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Prp8 intein in fungal pathogens: target for potential antifungal drugs

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Abstract Inteins are self-splicing intervening sequences in proteins, and inteins of pathogenic organisms can be attractive drug targets. Here, we report an intein in important fungal pathogens including *Aspergillus fumigatus*, *Aspergillus nidulans*, *Histoplasma capsulatum*, and different serotypes of *Cryptococcus neoformans*. This intein is inside the extremely conserved and functionally essential Prp8 protein, and it varies in size from 170 aa in *C. neoformans* to 819 aa in *A. fumigatus*, which is caused by the presence or absence of an endonuclease domain and a putative tongs subdomain in the intein. Prp8 inteins of these organisms were demonstrated to do protein splicing in a recombinant protein in *Escherichia coli*. These findings revealed Prp8 inteins as attractive targets for potential antifungal drugs to be identified using existing selection and screening methods.

Keywords: Intein; Protein splicing; Prp8 protein; Fungal pathogen; Drug target

1. Introduction

Inteins are protein intervening sequences that can self-excise through protein splicing, which also joins the flanking sequences (N- and C-exteins) with a peptide bond to produce the mature host protein (spliced protein) [1–4]. Most inteins have a homing endonuclease domain that initiates intein homing [5,6], and split inteins exist in two fragments and do protein *trans*-splicing [7,8]. No biological role benefiting the host organism has been determined for inteins, but intein's protein splicing function is required for and could potentially regulate production of the mature host protein.

Inteins have been found in certain mycobacterial pathogens and suggested as attractive targets for potential antimycobacterial drugs (reviewed in [9]). Because no intein has been found in animals including human, drugs targeting inteins are less likely to have toxic side effects. Systems for in vivo selection and in vitro screening have been developed to find such drugs. For example, coding sequence of a mycobacterial RecA intein was inserted in a thymidylate synthase gene from bacteriophage T4, and the resulting fusion protein could complement a *Escherichia coli* thyA mutant if the intein could self-excise through protein splicing. This allows positive growth selection against the protein splicing, because the thyA complementation leads to growth inhibition in the presence of thymine and trimethoprim [10]. Other genetic methods have involved the CcdB cytotoxic protein [11] and a quinolone-sensitive GyrA protein [12]. An in vitro screening system has been developed for identifying inhibitors of intein inserted in a green fluorescence protein [13].

An intein was also found in the fungal pathogen *Cryptococcus neoformans* and suggested as potential drug target [14], although its protein splicing activity was not demonstrated. Its host protein, Prp8, is an extremely conserved large protein and a critical component of the catalytic core of spliceosome [15]. The spliceosome is a large ribonucleoprotein complex and catalyzes the removal of introns from pre-mRNAs. The Prp8 protein is essential for RNA splicing, and evidences showed that it stabilizes tertiary RNA interactions, facilitates formation of the catalytic core, and acts as a protein cofactor to the RNA enzyme. The host organism *C. neoformans* has various strains classified into different serotypes (A, B, C, D, and AD) and varieties known as *grubii* (serotype A), *neoformans* (serotype D), and *gattii* (serotypes B and C). The Prp8 intein would be a more attractive drug target, if it is active in protein splicing and present also in other important fungal pathogens and in different serotypes of *C. neoformans*.

Here, we report the finding and characterization of Prp8 inteins from a wide range of important fungal pathogens, including *Aspergillus fumigatus*, *Aspergillus anidulans*, *Histoplasma capsulatum*, and different serotypes of *Cryptococcus neoformans*. We also demonstrate that Prp8 inteins from these organisms were active in protein splicing when inserted in a model host protein in *E. coli*. We discuss the usefulness of Prp8 inteins as attractive targets for potential antifungal drugs to be identified using existing selection and screening methods.

2. Materials and methods

2.1. Gene cloning and sequence analysis

Fungal cells and/or their genomic DNAs were obtained from other researchers (M. J. Bidochka, M. Momany, A. Sil, and J. Yu) and from the University of Alberta Microfungus Collection & Herbarium (UAMH). Coding sequences of Prp8 inteins and their flanking exteins (partial) were amplified from total genomic DNA by doing polymerase chain reaction (PCR) using degenerate and precise primers. Degenerate oligonucleotide primers for Prp8 sequences of *Aspergillus* and *Histoplasma* included 5′-ATGAAGAGCAAYCCNTTYTGGTGGAC-3′ and 5′-GCATTCGTGAGYTTYTTRAAYTTCAT-3′. Degenerate primers for *Cryptococcus* included 5′-GCTCTCGGTGGTGTTGAGGGTATHYTNGARCA-3′ and 5′-TGGGCGTTGGTCAGTCGYTTRTTYTTCAT-3′. The degenerate site nucleotides are Y (C and T), R (A and G), H (A, C, and T), and N (A, C, G, and T). The amplified DNAs

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were cloned in a pDrive plasmid vector (Qiagen) and subjected to automated DNA sequencing. GenBank searches and protein sequence alignments were performed using the BLAST search program [16] and the Clustal W program [17], respectively.

2.2. Protein splicing analysis in E. coli cells

To construct gene expression plasmids, Prp8 intein coding sequences were inserted in the previously reported expression plasmid pMST [18] between *Xho*I and *Age*I sites, replacing the *Ssp* DnaB intein coding sequence of pMST. Protein production in *E. coli* cells, gel electrophoresis and Western blot analysis were performed as before [19]. Briefly, cells containing the expression plasmid were grown in liquid Lurie Broth (LB) medium at 37 °C to late log phase (A_{600}, 0.5). IPTG was added to a final concentration of 0.8 mM to induce production of the recombinant protein, and the induction was continued at 37 °C for 3 h or at room temperature overnight. Cells were then harvested and lysed in SDS- and DTT-containing gel loading buffer in a boiling water bath before electrophoresis in SDS–polyacrylamide gel. Western blots were carried out using anti-thioredoxin antibody (Invitrogen) and the enhanced chemi-luminescence detection kit (ECL). Intensity of protein band was estimated using a gel documentation system (Gel Doc 2000 with Quantity One software, Bio-Rad).

3. Results

3.1. Prp8 intein in fungal pathogens

A suspected intein-containing fragment of *Prp8* gene, which corresponds to insertion site of the previously identified Prp8 intein of *C. neoformans* [14], was PCR-amplified from genomic DNAs of a number of fungal species and strains listed in Table 1. The resulting DNA fragments from some organisms exhibited a size larger than predicted for an intein-less *Prp8* gene, indicating the presence of intein-coding sequences. These organisms included *A. fumigatus*, *A. nidulans* (strains A28, 273, and FGSC A4), *H. capsulatum* (strains G217B and 186AR), and *C. neoformans* (strains AmMs229 and YBC81). The DNA fragments were cloned and their sequences determined, which revealed Prp8 inteins of various sizes (Fig. 1). Some of the intein sequences were also found in searches of databases and partial genome sequences of corresponding organisms (Table 1). A number of other *Aspergillus* species (*flavus*, *niger*, *oryzae*, *parasiticus*, *terreus*, and *ustus*) produced DNA sizes corresponding to intein-less Prp8 gene, indicating absence of the Prp8 intein (data not shown).

Prp8 inteins of the different pathogens have the same corresponding insertion site in Prp8 protein, but their sizes differ greatly. In particular, the 819-aa *Afu* Prp8 intein is nearly 5 times as large as the 170-aa *Cne* Prp8 intein. These size differences are mostly due to the presence or absence of a homing endonuclease domain in the intein, based on sequence alignment and comparison of Prp8 inteins of different organisms (Fig. 2). The structural domains of Prp8 inteins (splicing domain, endonuclease domain, and putative tongs subdomain) were predicted through comparison with the *Sce* VMA intein whose crystal structure is known [20,21]. Conserved sequence blocks (A through G, N) were identified using consensus sequences of known inteins [6]. A homing endonuclease domain is present in Prp8 inteins of *A. fumigatus*, *A. nidulans*, and *H. capasilatus*, but absent in Prp8 inteins of different *C. neoformans* strains and varieties (Fig. 2A). The *Afu* Prp8 intein has a 222-aa sequence (Fig. 2B) that has no counterpart in Prp8 inteins of the other organisms, including the closely related *A. nidulans*. This 222-aa sequence is located immediately after the conserved sequence block B and corresponds in position to the tongs subdomain in the crystal structure of *Sce* VMA intein [21]. However, its sequence shows no apparent similarity to and is over three times longer than the 69-aa tongs subdomain of *Sce* VMA intein.

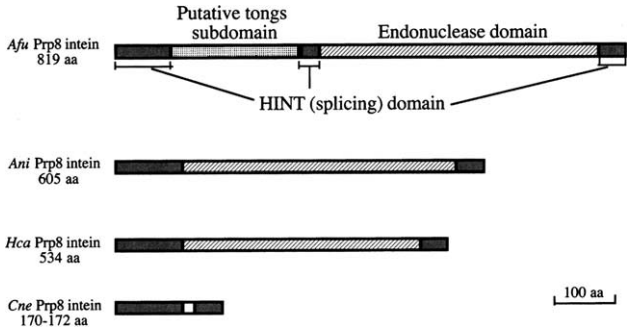

Fig. 1. Schematic comparison of Prp8 inteins. Structural domains of Prp8 inteins are illustrated, which include three parts of the protein splicing or HINT domain (gray boxes), the endonuclease domain (hatched boxes), the putative tongs subdomain (dotted box), and linker sequence (open box).

The splicing domain (HINT domain) of Prp8 intein is highly conserved among the different organisms (Fig. 2A), after excluding the homing endonuclease domain and the putative tongs subdomain. Sequence identities of the HINT domain are 78% between the *Aspergillus* species, ~73% between *Aspergillus* and *Histoplasma*, ~47% between *Aspergillus* and *Cryptococcus*, and over 85% among different *Cryptococcus* strains. The homing endonuclease domain is 387–455 aa long and

Table 1
Prp8 intein distribution in fungal organisms

Organism		Intein size (aa)	Source of sequence information
Aspergillus	*fumigatus*	819	This work
	nidulans	605	This work
	flavus, *niger*, *oryzae*, *parasiticus*, *terreus*, *ustus*	Absent	This work
Histoplasma	*capsulatum*	534	This work
Cryptococcus neoformans	Serotype D, neoformans	172	This work: strain AmMs229, ATCC66031 Butler et al. [14]: strain JEC21
	Serotype A, grubii	171	This work: strain YBC81, ATCC76484 InBase (www.neb.com/inteins/): strain PHLS 8104 Also GenBank AY422974
	Serotype AD	172	InBase (www.neb.com/inteins/): strain CBS132
	Serotype C/B gattii	170	InBase (www.neb.com/inteins/): *C. bacillisporus* Also GenBank AY422975

A HINT domain sequence alignment

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Afu   fwekaCLAKGTRLLRYDGSEIEVQDVKEGDLLLGPDGGPRRAFNIVNGKDRLYRIKIGGSKEDLVVTPNHILVLHR----(TSD, 222 aa)---GDD
Ani   fwekaCLANGTQLLRYDGTKVNVEDVKEGDLLLGPDGGPRRAFNVVSGKDRLYRIKIDGDKEDLVVTANHILVLHRAKAMNTSVCFDRSKEQQGGAGEQ
Hca   fwekaCLAKGTQLLRYDGTKVGVENVREGDLLLGPDGEPRRAFNIVSGRDRLYRISIDADKEDLVVTPNHILVLHH----------EKEN------QKR
Cne   fwekaCLQNGTRLLRADGSEVLVEDVQEGDQLLGPDGTSRTASKIVRGEERLYRIKTHEGLEDLVCTHNHILSMYK----------ERSGSE----RAH
Cgr   fwekaCLQNGTRLLRADGSEVLVEDVQEGDQLLGPDGTSRTASKIVRGEERLYRIKTHEGLEDLVCTHNHILSMYK----------ERFGRE----GAH
Cga   fwekaCLQNGTRLLRADGSEILVEDVQEGDQLLGPDGTSRTASKIVRGEERLYRIKADE-LEDLVCTHNHILSLYK----------KRSGSE----QDP
      *******  **.*** **. . *. *.*** ******  * *  .* * .*****      **** * ****. .
               A                                                          B

Afu   DELPQVSAEERYDTVEMTAAEFASL------(ED, 455 aa)-----RLIPQTHSFMIKDISLEPEATEWAGFRVDKDQLYLRHDYLVLHNsgfee
Ani   LDISEVSAAERYDTVEMTAAEFAAL------(ED, 444 aa)-----RLIPQSHSFAIKDISLESEATEWAGFRVDKDQLYLRHDYVVLHNsgfee
Hca   QSELSASATERYDTVEMTAADFAAL------(ED, 387 aa)-----SPVSQAHSFTIKGIHLESEMTEWAGFRVDKDQLYLRHDFLVLHNsgfee
Cne   SPSADLSLTDSHERVDVTVDDFVRLPQQEQQKYQLFRSTASVRHERPSTSKLDTTLLRINSIELEDEPTKWSGFVVDKDSLYLRHDYLVLHNsgfee
Cgr   SPSAGTSLTESHERVDVTVDDFVRLPQQEQQKYKLFRSTDFVRREQPSASKLATLLH-IMSIQLEEKPTKWSGFVVDKDSLYLRHDYLVLHNsgfee
Cga   SPSTDLSSTDSYERVDVTVDDFVRLPQQEQQKYRLFRSTGFKRADQPSTSSLATLLH-INSIELEEEPTKWSGFVVDKDSLYLRYDYLVLHNsgfee
            *  .  . *..*  .*  *                                .    *  * ** * * *.** **** ******..*********
                                                                                F          G
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B Putative tongs subdomain (TSD) Sequence of *Afu* Prp8 intein

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EKRARNVYTGPSVQGHIQRSENGHGNLPMLSSSPAAAHHPNNLVKNRGDFWSALKSAIAWVLHAERSSTGANMVRNVLNGTVGLTAHKESYTVTNPQQKG
VYYTYVWGNPQRTSIKGHRDHPPVFLPTKEDAFSAAIAKSRELYSQSEVTLATLRQRFLAKSADGKGGEILVDANLPNIFLLWDKNRSNLKFRVLCSRNF
KTYGRVYTFESMPSTNAEEPGY
```

C Endonuclease domain (ED) sequence alignment

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Afu   STEERSRYRVFRCPGFELPEQPVPVNPYFLGLWLGDDNHEKTTNHNIHEENVREFLVNHAAELDMYLAWQGLIDYATVAN-PAPMMVRLPPTN
Ani   HPQERSWYRAIRCPGFELPEQDVPVNPYFLGLWLGDESRNQSAIYSNHEEALREFLVSHAAELDMHLVYHGQSAYSTVCNKDRPTNKRIGPAN
Hca   DPEERRWYRLFRSPGFELGQQNVPIDPYFVGFWLCDGIRASTTIYTSPEEATREFIINHAAELDLQLASKE----------------------
        .**  **  *.***** .* **. ***.* ** *  .  ..     **  ***.. ******. *  .
                                  C

Afu   PDTIEHRPVVCQARQSIRKLRLAAKNIAQPEVVL----STSPRPESQMQPKRELPSNTETALRSEAEASSISAILDSKAGHSSLDTGDPNSDV
Ani   -QTQTVRPTIRQTRRTIRQQRLAAEHAAAEYTTQRETASLTPLLESPTSDKHGLLSSVETPGRLSDSVTTELPMSRSASAMRSIRTASG----
Hca   ---YMQHPVRRVARQTILEQRLAVQCTAPQETDG-----------------SLLSHILQKAAKSGLASSTRTMSTS-------RNRQP----
            .*    .*..*   ***    *                        * *            ..   .  *        .

Afu   VPESIPNDVADFGLDGVPELTSSGFSELTSDSELMRLIEQVERSSQGSTEEPSQASVVEQEADLNLLETDSEDEEADSADDDEFGDPEASEFR
Ani   --LSEFNDVTNVSAS-MPDIQNSGIKNQG---RIAKVTRQQD--SKG-EVDFRQQYSQAIKDDLELLETDIED---DVASSDEIEDVCVVGSE
Hca   ----LSETSAATSMNILPGFASNSTSVVSPGIDSHEILSLRN--------SCSQLVQIAEKSGLREECMINPP---SSRE-DLVLDLFDTHIE
               .      .*                  .                 *         *                 *   *

Afu   PEPESQLSQSHFSNRRRNHRLRTGRRVYGDLNGEEEGILLDQIVEQSEG-SRVNSLLRALDALGIIAQKGTGPETNRKHIPSIYMKNSRSVRL
Ani   NELIGSEKQDQSGRRRQIHRLRTGHRGYGDLSDDEQEQLLDSVVERYAGDSRLNTLQQELSKMGILNPE-TGPINDKKRIPQVFMQNSRSVRL
Hca   ADEIQGLDENLTG---QKHRLRTGCRAYGDLTVDEEGQILDNIISRPVGTPDIGTLLRALEELGLPTNRTEGHGVENKRIPLMYMKSSRSIRL
       .      .       . ****** * ****  .*.  .** .. .  *   ...* . *  .*.      *    *.** ..*. ***.**
                                                                                    D

Afu   AVLAGLIDSDGWYVYPENVLGFAQSERWHSKLFWDVVALARSLGLSVLTKRRMMWNPARTERYPQLFAQISGNVAEVPCLIARKKGVE
Ani   SVLAGLLDSDGWYIYPENMFGFAQSELCHKELFWDVVTLARSLGFGVWTKKRMMPDPTGKRMSPMLVAQISGDLAEIPCVLARKKAMP
Hca   ALLAGLIDSDGWYCQPQNTFCFGESERISPTLFWDIVTLARSLGLSVSTEQHTMRSPACTAFKPRFVAQISGNVAEVTCLLARKRGVK
      ..****.******  *.*   * .**      ****.*.******  * * .. *  *.     *   ***** .**. *..***. .
          E                                  H
```

Fig. 2. Protein sequence comparison of Prp8 inteins. (A) Sequence alignment of splicing (HINT) domain, with a 5-aa extein sequence on each side of the intein shown in lower case letters. Positions of the endonuclease domain (ED) and the putative tongs subdomain (TSD) are indicated, with the numbers of amino acids shown in parenthesis. Conserved intein sequence motifs (blocks A, B, F, and G) are underlined. (B) Sequence of the putative tongs subdomain of *Afu* Prp8 intein. *C*. Sequence alignment of endonuclease domain. Conserved sequence motifs (blocks C, D, E, and H) are underlined. The compared sequences are from *A. fumigatus* (*Afu*), *A. nidulans* (*Ani*), *H. capsilatum* (*Hca*), *C. neoformans* var. neoformans (*Cne*), *C. neoformans* var. grubii (*Cgr*), and *C. neoformans* var. gattii (*Cga*). Symbols: -, represent gaps introduced to optimize the alignment; *, and. mark positions of identical and similar amino acids, respectively.

shows 32–41% sequence identity and 47–55% sequence similarity among the different *Aspergillus* and *Histoplasma* species. Presence of conserved sequence blocks, especially blocks C and E, identified them as DOD (or LAGLIDADG) type endonucleases. The Prp8 inteins are not similar to other known inteins in host protein and in insertion site, and they showed less than 20% sequence identity to other inteins.

3.2. *Protein splicing activity of Prp8 intein in* E. coli

To test the Prp8 inteins for protein splicing activity in *E. coli*, each intein coding sequence was inserted in a fusion gene on a plasmid. In the resulting fusion protein, the Prp8 intein (plus 5 aa of its native extein sequence on each side) was flanked by a maltose binding protein at its N-terminus and a thioredoxin at its C-terminus (Fig. 3). Similar fusion protein constructs had

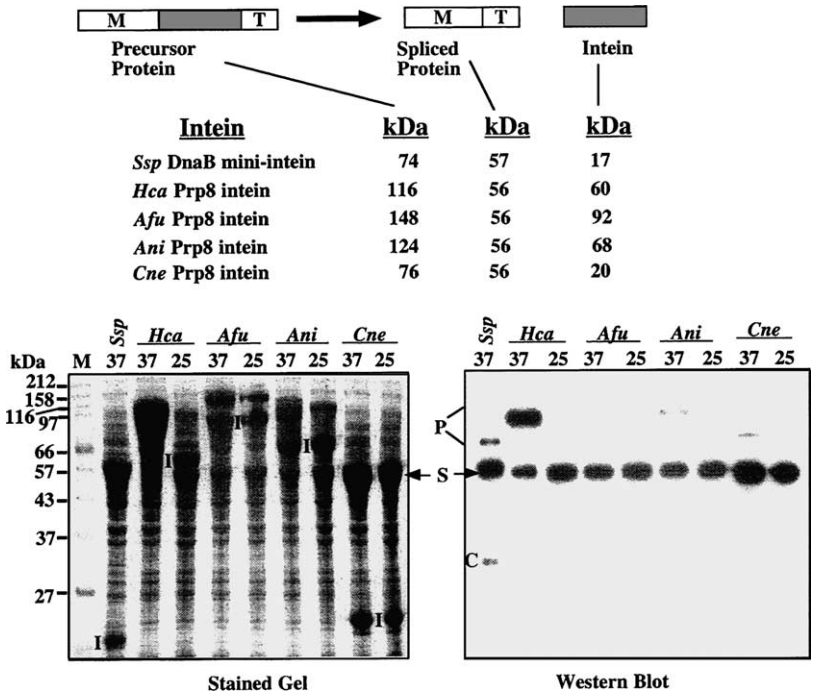

Fig. 3. Protein splicing activity of Prp8 inteins. (Top) Schematic illustration of the fusion-protein construct consisting of maltose binding protein sequence (M), intein sequence (gray box), and thioredoxin sequence (T). (Middle) Predicted sizes of protein products from fusion-protein constructs containing the specified inteins. The *Ssp* DnaB mini-intein was included as a known standard for identifying the spliced protein [18]. (Bottom) Observation of protein splicing. The specified fusion-proteins were produced in *E. coli* either at 37 °C or at 25 °C (room temperature) as indicated. Total cellular proteins were resolved by SDS–PAGE and visualized by Coomassie Blue staining (left panel) or Western blotting (right panel) using anti-thioredoxin antibody. Positions of precursor protein, spliced protein, and excised intein were indicated with letters P, S, and I, respectively. Letter C marks a putative product resulting from cleavage at the C-terminus of intein.

been used in previous studies of other inteins [18,19,22,23], thus the protein splicing products could readily be identified in SDS–PAGE and Western blotting. The precursor protein, spliced protein, and excised intein were identified by their predicted sizes. The precursor and spliced proteins were also identified through antibody recognition of their thioredoxin part.

Efficiency of protein splicing was estimated by comparing protein band intensities of the precursor and spliced proteins on Western blot. As seen in Fig. 3, each of the Prp8 inteins showed efficient protein splicing at room temperature, and the precursor protein was converted completely into the spliced protein. At 37 °C, Prp8 inteins from *A. fumigatus*, *A. nidulans*, and *C. neoformans* showed efficient protein splicing, and little or no precursor protein remained. The *Hca* Prp8 intein from *H. capsulatum*, however, showed significantly less protein splicing at 37 °C, because approximately 75% of the precursor protein remained unspliced.

4. Discussion

We have found Prp8 inteins in a number of fungal species but not in many others (see Table 1), which indicates a wide but sporadic distribution. Prp8 inteins of the different fungal species are clearly homologous, based on their identical insertion site in Prp8 protein and high degrees of sequence conservation. The Prp8 intein sequence of *A. fumigatus* (also *A. nidulans*) is more similar to that of *H. capsulatum* than either is to that of *C. neoformans*, which is consistent with the estimated evolutionary distances of these organisms. For example, the filamentous fungus *A. fumigatus* appears distantly related to the basidiomycete fungus *C. neoformans* and more closely related to the ascomycete fungus *H. capsulatum* (also known as *Ajellomyces capsulatus*), based on nucleotide differences in the ITS1-5.8S-ITS2 region of nuclear rRNA genes [24]. The absence of Prp8 intein in many other *Aspergillus* species, together with the finding of a homing endonuclease domain in some Prp8 inteins, suggests that this intein originated in the different fungal species through lateral gene transfers, which were perhaps catalyzed by the intein-contained homing endonuclease. Therefore, the *Cne* Prp8 intein most likely had the homing endonuclease domain and later lost it.

The *Hca* Prp8 intein is unusual, because it has an extra sequence predicted to be a putative tongs subdomain. Although this prediction was based only on its same location as the tongs subdomain in the crystal structure of *Sce* VMA intein, a sequence similarity is not necessary for this prediction to be correct. The tongs subdomain of *Sce* VMA intein was believed to participate in the binding of substrate DNA by the site-specific homing endonuclease of that intein [21]. If the putative tongs subdomain of *Hca* Prp8 intein has a similar function, it may not necessarily have a similar sequence, because it is expected to bind a very different substrate DNA at a very different intein insertion (homing) site. If our prediction is correct, the putative tongs subdomain of *Hca* Prp8 intein is three times longer than the tongs subdomain of *Sce* VMA intein, which has implications on the endonuclease function and intein evolution.

We have demonstrated for the first time that Prp8 inteins are active for protein splicing, at least in *E. coli*. This makes it likely that Prp8 inteins can actively self-excise in vivo, which implies that these inteins are not inactive sequences tolerated in mature Prp8 protein. The *Hca* Prp8 intein from *H. capsulatum* showed significantly lower splicing activity at 37 °C in *E. coli*, which could be potentially interesting if this happens also in its native Prp8 protein in vivo. A shift from 25 to 37 °C is known to stimulate the dimorphic transition of this fungus from the multicellular mycelial form to the unicellular yeast form [25,26]. The demonstration of protein splicing in a non-native protein in *E. coli* suggests that Prp8 inteins may be used in some of the existing selection or screening systems for identifying intein inhibitors [9], which may produce or lead to potential antifungal drugs. Because Prp8 protein has an essential cellular function, inhibiting Prp8 inteins should effectively kill the pathogens.

The finding of Prp8 inteins in several important fungal pathogens further increases the attractiveness of Prp8 inteins as drug targets. *A. fumigatus*, *H. capsulatum*, and *C. neoformans* are among the most significant opportunistic fungal pathogens [26,27], and *A. nidulans* has been associated with infections in humans and animals [28]. These environmental fungal pathogens exist widely in soil and/or on decaying organic matters, often become airborne in and out doors, and when inhaled into the lung, can cause invasive or non-invasive diseases. Because these opportunistic fungal pathogens primarily infect immunocompromised individuals, controlling them has become more urgent due to the pandemic of AIDS and the widespread use of immunosuppressive therapy.

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