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Distribution, expression and expansion of *Aspergillus fumigatus* LINE-like retrotransposon populations in clinical and environmental isolates

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ABSTRACT

Functional genomic analysis of the mould pathogen *Aspergillus fumigatus* has identified multiple secondary metabolism genes upregulated in the host niche. Intriguingly, transcriptomic analyses of infectious germlings, germinating spores and mutants lacking the LaeA methyltransferase reveal differential expression of transposable elements (TEs), which often flank secondary metabolite gene clusters. In this study we investigate, in clinical and environmental isolates, the structure and distribution of a specific class of *A. fumigatus* long interspersed nuclear element (LINE)-like retrotransposons occupying subtelomeric loci in the *A. fumigatus* genome, and probe their stability in response to laboratory- and host-imposed stresses. *In silico* analyses revealed that this class belongs to the *Tad* clade of LINE-like elements. Southern blotting with a LINE-specific probe in clinical and environmental isolates revealed a high variability in the insertion pattern between strains and active transcription of LINE-like element(s) was discernable, in the type strain Af293, by RT-PCR. One out of 14 tested clinical isolates did not contain any LINEs at all, arguing against an absolute requirement for LINE-mediated activities in human infections. Finally, we found preliminary evidence of an association between mycovirus-infection and the expansion of *Tad*-element populations in discrete *A. fumigatus* genomes.

1. Introduction

Post-genomic analyses of filamentous fungi have revealed a plethora of complex genetic loci governing secondary metabolite biosynthesis (Inglis et al., 2013; Perrin et al., 2007). As the genomic context of secondary metabolism gene clusters, and the expression of their composite genes, has come under closer scrutiny clues to their evolutionary origins and regulatory control have emerged. An important, but largely unanswered question, relates to the mechanism by which co-ordinate regulation of colocalised genes is achieved.

In the major mould pathogen of humans, *Aspergillus fumigatus*, several secondary metabolites have been shown to impact pathogenicity of the fungus in whole animal models of disease (Bok et al., 2006; Cramer et al., 2006; Heinekamp et al., 2012; O'Hanlon et al., 2011; Pardo et al., 2006; Tsai et al., 1998) and mutants lacking a general regulator of secondary metabolite gene expression, the LaeA methyltransferase, are defective in murine pathogenicity (Bok et al., 2005).

Analyses of the genome sequences of the strains Af293 (Nierman et al., 2005) and A1163 (Fedorova et al., 2008), coupled with transcriptomic analyses of *A. fumigatus* pathogenicity (McDonagh et al., 2008) and LaeA-mediated chemical diversity (Perrin et al., 2007), have revealed that genes preferentially employed during colonisation of the mammalian niche tend to be clustered in subtelomeric genomic islands which are highly enriched in secondary metabolite clusters and subject to LaeA regulation.

Transposons are often found within or in close proximity to secondary metabolite clusters in subtelomeric regions of aspergillus chromosomes (Fedorova et al., 2008). From a transcriptional perspective, it has been observed that LaeA-regulated secondary metabolite clusters are enriched in TEs (Bok and Keller, 2004; Perrin et al., 2007) and a significant number of transposons are upregulated during *A. fumigatus* germination (Lamarre et al., 2008), which occurs during early stages of lung infection. While there is no evidence for a functional connection between LaeA and transposon activity, it has been proposed that transposons might promote the genetic diversity of secondary metabolite clusters in aspergilli due to their repetitive nature and mutagenic activity, leading to rearrangements, insertions, deletions,

Abbreviations: LINE, long interspersed nuclear element; LLE, LINE-like element; LSP, LINE-specific probe; non-LTR, non long terminal repeat; TE, transposable element.

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duplications and related processes. Resulting changes in the structure and/or regulation of secondary metabolite clusters might facilitate adaptations of *A. fumigatus* to environmental niches such as those encountered in the infected host. (Fedorova et al., 2008; Perrin et al., 2007).

Reports providing more direct evidence for the role of TEs in adpative gene regulation exist in fungi. In *Schizosaccharomyces pombe*, for example, sequences in the TE Tf2 act as a promoter that is necessary for adaptation to hypoxia (Sehgal et al., 2007). In *Aspergillus nidulans*, the deletion of transposon-rich sequences flanking the *A. nidulans* penicillin cluster resulted in the reduction of penicillin synthesis by up to 75% (Shaaban et al., 2010), which suggests that TEs interact with regulatory processes acting on secondary metabolite clusters in a yet to be specified manner, for example by serving as boundary elements or influencing heterochromatin organisation.

In order to commence a detailed survey of TEs in *A fumigatus* genomes, we here present a full description of the previously uncharacterised class of LINE-like elements (LLEs) detected in the sequenced *A. fumigatus* genomes. Furthermore, we mapped the genomic locations of these elements; compared their insertion patterns in clinical and environmental isolates and asked if they are mobilised under stressful conditions to understand if LLEs might play a role in helping *A. fumigatus* to adapt to challenging environments such as those encountered within the mammalian niche.

2. Materials and methods

2.1. Bioinformatic analyses

All of the named bioinformatics servers were last accessed in May 2013.

2.1.1. BLAST search for LLEs

To retrieve sequence data of LLEs, the BLASTN functionality of the basic local alignment search tool (BLAST) programme WU BLAST 2.0 (Gish, W., 1996–2003, http://blast.wustl.edu), as it was available at the *Aspergillus* Genome Database (*Asp*GD, http://www.aspgd.org/, Arnaud et al., 2010, now licensed by Advanced Biocomputing, LLC), was used. The complete genome sequences of the strains Af293 and A1163 were used as query genomes. A conserved region of 592 base pairs (bp) of the LLEs in question, called LINE-specific Probe (LSP, Supplementary Sequences I) was used to interrogate the genome sequences. Parameters used were: BLOSUM62 scoring matrix; word length: 11; *E*-value cutoff: 10.

2.1.2. Multiple alignments and phylogenetic tree construction

The coordinates from the hits returned in the previous paragraph were used to retrieve sequences extending another 5500 bp upstream and another 1500 bp downstream, yielding a total of 7592 bp of sequence information (Supplementary Sequences I). The *A. fumigatus* Genome Map Viewer at the NCBI (http://www.ncbi.nlm.nih.gov/projects/genome/guide/aspergillus/index.html) was used for this purpose. Next, multiple DNA sequence alignments between these elements were performed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/, Goujon et al., 2010; Sievers et al., 2011) using the default settings. For the phylogenetic analysis, the analysis by Malik et al. (1999), was re-executed (EMBL accession DS36752) this time including *A. fumigatus* LLE #8_2.0 of this study and the non-LTR elements Zorro-3 (Goodwin et al., 2001, GenBank accession AF273027) and Ylli (Casaregola et al., 2002, EMBL accession AJ319752). As the reverse transcriptase (RT) domain of *A. fumigatus* LLE #8_2.0 amino acids 501–755 of ORF2 were used as they were determined by the Pfam 26.0 database at the Sanger Institute (http://pfam.sanger.ac.uk/, Finn et al., 2010), using the default settings with an E value of 1.0. A multiple sequence alignment was constructed with Clustal Omega using the default settings and subsequently fed into the phylogenetic analysis software MEGA 5.2.2 (Tamura et al., 2011) to construct a neighbour-joining phylogenetic tree with bootstrap support (1000 replications). Pre-alignment and post-alignment fasta files are provided as Supplementary material.

2.1.3. Searches for conserved domains

LLE sequences were used to perform homology-based searches with Pfam 26.0 using the default settings with an *E* value of 1.0.

2.1.4. Databases, automated TE annotation and splice site prediction

A. fumigatus ORF annotations were sourced from AspGD and CADRE (http://www.cadre-genomes.org.uk/index.html, Mabey et al., 2004) and were based on the software TransposonPSI (http://transposonpsi.sourceforge.net/, developed and maintained by Brian Haas, Broad Institute, Cambridge, Massachusetts, USA). For splice site prediction the NetAspGene 1.0 Server (http://www.cbs.dtu.dk/services/NetAspGene/, Wang et al., 2009) was used.

2.2. Fungal strains and culture conditions

2.2.1. Fungal culture

Unless otherwise stated, media used were *Aspergillus* Complete Media (ACM) according to Pontecorvo et al., 1953 (1% glucose, 0.5% yeast extract, 1% vitamin solution and 5 mM ammonium tartrate at pH 6.5) with 1% Agar No. 3 (Oxoid, UK) added for solid media. A standard growth temperature of 37 °C was employed throughout.

2.2.2. Growing and harvesting spores

Spores were streaked onto a 90 mm Petri dish containing solid ACM and then cultured for 2 days. Harvesting was performed by pouring 20 ml of autoclaved dH_2O onto the lawn of fungal spores and resuspending the spores, giving a typical yield of 5×10^9 spores. The spore suspension was filtered with Miracloth (Calbiochem, UK). Following spore enumeration with a conventional haemocytometer, the filtrate was used directly for growing mycelium.

2.2.3. Growing and harvesting mycelium

$\sim 5 \times 10^9$ spores were inoculated into a bottle with 200 ml of liquid ACM and then grown for 24 h on a shaker at 120 rotations per minute (rpm). Mycelium was harvested by filtering the suspension with Miracloth (Calbiochem) and snap freezing the mycelium in liquid nitrogen. Long term storage was at −80 °C.

2.2.4. Fungal strains

Af293, Fungal Genetics Stock Center (FGSC) A1100, is a clinical isolate sequenced by Nierman et al. (2005). A1163 was derived from CEA17 by ectopically inserting a *pyrG* gene from *A. niger* (d'Enfert, 1996). CEA17 is a uracil auxotroph derived from the clinical isolate CEA10, Centraalbureau voor Schimmelcultures (CBS) 144.89. American Type Culture Collection (ATCC) 46645 was obtained from Hubertus Haas, Medical University of Innsbruck, Austria. Af237y was produced from a wild type strain by random insertional mutagenesis (Afauvre-Brown et al., 1998). Af237y#8 is an isogenic strain transfected with a partitivirus (Bhatti et al., 2011). A56 is a clinical isolate, naturally infected with a chrysovirus (Jamal et al., 2010). 6(1) is a cured strain isogenic to A56. Other clinical isolates are respiratory patient samples obtained from hospitals in and around London, UK. A, B and C indices refer to the same strain but different morphologies, which often arises as a result of virus infection. Strains 22.4, 16.1, 7.3, 10.2 are environmental isolates sourced by Chris Thornton (University of Exeter). They are uncharacterised and unpublished.