via the α-aminoadipate pathway (Arst et al. 1973; Weidner et al 1997; Busch et al. 2003). This pathway includes the Ppant-dependent α-aminoadipate semialdehyde reductase. Consequently, *cfwA2* is supposed to be auxotrophic for lysine at the restrictive temperature; but

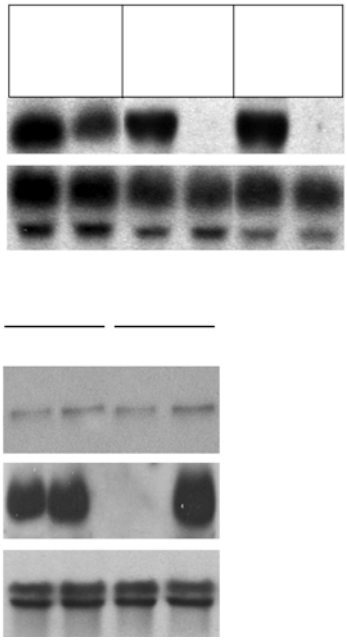

Fig. 3A, B Expression of *mirA* and *npgA* in *A. nidulans*. **A** Expression of *mirA* in *wt* (*left*) and *cfwA2* (*center*) at 37 °C (*top panel*) and 25 °C (*bottom panel*). **B** Expression of *npgA* in *wt* (*left*) and ΔsreA (*right*). Fungal strains were grown for 15 h in low-iron AMM (*top panel*) or AMM with iron (*middle panel*). Total RNA was isolated from the harvested mycelia and subjected to Northern analysis. Hybridization with *acnA* served as a control for the loading and quality of RNA (*at right in A*; *bottom panel in B*)

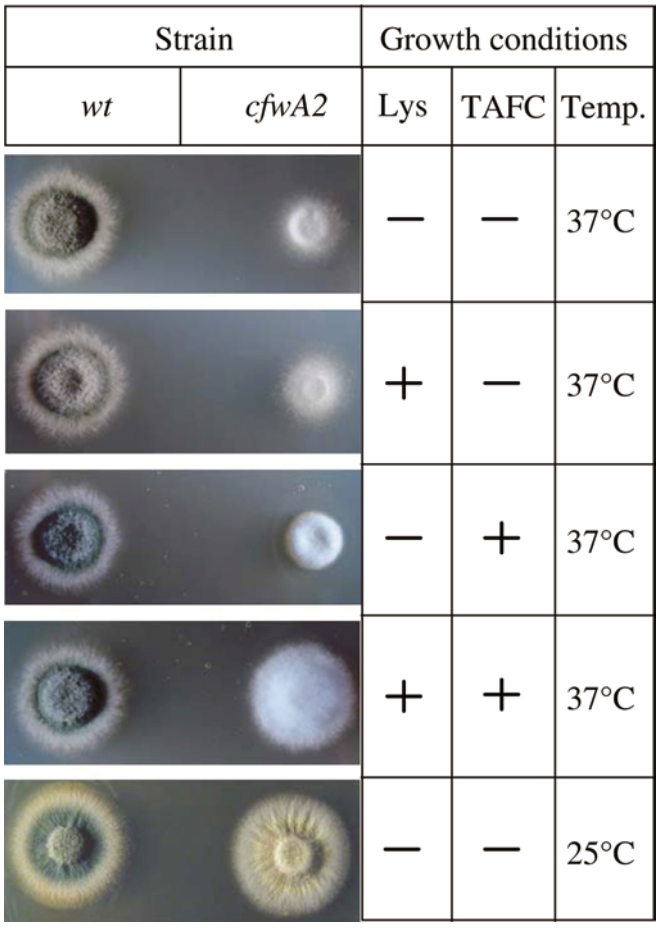

Fig. 4 Growth phenotypes of *A. nidulans wt* and *cfwA2* at 37 °C and 25 °C. Aliquots of 10^6 conidia of the respective fungal strains were point-inoculated on AMM supplemented *as indicated* with 10 μM TAFC and/or 10 mM lysine (*Lys*) and incubated for 48 h. Please note that *cfwA2* has yellow conidia instead of the green *wt* conidia, due to carrying the *ya2* allele (O'Hara and Timberlake 1989)

supplementation with lysine did not repair the growth defect (Fig. 4). In *A. nidulans*, siderophore biosynthesis is essential for viability; and the growth defect of mutants defective in siderophore synthesis can be cured by supplementation with TAFC or FC (Eisendle et al. 2003). However, supplementation with TAFC did not cure the growth defect of *cfwA2*. In contrast, supplementation with TAFC plus lysine led to a *cfwA2* growth rate similar to that of *wt* but did not compensate the pigmentation defect (Fig. 4). Replacement of TAFC with FC or performing the experiment at 42 °C yielded the same results (data not shown). These data demonstrate that the growth defect of *cfwA2* at the restrictive temperature is mainly due to impaired biosynthesis of siderophores and lysine.

In all experiments comparing *wt* and *cfwA2*, it has to be considered that these *A. nidulans* strains are not isogenic. Nevertheless, this fact seems not to interfere with the interpretation of the data because: (1) *cfwA2* behaves like *wt* at the permissive temperature of 25 °C, with respect to siderophore biosynthesis and regulation of expression of *mirA* and (2) the alleles *yA2* and *pabaA1*, present in *cfwA2*, do not interfere with the regulation of siderophore biosynthesis in other genetic backgrounds at a growth temperature of 37 °C (H. Haas, unpublished data).

In conclusion, NpgA seems to be a broad-range PPTase required for the modification of at least five different enzymes involved in both primary and secondary metabolism and, obviously, these functions cannot be complemented by a putative second PPTase. It is required for: (1) pigment synthesis, where the most likely impaired enzyme is the polyketide synthase WA, which is essential for pigment synthesis (Mayorga and Timberlake 1992), (2) lysine biosynthesis, with the enzyme most probably affected being the α-aminoadipate semialdehyde reductase, (3) biosynthesis of the siderophores TAFC and FC (in the latter case the putative enzyme compromised is the NRPS SidC), and (4) penicillin biosynthesis, where the impaired enzyme is probably the NRPS δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase. Additionally, the *cfwA2* allele might negatively affect another enzyme involved in penicillin biosynthesis: isopenicillin *N* synthase (IpnA). IpnA is a nonheme iron(II)-dependent oxygenase (Schofield et al. 1997). The *cfwA2* strain is iron-starved, due to impaired siderophore biosynthesis. Consequently, the iron-dependent IpnA might be inactive due to lack of iron. Moreover, we previously showed that various genes encoding proteins in need of iron-containing cofactors—like aconitase

(*acoA*), homoaconitase (*lysF*), and cytochrome *c* (*cycA*)—are transcriptionally downregulated in response to iron depletion (Oberegger et al. 2002b); and the same might apply to *ipnA*.

Acknowledgements We thank Dr. Geoffrey Turner for providing AJC12:36. We also thank Dr. Paul Illmer and Gerlinde Häninger for their help in HPLC analysis of siderophores. This work was supported in part by Austrian Science Foundation grant FWF-P15959-B07 (to H.H.) and Austrian National Bank (OENB) grant 8750 (to H.H.).

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