ELSEVIER

FEMS Microbiology Letters 248 (2005) 83–91

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

The expression of selected non-ribosomal peptide synthetases in *Aspergillus fumigatus* is controlled by the availability of free iron

Kathrin Reiber [a], Emer P. Reeves [a], Claire M. Neville [a], Robert Winkler [a,b], Peter Gebhardt [a,b], Kevin Kavanagh [a], Sean Doyle [a,*]

[a] *National Institute for Cellular Biotechnology, Department of Biology, National University of Ireland, Maynooth, Co. Kildare, Ireland*
[b] *Hans-Knoell-Institute for Natural Product Research, Department of Biostructure Chemistry, Jena, Beutenbergstr. 11, D-07745, Germany*

Received 7 April 2005; received in revised form 12 May 2005; accepted 16 May 2005

First published online 31 May 2005

Edited by G.M. Gadd

Abstract

Three non-ribosomal peptide synthetase genes, termed *sidD*, *sidC* and *sidE*, have been identified in *Aspergillus fumigatus*. Gene expression analysis by RT-PCR confirms that expression of both *sidD* and *C* was reduced by up to 90% under iron-replete conditions indicative of a likely role in siderophore biosynthesis. *SidE* expression was less sensitive to iron levels. In addition, two proteins purified from mycelia grown under iron-limiting conditions corresponded to SidD (~200 kDa) and SidC (496 kDa) as determined by MALDI ToF peptide mass fingerprinting and MALDI LIFT-ToF/ToF. Siderophore synthetases are unique in bacteria and fungi and represent an attractive target for antimicrobial chemotherapy.

Keywords: NRPS; Iron; Siderophore; MALDI ToF; Secondary metabolites; Proteomics; 4′-PPTase

1. Introduction

The fungus *Aspergillus fumigatus* is receiving increasing attention as a significant cause of mortality in immunocompromised individuals such as bone marrow and stem cell transplant patients [1]. The near-completion of the *A. fumigatus* genome sequencing effort and resultant data [2] has greatly facilitated the search for genes which may be involved in mediating organismal pathogenicity and a number of reports have shown that either specific gene deletion or silencing may downregulate the virulence of *A. fumigatus* [3–5]. Hissen et al. [6] have convincingly argued that siderophores produced by the fungus are involved in iron acquisition from transferrin and that, at least in vitro, they may be responsible for organism growth and survival in human serum.

Most members of the family Ascomycota (e.g., *A. fumigatus*) produce hydroxamate-type siderophores. Hydroxamates can be sub-divided into four groups: rhodotorulic acid, fusarinines, coprogens and ferrichromes. *A. fumigatus* produces ferrichrome class siderophores (e.g., ferricrocin), which are cyclic peptides [7]. In addition, a number of siderophores (primarily triacetylfusarinine C and ferricrocin) were purified from *A. fumigatus* culture medium after 8 h of growth in medium containing human serum [6].

In *Aspergillus nidulans*, siderophore biosynthesis commences with the N^5-hydroxylation of ornithine, followed by transacylation and subsequent covalent assembly of the modified ornithine residues with or without further amino acids (e.g., serine, alanine and glycine),

* Corresponding author. Tel.: +353 1 7083858; fax: +353 1 7083845.
E-mail address: sean.doyle@nuim.ie (S. Doyle).

0378-1097/$22.00
doi:10.1016/j.femsle.2005.05.028

catalyzed by non-ribosomal peptide synthetases (NRPS) [8,9]. NRPS are multifunctional enzymes, which operate via a thiotemplate mechanism [10] and consist of semi-autonomous units or modules (Fig. 1), co-linearly arranged to the structure of the peptide product. A typical module minimally consists of an adenylation domain (A-domain), responsible for the activation of its cognate substrate amino acid as an amino acyl adenylate, a downstream located peptidyl carrier protein (PCP or P) or thiolation domain and an upstream positioned condensation domain (C-domain) [11]. Siderophores are predominantly released by cyclization of the peptide product, often catalyzed by cyclotrimerizing TE-domains (e.g., enterobactin, yersiniabactin) or variants of the condensation domains, termed as cyclization domains [12,13]. Furthermore, the iterative (repetitive) use of the enzyme template, as well as a non-linear structure (e.g., –P–C–P–C–), seems to be characteristic of siderophore synthetases. S*idC*, an NRP synthetase involved in ferricrocin biosynthesis in *A. nidulans*, encodes a 525.5 kDa protein comprising three adenylation, five condensation and five thiolation domains [9,14]. In spite of only three complete modules, the entire hexapeptides of the cyclic hydroxamate-type siderophores, such as triacetylfusarinine C and ferricrocin are formed by repeated use of modules. Furthermore, *sidA* encodes an L-ornithine-N^5-monooxygenase which catalyzes the first step in the siderophore biosynthesis pathway in *A. nidulans* [9]. Deletion of the *sidA* gene completely inhibited siderophore biosynthesis in *A. nidulans* and resulted in severely diminished fungal viability. The *sidA* ortholog in *A. fumigatus* has been recently reported and deletion mutants show a distinct reduction in virulence [3]. *Ustilago maydis*, an infectious agent of maize, scavenges iron using extracellular hydroxamate siderophores, where siderophore production is initiated by the *sid1* gene. However, analysis of siderophore mutants in *U. maydis* suggests that the siderophore biosynthetic pathway is not involved in the infection of maize [15]. More recently, Yuan et al. [16] have identified an NRP synthetase gene (*sid2*) involved in ferrichrome biosynthesis in *U. maydis*. Expression of *sid2* was upregulated (×2.5) in low-iron compared to high-iron media.

We have detected a number of putative NRPS open reading frames in the genome of *A. fumigatus* and recently demonstrated in vitro NRPS (termed Pes1/SidB) activation by a functional 4′-phosphopantetheinyl transferase [17]. Evidence is now presented that at least three distinct NRPS genes are susceptible to regulation of expression by the level of free iron present in the culture medium.

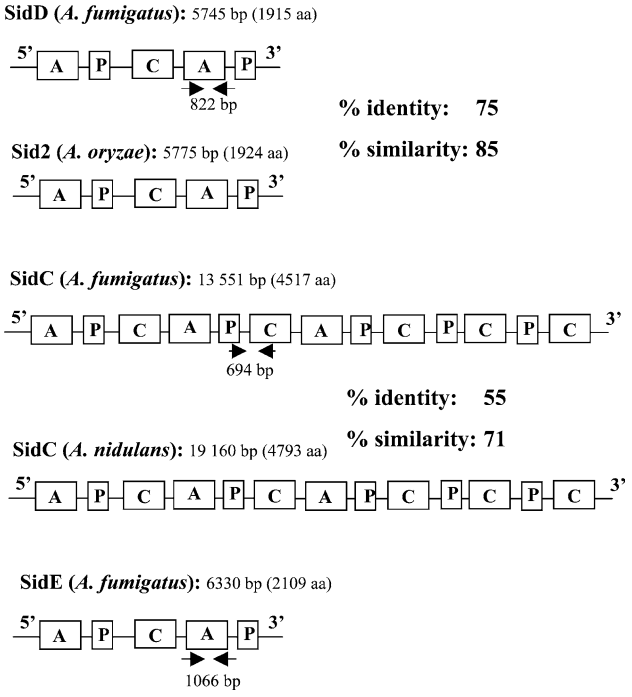

Fig. 1. Modular organization of three non-ribosomal peptide synthetase genes from *Aspergillus fumigatus*: *sidD*, *C* and *E*, and relevant orthologs (i.e., *sid2* of *A. oryzae* and *sidC* of *A. nidulans*). Where available, the degree of identity and similarity is indicated. Single catalytic units of NRPS are indicated as follows: A, adenylation; P, thiolation and C, condensation domain. The position of PCR amplified and sequenced regions is marked by arrows and has been deposited in GenBank. Preliminary data were obtained from http://www.tigr.org.

2. Materials and methods

2.1. Microorganisms and culture media

Aspergillus fumigatus ATCC 26933 (obtained from the American Type Culture Collection, Manasas, VA, USA) was used in this study and maintained as spore suspensions containing 50% (v/v) glycerol at −80 °C. The fungus was grown in a mineral salt medium (pH 6.8) composed of 25 g/l glucose, 3.5 g/l $(NH_4)_2SO_4$, 2.0 g/l KH_2PO_4, 0.5 g/l $MgSO_4$ and 8 mg/l $ZnSO_4$ and supplemented with different concentrations of $Fe(III)Cl_3$, as required. *A. fumigatus* cultures were set up in 500 ml shaking flasks at 230 rpm, previously treated to ensure that all traces of iron were removed from the glassware [6]. Medium (250 ml) was inoculated with *A. fumigatus* conidia at a final concentration of 10^7 per ml and flasks were incubated at 37 °C and 230 rpm.

2.2. Bioinformatic analyzes

Preliminary *A. fumigatus* sequence data were obtained from The Institute for Genomic Research website at http://www.tigr.org. The unannotated *A. fumigatus* genome was interrogated, via a BLAST program, for non-ribosomal peptide synthetase encoding open reading frames (ORFs) using the *Acremonium chrysogenum*