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Creating and screening *Cochliobolus heterostrophus* non-ribosomal peptide synthetase mutants

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ABSTRACT

An exhaustive characterization of the set of non-ribosomal peptide synthetase (NRPS) genes of the corn pathogen, *Cochliobolus heterostrophus*, and the small molecule peptides produced by the enzymes they encode, has been undertaken to ascertain the role of the peptide metabolites in the fungal cell. To date, the NRPS method of peptide biosynthesis has been described for filamentous ascomycete fungi (and to a limited extent, for basidiomycete fungi) and for bacteria, only. In addition to structural diversity, non-ribosomal peptides have a broad spectrum of biological activities, many are useful in medicine, agriculture, industry, and biological research. However, to suggest that inter-organismal activities is their primary function is likely incorrect; in fact, the physiological significance of these peptides to the producing fungi is largely unknown. We document that NRPS enzymes are purveyors of small molecules for both basal metabolism and for specialized environmental niches and that some are conserved, but most are not.

Introduction

Non-ribosomal peptide synthetases (NRPSs) are multimodular enzymes that produce non-ribosomal peptides (NRPs) through a thiotemplate mechanism, independent of the organelles best known for protein synthesis, the ribosomes. These enzymes are usually thought of as participants in secondary metabolism and are among the largest enzymes known. To date, the NRPS method of peptide biosynthesis has been described mostly for filamentous ascomycete fungi (an exception being siderophore biosynthesis in basidiomycetes such as *Ustilago*) and for bacteria.

The diversity of possible NRP products is potentially limitless as NRPs can be composed of D- and L-amino acids, protein and non-protein amino acids, hydroxy acids, ornithine, β-amino acids, and other unusual constituents (Schwarzer *et al.* 2003). Furthermore, NRPs can be linear, cyclic, or branched cyclic, and may be modified by glycosylation, N-methylation or acylation. In addition to structural diversity, NRPs have an astonishingly broad spectrum of biological activities, many of which have been useful in medicine, agriculture, industry, and biological research (for reviews see Kleinkauf & von Doehren 1996; Schwarzer & Marahiel 2001; Stachelhaus & Marahiel 1995; von Dohren *et al.* 1997).

Products made by NRPS enzymes include well-known antibiotics (penicillin), immunosuppressants (cyclosporin), antitumour agents (actinomycin, bleomycin) and toxins involved in pathogenesis (HC-toxin, enniatin, AM-toxin). However, despite the fact that the activities of the peptide products, with respect to their interactions with other organisms, are well documented, the physiological significance of these small peptides to the producing fungi is largely unknown. Some proposed activities include roles as signal molecules for coordination of growth and differentiation (hyphal fusion, aerial

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hyphae formation, conidiation, sexual reproduction, pigments, 'scents') (Hahn & Dubnau 1991; Horinouchi & Beppu 1990; Marahiel *et al.* 1997; Schaeffer 1969), as aids in the breakdown of metabolic products (Davies 1990), as defense compounds that kill competing microorganisms (Vining 1990), as 'reward' molecules, such as siderophores, which assist in nutrient uptake (Challis *et al.* 2000), or as virulence effectors (Haese *et al.* 1993; Johnson *et al.* 2000; Panaccione *et al.* 1992; Scott-Craig *et al.* 1992).

Cross-genome comparisons indicate that most filamentous ascomycetes carry many genes encoding NRPSs (Table 1). Most non-ribosomal peptide synthetase genes (*NPS*) are not conserved from one species to the next, although some appear to be discontinuously distributed. For example, *Cochliobolus heterostrophus* carries four *NPS* genes that are common to other filamentous ascomycetes, whereas the remaining eight *NPS* genes may or may not have a counterpart in one or more additional fungal species. To date no *NPS* has been found in the yeasts, except for one in *Schizosaccharomyces pombe* (*sib1*, encoding a siderophore) (Schwecke *et al.* 2006). If, as predictions suggest, there are over 100 K different fungal species on the planet (Hawkesworth 1991) a large percentage of which are filamentous ascomycetes, each carrying 10–20 *NPS* genes, the capacity for biosynthesis of, and the diversity of, small molecules is astonishing.

Our laboratory is involved in an exhaustive functional characterization of *NPS* genes to ascertain the role of the peptide secondary metabolite products of the enzymes in (and for) the fungal cell. We hypothesize that the metabolites produced by NRPSs can have a significant impact on the lives of their producers and, although associated with secondary metabolism, in some cases are purveyors of small molecules for basal metabolism. Thus, these molecules play more diverse roles than previously revealed. We suggest that these molecules are also required in particular environmental niches, often, but not always, associated with stress conditions [e.g. low or high iron, oxidative or nitrosative stress, on the plant host, when mating, when the fungal population is too high (or too low), etc].

Table 1 – Number of non-ribosomal peptide synthetase genes in selected fungal ascomycete genomes

Species	# of *NPS*s	# NPS/PKS[a] hybrids	# PKS/NPS hybrids	Reference
C. heterostrophus	12 + 1 pseudogene	1	0	Lee *et al.* (2005) Oide *et al.* (2006)
A. brassicicola	8			Kim *et al.* (2007), C. Lawrence (pers. comm.) Bushley & Turgeon, unpubl.
S. nodorum	~18			Bushley & Turgeon, unpubl.
F. graminearum	19			MIPS
F. verticillioides	20		3	Lee *et al.* (2005)
B. cinerea	12		3	Lee *et al.* (2005)
N. crassa	3	0	0	Galagan *et al.* (2003)
M. grisea	6	0	8	Dean *et al.* (2005)
S. pombe	1	0	0	Schwecke *et al.* (2006)

a blank = unknown.

Experimental organism

The experimental organism is *Cochliobolus heterostrophus*, the filamentous ascomycete pathogen of maize, an especially appropriate subject for a study of *NPS* gene (*ChNPS*) function because it belongs to a genus renowned for its ability to produce secondary metabolites, including NRPs (Walton *et al.* 2004; Yoder *et al.* 1997). Closely related genera include *Alternaria*, *Setosphaeria*, and *Pyrenophora* spp., genera best known for their host-selective toxin-producing abilities. Among these, *C. heterostrophus* is unsurpassed in terms of genetic tractability (both conventional and molecular) and very efficient homologous recombination between transforming DNA and target genomic sequences, which facilitates functional analysis by site-specific gene deletion (80–100 %). The *C. heterostrophus* genome is being sequenced by the Joint Genome Institute. *C. heterostrophus* is deposited in ATCC.

NPS genes in *Cochliobolus heterostrophus*

Twelve *NPS* genes (and one pseudogene) have been found in the *Cochliobolus heterostrophus* genome, only one of which is required for normal virulence of the fungus to maize (Lee *et al.* 2005). When first discovered, this was an unexpected finding, as before this work, virulence/pathogenicity roles were among the most 'popular' suggested roles for NRP metabolites, at least for fungal plant pathogens. What then are the remainder of these small molecules doing?

Organization of NRPS proteins

In terms of enzyme organization, a minimal NRPS module is composed of an adenylation (A), a thiolation [T, also called peptidyl carrier protein (PCP)], and a condensation (C) domain. The A domain is required for amino acid substrate recognition and activation. The T domain is the site for 4′ phosphopantetheine (4′PP) cofactor binding; the holo-enzyme then activates aminoacyl substrates to form a thioester bond. The C domain, typically found after each A–T module functions in peptide bond formation and elongation of the nascent peptide (Fig 1).

Generally, the number and order of modules determine the length and structure of the resulting NRP. In addition to A, T, and C domains, an N-methyl transferase (M) domain that methylates the amino acid specified by the A domain may be between the A and T domains, and an epimerase (E) domain that changes an amino acid from L- to D-form may be between the T and C domains. In some NRPSs, a thioesterase domain is found at the C-terminal end of the protein for