Cell PRESS

Chemistry & Biology
Article

Biosynthesis of the Cyclooligomer Depsipeptide Beauvericin, a Virulence Factor of the Entomopathogenic Fungus *Beauveria bassiana*

Yuquan Xu,[1] Rousel Orozco,[2] E.M. Kithsiri Wijeratne,[1] A.A. Leslie Gunatilaka,[1,3] S. Patricia Stock,[2,3] and István Molnár[1,3,*]
[1]Southwest Center for Natural Products Research and Commercialization, Office of Arid Lands Studies, The University of Arizona, 250 E. Valencia Road, Tucson, AZ, 85706-6800, USA
[2]Department of Entomology, The University of Arizona, 1140 E. South Campus Drive, Tucson, AZ 85721-0036, USA
[3]Bio5 Institute, The University of Arizona, 1657 E. Helen Street, Tucson, AZ 85721, USA
*Correspondence: imolnar@cals.arizona.edu
DOI 10.1016/j.chembiol.2008.07.011

SUMMARY

Beauvericin, a cyclohexadepsipeptide ionophore from the entomopathogen *Beauveria bassiana*, shows antibiotic, antifungal, insecticidal, and cancer cell antiproliferative and antihaptotactic (cell motility inhibitory) activity in vitro. The *bbBeas* gene encoding the BbBEAS nonribosomal peptide synthetase was isolated from *B. bassiana* and confirmed to be responsible for beauvericin biosynthesis by targeted disruption. BbBEAS utilizes D-2-hydroxyisovalerate (D-Hiv) and L-phenylalanine (Phe) for the iterative synthesis of a predicted *N*-methyl-dipeptidol intermediate, and forms the cyclic trimeric ester beauvericin from this intermediate in an unusual recursive process. Heterologous expression of the *bbBeas* gene in *Escherichia coli* to produce the 3189 amino acid, 351.9 kDa BbBEAS enzyme provided a strain proficient in beauvericin biosynthesis. Comparative infection assays with a BbBEAS knockout *B. bassiana* strain against three insect hosts revealed that beauvericin plays a highly significant but not indispensable role in virulence.

INTRODUCTION

Beauvericin is a cyclooligomer depsipeptide ionophore that transports monovalent cations across membranes as a free carrier uncoupling oxidative phosphorylation (Steinrauf, 1985). Beauvericin displays a diverse array of biological activities in vitro. It shows moderate antifungal and antibiotic activity (Hamill et al., 1969), and potentiates other antifungal agents in combination therapies (Zhang et al., 2007). Importantly, beauvericin displays a broad-spectrum antiproliferative activity against different human cancer cell lines by activating calcium-sensitive cell apoptotic pathways (Jow et al., 2004). It also inhibits the directional cell motility (haptotaxis) of cancer cells at subcytotoxic concentrations (Zhan et al., 2007): haptotaxis is essential for the formation of new blood vessels in tumors (angiogenesis), invasion of other tissues by cancer cells, and metastasis (Carmeliet, 2003).

Beauveria (teleomorph: *Cordyceps*) *bassiana* (Hypocreales) is a broad host range facultative entomopathogen that plays an important role in the control of insect populations in nature. During its pathogenic phase, the developing hyphae of *B. bassiana* directly penetrate the insect integument by producing extracellular enzymes (Fan et al., 2007). Virulence factors that modulate the insect immune system, and those that disable and finally kill the host, are also expected to contribute to the pathogenic armamentarium of *B. bassiana*. Filamentous fungi with a pathogenic lifestyle often produce a large variety of polyketide and nonribosomal peptide natural products that act as immunosuppressors, and general or host-specific toxins (von Döhren, 2004). Thus, *B. bassiana* produces the cyclooligomer nonribosomal depsipeptides beauvericin and bassianolide, the diketomorpholine bassiatin, the cyclic peptides beauverolides, the dibenzoquinone oosporein, and the 2-pyridone tenellin (Figure 1). Tenellin, a polyketide nonribosomal peptide, was shown not to be involved in insect pathogenesis (Eley et al., 2007). Confirming earlier reports on the in vitro toxicity of bassianolide to insects (Champlin and Grula, 1979), we have recently shown that a bassianolide-nonproducer strain of *B. bassiana* is significantly less virulent against selected insect hosts (unpublished data). However, the in vivo significance of beauvericin for the producer organism has remained controversial up to now, as purified beauvericin was shown to be toxic only to some insect larvae (Champlin and Grula, 1979; Hamill et al., 1969).

Nonribosomal peptides are assembled in a process of stepwise condensations on nonribosomal peptide synthetases (NRPSs). Incorporation of each precursor into the product requires a separate NRPS module, with each module featuring enzymatic domains for the activation (A: adenylation domain), covalent thioester tethering (T: thiolation domain), and condensation (C domain) of the precursors (Finking and Marahiel, 2004). Further domains in the NRPS modules might edit the enzyme-bound precursors or intermediates. The product is released (and cyclized, if necessary) by a penultimate TE (thioesterase), R (reductase), or C domain (Kopp and Marahiel, 2007). According to the classical NRPS paradigm, each module and each active site domain is used only once during product assembly (Finking and Marahiel, 2004). By contrast, NRPSs that synthesize cyclooligomer peptides assemble oligopeptide monomer intermediates by the programmed iterative use of their modules. These monomers are then used in a recursive

 ©2008 Elsevier Ltd All rights reserved

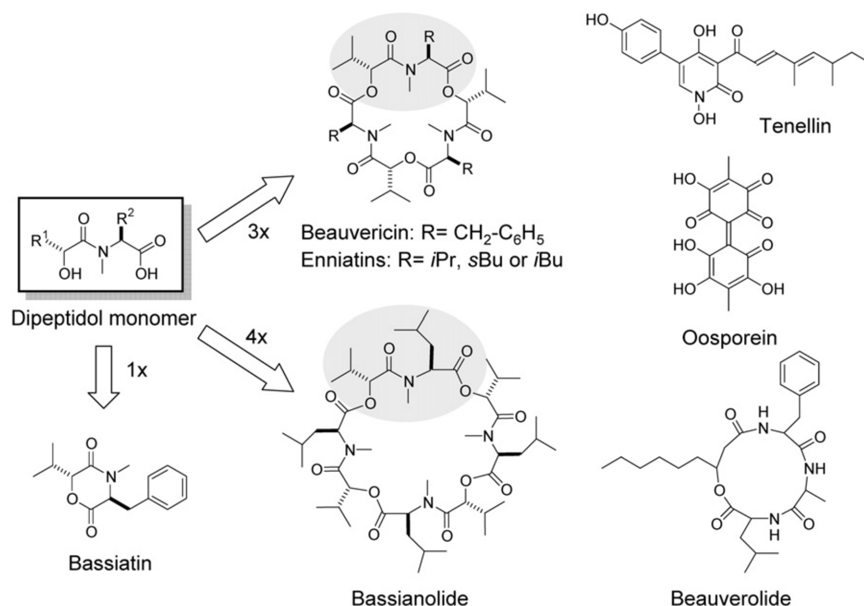


Figure 1. Secondary Metabolites of *B. bassiana*

The structures of enniatins from *Fusarium* sp. and a generalized structure of the dipeptidol monomer intermediate are shown.

oligomerization and cyclization process (Kopp and Marahiel, 2007). NRPSs that synthesize depsipeptides incorporate 2-hydroxycarboxylic acids into their products, and may form ester instead of amide bonds.

An impressive series of publications from the Zocher lab describe the cloning and enzymatic characterization of the cyclooligomer depsipeptide synthetase (CODS) for enniatin biosynthesis from *Fusarium equiseti* (synonym: *F. scirpi*) (Glinski et al., 2002; Hacker et al., 2000; Haese et al., 1993; Hornbogen et al., 2007; Krause et al., 2001; Pieper et al., 1995). Although the beauvericin synthetase has also been purified from *B. bassiana* (Peeters et al., 1988), the encoding gene has hitherto not been identified. In the present study, we have cloned the NRPS-encoding gene for the beauvericin synthetase of *B. bassiana.* Targeted inactivation of this gene allowed the evaluation of the in vivo role of beauvericin in insect pathogenesis. A highly efficient heterologous production system was also developed by the expression of the beauvericin synthetase in an apparently functional form in *Escherichia coli*.

RESULTS

Isolation of the Beauvericin Biosynthetic Locus

We have used several different pairs of PCR primers for the selective amplification of NRPS gene segments from *B. bassiana* genomic DNA. Because the beauvericin synthetase was expected to activate D-2-hydroxyisovalerate (D-Hiv), we sought increased selectivity by designing two primer sets to amplify A-domain-encoding sequences with high similarity to the D-Hiv-activating A_1 domain of the ESYN enniatin synthetase from *F. equiseti* (GenBank accession number CAA79245). Two distinct amplification products from reactions with the EA1f-EA1r primer pair, EA1042 and EA1046, were indeed found to encode A-domain segments highly similar (64.6% and 69.7% identity, respectively) to the A_1 domain of ESYN. As all the other amplified sequences were significantly less similar to ESYN, we expected EA1042 and EA1046 to represent those NRPS A domains that might activate D-Hiv. EA1042 was later found to be derived from the *bbBsls* gene encoding the bassianolide synthetase of *B. bassiana* (unpublished data). This report describes the characterization of the EA1046-related NRPS as the beauvericin synthetase.

First, we sought proof that the EA1046-related mRNA is present in *B. bassiana* cells under beauvericin production conditions. We have used RT-PCR with a primer pair specific for the EA1046 (but not the EA1042) sequence and total RNA from beauvericin-producing cultures, and observed the specific amplification of the expected product (results not shown). Next, we have isolated the EA1046-related locus from a genomic DNA library of *B. bassiana*. Fosmids hybridizing with EA1046 as the probe did not overlap with those hybridizing with EA1042, proving that the two amplification products originated from distinct genomic loci. Two representative EA1046-homologous fosmids were sequenced to yield a 33,475 bp contig (Figure 2) centered on a 9,570 bp gene (*bbBeas*) encoding a putative CODS. The contig contains a further nine open reading frames, three of which encode putative carboxylic acid oxidoreductases related to branched-chain amino acid catabolism (Table 1). Thus, the *kivr* gene encodes a novel NADPH-dependent 2-ketoisovalerate reductase (KIVR) proposed to convert 2-ketoisovalerate from valine catabolism or pyruvate metabolism to D-Hiv (Figure 2). Disruption of *kivr* abolishes the biosynthesis of beauvericin and bassianolide, and cyclodepsipeptide production can be rescued by complementation with D-Hiv (unpublished data). The translation of the predicted pseudogene *orf4* would yield an N- and C-terminally truncated protein segment with high similarity to glycolate oxidases and lactate monooxygenases. Oxidizing short-chain 2-hydroxycarboxylic acids, such an enzyme might have interfered with beauvericin production by depleting D-Hiv. Downstream of the NRPS-encoding gene, *orf7* codes for a putative methylmalonate semialdehyde dehydrogenase, another enzyme from valine catabolism. At the left border of the sequenced region, *orf1* encodes a putative Gal4-like transcriptional regulator with a Zn_2Cys_6 binuclear cluster DNA-binding domain. Similar regulators are often encoded in secondary metabolite biosynthetic gene clusters of fungi (Bergmann et al., 2007). Three conserved hypothetical proteins (ORF3, ORF5, and ORF8) and two deduced proteins that might be involved in protein folding (ORF2 and ORF6) are also encoded in the sequenced locus.

Disruption of the *bbBeas* Gene in *B. bassiana*

To ascertain the function of the CODS encoded by *bbBeas*, an internal fragment of this gene on the *B. bassiana* genome was

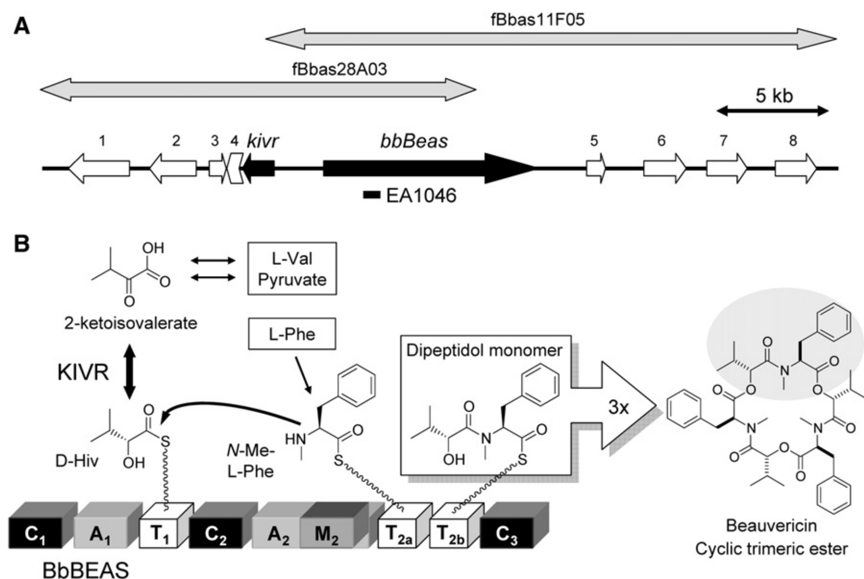


Figure 2. The *bbBeas* Locus and Proposed Biosynthesis of Beauvericin

(A) Putative open reading frames (see Table 1) are shown as white arrows, the *bbBeas* and *kivr* genes are drawn as black arrows, the two sequenced fosmids are indicated as two-way gray arrows, and the approximate position of the EA1046 PCR product is shown as a bold line.

(B) Model of the biosynthesis of beauvericin from primary metabolites on the BbBEAS beauvericin synthetase (domains not to scale).

replaced with the *bar* selectable marker gene using *Agrobacterium*-mediated transformation (Fang et al., 2004). Approximately 15% of the glufosinate-resistant progeny were found to originate from double homologous recombination (Figures 3A–3C). The isolates with the disrupted *bbBeas* gene showed unchanged growth rates and morphology. LC-MS analysis of fermentation extracts of wild-type *B. bassiana* and several ectopic integrant isolates revealed copious production of beauvericin and bassianolide. However, beauvericin production was specifically and completely arrested in all *bbBeas* knockout isolates, with a concomitant slight increase of bassianolide production (Figure 3D). Thus, the *bbBeas*-encoded CODS is responsible for the biosynthesis of beauvericin in *B. bassiana*, but not for that of bassianolide.

Comparative Sequence Analysis of the Beauvericin Synthetase

The *bbBeas* gene encodes a predicted NRPS (BbBEAS) of 3,189 amino acids in length. The deduced molecular mass of BbBEAS (351,889 Da) is about 100 kDa more than earlier estimates for the purified beauvericin synthetase (Peeters et al., 1988); a discrepancy of identical magnitude was noted for the initial size estimates versus the deduced size of the ESYN enniatin synthetase (Haese et al., 1993; Pieper et al., 1995). BbBEAS shows 60% identity and 75% similarity to ESYN (GenBank accession number CAA79245), and 65% identity and 79% similarity to the BbBSLS bassianolide synthetase of *B. bassiana* ATCC 7159 (unpublished data). A similar level of identity is also noted with the NRPS-homologous part of the bassianolide synthetase of *Xylaria* sp. BCC 1067 (GenBank accession number ABR28366), whose characterization coincided with our work (Jirakkakul et al., 2008). Regardless of synthesizing hexa- or octadepsipeptides, all these CODSs feature identical module and domain organizations: $C_1A_1T_1$-$C_2A_2M_2T_{2a}T_{2b}$-C_3 (Figure 2). The A_1 domains within the first modules of the beauvericin, bassianolide, and enniatin synthetases are all expected to activate D-Hiv as an adenylate and install it as a thioester onto the T_1 domains of the same modules, as demonstrated for ESYN (Haese et al., 1994; Pieper et al., 1995). The enniatin and the bassianolide synthetase A_2 domains activate and load branched-chain amino acids onto the twin T_2 domains within module 2. In contrast, the BbBEAS beauvericin synthetase A_2 domain was shown to be specific for Phe (Peeters et al., 1988; Xu et al., 2007). The amino acid constituents of fungal cyclooligomer depsipeptides are all modified by *N*-methylation, accomplished at the amino acyl thioester stage by SAM-dependent *N*-methyltransferase domains in NRPSs (Billich and Zocher, 1987; Hacker et al., 2000). Indeed, integrated *N*-methyltransferase domains responsible for this editing function were first identified from the enniatin and the beauvericin synthetases (Billich and Zocher, 1987; Peeters et al., 1988). The *N*-methyltransferase domains (M_2) of the CODSs display three well-conserved core motifs (Schwarzer et al., 2003), and are inserted into a flexible loop region between core motifs 8 and 9 of the A_2 domains (Figure 2). This "stuffer" region often accommodates different "editing" domains in diverse NRPSs. Dipeptidol formation from D-Hiv and the *N*-Me-amino acid, both presented as thioesters on the phosphopantetheinyl arms of T domains, is catalyzed by the condensation domain C_2 in CODSs (Figure 2). The beauvericin, bassianolide, and enniatin synthetases also feature both an N-terminal C_1 and a C-terminal C_3 domain each that might play a role in the recursive cyclooligomerization process characteristic of fungal CODS (Glinski et al., 2002; Haese et al., 1993). The C_1 and C_3 domains show overall similarity to condensation domains, but their core motifs are substantially divergent from the canonical forms (Schwarzer et al., 2003). However, they retain a modified form of the active site signature (S**H**xxV**D** in C_1 and S**H**ALY**D**G in C_3 domains versus H**H**xxx**D**G for the canonical motif) incorporating the His active site and the conserved Asp that stabilizes the active site architecture.

Heterologous Production of Beauvericin in *E. coli*

Expression of active fragments of ESYN have been demonstrated using *E. coli* and *Saccharomyces cerevisiae* as hosts (Billich and Zocher, 1987; Hornbogen et al., 2007), but the heterologous expression of a fungal CODS in a functional form has hitherto not been reported. We have used the T7 promoter-based dual expression vector pACYCDuet-1 for the coregulated expression of the intronless *bbBeas* gene with the phosphopantetheinyl transferase-encoding gene *sfp* from *Bacillus subtilis* (Quadri et al., 1998). The resulting expression construct (Figure 4) was introduced into several *E. coli* expression hosts and used for

 ©2008 Elsevier Ltd All rights reserved

Table 1. Databank Similarities of the Deduced Proteins from the Beauvericin Locus

Gene Models (Start–end, bp)	Protein Length (aa)	Closest GenBank Homolog, Organism,[a] Function of the Homolog	Identity/ Similarity (%)	Conserved Domain	E Value[b]
orf1[c] (1–2,705)	749	XP_001556512, *B. fuckeliana*, hypothetical protein	37/54[d]	cd00067, GAL4-like Zn_2Cys_6 binuclear cluster DNA-binding domain	$2e^{-07}$
orf2[c] (3,519–5,598)	623	XP_380656, *G. zeae*, cyclophilin with WD40 domain	79/90	cd01927, cyclophilin-type peptidyl-prolyl *cis-trans* isomerase; and cd00200, WD40 domain	$2e^{-72}$ $1e^{-19}$
orf3 (6,216–6,956)	228	XP_380657, *G. zeae*, hypothetical protein	56/75	pfam04750, FAR-17a/ AIG1-like protein	0.002
orf4[c,e] (7,041–7,751)	NA[e]	XP_001823913, *A. oryzae*, glycolate oxidase	47/66[e]	cd03332, L-lactate 2-monooxygenase FMN-binding domain	$3e^{-63}$
kivr[c] (7,882–9,270)	462	XP_382715, *G. zeae*, ketopantoate reductase	43/61	PRK06522, 2-ketopantoate reductase	$7e^{-21}$
bbBeas (11,511–21,080)	3189	CAA79245, *F. equiseti*, enniatin nonribosomal peptide synthetase	60/75		
orf5 (23,275–24,112)	212	XP_380668, *G. zeae*, hypothetical protein	38/57	ND	NA
orf6 (25,822–27,691)	557	XP_380667, *G. zeae*, calreticulin	75/86	pfam00262, calreticulin family	$2e^{-113}$
orf7 (28,615–30,425)	584	EAA35465, *N. crassa*, methylmalonate semialdehyde dehydrogenase	81/90	pfam00171, aldehyde dehydrogenase family	$3e^{-120}$
orf8 (31,637–33,475)	612	EAQ93245, *C. globosum*, hypothetical protein	30/40[d]	ND	NA

NA, not applicable; ND, not detected.
[a] Genus abbreviations: *A*, *Aspergillus*; *B*, *Botryotinia*; *C*, *Chaetomium*; *F*, *Fusarium*; *G*, *Gibberella*; *N*, *Neurospora*.
[b] E value for the match against the conserved domain.
[c] Reverse complement.
[d] Similarity on a shorter protein segment only.
[e] Predicted pseudogene. Identity and similarity percentages are from the deduced protein sequence regardless of internal stop codons.

the biocatalytic conversion of D-Hiv to beauvericin. Low-level production (0.1 mg/l of expression culture) of a substance comigrating with beauvericin was detected with BL21(DE3) as the host and protein expression at 20°C. No product was formed with expression cultures above 25°C, or without supplementation with D-Hiv (*E. coli* is not known to produce this metabolite). Heterologous production of beauvericin was improved by utilizing *E. coli* Origami B(DE3): this host facilitates proper protein folding by promoting cytoplasmic disulfide bond formation. Further improvement was gained by reducing the cultivation temperature to 16°C, supplementing L-Phe to the bioconversions, and increasing the length of the reaction to 48 hr. The identity of the isolated product was confirmed by detecting the appropriate molecular ion in high-resolution mass spectrometry (Figure 4). The biocatalytic conversion of D-Hiv and L-Phe to fully elaborated beauvericin shows that BbBEAS is functional in *E. coli*. Further, the yield of beauvericin with *E. coli* reached approximately 8 mg/l of expression culture, around 40% of that with *B. bassiana* (approximately 20 mg/l).

Beauvericin Is a Virulence Factor of *B. bassiana*

We have compared the virulence of *B. bassiana* ATCC 7159 (wild-type) with the *bbBeas* KO beauvericin synthetase knockout strain and the *kivr* KO knockout strain (the latter is deficient in both beauvericin and bassianolide production owing to the specific disruption of the 2-ketoisovalerate reductase-encoding gene). Last-instar larvae of the greater wax moth *Galleria mellonella*, the corn earworm *Helicoverpa zea*, and the fall armyworm *Spodoptera exigua* were used as insect hosts. *G. mellonella* was selected considering its previous use for the evaluation of tenellin as a virulence factor in *B. bassiana* (Eley et al., 2007). *Spodoptera* cells were found to be sensitive to beauvericin in vitro (Fornelli et al., 2004), whereas *H. zea* was described to be resistant to purified beauvericin but sensitive to bassianolide (Champlin and Grula, 1979). Gratifyingly, larval mortality was significantly reduced ($p < 0.05$) when comparing the mutant *B. bassiana* strains to the wild-type in all three insect hosts (Figure 5). The highest cumulative mortality was observed in *S. exigua* challenged with the wild-type strain at a dose of

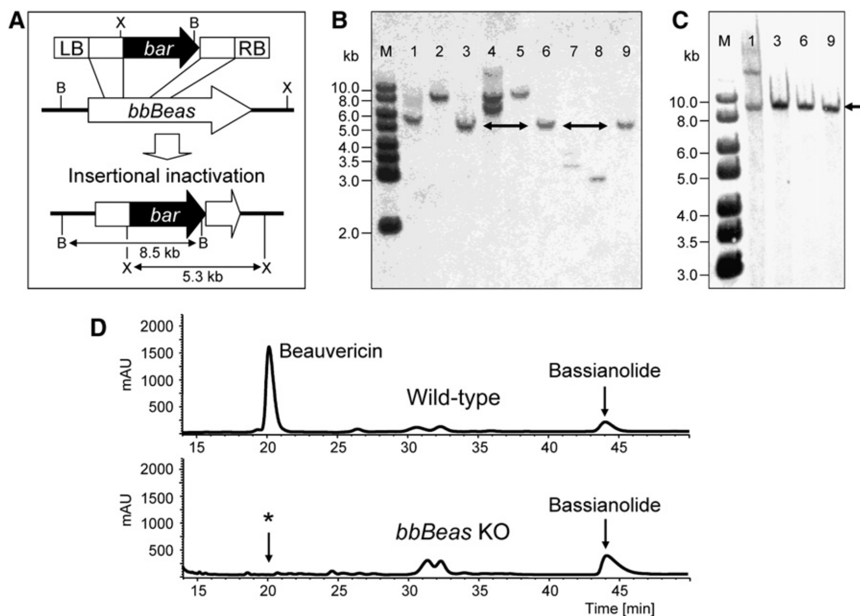


Figure 3. Insertional Inactivation of the *bbBeas* Gene

(A) Scheme for the double homologous recombination between the T-DNA and the *B. bassiana* genome. LB and RB, left and right borders of the T-DNA; B, BamHI restriction site; X, XhoI restriction site.

(B) Southern hybridization against XhoI-digested total DNA of several transformants. The arrows show the expected hybridizing fragment found in isolates 3, 6, and 9. M, marker.

(C) Southern hybridization against BamHI-digested total DNA preparations of selected isolates from (B). The arrow shows the expected hybridizing fragment.

(D) Cyclooligomer depsipeptide production in *B. bassiana* ATCC 7159 (wild-type) and the *bbBeas* knockout mutant (*bbBeas* KO). *, the expected position of the beauvericin peak (absent).

1×10^6 conidia/ml (98.1% ± 2.5, day 7). Wild-type *B. bassiana* was also highly virulent against *G. mellonella*, with *H. zea* showing the most resistance to this fungus. By contrast, both mutant strains exhibited very low virulence against all three insects even at the highest dose (Figure 5), irrespective of the previously described sensitivity or resistance of these hosts to purified beauvericin. Clear signs of fungal infection and subsequent mortality were delayed with both mutants in all hosts. However, there was no clear tendency for decreased mortality of the insects challenged with the strain deficient in the production of both beauvericin and bassianolide (*kivr* KO) as compared to the beauvericin-nonproducer mutant.

DISCUSSION

The synthesis of cyclooligomer depsipeptides by both filamentous fungi and bacteria significantly deviates from the collinear and processive logic of canonical NRPSs (Schwarzer et al., 2003; von Döhren, 2004). Thus, CODSs execute a parallel synthetic scheme that involves the programmed iterative synthesis of peptidol monomer intermediates from amino acid and 2-hydroxycarboxylic acid precursors (Figure 2). The monomers undergo recursive ligations and macrolactone ring closure in a concerted, ester bond-forming cyclooligomerization process. Bacterial CODSs hold the first monomer unit on their TE domain

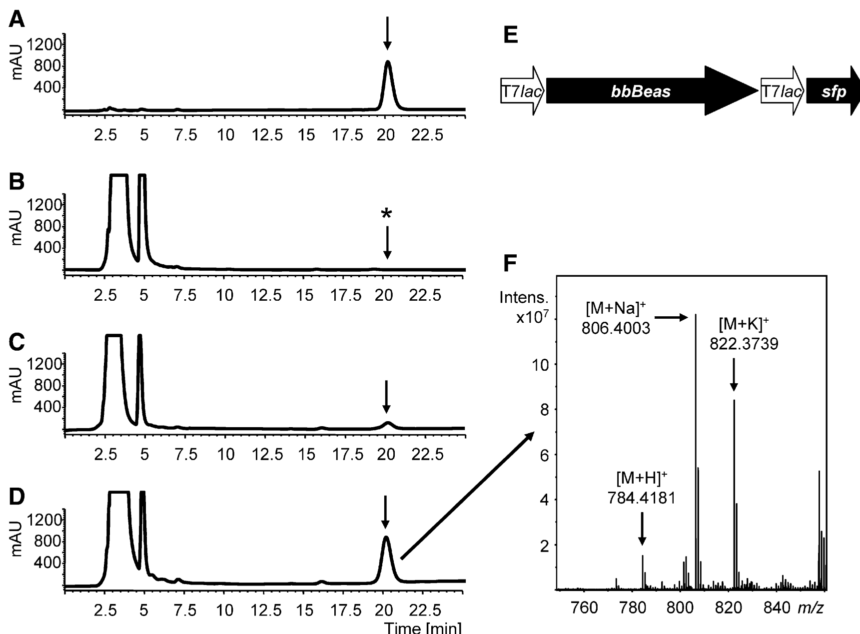


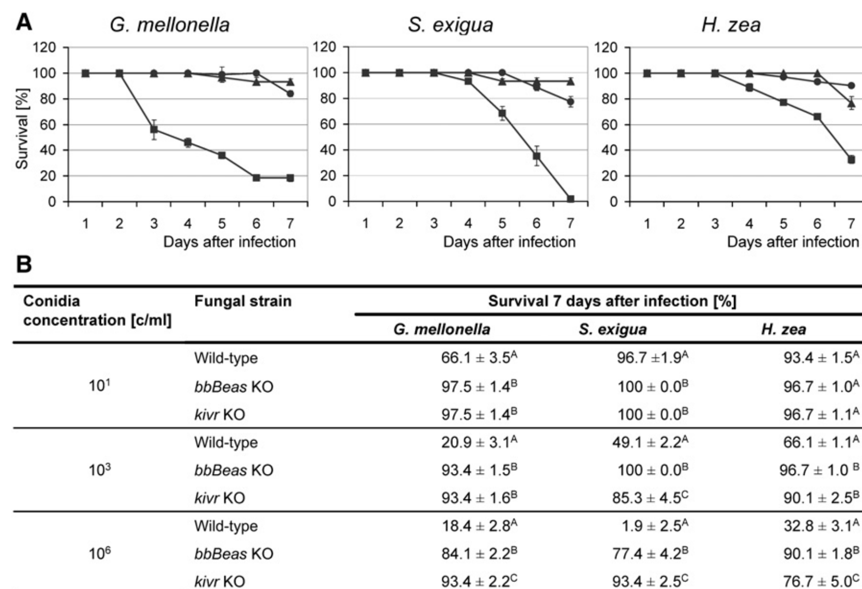
Figure 4. Heterologous Production of Beauvericin

(A–D) HPLC traces of (A) authentic beauvericin isolated from *B. bassiana* fermentations; (B) an organic extract of *E. coli* Origami B(DE3) [pACYC-Duet-1] biotransformation reaction with D-Hiv and L-Phe; (C) an organic extract of *E. coli* BL21(DE3) [pACYC-*bbBeas-sfp*] 20 hr biotransformation reaction with D-Hiv; and (D) an organic extract of *E. coli* Origami B(DE3) [pACYC-*bbBeas-sfp*] 48 hr biotransformation reaction with D-Hiv and L-Phe. The arrows indicate the beauvericin peak; absence of this peak is emphasized with an asterisk.

(E) Schematic representation of the expression cassette in pACYC-*bbBeas-sfp*.

(F) FTICR-MS spectrum of beauvericin isolated from the extract of the reaction shown in (D).

 ©2008 Elsevier Ltd All rights reserved



Conidia concentration [c/ml]	Fungal strain	Survival 7 days after infection [%]		
		G. mellonella	*S. exigua*	*H. zea*
10^1	Wild-type	66.1 ± 3.5^A	96.7 ±1.9^A	93.4 ± 1.5^A
	bbBeas KO	97.5 ± 1.4^B	100 ± 0.0^B	96.7 ± 1.0^A
	kivr KO	97.5 ± 1.4^B	100 ± 0.0^B	96.7 ± 1.1^A
10^3	Wild-type	20.9 ± 3.1^A	49.1 ± 2.2^A	66.1 ± 1.1^A
	bbBeas KO	93.4 ± 1.5^B	100 ± 0.0^B	96.7 ± 1.0^B
	kivr KO	93.4 ± 1.6^B	85.3 ± 4.5^C	90.1 ± 2.5^B
10^6	Wild-type	18.4 ± 2.8^A	1.9 ± 2.5^A	32.8 ± 3.1^A
	bbBeas KO	84.1 ± 2.2^B	77.4 ± 4.2^B	90.1 ± 1.8^B
	kivr KO	93.4 ± 2.2^C	93.4 ± 2.5^C	76.7 ± 5.0^C

Figure 5. Virulence of *B. bassiana* Strains Against Selected Insects

(A) Time course of the survival of *G. mellonella*, *S. exigua*, and *H. zea* larvae infected with *B. bassiana* at 10^6 conidia/ml. Squares, *B. bassiana* ATCC 7159 wild-type; circles, beauvericin-nonproducer strain *bbBeas* KO; triangles, beauvericin and bassianolide-nonproducer strain *kivr* KO.

(B) Endpoint survival assays. Significantly different means of survival ($p < 0.05$) for a given insect challenged with the three fungal strains at a given conidial suspension concentration are emphasized with different superscript letters. Error bars and values signify standard deviations.

as an acyl-*O*-thioester. Oligomerization and cyclization are catalyzed by this recursive TE domain in cooperation with the preceding T domain (Kopp and Marahiel, 2007). Cyclooligomerization has probably evolved independently in fungal NRPSs. Instead of TE domains, these CODSs feature two characteristic domains at their N and C termini (the C_1 and C_3 domains, respectively) both with overall similarity to condensation domains, and also feature two consecutive T domains within their second modules. Zocher and colleagues proposed (Glinski et al., 2002; Haese et al., 1993) that one dipeptidol monomer is transiently parked on one of the two T_2 domains while a second dipeptidol is assembled on the other. These monomers would then be ligated and cyclized by the C_1 and C_3 domains (Figure 2). Thus, the C_1 and/or the C_3 domains should function as recursive condensation catalysts that form both inter- and intramolecular ester bonds. Fittingly, the C_3 domains of the fungal CODSs are most similar to terminal C domains that catalyze product release by intramolecular cyclization in other fungal NRPSs such as the *Tolypocladium inflatum* cyclosporine synthetase (GenBank accession number CAA82227, 42% identity/61% similarity to the C_3 domain of BbBEAS). However, the C_1 domains of the fungal CODSs have no close (>30% identity) database homologs. The C_1 and C_3 domains feature slightly modified active site signatures (S**H**xxV**D** in C_1 and S**H**ALY**D**G in C_3 domains). The BbBEAS C_1 domain is even more irregular in missing the His that is essential for condensation catalysis (HLxxxD, compared to the C domain consensus H**H**xxx**D**). Similar mutations were previously shown to inactivate condensation domains (Bergendahl et al., 2002). However, this change should have no effect on the function of the C_1 domain of BbBEAS, considering that this is the only synthetase for beauvericin production in *B. bassiana*, and that beauvericin production can be reconstituted in *E. coli* using the *bbBeas* gene. Thus, C_1 domains might indeed serve a function different from peptide bond formation. As of now, however, there is no experimental evidence for the role of either the C_1 or the C_3 domains in fungal cyclodepsipeptide synthesis.

The structurally related cyclooligomer depsipeptides of Sordariomycetes might be classified according to their oligomerization state: hexadepsipeptides like beauvericin and enniatin are assembled from three dipeptidol monomers; octadepsipeptides like bassianolide are formed from four monomers; and diketomorpholines like bassiatin correspond to a single monomer (Figure 1). Comparisons of the primary amino acid sequences of the corresponding synthetases, or separately their C_1 or C_3 domains, failed to provide any sequence clues for the programming of the number of ligations during cyclooligomerization. Similarly, it is currently not possible to predict product chain length from the primary amino acid sequences of other iterative enzymes like bacterial type II polyketide synthases (PKSs), fungal PKSs, or type III polyketide (chalcone) synthases. Chain length (oligomerization state) is probably determined by the shape and size of the active site cavities where condensation or cyclooligomerization occurs.

The biosynthesis of 2-hydroxycarboxylate precursors in bacteria and fungi may represent another example of convergent evolution. Bacterial depsipeptide synthetases employ A domains that load 2-ketocarboxylates as β-ketoacyl thioesters onto their cognate T domains, and use chiral ketoacyl reductase domains for the in situ synthesis of 2-hydroxycarboxylate thioesters (Fujimori et al., 2007; Magarvey et al., 2006). In contrast to this on-demand strategy, fungal CODS A domains utilize free 2-hydroxycarboxylates, preformed by chiral, dissociated, monofunctional, NADPH-dependent enzymes (Lee et al., 1992; Pieper et al., 1995). Thus, the 2-ketoisovalerate reductase of *B. bassiana*, whose encoding gene, *kivr*, is clustered with *bbBeas*, produces D-Hiv that is proposed to be activated by the A_1 domains of both the beauvericin and the bassianolide synthetases in *B. bassiana*. While this manuscript was in preparation, a reductase domain similar to KIVR has been found appended to the C terminus of the *Xylaria* sp. bassianolide synthetase (Jirakkakul et al., 2008).

Previous work has shown that the amino acids that line the active site cavities of bacterial A domains can be reliably predicted using sequence alignments with two structurally characterized A domains as the anchors. Further, the derived 10 amino acid "signatures" can be used for the a priori prediction of the substrate specificities of bacterial A domains (Challis et al., 2000;

 ©2008 Elsevier Ltd All rights reserved

Table 2. Specificity-Conferring Signatures of NRPS Adenylation Domains

Synthetase[a]	*Domain*	*Specificity*[b]	235[c]	236	239	278	299	301	322	330	331	517
BbBEAS	A_1	D-Hiv	G	A	L	M	I	V	G	S	I	K
BbBSLS	A_1	D-Hiv	G	A	L	M	**V**	V	G	S	I	K
XsBSLS	A_1	D-Hiv	G	A	L	L	**V**	V	G	**T**	I	K
FeESYN	A_1	D-Hiv	G	A	L	H	**V**	V	G	S	I	K
Proposed fungal D-Hiv signature			G	A	L	x	I/**V**	V	G	S/**T**	I	K
StVlm1	A_1	Kiv	**A**	A	L	W	I	A	V	S	G	K
BcCesB	A_1	Kiv	V	**G**	F	W	**V**	A	V	S	D	K
KsKtzG	A	Kiv	V	T	Y	F	N	G	P	S	G	K
Bacterial Phe signature			D	**A**	**W**	T	I	A	A	V	C	K
BbBEAS	A_2	Phe	D	G	Y	I	M	A	A	V	M	K
AfPSES	A_1	Phe	D	**A**	Y	T	M	A	A	**I**	C	K
CpEALS	A_2	Phe	D	L	V	G	M	A	A	V	G	K
AfGliP	A_2	Phe	D	Y	N	T	Y	T	A	**I**	C	K
BbBSLS	A_2	Leu	D	G	Y	I	I	**G**	**G**	V	F	K
XsBSLS	A_2	Leu	D	**A**	**W**	**L**	V	**G**	A	V	M	K
FeESYN	A_2	V(LI)	D	G	**W**	F	I	**G**	I	**I**	I	K
FsESYN	A_2	I(LV)	D	G	**W**	F	A	**G**	V	M	I	K

Amino acids identical to those in the A-domain signatures of BbBEAS are shown in white font on a black background. Amino acids similar (V = I = L, A = G, S = T, W = Y = F) to those in the A-domain signatures of BbBEAS are shown in bold type.

[a] NRPS abbreviations (and GenBank accession numbers): BbBSLS, *B. bassiana* bassianolide synthetase (unpublished data); XsBSLS, *Xylaria* sp. bassianolide synthetase (ABR28366); FeESYN, *F. equiseti* enniatin synthetase (CAA79245); StVlm1, *Streptomyces tsusimaensis* valinomycin synthetase subunit 1 (ABF61888); BcCesB, *Bacillus cereus* cereulide synthetase B subunit (ABK00751); KsKtzG, *Kutzneria* sp. kutzneride synthetase G subunit (ABV56585); AfPSES, *A. fumigatus* pseurotin A synthetase (ABS87601); CpEALS, *Claviceps purpurea* ergot alkaloid synthetase (O94205); AfGliP, *A. fumigatus* gliotoxin synthetase (ABE60889); FsESYN, *F. sambucinum* enniatin synthetase (CAA88634). Bacterial Phe signature according to Stachelhaus et al. (1999); fungal D-Hiv signature as described in the text.

[b] A-domain specificities: D-Hiv, D-2-hydroxyisovalerate; Kiv, 2-ketoisovalerate; V(LI), valine preferred, leucine and isoleucine accepted; I(LV), isoleucine preferred, leucine and valine accepted.

[c] Numbering according to PheA (Challis et al., 2000; Stachelhaus et al., 1999). Specificity signatures were derived by NRPSpredictor (Rausch et al., 2005).

Stachelhaus et al., 1999). Prediction of substrate specificity is, however, not currently possible in fungal NRPSs. The fungal "code," if such exists, seems to be divergent from the one used by bacteria, and deriving new correlations is hindered by the scarcity of fungal A domains with known substrate specificities (Schwarzer et al., 2003; von Döhren, 2004). We have used the support vector machine-based program NRPSpredictor (Rausch et al., 2005) to extract the 10 amino acid signatures of the fungal CODSs (Table 2). Replacement by Gly, Ala, Val, or Asn of the highly conserved Asp230 that anchors the substrate amines is a hallmark of A domains recognizing non-amino acid substrates (Schwarzer et al., 2003). Accordingly, Gly is featured at this position in all the D-Hiv-activating A_1 domains. The A_1 domain signatures of these synthetases are highly similar to each other (Table 2). The derived consensus signature, GALx(I/V)VG(S/T)IK, represents to our knowledge for the first time a fungal A-domain "codon" for the 2-hydroxycarboxylate precursor D-Hiv. No significant similarity of this fungal D-Hiv signature is evident with that of the 2-ketoisovalerate-activating A domains of bacterial depsipeptide NRPSs (Fujimori et al., 2007; Magarvey et al., 2006) (Table 2).

A comparison of the A_2 domain specificity signature of BbBEAS with the bacterial Phe code was unproductive (Table 2). Comparison with the derived signatures of the three currently identified fungal A domains that activate Phe was also equivocal. Whereas the signature for the Phe-activating A domain of the *Aspergillus fumigatus* pseurotin synthetase is rather similar to that of BbBEAS, the signature of the gliotoxin synthetase from the same *Aspergillus* species is highly different. This discrepancy might indicate the existence of synonymous nonribosomal "codons" for Phe activation in fungi: similar degeneracy was also noted in the prokaryotic nonribosomal code (Schwarzer et al., 2003; von Döhren, 2004). Surprisingly, the BbBEAS A_2 domain signature is very similar to those of the Leu-activating A_2 domains from the two identified bassianolide synthetases. This similarity did not extend to the branched-chain amino acid activating A_2 domains of the enniatin synthetases from two *Fusarium* sp. It has been observed that A domains within a single biosynthetic pathway are often very similar to each other in spite of activating different amino acids (Wenzel et al., 2006). If nature switches amino acid specificity by initially mutating the "codon" residues only, then specificity signatures of orthologous A domains might only be minimally altered to accommodate a new substrate. The resulting convergent evolution would lead to a higher degeneracy of the code and might confound a priori predictions. Alteration of substrate specificity by such "keyhole surgery" has been demonstrated in the lab (Finking and Marahiel, 2004).

In spite of formidable technical difficulties, heterologous production of secondary metabolites is increasingly feasible (Halo et al., 2008; Zirkle et al., 2004). Fungal PKSs, NRPSs, and even

 ©2008 Elsevier Ltd All rights reserved

Cell PRESS

complete biosynthetic gene clusters have been expressed in *Aspergillus* hosts with the concomitant production of the corresponding metabolites (Halo et al., 2008). Although production of dissected NRPS domains in bacterial hosts is practicable (Haese et al., 1994), in vivo production of fungal nonribosomal peptide natural products in bacterial hosts is less straightforward. As an alternative, in vitro reconstitution of fungal nonribosomal peptide biosynthesis using recombinant enzymes of up to 236 kDa has been demonstrated (Balibar and Walsh, 2006). Here we demonstrate the highly efficient in vivo production of beauvericin in a biocatalytic process using a recombinant *E. coli* strain for the expression of the *bbBeas* gene encoding the 3189 amino acid, 351.9 kDa beauvericin synthetase (Figure 4). Supplementation of the bioconversion reactions with D-Hiv was essential. Further improvements in beauvericin production could be realized with an *E. coli* host strain that facilitates protein folding; cultivation at a reduced temperature during protein expression; and supplementation with L-Phe during an extended bioconversion reaction (Figure 4). Remarkably, beauvericin titers in *E. coli* bioconversions reached about 40% of that from fermentations with the native producer fungus, with a shortened process time (3 days for *E. coli* versus 5–7 days for *B. bassiana*). To the best of our knowledge, this is the first example of the production of a fungal cyclooligomer depsipeptide in a surrogate host.

The physiological roles of natural products in their producer organisms are surprisingly unclear (Turgeon et al., 2008). Although a few fungal host-selective toxins have been shown to contribute to pathogenesis, these represent only a tiny portion of the astonishingly large deduced secondary metabolome "encoded" in fungal genomes (Turgeon et al., 2008). Systematic inactivation of PKS or NRPS genes in fungi with sequenced genomes often reveals only a limited role of these metabolites in pathogenicity (Turgeon et al., 2008). Thus, engineered *F. avenaceum* strains that do not produce enniatin were shown to be less virulent, but production of enniatin did not correlate with virulence in a wider panel of *Fusarium* strains (Herrmann et al., 1996). Investigations into the virulence factors of the insect pathogen *B. bassiana* focused on lytic enzymes that provide entry into the insect host by degrading the cuticle (Fan et al., 2007). In addition to these enzymes, *B. bassiana* also produces varied secondary metabolites (Figure 1). Among these, tenellin was shown to have no significant role in insect pathogenesis (Eley et al., 2007). We have recently demonstrated that the virulence of a bassianolide-nonproducer *B. bassiana* strain is severely compromised (unpublished data). By contrast, up to now the in vivo role of beauvericin in entomopathogenesis has been controversial. Beauvericin has been isolated from silkworm larvae killed by *B. bassiana* (Kwon et al., 2000), showing that its biosynthesis coincides with infection. Isolated beauvericin was toxic to a *Spodoptera* cell line (Fornelli et al., 2004), but was apparently well tolerated by *H. zea* (Champlin and Grula, 1979). Here we compared the virulence of wild-type *B. bassiana* with that of two near-isogenic mutant strains against three insects with reportedly different sensitivities to beauvericin, and monitored insect survival upon a fungal challenge at three different doses (Figure 5). Whereas the beauvericin-nonproducer strain remained marginally infectious, its virulence was severely reduced in all three insect models, including *H. zea* which was previously described as insensitive to synthetic beauvericin (Champlin and Grula, 1979). A *B. bassiana* strain that is deficient in both beauvericin and bassianolide production was similarly compromised in its virulence. However, it did not suffer an increased handicap in pathogenicity compared to the *bbBeas* KO strain in any of the insect models (Figure 5), suggesting that the contribution of these two cyclodepsipeptides to *B. bassiana* virulence is not additive. Herein we propose that beauvericin is a significantly contributing, albeit not indispensable, factor for the virulence of *B. bassiana* against its large variety of insect hosts. However, the physiological roles of beauvericin, and the other secondary metabolites of *B. bassiana*, are likely to be multifaceted. In natural settings, *B. bassiana* has to protect the insect cadaver as a nutrient source against invasion by other competing pathogens and saprophytes. Moreover, *B. bassiana* can also adopt saprophytic, endophytic, or rhizosphere colonizer lifestyles that might demand a secondary metabolome with divergent biotic effector and abiotic stress mitigator activities (Turgeon et al., 2008).

SIGNIFICANCE

Fungal cyclooligomer depsipeptides exhibit potent antibiotic, antifungal, herbicidal, insecticidal, and nematicidal activities, and inhibit multidrug resistance, cancer cell proliferation, and cell motility. These metabolites are biosynthesized by nonribosomal peptide synthetases (NRPSs) that use highly unconventional biosynthetic mechanisms. The identification of the beauvericin synthetase-encoding *bbBeas* gene from the entomopathogen *Beauveria bassiana* allowed us to generalize a proposed reaction sequence for fungal cyclodepsipeptide biosynthesis. This includes the programmed iterative use of modules for the assembly of dipeptidol monomers from amino acid and hydroxycarboxylic acid precursors, and the use of these monomers in a recursive, inter- and intramolecular ester bond-forming oligomerization and cyclization process supported by twin thiolation and unusual N- and C-terminal condensation domains. Comparison of fungal and bacterial pathways for the production and activation of hydroxycarboxylate precursors, and those for the cyclooligomerization of the peptidol monomers, showed evidence of convergent evolution. Comparative sequence analysis of adenylation domains revealed a fungal specificity signature for D-2-hydroxyisovalerate (D-Hiv). Heterologous expression of the *bbBeas* gene in *E. coli* to produce the 351.9 kDa beauvericin synthetase demonstrated, to our knowledge for the first time, the feasibility of the heterologous in vivo production of a fully elaborated fungal cyclooligomer depsipeptide. Comparative insect infection assays using a beauvericin-nonproducer *B. bassiana* strain, created by targeted gene disruption, revealed that beauvericin is a bona fide virulence factor that plays a significant albeit not indispensable role in entomopathogenesis. These results will facilitate the deciphering of the mechanistic details of cyclooligomer depsipeptide biosynthesis in fungi, and enable the use of combinatorial biosynthesis for the production of novel cyclodepsipeptide unnatural natural products. The unambiguous identification of a fungal secondary metabolite as an important virulence factor contributes to our understanding of the ecological role of these metabolites.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions

B. bassiana ATCC 7159 was maintained on potato dextrose agar (PDA). *E. coli* Epi300 and fosmid pCCFOS1 (Epicenter) were used for *B. bassiana* genomic library construction, *E. coli* DH10B and plasmid pJET1 (Fermentas) served for routine cloning and sequencing, and *E. coli* strains BL21(DE3) and Origami B(DE3) with plasmid pACYCDuet-1 (Novagen) were utilized for heterologous expression. *Agrobacterium tumefaciens* LBA4404 (Invitrogen) with the plasmid pAg1-H3 (Zhang et al., 2003) and the *bar* (glufosinate resistance) selectable marker gene from pCB1635 (Sweigard et al., 1997) were used for *Beauveria* transformation.

PCR, Genomic Library Construction, and Screening

A-domain-homologous sequences were amplified from *B. bassiana* using four different primer pairs as described in Supplemental Data available online. RT-PCR was used to confirm the presence of transcripts with A-domain-homologous sequences as described in Supplemental Data. To generate a fosmid library with the CopyControl Fosmid Library Production kit (Epicenter), *B. bassiana* genomic DNA was physically sheared and the 40 kb fraction was isolated by pulsed-field gel electrophoresis. Clones (3456) of this library were arrayed in microtiter plates for ~3.5× coverage of the genome, and used for colony hybridizations with the digoxigenin-labeled (Roche Applied Science) PCR products EA1046 and separately EA1042.

Sequencing and Sequence Analysis

Double-stranded DNA templates for sequencing were generated in pJET1 by cloning fragments of ~2 kb from partial HaeIII digests of fosmids fBbass28A03 and fBbass11F05. DNA sequencing was done at the University of Arizona Genomic Analysis and Core Technology facility. Sequencher 4.7 (Gene Codes) and the VectorNTI suite 9.0 (Invitrogen) were used for sequence assembly and analysis. FGENESH (Softberry) was used to build HMM-based gene models with the *F. graminearum* training set. Gene models were manually curated using exon/intron boundary predictions from SPLICEVIEW (http://zeus2.itb.cnr.it/~webgene/wwwspliceview.html/) and multiple sequence alignments. The UMA algorithm was used for the prediction of domain boundaries in NRPSs (Udwary et al., 2002).

Disruption of the *bbBeas* Gene

The disruption cassette (see Supplemental Data) contained the *bar* selectable marker cloned in between two PCR-amplified DNA fragments covering the 5′ and 3′ regions of the *bbBeas* gene. This disruption cassette was used for *Agrobacterium*-mediated transformation as described (Eley et al., 2007; Fang et al., 2004). Genomic DNA isolated from glufosinate-resistant *B. bassiana* transformants was used for Southern hybridizations with the digoxigenin-labeled (Roche Applied Science) *bar* gene as the probe. Three ectopic integrant and three homologous replacement isolates each were evaluated independently for beauvericin and bassianolide production by fermentation (Xu et al., 2007).

Heterologous Production of Beauvericin

The pACYC-*bbBeas-sfp* expression construct was based on pACYCDuet-1 and assembled as described in Supplemental Data. Starter cultures of *E. coli* BL21(DE3) or Origami B(DE3)-based expression strains were grown overnight at 37°C in LB medium (3 ml) with shaking at 250 rpm. Main-stage cultures (200 ml, LB medium) were grown at 37°C with shaking at 250 rpm to an OD_{600} of 1.0, and transferred to 16°C for induction with IPTG (200 μM, final concentration). Incubation with shaking at 250 rpm was continued for 16 hr at 16°C. Chloramphenicol (12.5 μg/ml, final concentration) was supplemented to all cultures. The cells were collected by centrifugation, and resuspended in fresh LB medium (20 ml) supplemented with D-Hiv and L-Phe (each at 15 mM, final concentrations). The resultant biotransformation reactions were incubated with shaking at 250 rpm for 48 hr at 16°C.

Extraction and Analysis of Beauvericin

Beauvericin was extracted from *B. bassiana* and *E. coli* fermentations and analyzed by HPLC as described (Xu et al., 2007). Accurate mass measurements were done on a Bruker Apex 9.4T FTICR-MS instrument with electrospray ionization to generate the singly charged ions. Beauvericin isolated from *E. coli* biocatalytic reactions, *m/z*: $[M+H]^+$ $C_{45}H_{58}N_3O_9$, calculated 784.4168, measured 784.4181; $[M+Na]^+$ $C_{45}H_{57}N_3O_9Na$, calculated 806.3987, measured 806.4003; $[M+K]^+$ $C_{45}H_{57}N_3O_9K$, calculated 822.3736, measured 822.3739.

Insect Pathogenesis Assays

Aerial conidia were harvested in Triton X-100 (0.05%) from *B. bassiana* strains grown on PDA plates at 28°C for 14 days, and filtered through a 100 μm mesh size cell strainer (BD Falcon). Conidial concentrations were adjusted to 10^1, 10^3, and 10^6 conidia/ml using a hemocytometer. Conidia viability was assessed by outgrowth on PDA plates. Germination rates ranged from 90% to 100%. Last-instar larvae of *G. mellonella* (Timberline Fisheries), *H. zea*, and *S. exigua* (both from Benzon Research) were inoculated by immersion in 10 ml conidial suspensions for 10 s, and transferred individually into the wells of a 12-well plate that contained one filter paper disc (Whatman no. 1). Well plates were incubated at 20°C with a 16:8 hr photoperiod under humid conditions. Controls consisted of larvae treated with water only. Ten larvae of each insect species were used for each *B. bassiana* strain at each inoculum concentration, and the experiments were repeated twice. The larvae were checked for mortality daily for 7 days. Data were analyzed using SigmaStat 3.5 (Systat Software) for analysis of variance (ANOVA) with the Student-Newman-Keuls test to compare means, and correcting for control mortality with Abbott's formula.

ACCESSION NUMBERS

The sequence of the beauvericin locus has been deposited in GenBank under accession number EU886196.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and Supplemental References, and can be found with this article online at http://www.chembiol.com/cgi/content/full/15/9/898/DC1/.

ACKNOWLEDGMENTS

Financial support was provided by the Arizona Biomedical Research Commission, and the Center of Insect Science, University of Arizona. R.O. acknowledges support of the Bio5 Institute, University of Arizona. FTICR-MS service was provided by A. Somogyi at the University of Arizona.

Received: May 8, 2008
Revised: July 16, 2008
Accepted: July 22, 2008
Published: September 19, 2008

REFERENCES

Balibar, C.J., and Walsh, C.T. (2006). GliP, a multimodular nonribosomal peptide synthetase in *Aspergillus fumigatus*, makes the diketopiperazine scaffold of gliotoxin. Biochemistry *45*, 15029–15038.

Bergendahl, V., Linne, U., and Marahiel, M.A. (2002). Mutational analysis of the C-domain in nonribosomal peptide synthesis. Eur. J. Biochem. *269*, 620–629.

Bergmann, S., Schumann, J., Scherlach, K., Lange, C., Brakhage, A.A., and Hertweck, C. (2007). Genomics-driven discovery of PKS-NRPS hybrid metabolites from *Aspergillus nidulans*. Nat. Chem. Biol. *3*, 213–217.

Billich, A., and Zocher, R. (1987). N-methyltransferase function of the multifunctional enzyme enniatin synthetase. Biochemistry *26*, 8417–8423.

Carmeliet, P. (2003). Blood vessels and nerves: common signals, pathways and diseases. Nat. Rev. Genet. *4*, 710–720.

Challis, G.L., Ravel, J., and Townsend, C.A. (2000). Predictive, structure-based model of amino acid recognition by nonribosomal peptide synthetase adenylation domains. Chem. Biol. *7*, 211–224.

Champlin, F.R., and Grula, E.A. (1979). Noninvolvement of beauvericin in the entomopathogenicity of *Beauveria bassiana*. Appl. Environ. Microbiol. *37*, 1122–1126.

 ©2008 Elsevier Ltd All rights reserved

Eley, K.L., Halo, L.M., Song, Z., Powles, H., Cox, R.J., Bailey, A.M., Lazarus, C.M., and Simpson, T.J. (2007). Biosynthesis of the 2-pyridone tenellin in the insect pathogenic fungus *Beauveria bassiana*. ChemBioChem *8*, 289–297.

Fan, Y., Fang, W., Guo, S., Pei, X., Zhang, Y., Xiao, Y., Li, D., Jin, K., Bidochka, M.J., and Pei, Y. (2007). Increased insect virulence in *Beauveria bassiana* strains overexpressing an engineered chitinase. Appl. Environ. Microbiol. *73*, 295–302.

Fang, W., Zhang, Y., Yang, X., Zheng, X., Duan, H., Li, Y., and Pei, Y. (2004). *Agrobacterium tumefaciens*-mediated transformation of *Beauveria bassiana* using an herbicide resistance gene as a selection marker. J. Invertebr. Pathol. *85*, 18–24.

Finking, R., and Marahiel, M.A. (2004). Biosynthesis of nonribosomal peptides. Annu. Rev. Microbiol. *58*, 453–488.

Fornelli, F., Minervini, F., and Logrieco, A. (2004). Cytotoxicity of fungal metabolites to lepidopteran (*Spodoptera frugiperda*) cell line (SF-9). J. Invertebr. Pathol. *85*, 74–79.

Fujimori, D.G., Hrvatin, S., Neumann, C.S., Strieker, M., Marahiel, M.A., and Walsh, C.T. (2007). Cloning and characterization of the biosynthetic gene cluster for kutznerides. Proc. Natl. Acad. Sci. USA *104*, 16498–16503.

Glinski, M., Urbanke, C., Hornbogen, T., and Zocher, R. (2002). Enniatin synthetase is a monomer with extended structure: evidence for an intramolecular reaction mechanism. Arch. Microbiol. *178*, 267–273.

Hacker, C., Glinski, M., Hornbogen, T., Doller, A., and Zocher, R. (2000). Mutational analysis of the N-methyltransferase domain of the multifunctional enzyme enniatin synthetase. J. Biol. Chem. *275*, 30826–30832.

Haese, A., Schubert, M., Herrmann, M., and Zocher, R. (1993). Molecular characterization of the enniatin synthetase gene encoding a multifunctional enzyme catalyzing *N*-methyldepsipeptide formation in *Fusarium scirpi*. Mol. Microbiol. *7*, 905–914.

Haese, A., Pieper, R., von Ostrowski, T., and Zocher, R. (1994). Bacterial expression of catalytically active fragments of the multifunctional enzyme enniatin synthetase. J. Mol. Biol. *243*, 116–122.

Halo, L.M., Marshall, J.W., Yakasai, A.A., Song, Z., Butts, C.P., Crump, M.P., Heneghan, M., Bailey, A.M., Simpson, T.J., Lazarus, C.M., and Cox, R.J. (2008). Authentic heterologous expression of the tenellin iterative polyketide synthase nonribosomal peptide synthetase requires coexpression with an enoyl reductase. ChemBioChem *9*, 585–594.

Hamill, R.L., Higgens, C.E., Boaz, M.E., and Gorman, M. (1969). The structure of beauvericin, a new depsipeptide antibiotic toxic to *Artemia salina*. Tetrahedron Lett. *10*, 4255–4258.

Herrmann, M., Zocher, R., and Haese, A. (1996). Effect of disruption of the enniatin synthetase gene on the virulence of *Fusarium avenaceum*. Mol. Plant Microbe Interact. *9*, 226–232.

Hornbogen, T., Riechers, S.P., Prinz, B., Schultchen, J., Lang, C., Schmidt, S., Mügge, C., Turkanovic, S., Süssmuth, R.D., Tauberger, E., and Zocher, R. (2007). Functional characterization of the recombinant *N*-methyltransferase domain from the multienzyme enniatin synthetase. ChemBioChem *8*, 1048–1054.

Jirakkakul, J., Punya, J., Pongpattanakitshote, S., Paungmoung, P., Vorapreeda, N., Tachaleat, A., Klomnara, C., Tanticharoen, M., and Cheevadhanarak, S. (2008). Identification of the nonribosomal peptide synthetase gene responsible for bassianolide synthesis in wood-decaying fungus *Xylaria* sp. BCC1067. Microbiology *154*, 995–1006.

Jow, G.-M., Chou, C.-J., Chen, B.-F., and Tsai, J.-H. (2004). Beauvericin induces cytotoxic effects in human acute lymphoblastic leukemia cells through cytochrome *c* release, caspase 3 activation: the causative role of calcium. Cancer Lett. *216*, 165–173.

Kopp, F., and Marahiel, M.A. (2007). Macrocyclization strategies in polyketide and nonribosomal peptide biosynthesis. Nat. Prod. Rep. *24*, 735–749.

Krause, M., Lindemann, A., Glinski, M., Hornbogen, T., Bonse, G., Jeschke, P., Thielking, G., Gau, W., Kleinkauf, H., and Zocher, R. (2001). Directed biosynthesis of new enniatins. J. Antibiot. (Tokyo) *54*, 797–804.

Kwon, A.C., Bang, E.J., Choi, S.U., Lee, W.C., Cho, S.Y., Jung, I.Y., Kim, S.Y., and Lee, K.R. (2000). Cytotoxic cyclodepsipeptides of *Bombycis corpus* 101A. Yakhak Hoechi *44*, 115–118.

Lee, C., Gorisch, H., Kleinkauf, H., and Zocher, R. (1992). A highly specific D-hydroxyisovalerate dehydrogenase from the enniatin producer *Fusarium sambucinum*. J. Biol. Chem. *267*, 11741–11744.

Magarvey, N.A., Ehling-Schulz, M., and Walsh, C.T. (2006). Characterization of the cereulide NRPS α-hydroxy acid specifying modules: activation of α-keto acids and chiral reduction on the assembly line. J. Am. Chem. Soc. *128*, 10698–10699.

Peeters, H., Zocher, R., and Kleinkauf, H. (1988). Synthesis of beauvericin by a multifunctional enzyme. J. Antibiot. (Tokyo) *41*, 352–359.

Pieper, R., Haese, A., Schröder, W., and Zocher, R. (1995). Arrangement of catalytic sites in the multifunctional enzyme enniatin synthetase. Eur. J. Biochem. *230*, 119–126.

Quadri, L.E.N., Weinreb, P.H., Lei, M., Nakano, M.M., Zuber, P., and Walsh, C.T. (1998). Characterization of Sfp, a *Bacillus subtilis* phosphopantetheinyl transferase for peptidyl carrier domains in peptide synthetases. Biochemistry *37*, 1585–1595.

Rausch, C., Weber, T., Kohlbacher, O., Wohlleben, W., and Huson, D.H. (2005). Specificity prediction of adenylation domains in nonribosomal peptide synthetases (NRPS) using transductive support vector machines (TSVMs). Nucleic Acids Res. *33*, 5799–5808.

Schwarzer, D., Finking, R., and Marahiel, M.A. (2003). Nonribosomal peptides: from genes to products. Nat. Prod. Rep. *20*, 275–287.

Stachelhaus, T., Mootz, H.D., and Marahiel, M.A. (1999). The specificity-conferring code of adenylation domains in nonribosomal peptide synthetases. Chem. Biol. *6*, 493–505.

Steinrauf, L.K. (1985). Beauvericin and the other enniatins. Met. Ions Biol. Syst. *19*, 139–171.

Sweigard, J., Chumley, F., Carroll, A., Farrall, L., and Valent, B. (1997). A series of vectors for fungal transformation. Fungal Genet. Newsl. *44*, 52–53.

Turgeon, B.G., Oide, S., and Bushley, K. (2008). Creating and screening *Cochliobolus heterostrophus* non-ribosomal peptide synthetase mutants. Mycol. Res. *112*, 200–206.

Udwary, D.W., Merski, M., and Townsend, C.A. (2002). A method for prediction of the locations of linker regions within large multifunctional proteins, and application to a type I polyketide synthase. J. Mol. Biol. *323*, 585–598.

von Döhren, H. (2004). Biochemistry and general genetics of nonribosomal peptide synthetases in fungi. Adv. Biochem. Engin. Biotechnol. *88*, 217–264.

Wenzel, S.C., Meiser, P., Binz, T.M., Mahmud, T., and Müller, R. (2006). Nonribosomal peptide biosynthesis: point mutations and module skipping lead to chemical diversity. Angew. Chem. Int. Ed. Engl. *45*, 2296–2301.

Xu, Y., Zhan, J., Wijeratne, E.M., Burns, A.M., Gunatilaka, A.A., and Molnár, I. (2007). Cytotoxic and antihaptotactic beauvericin analogues from precursor-directed biosynthesis with the insect pathogen *Beauveria bassiana* ATCC 7159. J. Nat. Prod. *70*, 1467–1471.

Zhan, J., Burns, A.M., Liu, M.X., Faeth, S.H., and Gunatilaka, A.A.L. (2007). Search for cell motility and angiogenesis inhibitors with potential anticancer activity: beauvericin and other constituents of two endophytic strains of *Fusarium oxysporum*. J. Nat. Prod. *70*, 227–232.

Zhang, A., Lu, P., Dahl-Roshak, A.M., Paress, P.S., Kennedy, S., Tkacz, J.S., and An, Z. (2003). Efficient disruption of a polyketide synthase gene (pks1) required for melanin synthesis through *Agrobacterium*-mediated transformation of *Glarea lozoyensis*. Mol. Genet. Genomics *268*, 645–655.

Zhang, L., Yan, K., Zhang, Y., Huang, R., Bian, J., Zheng, C., Sun, H., Chen, Z., Sun, N., An, R., et al. (2007). High-throughput synergy screening identifies microbial metabolites as combination agents for the treatment of fungal infections. Proc. Natl. Acad. Sci. USA *104*, 4606–4611.

Zirkle, R., Ligon, J.M., and Molnár, I. (2004). Heterologous production of the antifungal polyketide antibiotic soraphen A of *Sorangium cellulosum* So ce26 in *Streptomyces lividans*. Microbiology *150*, 2761–2774.