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Tenellin acts as an iron chelator to prevent iron-generated reactive oxygen species toxicity in the entomopathogenic fungus Beauveria bassiana

Jiraporn Jirakkakul1, Supapon Cheevadhanarak1, Juntira Punya2, Chanikut Chutrakul2, Jittisak Senachak2, Taridaporn Buajarern2, Morakot Tanticharoen1 and Alongkorn Amnuaykanjanasin2.∗

1School of Bioresources and Technology, King Mongkut's University of Technology Thonburi (KMUTT), Bangkok 10140, Thailand and 2Bioresources Technology Unit, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency, Pathumthani 12120, Thailand

∗Corresponding author. Bioresources Technology Unit, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), 113 Thailand Science Park, Thanon Phahonyothin, Tambon Khlong Nueng, Amphoe Khlong Luang, Pathumthani 12120 Thailand; Tel: +66-2-5646700 ext. 3248; Fax: +66-2-5646707; E-mail: alongkorn@biotec.or.th

One-sentence summary: Tenellin played an important role in iron homeostasis in the ferricrocin-deficient Beauveria bassiana.

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ABSTRACT

Iron is an essential element for life. However, the iron overload can be toxic. Here, we investigated the significant increase of tenellin and iron–tenellin complex production in ferricrocin-deficient mutants of Beauveria bassiana. Our chemical analysis indicated that the ferricrocin-deficient mutants T1, T3 and T5 nearly abolished ferricrocin production. In turn, these mutants had significant accumulation of iron–tenellin complex in their mycelia at 247–289 mg g⁻¹ cell dry weight under iron-replete condition. Both tenellin and iron–tenellin complex were not detected in the wild-type under such condition. Mass analysis of the mutants’ crude extracts demonstrated that tenellin formed a 3:1 complex with iron in the absence of ferricrocin. The unexpected link between ferricrocin and tenellin biosynthesis in ferricrocin-deficient mutants could be a survival strategy during iron-mediated oxidative stress.

Key words: Beauveria bassiana; ferricrocin; tenellin; iron chelator; iron-generated reactive oxygen species; intracellular siderophore

INTRODUCTION

Iron is an essential mineral. Its redox properties play important roles in basic biological processes, for example, cellular respiration, oxidative stress detoxification and the synthesis of deoxyribonucleotides, amino acids and lipids (Haas, Eisendle and Turgeon 2008; Canessa and Larrondo 2013). However, an excessive amount of iron can be cytotoxic as a result of the reactive oxygen species (ROS) generated from the Fenton’s reaction (Halliwell and Gutteridge 1984; Heli et al., 2011). Therefore, iron homeostasis is tightly regulated. Iron homeostasis in fungi is regulated by controlling iron uptake, intracellular storage and iron utilization (Ong et al., 2006; Haas, Eisendle and Turgeon 2008; Canessa and Larrondo 2013). Siderophores are low molecular weight iron-chelating compounds with a high affinity for ferric ion (Fe(III)). Siderophores are synthesized by various
microorganisms. Extracellular siderophores play a crucial role in iron uptake, whereas intracellular siderophores contribute to iron storage (Renshaw et al., 2002; Haas 2003). In iron-limited conditions, most fungi excrete extracellular siderophores for iron acquisition, for example, coprogen from Cochliobolus heterostrophus and Magnaporthe grisea, and triacylufusarinine C from Aspergillus nidulans and A. fumigatus (Lee et al., 2005; Eisendle et al., 2006; Oide et al., 2006). However, intracellular siderophores bind iron to prevent the deleterious effects of free iron and are involved in the iron storage and supply of fungal cells, such as ferricrocin from Neurospora crassa (Matzanke et al., 1987) and A. nidulans (Eisendle et al., 2006; Wallner et al., 2009).

The fungus Beauveria bassiana has been widely used for the biological control of several insect pests, including coleopterans, hemipterans and lepidopterans (Alves et al., 2002). Beauveria bassiana may kill its insect host through two main mechanisms, propagation and toxin production (Bidochema, Kamp and De Croos 2000; Charnley 2003). Tenellin is a yellow pigment that can be isolated from Beauveria mycelia (El Basyouni and Vining 1966). The tenellin structure contains a 2-pyridone (Eley et al., 2007). Some of pyridone compounds have interesting activities such as leporin A from A. leporis which is toxic to lepidopteran insect pests (TePaske et al., 1991). Tenellin can inhibit equine erythrocyte membrane ATPase activity (Jeffs and Khachatourians 1997). However, tenellin is not involved in Galleria mellonella pathogenesis of B. bassiana (Eley et al., 2007). In this study during the course of our study of siderophore biosynthesis, we found an unexpected link between ferricrocin and tenellin biosynthesis in ferricrocin-deficient mutants. The new biological property of tenellin, which acts to alleviate iron-generated oxidative stress in ferricrocin-deficient mutants, was reported.

**MATERIALS AND METHODS**

**Fungal strain and culture conditions**

Beauveria bassiana strain BCC2660 was obtained from the BIOTEC Culture Collection in Thailand. The fungal strain was maintained on potato dextrose agar (PDA; Difco, USA). All mutants were maintained on PDA containing 200 μg ml⁻¹ hygromycin B (Roche Applied Science, USA), at 25°C. To perform an assay under iron-limited and iron-replete conditions, 1 x 10⁷ conidia ml⁻¹ of B. bassiana BCC2660 (wild-type) or mutants were inoculated into 100 ml of the iron-limited condition and MM agar containing 10 μM FeSO₄ (Sigma-Aldrich) (MMfe) for the iron-replete condition.

Escherichia coli strain DH5α was used for bacterial transformation and recombinant plasmid propagation.

**Identification of a putative siderophore synthetase gene from B. bassiana BCC2660**

A pair of degenerate oligonucleotide primers CCPSII and CS, which target highly conserved motifs of adenyllyl (A) domains in known fungal NRPSs (Chutrakul 2003), were used to amplify putative NRPS gene fragments using the cDNA of B. bassiana BCC2660 that infected the aphid Myzus persicae as a template. An inverse PCR method (Ochman, Gerber and Hartl 1988) was used for chromosomal walking of one of the identified NRPS genes that has similarity to siderophore synthetase, designated sidC. Briefly, inverse PCR of SidC was performed using the sidC-specific primers: SidC₃'-F_INV (5'-TTGTTGAGGGGACAAAGC -3') and SidC₃'-R_INV (5'-TGGTTGAGGGGACAAAGC -3') and PsI-restricted, self-ligated genomic DNA of B. bassiana BCC2660 as a template. The partial sequence of sidC was submitted to GenBank with accession no. JQ991001.

**Construction of sidC-silencing vector**

The plasmid pSilent-1 (Nakayashiki et al., 2005) was used to construct the sidC-silencing vector (pS_sidC). A 388 bp sidC fragment was amplified by PCR by using B. bassiana BCC2660 genomic DNA as a template. Two primer pairs were used to amplify a sense and antisense orientation of this fragment. The first pair included a forward primer with an XhoI site at its 5'-end (sFXholSidC: 5'-ATCCCTGAGTGGCTACAGGTCAGACT-3'; Xhol site is underlined) and a reverse primer (sSSidC: 5'-TGGAGTTCACTGGCAACGACA-3'). This pair amplified the sidC fragment, which was inserted into pSilent-1 at Xhol and SnaBl sites as the sense orientation of the sidC fragment. The second pair of primers included a forward primer with an Apal site (sFAPsidC: 5'-TTAAGGCCCCTCTGCTACAGGTCACCAGACT-3'; Apal site is underlined) and a reverse primer with a KpnI site (sKpnSidC: 5'-TATAGTACCTGAGTGGCATGGCAACGACA-3'; KpnI site is underlined). This second primer pair amplified the same sidC fragment, which was later cloned into the Apal and KpnI sites of the first recombinant plasmid, thus inserting an inverted repeat (the antisense orientation) of this 388 bp sidC fragment. The pS_sidC was constructed to express short hairpin RNA, a mediator of siderophore synthetase gene silencing.

**Protoplast preparation and B. bassiana transformation**

B. bassiana was grown in Sabouraud dextrose broth with 1% yeast extract for 48 h under a static condition. A thin layer of young mycelia found floating on the medium surface was collected. The mycelia were rinsed with 0.8 M NaCl (Sigma-Aldrich) in sodium phosphate buffer (pH 5.8) and then fragmented by vortexing with 0.5 mm diameter glass beads in 10 ml of the same buffer. The protoplasts were isolated and used for fungal transformation according to a previously described method (Tilburn et al., 1983) with some modifications. In brief, fragmented mycelia were digested in 50 ml of the wall-lysing enzyme mixture ‘Glucanex’ (40 mg ml⁻¹) (Novozyme, Denmark), and 0.1% β-mercaptoethanol (Sigma-Aldrich) in 0.8 M NaCl/10 mM sodium phosphate buffer (pH 5.8) for 2 h at 30°C, 130 rpm. The fungal protoplasts were harvested by centrifugation at 1000 g for 15 min at 4°C. The pellet was washed three times with 1 ml of protoplast washing buffer [1 M sorbitol (Fluka, UK), 10 mM Tris-HCL pH 7.5] and resuspended in resuspension buffer [1 M sorbitol and 10 mM CaCl₂ (Riedel-de Haen, Germany) in 10 mM Tris-HCL pH 7.5] for a final concentration of 2 x 10⁶ protoplasts ml⁻¹. An 800 μl aliquot of the protoplast suspension was mixed with 10 μg of pS_sidC at room temperature. Then, the DNA-protoplast mixture was added to 200 μl of 60% polyethylene glycol-4000 (PEG-4000) (Sigma-Aldrich), CaCl₂ solution [60% PEG-4000 (w/v), 10 mM CaCl₂ and 10 mM Tris-HCL pH 7.5] and placed on ice for 30 min. After incubation, 1 ml of 60% PEG4000/CaCl₂ solution was added to the mixture and further incubated at room temperature for 1 min. Then, the transformation mixture was mixed with 10 ml of molten malt extract top agar (Oxoid, UK) containing 0.7% agar (Difco) and 0.6 M sucrose (Univar, USA) and overlaid on malt extract agar containing 1.5% agar, 0.6 M sucrose and 600 μg ml⁻¹ hygromycin B. The cultures were incubated at 25°C for 7-10 days until colonies appeared. Three rounds of selection for hygromycin-resistant colonies were performed.
To determine the presence of the sidC-silencing cassette and the hygromycin resistance cassette in mutants, PCR analysis was conducted with two pairs of primers. The first primer pair, i.e. ITF (5′-GTACCCACTGGAATTGTGTGGCCATGC-3′) and SidCF (5′-CTTGCTACAGGTCCACGACT-3′), was used to determine the presence of the SidC-silencing cassette, whereas the second pair, namely Ex_HygF (5′-TGAACTCAGCCGACGTCTG-3′) and Ex_HygR (5′-CTTTAGACAGCCATCGTCC-3′), was used to amplify the hygromycin resistance cassette.

**HPLC analysis of the metabolic profile**

An individual culture of *B. bassiana* wild-type or mutant was grown on a cellophane sheet laid on top of MM or MMFe. The culture was incubated at 25 °C for 30 days. The harvested mycelia were then lyophilized and extracted with 50 ml of methanol for two days. After discarding the mycelia, the methanol fraction was concentrated under reduced pressure to obtain a crude extract. HPLC analysis was conducted by using a reverse-phase column (VertiSep HPLC Column; Vertical Chromatography, Thailand) and diode array detector (996 Photodiode Array Detector, Waters). The crude extract was dissolved in methanol to a final concentration of 10 mg ml⁻¹. Metabolite separation was performed on a VertiSep HPLC Column. HPLC analysis was performed at a flow rate of 0.8 ml min⁻¹ at 210 nm with a water-acetonitrile step gradient as follows: 0 min/2% acetonitrile, 14 min/60% acetonitrile, 16 min/60% acetonitrile, 19 min/100% acetonitrile, 50 min/100% acetonitrile, 51 min/50% acetonitrile, 60 min/50% acetonitrile and 64 min/2% acetonitrile. Purified desferricrocin (Fe-free form) was obtained from Genaxxon Bioscience (Germany). Purified tenellin was isolated from *Cordyceps* sp. BCC18179 (a kind gift from the Biosources Research Laboratory at BIOTEC). Desferricrocin, ferricrocin, tenellin and iron–tenellin complex quantification was performed by single-point internal standard method using 2-nitrobenzaldehyde (Sigma-Aldrich) as an internal standard.

**Mass analysis**

Ferricrocin, tenellin and iron–tenellin complex were partially isolated from the mycelia of wild-type and mutants cells by preparative TLC before mass analysis. In brief, the crude mycelial extracts were prepared, spotted on a TLC plate (TLC silica gel 60 F254 25 aluminum sheets 20 × 20 cm, Merck, Germany) and developed by using a freshly prepared solvent chloroform/methanol/water (70:24:4) system as previously reported (Konetschny-Rapp et al., 1988). The expected band was scraped off, and the compound was eluted from the silica gel with methanol. The methanol fraction was then concentrated under reduced pressure to obtain a partial purification of the compound. ESI-TOF mass spectra were determined by using a microTOF mass spectrometer (Bruker, Germany).

**RESULTS**

**Isolation of ferricrocin synthetase-silenced mutants**

Transformation of *B. bassiana* BCC2660 with the plasmid pS_sidC generated three independent transformants T1, T3 and T5. The transformants were verified for the presence of a 491 bp fragment of sidC-silencing cassette and a 960 bp fragment of Hyg cassette in their genomes using PCR analysis (Fig. 1).

The mutants were analyzed for phenotypic changes in comparison to the wild-type. The T1, T3 and T5 mutants showed similar phenotypes. The mutants’ colonies had yellow pigment around the colony center (older region) under iron-limited condition and intense orange pigment under iron-replete culture (Fig. 2a and b). Also, the hyphae of T1, T3 and T5 mutants showed closely spaced septa and swollen and highly vacuolated when compared with the wild-type (Fig. 2c). Notably, the vacuoles in many regions of the mutant hyphae had a green–yellow color, which was undetected in the wild-type.

**The increasing of tenellin in ferricrocin-deficient mutants**

Our chemical analysis of mycelial extracts showed striking differences between the mutants’ extracts and that of wild-type (Fig. 3). HPLC analysis indicated that under iron-limited conditions, both desferricrocin and ferricrocin were detected in the wild-type mycelia at retention times of 10.3 and 10.8 min, respectively. However, under iron-replete conditions, the wild-type mycelia had only ferricrocin and the production of ferricrocin was highly induced by approximately 2.9-fold in comparison to that of the iron-limited condition (Fig. 3b). In contrast to the wild-type, the HPLC chromatograms of the T1, T3 and T5 mutants showed huge reduction of ferricrocin by 97, 98 and 98% decrease under iron-replete condition, respectively, compared to those of the wild-type and none of desferricrocin. Furthermore, the HPLC chromatogram of the mutants showed extra peaks corresponding to tenellin at a retention time of 24.4 min and iron–tenellin complex at a retention time of 35.4 min under the iron-limited and iron-replete conditions, respectively (Fig. 3a). The tenellin content in the mutants was reduced under iron-replete condition in correlation with the increased iron–tenellin complex (Fig. 3b). Both tenellin and iron–tenellin complex were not detected in the wild-type.

Our LC-MS MS analysis verified ion masses of the three predominant compounds. The first one, at a retention time of 10.8 min in the wild-type extract, was identical to the mass of ferricrocin ([C₉₀O₁₃N₈H₄Fe(III)]: 770.58 Da (Hissen et al., 2004) (Fig. 4c). The second compound at 24.4 min in the
Figure 2. Comparison of growth characteristics between the wild-type and mutants. A colony color difference was observed between mutants T1, T3 and T5 and the wild-type grown on (a) MM agar and (b) MM + 10 μM FeSO₄ (MMFe) agar at 25°C for two weeks. Bar, 1 cm. (c) The mutants’ hyphae were strikingly different from those of the under the iron-replete condition. Major differences included the presence of yellow–blue vesicles. Arrows indicate yellow–blue vacuoles. Bar, 4 μm.

mutants’ extract was identical to tenellin (C₂₁H₂₃NO₅): 369.16 Da, (CSID:10207541, http://www.chemspider.com/Chemical-Structure.10207541.html) (Fig. 4d). Lastly, the ion mass of iron–tenellin complex [Fe (III) + (tenellin-H)₃ + Na]⁺ was detected at m/z 1183.44 in the partially purified mycelial fraction of mutants (Fig. 4e). In addition, a coordination complex between tenellin and iron was demonstrated by comparison of chemical analysis between the purified mycelial fraction of the mutants and in vitro preparation mixture of three moles of tenellin and one mole of FeCl₃. The yellow solution of tenellin changed to the red–orange color after mixing with FeCl₃ solution. The formation of an iron–tenellin complex was first determined by the absorption at approximately 424 nm, which is the characteristic UV spectrum of iron-binding compounds. As desferricrocin binds to iron to form ferricrocin, the UV spectrum exhibited the absorbance increase at 426 nm. The UV spectra of the desferricrocin, ferricrocin, tenellin and iron–tenellin complex are shown in Fig. 5. All the chemical data, including masses, retention time and UV absorption property, indicated that the red-orange pigment from the mutants’ mycelial extracts was iron–tenellin complex. These data also indicated that three molecules of tenellin, each of which acts as a bidentate ligand, bind to an iron molecule, therefore suggesting that the stoichiometry of the iron:tenellin complex is 1:3.

DISCUSSION

The current work reveals a new biological function of tenellin in decreasing iron-induced oxidative stress by acting as an iron (III) chelating compound in iron-overload condition. In A. nidulans, ferricrocin is also a major iron storage compound in hyphae. When intracellular iron became excessive, the expression of iron storage ferricrocin synthetase gene, sidC, was up-regulated (Eisendle et al., 2003).

In fungi, two principal mechanisms for iron storage are mediated by the vacuole and siderophore (Haas, Eisendle and Turgeon 2008). The comparison between the B. bassiana wild-type and the ferricrocin-deficient mutants has highlighted the roles of the intracellular siderophore ferricrocin in this fungus. For the wild-type under an iron-replete condition, a high amount of ferricrocin was accumulated in the mycelia. On the other hand, the lack of desferricrocin (iron-free form) in the mutants may result in an iron homeostasis imbalance and free
Figure 3. HPLC analysis of the wild-type and mutants. (a) HPLC chromatogram of methanol extract from mycelia of the B. bassiana wild-type and mutants that were grown on a cellophane sheet laid on top of MM or MMFe at 25°C for 30 days. HPLC analysis unambiguously showed that all three mutants were markedly reduced in desferricrocin and ferricrocin production and in turn increased in tenellin production under the iron-limited condition (MM). In the iron-replete condition, only ferricrocin was detected in the wild-type, whereas iron (III)–tenellin complex was detected in crude extracts from the mutants. (b) The accumulation of desferricrocin, ferricrocin, tenellin and iron–tenellin complex in mycelia was quantified. The data represent the mean from three experiments. Error bars are standard errors from the three experiments.
radical generation from free iron via Fenton reaction (Halliwell and Gutteridge 1984; Heli et al., 2011). Under oxidative stress, cells exhibit various dysfunctions in response to lesions caused by ROS to lipids, protein and DNA such as membrane damage, protein dysfunction and impaired DNA damage, respectively (Ercal, Gurur-Orhan and Aykin-Burns 2001). In the ferricrocin-deficient mutants, the irregular hyphal forms included closely spaced septa, hyphal swelling and highly vacuolated hyphae, none of which were found in the wild-type. These hyphal irregularities may be a consequence of oxidative stress from an increased production of ROS such as the hydroxyl radical (HO•), which is generated from intracellular free iron.
We clearly observed that iron–tenellin complex was accumulated in mycelia of the mutants instead of ferricrocin in the wild-type particularly in the iron-replete condition. Tenellin contains a hydroxamic acid functional group (Williams and Sit 1982). The hydroxamic acid group is an effective metal binding agent (Farkas et al., 2007). Therefore, chemical structure of tenellin enables tenellin to bind with iron as we showed here that tenellin could bind to an uncoordinated iron atom in B. bassiana hyphae. One ferric iron can form complex with three tenellin molecules. In our in vitro experiment, the yellow color of tenellin changed to the red–orange when a tenellin solution was mixed with Fe (III), which was the same color pattern as we found in the ferricrocin-deficient mutants. The formation of an iron–tenellin complex at a ratio of 1:3 in the mutants was strongly supported by our chemical analysis. The binding of tenellin to excessive unbound iron can shade the free iron from Fenton reaction and protect the cell from iron-mediated free radical damage.

In conclusion, we have demonstrated that ferricrocin is an intracellular siderophore in the entomopathogen B. bassiana. Consequently, ferricrocin deficiency can induce tenellin production, which has a novel biological property as an iron chelator to minimize the intracellular level of the Fenton reaction, as shown in this study. Tenellin production appeared to be specifically induced, perhaps to protect the cells from oxidative stress caused by iron excess. We propose that ferricrocin contributes to iron storage in B. bassiana BCC2660. In the wild-type, desferricrocin acts as the principal iron storage molecule. Desferricrocin production is highly induced under iron excess, in which desferricrocin binds to Fe (III) to form ferricrocin. The increased level of desferricrocins protects the fungal cells from free radicals generated by the free iron. Therefore, ferricrocins accumulate in the fungal hyphae grown on MM + FeSO₄. In mutants, drastic reduction in desferricrocin production greatly affects intracellular iron storage, causing iron homeostasis imbalance. The free iron can accumulate in the hyphae and consequently generate iron-induced oxidative stress. The iron-generated ROS induces tenellin production for detoxification and prevention of oxidative stress in the mutants by acting as an iron chelator. Iron chelating property of tenellin can be used in future application. Nevertheless, more comprehensive roles of ferricrocin in B. bassiana development and pathogenicity will be investigated in further study.

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