In Vivo Characterization of the Homing Endonuclease within the polB Gene in the Halophilic Archaeon Haloferax volcanii

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Abstract

Inteins are parasitic genetic elements, analogous to introns that excise themselves at the protein level by self-splicing, allowing the formation of functional non-disrupted proteins. Many inteins contain a homing endonuclease (HEN) gene, and rely on its activity for horizontal propagation. In the halophilic archaeon, Haloferax volcanii, the gene encoding DNA polymerase B (polB) contains an intein with an annotated but uncharacterized HEN. Here we examine the activity of the polB HEN in vivo, within its natural archaeal host. We show that this HEN is highly active, and able to insert the intein into both a chromosomal target and an extra-chromosomal plasmid target, by gene conversion. We also demonstrate that the frequency of its incorporation depends on the length of the flanking homologous sequences around the target site, reflecting its dependence on the homologous recombination machinery. Although several evolutionary models predict that the presence of an intein involves a change in the fitness of the host organism, our results show that a strain deleted for the intein sequence shows no significant changes in growth rate compared to the wild type.

Introduction

Inteins are parasitic genetic elements within open reading frames able to perform self-splicing at the level of the protein. The intein is transcribed and translated along with the gene in which it resides, and is subsequently excised from the protein between its two bordering exteins by an autocatalytic process, in which the exteins are joined together [1,2]. Homing Endonucleases (HENs) are a diverse class of site-specific DNases found in archaea, bacteria and lower eukaryotes, and in some of their respective viruses [3,4]. HENs are selfish genetic elements that reside within self splicing introns and inteins, and promote the horizontal propagation of their respective intron/intein into intron-less or intein-less alleles by cleaving the vacant target site to induce homologous recombination or reverse transcription. HENs recognize relatively long target sequences (14–40 bp), a fact that has made them a potential tool for gene therapy and genetic engineering [5].

The gene for DNA polymerase B is a known target for inteins in numerous organisms, their potential effect on host fitness has not been tested experimentally. Here we assayed the in vivo endonuclease activity encoded by the HEN located in the Hfx. volcanii polB gene. We also generated a strain that was cured of the polB intein and tested its fitness.

Results and Discussion

The polB gene of Hfx. volcanii is annotated in InBase as having a putative HEN. It has been proposed that the presence of an intein involves a change in the fitness of the host organism [1], but this has not been tested experimentally. Here we assayed the in vivo endonuclease activity encoded by the HEN located in the Hfx. volcanii polB gene. We also generated a strain that was cured of the polB intein and tested its fitness.

Curing the intein is hampered by HEN activity

Although inteins are present in many essential genes in numerous organisms, their potential effect on host fitness has not been tested [1]. To determine whether the presence of an intein in the polB gene of Hfx. volcanii affects the fitness of this archaeon, we...
attempted to cure the *Hfx. volcanii* polB gene of its intein. By employing the ‘pop-in/pop-out’ strategy for allele exchange, previously developed for *Hfx. volcanii* ([7], see materials and methods and figure 2), a plasmid construct was generated containing a *polB* gene fragment (approximately 1700bp out of about 4000bp) that includes the original stop codon at the 3' end but not the intein (Figure 2A #1). Thus, an intein-less *polB* allele was created lacking the first 1000 nucleotides of this gene. The intein-less construct was created by overlap PCR (see materials and methods), cloned into the pTA131 vector [8], and the resulting suicide plasmid (pAN9, see Table 1 and Figure 2A #2), was transformed into the uracil auxotroph *Hfx. volcanii* strain WR532 (DRpyrE). Transformed colonies were selected for on a medium lacking uracil.

The plasmid integration via homologous recombination occurs at either flanking region (Figure 2A #2), resulting in two possible different arrangements. In the first alternative, integration occurs through homologous recombination in the region 5' to the intein, (Figure 2A #3) resulting in an intact *polB* gene lacking the intein, and a second copy containing only two 850 bp sequences surrounding the intein sequence. The second alternative, is that integration occurs 3' to the intein (Figure 2A #4), and results in an intact, intein-containing, *polB* sequence, followed by a second sequence, containing only 850 bp flanking the intein. In both cases one intact *polB* gene will be expressed, but one version will express a cured *polB* while the other will produce an intein-containing PolB, including its endogenous HEN, which will later be excised and might be active.

The uracil prototrophs of WR532 obtained after transformation were screened by PCR using primers from both sides of the intein (RP1 and RP2, see figure 2A #6 and Table S1). PCR was expected to yield, for each colony, two different-sized amplicons, regardless of the integration site: one copy containing an intein, (a larger PCR fragment), and the second, originating from the plasmid, harboring no intein, thus producing a smaller PCR band (Figure 2A #3–4). Instead, seven out of eight colonies yielded only one band, corresponding to the w.t. length (about 1600bp), indicating that the intein sequence was present in both locations (Figure 2A #5). This observation was validated by two additional PCR reactions, using different primer sets. In each of these
Figure 2. The polB 'pop-in'/'pop-out' experiment. A. 1. The genomic region containing the w.t. polB sequence, indicating the fragments amplified and cloned to create pAN9. Arrows indicate primer binding sites. 2. The suicide vector pAN9, which contains 1700bp of the polB gene, without the intein. Striped boxes indicate sequence originating from the plasmid. Arrows indicate primer binding sites. 3+4. Two alternative expected 'pop-in' arrangements, following selection for plasmid integration. The integration of the plasmid is forced by selecting for ura+ colonies. The plasmid can integrate, by a single homologous recombination event either by the region 5’ to the intein – resulting in arrangement 3, or through the 5’ region resulting in arrangement 4. 5. The 'pop-in' obtained in this experiment, in 7 out of 8 'pop-in' colonies examined. I and II: two different PCR products (see figure 2B). 6. The desired 'pop-out' state. B. Agarose gel electrophoresis of PCR amplicons obtained from intein 'pop-in' candidates, using primers RP1 and RP2. Lane 1 – wild type, lane 2 and 4 – 'pop in' with an intein duplication see figure 2 A #3,4. Lane 3 – expected 'pop-in', see figure 2 A #5. C. Agarose gel electrophoresis of PCR amplicons obtained from intein 'pop-out' candidates, using primers RP1 and RP2, see figure 2 A #6. Lane1 – w.t. cells; lanes 2,4,5,6,8,9,10 – 'pop out' back to the w.t. state; lanes 3 and 7 – deletion of the intein. D. A growth curve comparing the wild type WRS32 to its intein-cured derivative.

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Table 1. Plasmids used in this study.

| Plasmid      | Description                                                                 | Primers used for the construction | Source or reference |
|--------------|-----------------------------------------------------------------------------|-----------------------------------|---------------------|
| pTA131       | pBluescript II containing the Hfx. volcanii pyrE2 gene, used for 'pop-in' 'pop-out' experiments |                                   | [8]                 |
| pAN9- pTA131 | Hfx. volcanii polB intein flanking regions cloned into pTA131.              | AP58,AP59, AP60,AP61              | This study          |
| pTA354       | E. coli/Hfx. volcanii shuttle vector with pyrE2 marker. Contains 946-bp BmgBI-EcoRV fragment of pTA250 with pHV1/4 replication origin inserted at polB site. |                                   | [18]                |
| pRL1         | 850 bp flanking regions on each side of the HEN recognition site/intein insertion site, cloned into pTA354. | The insert cut from pAN9         | This study          |
| pRL2         | 500 bp flanking regions on each side of the HEN recognition site/intein insertion site, cloned into pTA354 | RP7, RP8                          | This study          |
| pRL3         | 250 bp flanking regions on each side of the HEN recognition site/intein insertion site, cloned into pTA354 |                                   | This study          |
| pRL4         | 850 bp flanking regions on each side of the HEN recognition site/intein insertion site, with an altered HEN recognition site. | RP1-12                            | This study          |
| pGEM-T-easy  |                                                               |                                   | Promega             |

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tions. This plasmid was generated since it had been demonstrated in vitro that HENs can easily tolerate single synonymous substitutions, but not non-synonymous ones [11,12]. Since homing is a gene conversion process, which requires homologous recombination, the length of flanking regions upstream and downstream of the cleavage sites should affect homing efficiency, should homologous recombination be the primary mechanism in vivo.

Reason suggested that since the chromosome-encoded HEN was highly active on chromosomal sites (see above), it should also cleave and gene-convert a plasmid-encoded target site.

The largest construct (pRL1) contains approximately 1700 bp of the Hfx. volcanii polB gene, providing flanking regions of approximately 850 bp at each side (Figure 3). Following transformation of pRL1 into Hfx. volcanii, transformant colonies were screened by colony PCR using primers M13-F and RP2 (Figure 3 and Table S1). Nearly 90% of colonies screened (44 out of 49 colonies analyzed) yielded a PCR product matching the size of the intein-containing polB allele. About 10% of the colonies displayed PCR products of a smaller size, corresponding to the original construct (Figure 3B and Table S2). Sequencing of selected plasmids from each category verified those findings.

Even single non-synonymous mutations in the target site dramatically reduce HEN activity [11,12]. In a fourth construct (pRL4) the homing site was changed by introducing non-synonymous substitutions, one codon before the homing point and one codon after the homing site (Figure 3C). This change reduced intein homing to only 27% of the colonies, despite having flanking regions that were 850 bp long on both sides, as in pRL1. This level of specificity again supports a homing mechanism rather than homing-independent gene conversion.

Flanking regions shortened to approximately 500 bp on each side of the target site (pRL2) reduced the recombination efficiency from 90% to 67% (16/24). Additional shortening of the flanking sequences to approximately 250 bp each (pRL3) further reduced the efficiency of homing to 20% (8/39) (Figure 3A). These results confirm that the homing process observed is mediated by the homologous recombination machinery of Hfx. volcanii, which requires longer stretches of highly similar sequences.

A previous study regarding an archaeal HEN of the DOD family, 1-DmoI from the hyperthermophile archaeon Desulfurococcus mobilis, showed its in vivo activity when transformed into another archael species, Sulfolobus acidocaldarius [9]. In that study, the HEN was located in an rRNA intron. 1-DmoI invaded an intron-less sequence when supplied on a suicide vector, either by electroporation of the plasmid or by mating between neighboring Sulfolobus cells. Aagaard and coworkers also reported a fitness advantage for the cells containing the mobile intron. Interestingly, this was not the case in our study. Further work attempting to screen different growth media and environmental stresses for such a fitness effect on Hfx. volcanii should be performed.

Apart from its evolutionary importance, the polB HEN represents a highly valuable molecular genetic tool. Hfx. volcanii, serves as a genetic model organism for the domain of Archaea, [8,13,14] and has also recently become a target for DNA repair studies [15]. The existence of a highly efficient, specific endogenous endonuclease may facilitate the study of DNA double strand break repair, since the target sequence is specific, and does not exist elsewhere in the genome. Such a system has been very useful for studying DNA repair in yeast [16] and could help advance this field in the third, and sometimes neglected, domain of life.

Materials and Methods

Strains and culture conditions

The Hfx. volcanii strain used was WR532 (H26) ApyrE2 [14]. Construction of the intein-cured strain (HAN12) is described in the results.

Hfx. volcanii was routinely grown in rich (HY) medium containing (per liter): 150 g of NaCl, 36.9 g of MgSO4 \( \cdot \) 7H2O, 5 ml of a 1 M KCl solution, 1.8 ml of a 75-mg/liter MnCl2 solution, 5 g yeast extract (Difco) and Tris-HCl (pH 7.2) at a final concentration of 50 mM. After autoclaving and cooling, 5 ml of 10% (w/v) CaCl2 were added. Agar plates contained 18 g of Bacto Agar (Difco) per liter. Casamino Acids (CA) medium contains the same components of the HY medium except that the yeast extract is replaced by 5 g/liter of Casamino acids (Difco).

For counter-selection of uracil auxotrophs, 5-fluoroorotic acid (5-FOA) (United States Biological) was added to the medium at a final concentration of 100 µg/ml. When required, uracil was added to a final concentration of 50 µg/ml.

Figure 3. In vivo activity of the HEN extends to plasmid-located recognition sites. A. The percentage of intein invasion into four different constructs. The screen was performed by colony PCR and agarose gel electrophoresis. The numbers represent the average of two independent experiments. Bars represent standard error of the mean. B. Agarose gel electrophoresis of PCR amplicons from a colony transformed by pRL1, with primers R2P and M13-F (located on the plasmid). Lane 1- a site invaded by an intein corresponding to a length of approximately 3.3 kb; lane 2- a vacant site of about 2kb. C. The non-synonymous substitutions engineered in pRL4, arrow indicates intein integration site.

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Transformation

Transformation of *Hfx. volcanii* was carried out using the PEG method as described in [17]. Briefly, 1.5 ml of liquid culture were grown to OD<sub>600nm</sub> of 1.5, then centrifuged at 3500g for 5 minutes. The supernatant was discarded and the cells were resuspended in 200μl spheroplasting solution (1 M NaCl, 27 mM KCl, 50 mM Tris-HCl pH 7.2, 15% sucrose) and incubated at room temperature for 5 minutes. 20 μl of 0.5 M EDTA were added and cells were incubated at room temperature for 10 minutes. 10 μl of purified plasmid DNA were mixed with 15 μl spheroplasting solution and 5 μl of 0.5 M EDTA were added to the cells, followed by incubation of 5 minutes at room temperature. Subsequently, 240 μl of PEG solution (60% PEG 600 spheroplasting solution) was added and cells were incubated for 20 more minutes at room temperature. Following the incubation, 1 ml of regeneration solution (3.4M NaCl, 175 mM MgSO<sub>4</sub>, 34 mM KCl, 5 mM CaCl<sub>2</sub>, 50 mM Tris-HCl pH 7.2, 15% sucrose) was added and cells were centrifuged at 3500 g for 7 minutes. The supernatant was discarded and cells were resuspended in HY medium supplemented with 15% sucrose and incubated at 37°C for 3 more hours, then washed and plated on selective media.

Gene knockouts

The gene knockouts was performed according to the protocol described in [7,8]. In this method, the upstream and downstream flanking regions of the sequence to be exchanged are amplified by PCR and cloned together into the ‘suicide plasmid’ pTA131 that carries the *pyrE* selectable marker genetic and cannot replicate autonomously in *Hfx. volcanii*. The plasmids are then transformed into a *Hfx. volcanii pyrE* mutant and transformants, in which the plasmids have been integrated into the chromosome, are selected for on plates that lack uracil (‘pop-in’). Upon counter-selection on plates containing uracil and 5-fluoroorotic acid (5FOA), the only cells that survive are those in which the integrated plasmids have been excised by spontaneous intra-chromosomal homologous recombination (‘pop-out’), either restoring the wild-type gene or resulting in allele exchange.

Curing of the intein was performed by allele exchange using the ‘pop-in’-‘pop-out’ methodology as described above. The intein-less sequence was generated by separately amplifying the upstream and downstream regions of the wild type *polB* intein, using primers that generate an overlap of approximately 15 nucleotides between the 3' end of the upstream region and the 5' end of the downstream region (for primers see Table S1). The two parts were assembled using overlap PCR to generate an intein-less *polB* construct. The ‘pop-in’ and ‘pop-out’ strains were screened using pairs of external ‘intein short-up’ (RP1) and ‘intein short down’ (RP2) primers located approximately 150bp upstream of the intein and 150bp downstream of the intein.

Determination of intein presence on the exogenic target plasmids

The presence of an intein on the exogenic plasmids was tested by PCR using ‘intein short was conducted with the Phusion® DNA Polymerase (Finnzymes) according to the manufacturer’s protocol.

Determination of growth rates of the w.t and deletion strains

To compare the growth rates of the w.t strain and intein deletion strains, each strain was grown over-night in CA+uracil media at 42°C to the late log phase and then diluted to a fresh medium and left to shake at either 37°C, 42°C or 45°C. Turbidity of the culture (OD<sub>600nm</sub>) was measured every 3–5 hours using the Genesis 200 Workstation robot (Tecan).

Plasmids and primers

A list of all plasmids that were used in this study is given in Table 1. A list of all primers used in this study is given in Table S1.

Plasmids intended for gene knockout had their inserts cloned between the *HindIII-* *NcoI* restriction sites within the pTA131 multiple cloning site.

Supporting Information

Figure S1 Multiple sequence alignment of the PolB intein in different archaea. Colors denote conserved functional blocks. Hvo- *Haloferax volcanii*, Hwa- *Halopondichloris walsbyi*, Ton- *Thermococcus onnurineus* Tzi- *Thermococcus zilligii*. (PDF)

Figure S2 In vivo homing into the integrated plasmid (‘pop-in’). A. Agarose gel electrophoresis of PCR analysis on intein ‘pop-in’ candidates, using primers RP1 and RP2. All lanes ‘pop in’ with intein duplication see figure 2A #3,4. B. Agarose gel electrophoresis of PCR analysis on intein ‘pop-in’ candidates, see figure 2A stage 5. I- using RP6 and M13R. II- using M13F and RP5. The different lanes signify different annealing temperature. C. Agarose gel electrophoresis of PCR analysis on intein ‘pop-in’ candidates, to examine ‘pop-in’ arrangement, see figure 2A stages 3 and 4. Using primers RP4 and RP2 distinguishing between the two ‘pop-in’ arrangements. Lane 1- w.t.; lane 2 – intein ‘pop-in’ corresponding to the arrangement seen in figure 2 stage 3. D. A schematic representation of the *polB* region, following ‘pop-in’. Arrows represent primer binding sites used in C. (PPT)

Table S1 Primers used in this study. (DOC)

Table S2 Homing efficiencies for the different constructs per experiment. (DOC)

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Author Contributions

Conceived and designed the experiments: AN RL UG. Performed the experiments: AN RL. Analyzed the data: AN RL AB UG. Wrote the paper: AN RL RTP UG. Formulated hypothesis: AN AB RTP UG.

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