Exploring a Novel Genomic Safe-haven Site in the Human Pathogenic Mould *Aspergillus fumigatus*

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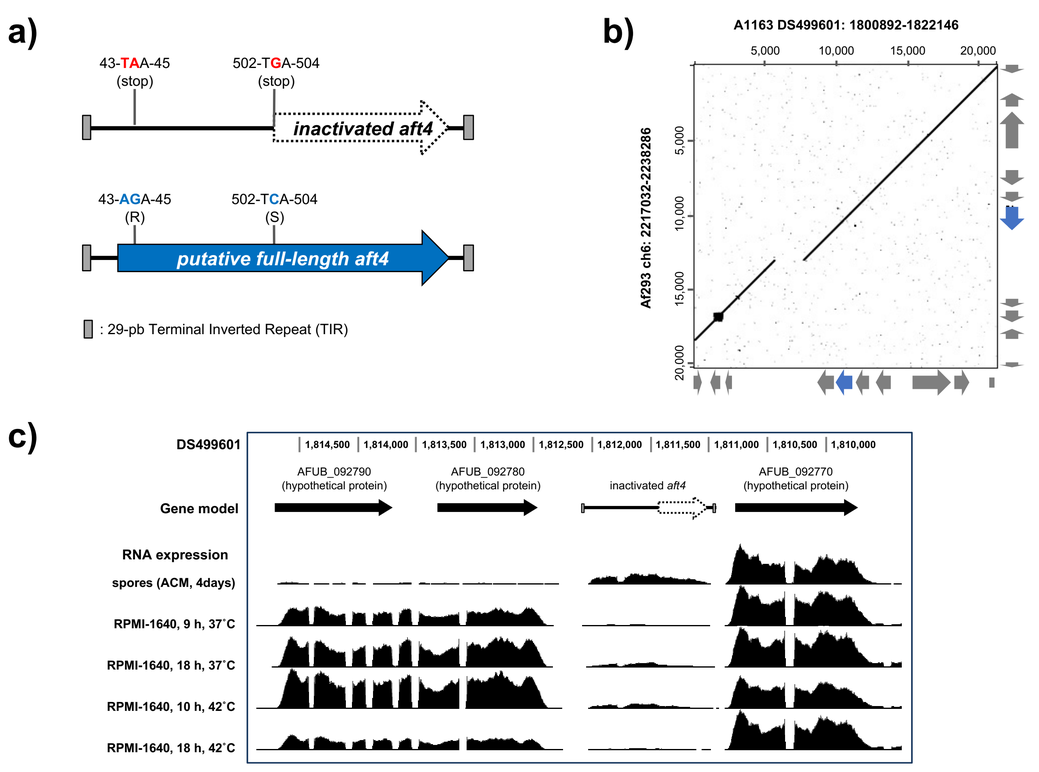
ABSTRACT

Development of novel molecular tools is necessary to fully explore the molecular landscape of the pathogenicity of *A. fumigatus*. In this study, we identified a new genomic safe-haven site that we term SH-*aft4* at the site of an inactive *Tc1/mariner* type transposable element (Panel A). Our analyses demonstrate that the deletion of the *aft4* element as well as the expression of a transgene construct from the *aft4* locus do not have any significant impact on the growth characteristics (Panel B) and the pathogenic properties (Panel C) of *A. fumigatus*. We also demonstrate that the *aft4* locus has a great potential to provide a robust integration site for expression of a transgenic construct in combination with the CRISPR-Cas9 mediated genome-editing system (Panel D). Furthermore, we show that SH-*aft4* is highly conserved in the genomes of a large number of clinical and environmental isolates of *A. fumigatus* (Panel E). Our results strongly suggest that SH-*aft4* locus can serve as a novel molecular tool for genetic manipulation of *A. fumigatus* to aid functional genomics studies of this important human fungal pathogen.

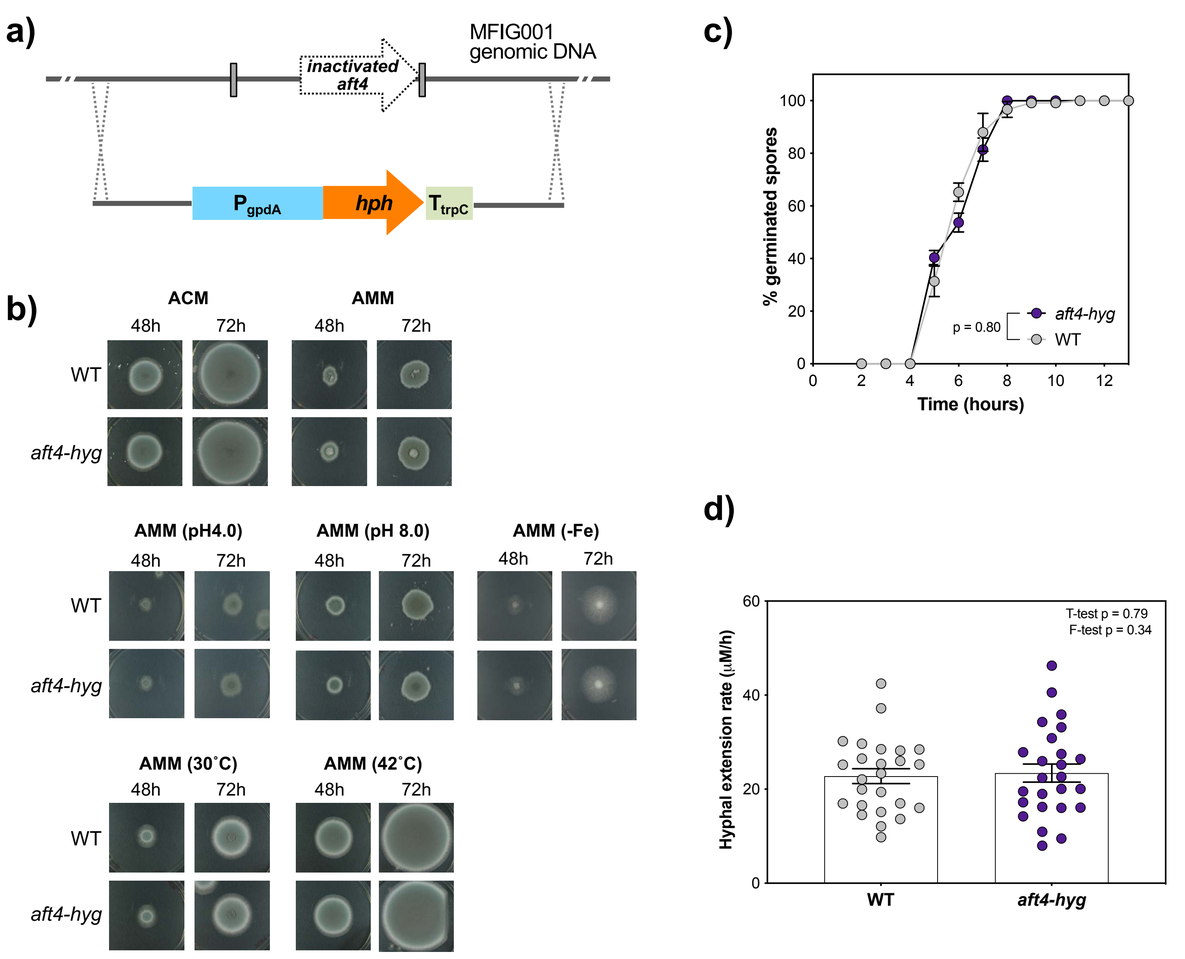
A Identification of the aft4 locus, encoding an inactive transposable element, as a potential genomic safe-haven site

The *aft4* locus was identified as a unique 1.3 kb nucleotide region with a moderate homology to the *Fusarium oxysporum* impala transposon. The *aft4* element is present in the genome of the *A. fumigatus* isolates in common laboratory use. The full-length ORF of the *aft4* transposase appear to have been genetically inactivated by several mutations but it lies in a transcriptionally active genomic region.



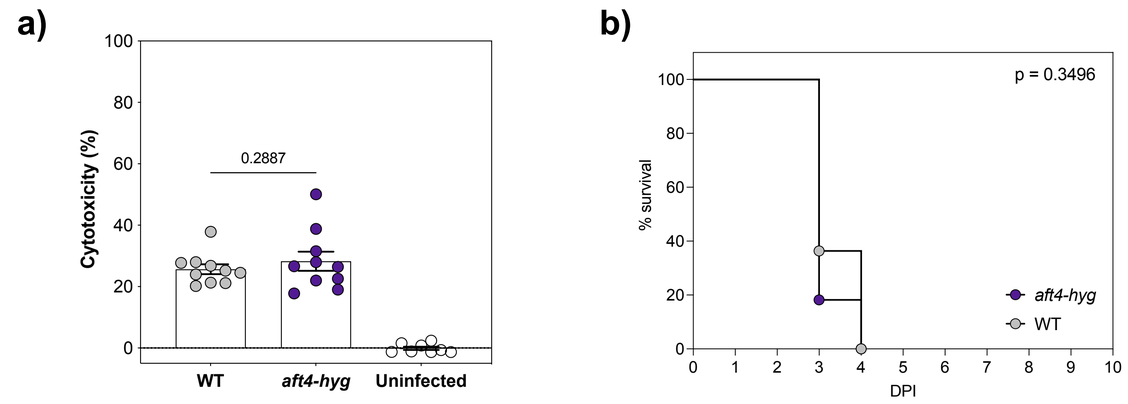
(a) Schematic representation of the *aft4* locus encoding a putative inactivated *Tc1/mariner* like transposable element. The full-length ORF can be generated from the inactivated *aft4* by introducing three nucleotide changes (T(43)A, A(44)G, G(503)C. **(b)** Dot plot analysis of the 20-kbp region surrounding the *aft4* element between *A. fumigatus* Af293 and A1163. **(c)** RNA-expression profiles of the *aft4* locus in *A. fumigatus* A1163 indifferent culture conditions.

B Deletion of the inactivated aft4 element does not have significant impact on growth characteristics



(a) Schematic overview of the construction of the *aft4-hyg* knockout mutant. **(b)** Colonial growth phenotypes of the wild-type (MFIG001:WT), and the *aft4* knockout mutant *(aft4-hyg)* grown on a solid ACM or solid AMM with an infection relevant stress. **(c, d)** Germination profiles and hyphal extension rates in a liquid RPMI-1640 at 37 ˚C. NS, *P*>0.05.

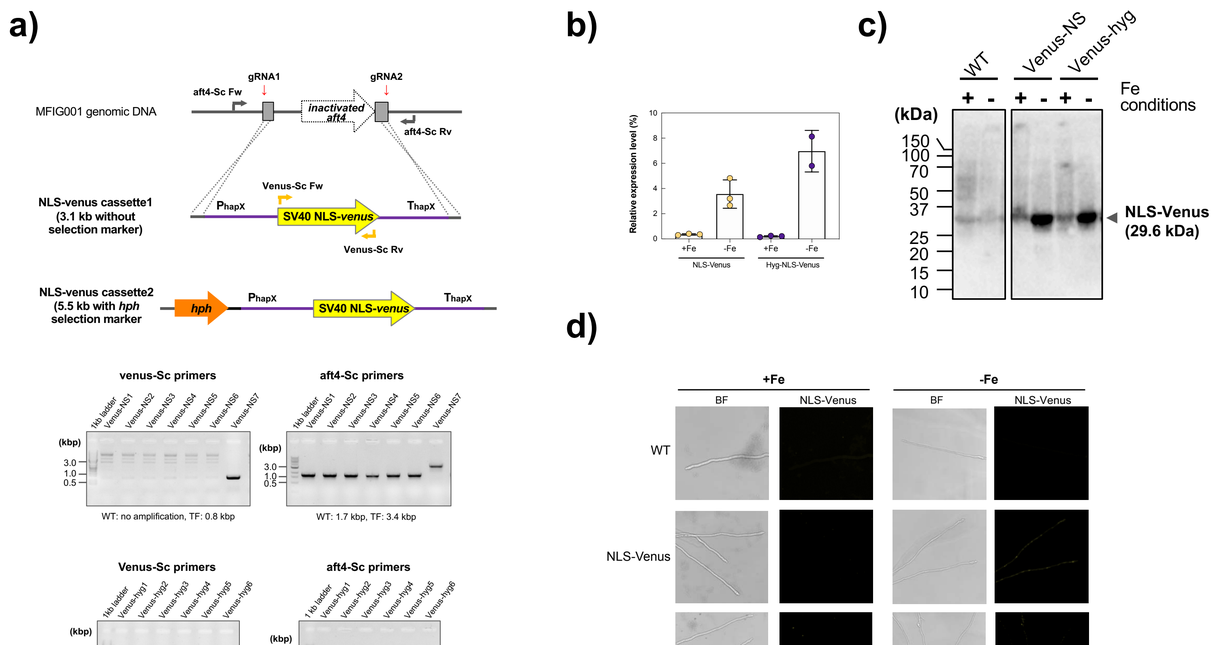
C Deletion of the inactivated aft4 element does not have significant impact on the pathogenicity



(a) Cytotoxicity of the wild-type (WT) and the *aft* knockout mutant (*aft4-hyg*). A549 epithelial cells were infected with the strains for 24 hours and their cytotoxicity was evaluated by measuring the release of lactose dehydrogenase (LDH) activity into the culture medium. **(b)** Effect of the deletion of the *aft4* element on virulence of *A. fumigatus* in a murine model of invasive pulmonary aspergillosis. Mice were rendered neutropenic by treatment with cyclophosphamide and challenged with 5.0 × 10^5 spores via intranasal route.

D The potential of the aft4 locus as a transgene expression site for functional genomics applications

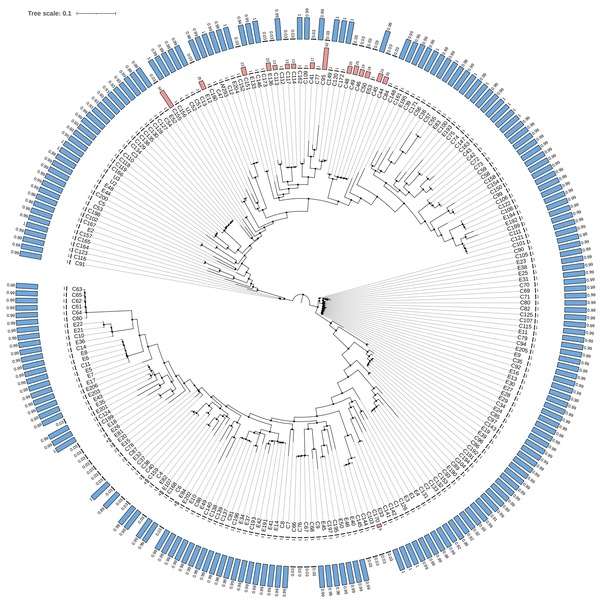
We examined the potential of SH-*aft4* as a safe-haven site for functional genomics studies in *A. fumigatus* by expressing a nuclear targeted yellow fluorescent protein (NLS-Venus) as an example. Our results demonstrate that the capability of SH-*aft4* for an efficient transgene integration, especially in combination with the CRISPR-Cas9 transformation system. Moreover, we are able to effectively express a transgene construct from SH-*aft4* under the control of a promoter which regulates the expression of the transcription factor *hapX* in an iron dependent manner.



(a) Targeted integration of the NLS-venus expression constructs into the *aft4* locus using the CRISPR-Cas9 mediated transformation. **b)** Relative expression levels of *NLS-venus* transcripts in the obtained transformants after 18 h grown under iron-replete (0.03 μM; +Fe) or starvation (-Fe) conditions. **(b)** Western blot analysis showing iron level dependent inducible-expression of the NLS-Venus protein from the *hapX* promoter region. **(d)** Fluorescent microscopy of the NLS-venus expressing mutants grown on 24 h under iron-replete or starvation conditions.

E The SH-aft4 locus is conserved as a single-copy element in a large subset of clinical and environmental isolates

Our bioinformatic analysis of the genome of 234 different *A. fumigatus* isolates that include 159 clinical and 75 environmental isolates revealed that the majority of the isolates possess SH-*aft4* as a single copy element. This indicates that SH-*aft4* can be used as a universal safe-haven site for most of *A. fumigatus* isolates.



A phylogenetic tree of a collection of 234 *A. fumigatus* clinical and environmental isolates was generated by RAxML. Copy number of *aft4* in red bars and % identity in blue bars