Fungal Genetics and Biology 46 (2009) 353–364

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

Biosynthesis of the cyclooligomer depsipeptide bassianolide, an insecticidal virulence factor of *Beauveria bassiana*

Yuquan Xu [a], Rousel Orozco [b], E.M. Kithsiri Wijeratne [a], Patricia Espinosa-Artiles [a], A.A. Leslie Gunatilaka [a,c], S. Patricia Stock [b,c], István Molnár [a,c,*]

[a] SW Center for Natural Products Research and Commercialization, Office of Arid Lands Studies, College of Agriculture and Life Sciences, The University of Arizona, 250 E. Valencia Rd., Tucson, AZ 85706-6800, USA
[b] Department of Entomology, College of Agriculture and Life Sciences, The University of Arizona, 1140 E. South Campus Dr., Tucson, AZ 85721-0036, USA
[c] Bio5 Institute, The University of Arizona, 1657 E Helen Street, Tucson, AZ 85721, USA

ARTICLE INFO

Article history:
Received 2 June 2008
Accepted 3 March 2009
Available online 11 March 2009

Keywords:
Nonribosomal peptide synthetase
Cyclic depsipeptide
Iterative biosynthesis
Agrobacterium-mediated transformation
Insect pathogenesis assay
Virulence

ABSTRACT

Beauveria bassiana is a facultative entomopathogen with an extremely broad host range that is used as a commercial biopesticide for the control of insects of agricultural, veterinary and medical significance. *B. bassiana* produces bassianolide, a cyclooligomer depsipeptide secondary metabolite. We have cloned the *bbBsls* gene of *B. bassiana* encoding a nonribosomal peptide synthetase (NRPS). Targeted inactivation of the *B. bassiana* genomic copy of *bbBsls* abolished bassianolide production, but did not affect the biosynthesis of beauvericin, another cyclodepsipeptide produced by the strain. Comparative sequence analysis of the BbBSLS bassianolide synthetase revealed enzymatic domains for the iterative synthesis of an enzyme-bound dipeptidol monomer intermediate from D-2-hydroxyisovalerate and L-leucine. Further BbBSLS domains are predicted to catalyze the formation of the cyclic tetrameric ester bassianolide by recursive condensations of this monomer. Comparative infection assays against three selected insect hosts established bassianolide as a highly significant virulence factor of *B. bassiana*.

1. Introduction

The identification of the filamentous fungus *Beauveria* (teleomorph: *Cordyceps*) *bassiana* (Hypocreales) as the causative agent of the white muscardine disease of *Bombyx mori* (domestic silkworm) by A. Bassi in 1835 provided the first scientifically proven link between a communicable disease and an isolated microbial pathogen, and contributed to the later development of the germ theory of diseases by Koch and Pasteur. The extremely broad host range (>700 insect hosts) of this fungus has since then been exploited to develop commercial biocontrol strategies against many agriculturally important insect pests and arthropod disease vectors (Samish et al., 2004). However, a detailed molecular level understanding of the pathogenic mechanisms of *B. bassiana* is still lacking. Production of an extracellular chitinase proved to be necessary for *B. bassiana* virulence (Fan et al., 2007). Further putative virulence factors include secreted lipases and proteases that might also take part in the degradation of the insect cuticle (Joshi et al., 1995). Secreted protein toxins like bassiacridin (Quesada-Moraga and Vey, 2004) and secreted, mildly toxic primary metabolites like oxalic acid (Kirkland et al., 2005) might also contribute to pathogenesis. On the other hand, filamentous fungi are known to be prolific producers of small molecule secondary metabolites (Keller et al., 2005), many of which might also take part in various biotic interactions including pathogenic processes. Thus, *B. bassiana* produces several secondary metabolites of varied structures, but the contribution of these metabolites to pathogenesis has been largely unknown. Pioneering work by the Simpson group showed that the polyketide – nonribosomal peptide hybrid metabolite tenellin is not involved in virulence (Eley et al., 2007). Conversely, we have recently shown that the cyclooligomer depsipeptide beauvericin is a significant but not essential virulence factor of *B. bassiana* (Xu et al., 2008). Several lines of evidence suggest that a second cyclooligomer depsipeptide, bassianolide, might also be important during insect pathogenesis. Purified bassianolide induces atony to the larvae of *Helicoverpa (Heliothis) zea* (Champlin and Grula, 1979) and is toxic to silkworm larvae (Suzuki et al., 1977). Bassianolide has been detected in the cadavers of silkworm larvae killed by *B. bassiana* infection (Kwon et al., 2000), demonstrating that the production of this metabolite is compatible and coincides with insect infection. Thus, bassianolide production is widely claimed in the biopesticide product literature to contribute to the effectiveness of commercial biological insecticide preparations containing the spores of *B. bassiana* and *Verticillium lecanii* (Suzuki et al., 1977). Apart from its insecticidal properties, purified bassianolide also inhibits acetylcholine-induced smooth muscle contraction (Nakajyo et al., 1983), and shows cytotoxic and moderate antiplasmo-

* Corresponding author. Fax: +1 520 741 0113.
E-mail address: imolnar@cals.arizona.edu (I. Molnár).

1087-1845/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.fgb.2009.03.001

dial and anti-mycobacterial activities *in vitro* (Jirakkakul et al., 2008).

Cyclooligomer nonribosomal depsipeptides are macrocyclic lactones derived from repeated structural units (“monomers”) that consist of amino acid and 2-hydroxycarboxylic acid building blocks. These monomers undergo oligomerization *via* head-to-tail condensation or ligation through side chains, followed by macrocycle closure (Kopp and Marahiel, 2007). Thus, bassianolide (Fig. 1) is an octadepsipeptide with a 24-membered macrolactone ring that is formed as the cyclic tetrameric ester of the dipeptidol monomer D-hydroxyisovalerate (D-Hiv) – *N*-methyl-L-leucine (*N*-Me-Leu). Other fungal octadepsipeptides like the PF1022 congeners (Fig. 1) are composed of different dipeptidol monomers, and show highly potent anthelminthic activity with no significant insecticidal activity (Scherkenbeck et al., 2002). Fungal hexadepsipeptides with an 18-membered macrolactone ring like beauvericin or the enniatins (Fig. 1) are formed as the cyclic trimeric esters of dipeptidol monomer intermediates. In particular, the prevalent hexadepsipeptide enniatin C is assembled from the same D-Hiv – *N*-Me-Leu dipeptidol monomer units as bassianolide, but differs in the number of monomer units ligated to form the final macrocycle. Beauvericin and the enniatins display a broad range of biological activities, including antibiotic, antifungal, anthelminthic, phytotoxic, cell antiproliferative, and cell motility inhibitory activities (Firakova et al., 2007; Xu et al., 2007).

Nonribosomal peptides are biosynthesized on multi-domain nonribosomal peptide synthetase (NRPS) enzymes by the thiotemplate mechanism (Finking and Marahiel, 2004; Fischbach and Walsh, 2006). Separate NRPS core domains activate the precursors (A = adenylation domain), tether these as covalent thioesters (T = thiolation domain), and catalyze their condensation (C domain) during product formation (Finking and Marahiel, 2004; Fischbach and Walsh, 2006). Enzyme-bound precursors and intermediates can also be tailored by additional domains of the NRPS to yield *N*-methylated (M domain), oxidized or reduced (Ox and KR domains), and epimerized (E domain) moieties. Depsipeptide synthetase NRPSs incorporate 2-hydroxycarboxylic acids beside amino acids, and thus might form ester instead of amide bonds. The synthetic and tailoring domains are repeated along the NRPS multienzymes and are organized into modules. Each of these modules is responsible for the selection, activation, condensation, and modification of a single precursor. In canonical NRPSs, each module and each active site domain is used only once in an assembly line fashion (processive NRPSs), although rare exceptions to this rule have been observed (Schwecke et al., 2006; Wenzel et al., 2005). In contrast, NRPSs for cyclooligomer depsipeptides house complete modules sufficient only for the biosynthesis of the peptidol monomer unit (Haese et al., 1993; Magarvey et al., 2006b), but evolved mechanisms that allow the iterative use of these modules and also provide for the recursive ligation (oligomerization) and final cyclization of two or more of these monomer units (Kopp and Marahiel, 2007).

The first fungal cyclooligomer depsipeptide synthetase (CODS) cloned and characterized was the enniatin synthetase ESYN from *Fusarium equiseti* (synonym: *Fusarium scirpi*) (Glinski et al., 2002; Haese et al., 1993; Hornbogen et al., 2007; Pieper et al., 1995). The PF1022 synthetase (PFSYN) has been purified and biochemically characterized from *Mycelia sterilia* (Weckwerth et al., 2000), but the sequencing of the corresponding gene has been reported only in the patent literature (Mido et al., 2001). We have recently cloned and heterologously expressed the BbBEAS beauvericin synthetase of *B. bassiana* (Xu et al., 2008). The *bbBeas* gene is clustered with a gene (*kivr*) encoding ketoisovalerate reductase, the enzyme responsible for the biosynthesis of D-Hiv in *B. bassiana* from 2-ketoisovalerate, an intermediate of L-valine metabolism (Xu et al., 2009). While this manuscript was in preparation, an orphan NRPS gene of the wood-decaying fungus *Xylaria* sp. has been shown to encode the NRPSXY bassianolide synthetase (Jirakkakul et al., 2008).

In an effort to generate a more detailed understanding of the mechanisms of cyclooligomer depsipeptide biosynthesis in fungi and to understand the role of these natural products as possible

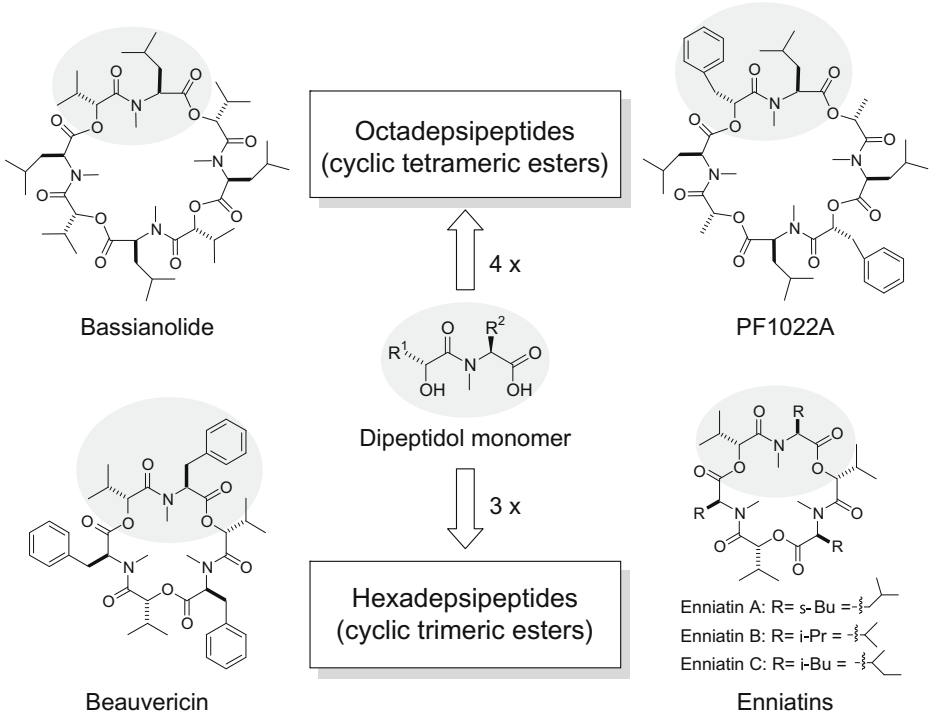

Fig. 1. Fungal cyclooligomer depsipeptides.