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Site Selection of Pyrococcus horikoshi RadA Intein

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Certificate of Approval

Site Selection of *Pyrococcus horikoshi* RadA Intein

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Site Selection of *Pyrococcus horikoshi* RadA Intein

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ABSTRACT

Inteins are polypeptides translated within host proteins that undergo an autocatalytic protein splicing reaction to join the flanking sequences, termed N- and C- exteins. Long considered molecular parasites, recent work has demonstrated that protein splicing can be regulated by environmental signals, representing an exciting new form of post-translational regulation. A landmark example comes from the homologous recombinase RadA of the archaeon *Pyrococcus horikoshii*, which splices in response to substrate single-stranded DNA. Interestingly, while the *P. horikoshii* RadA intein has been shown to splice exceptionally well in numerous foreign exteins reporters under all conditions tested, splicing is inhibited in native exteins in absence of ssDNA. In order to better understand this intriguing observation, *P. horikoshii* RadA intein site preference within the native extein context was studied by artificially moving the intein to different locations throughout the native sequence. Surprisingly, splicing was defective at all alternative locations within the native extein context. These results help us to better understand intein site selection as a whole, as well as suggests a complex coevolutionary interplay between intein and host.

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INTRODUCTION

Inteins are polypeptides that invade host genes at the DNA level and splice out at the protein level. They are found in a wide range of single celled organisms that span every domain of life. Despite the diversity of their hosts, all inteins are relatively similar in structure and have similar splicing mechanisms (Eryilmaz, *et al. 2014*). Inteins are often found within critical regions of host proteins, such as active sites, and it is generally expected that splicing is needed to occur in order for the host protein to become functional. Protein splicing does not require cofactors but can be regulated by a number of environmental signals, and in one example, by the presence of host protein substrate (Lennon et al. 2016).

Splicing most commonly occurs by a four-step mechanism in which the intein autocatalytically excises from the host protein, leaving behind nothing but a normal peptide bond linking the N-extein to the C-extein (Fig. 1).

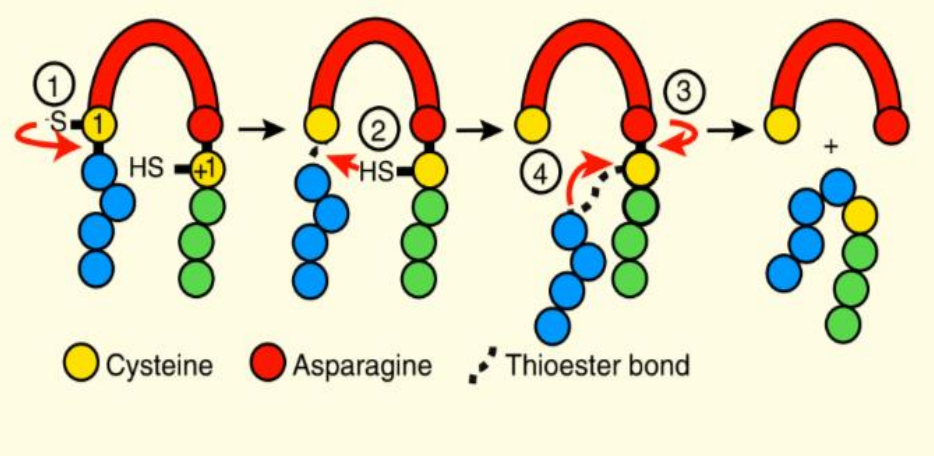

Figure 1. Class 1 intein splicing mechanism. Adapted from Lennon and Belfort, 2017 Current Biology, this figure depicts the four-step splicing mechanism of class 1 inteins with the red region representing the intein, the blue region representing the N-extein, and the green region representing the C-extein. The yellow cysteine to the right of the intein is the +1 nucleophile, while the yellow cysteine to the left is the first residue of the intein itself.

Exteins are the protein segments that flank the intein in the precursor protein prior to splicing. The mature protein consists of these two exteins ligated together and excludes the intein. In the first step of this reaction, the first amino acid of the intein attacks the peptide bond between itself and the last residue of the N-extein known as the -1 residue. This forms a thioester linkage which is then attacked by the first residue of the C-extein, known as the +1 nucleophile, creating a branched intermediate. This intermediate then releases the intein leaving a thioester bond between the two exteins. In the final step, the thioester bond rearranges itself into a normal peptide bond, leaving no evidence the intein was ever present (Lennon *et al.* 2017).

The use of inteins has many exciting applications in the fields of microbiology, bioengineering, genetic engineering, and medicine. Intein mediated self-cleaving affinity tags are widely used for efficient protein purification and protein labeling. In this application, an intein fuses the protein being purified to an affinity tag which can then be removed by intein splicing induced by temperature or pH. Working with inteins also provides a new means of biomolecule engineering by joining two separate protein segments both *in vitro* and *in vivo* with the use of *trans*-splicing inteins (Wood et al., 2014). One application of this split-intein system for protein ligation currently used in genetic research is the CRISPR/Cas9 system which has the potential for one day treating genetic diseases. Once in the cell, the Cas9 protein has the ability to locate and cut any gene within an organism in order to edit the host's genome allowing researchers to gain understanding of that gene's function. However, the use of CRISPR/Cas9 is unfortunately limited by the protein's large size which makes its delivery by recombinant adeno-associated virus much less efficient. Biologists have found a way around this limitation by

delivering the protein to its destination in two parts, then ligating them *in vivo* through intein-mediated *trans*-splicing (Truon et al, 2015).

Because inteins are present in many bacterial pathogens but not in humans or other multicellular eukaryotic hosts, there is great potential for inteins to be drug targets. One such example is *Mycobacterium tuberculosis* which contains four inteins including one in the cell's RecA protein (Zhang et al. 2010). This bacterium is a highly contagious pathogen responsible for large numbers of Tuberculosis infections in individuals all over the world. However, it has been found that widely used chemotherapy drug cisplatin, cis-[Pt(NH3)2Cl2], prevents intein splicing by binding to RecA providing an alternative strategy of fighting this bacterial pathogen by hindering the cell's ability to correct DNA damage (Chan *et al.* 2016). This is also a potential treatment option that would not contribute to the growing problem of antibiotic resistance. Because similar bacterial pathogens including *Cryptococcus neoformans*, which causes cryptococcosis, *Coxiella burnetii*, which causes Q fever, and *Mycobacterium leprae*, which causes leprosy, all contain inteins located within critical proteins, intein splicing inhibition is a promising treatment for a number human diseases (Zhang et al. 2010).

Inteins, present in about 50% of archaea and 25% of bacteria, were commonly thought to be molecular parasites that served no purpose in benefiting the host (Lennon *et al.* 2017). However, some characteristics of certain inteins do not appear to classify them as purely selfish DNA as there is now strong evidence to suggest some inteins have coevolved with their host protein to play a useful role in regulation. Roughly 70% of inteins are located within ATPase proteins (Lennon *et al.* 2017). When the intein is present, ATP cannot bind, and the protein is unable to perform its function. Once the intein splices, ATP is able to bind, and the protein becomes fully functional. The strategic location of these inteins coupled with the fact that their

splicing is sometimes contingent on environmental factors and the presence of different substrates creates an effective mechanism for post-translational protein regulation. Once the intein splices, the protein is able to respond to the environmental stressor that induced splicing.

The RadA recombinase protein used in this study is a DNA-dependent ATPase that catalyzes homologous DNA pairing and strand exchange which serves to increase genetic variability and repair DNA damage in archaeal genomes (Lennon *et al.* 2016). This protein is thought to be regulated by mostly post-translational means and is homologous to RecA found in bacteria and Rad51 found in single celled eukaryotic organisms. These proteins all share the same ATPase domain and ATP-binding P-Loop, however, RadA has a few structural differences which have been observed through electron microscopy and X-ray crystallography (Galkin *et al*, 2006, Topilina et al. 2015). Prior to splicing, RadA is able to bind to single-stranded DNA but ATP binding is blocked by the intein (Lennon et al. 2016, Topilina et al. 2015). Because RadA is synthesized with the intein located within an active site, the protein is inactive until splicing is induced.

This intein comes from hyperthermophile *Pyrococcus Horikoshii (Pho)*; an archaeon species that requires extremely high temperatures to achieve population growth. These high temperatures induce the *Pho* RadA intein to splice out allowing for gene expression. Until the proper environmental conditions are met, protein interactions between the C-extein and intein inhibit splicing, which in turn inhibits protein function and, ultimately, growth. A previous study determined temperature dependent disruption of the extein-intein interaction is mediated by remote residues within the tertiary structure of the protein (Topilina et al, 2015). This mechanism adds a layer of post-translational regulation, allowing the RadA protein to be synthesized in an inactive state, then quickly activated when a high enough temperature is reached and the RadA

protein is needed to respond. With this system, ATP is utilized by RadA when conditions are optimal for cell growth and conserved by the cell in conditions of cold shock. This suggests RadA coevolved with the intein in order to improve the energy efficiency of the host (Topilina et al. 2015).

Given RadA is a recombinase, it is particularly exciting that the presence of single-stranded DNA is also an effective trigger for intein splicing and accelerates this process. Single-stranded DNA is indicative of DNA damage and the cell must have a mechanism to correct this damage quickly. Without the intein allowing for the production of inactive RadA proteins, the cell would have to synthesize RadA once DNA damage was detected. Because DNA damage itself induces splicing and, thus, activation of RadA, the damage is corrected more quickly. Additionally, the presence of this substrate is about 50 times more effective in inducing the intein to splice than increased temperature alone, providing further evidence for the intein's role in post-translational regulation (Lennon et al. 2016).

Prior to induction, *Pho* RadA intein splicing is blocked by protein interactions between the intein and native C-extein (Topilina et al. 2015). For the protein to become functional, single-stranded DNA must be present or specific environmental factors met to disrupt these protein interactions and induce splicing. By contrast, when placed in almost any non-native extein context, a different host protein, splicing is not blocked and the intein autocatalytically splices out with great speed and accuracy leaving no residues or scaring.

The difference in *Pho* RadA intein splicing behavior in the native and non-native hosts is striking. As the only strict requirement for intein splicing is the presence of a +1 nucleophile, it is also intriguing that inteins so strongly cluster to certain areas of proteins. Despite this difference in behavior, it has not previously been studied how the intein would react to being inserted into

non-native locations within the native host protein. This study seeks to determine whether neighboring threonines can be utilized as the +1 nucleophile to enable splicing at various locations within the native extein context. This work helps us further understand the *Pho* RadA system, a landmark example of inteins as post-translational regulators, and informs the factors that control intein site selection in natural and engineered systems.

EXPERIMENTAL DESIGN

The *P. Horikoshii* plasmids were manipulated to contain genes for 10 mutated RadA proteins with the intein artificially inserted at various locations within the DNA sequence (Fig. 2).

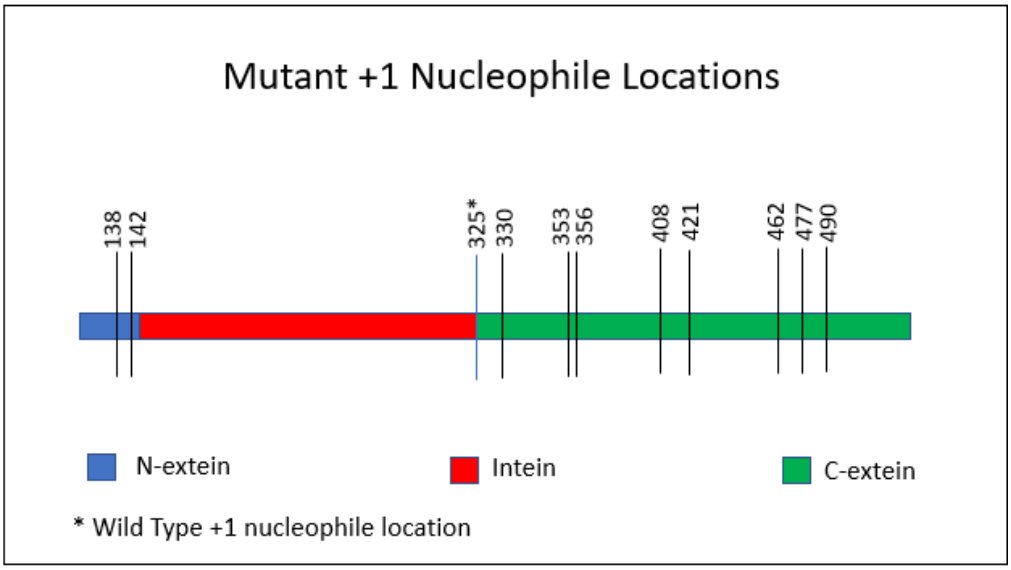

Figure 2. Mutant *Pho* RadA +1 nucleophile locations. Each line represents the location of an alternate threonine with the potential to be used as a +1 nucleophile for splicing upon relocation of the intein. These threonines are T138, T142, T330, T353, T356, T408, T421, T462, T477, and T490, respectively. The blue region represents the wild type N-extein, the red region represents the wild type location of the intein, and the green region represents the wild type C-extein.

The plasmids were also altered to give the proteins specific characteristics which allowed us to select for cells that successfully took up and retained this plasmid and purify the resulting proteins letter in the experiment. These proteins were then overexpressed in *Escherichia coli* (*E. coli*) cells, purified using Ni^{2+} chromatography, and tested for splicing competency. These alterations to the genome were achieved using pET vectors which were received prior to the experiment. A pET vector is a lab-created plasmid designed for protein expression of the cloning region which contained our gene of interest: *Pho* RadA. The expression of this gene is

highly regulated by T7 LacI which is acted upon by two different mechanisms in order to ensure the gene is only expressed when needed.

Thirteen different pET vectors were used for this experiment. The *Pho* RadA intein needs a nucleophile in the +1 position, the first amino acid in the C-extein, in order to achieve splicing. The three amino acids that can be utilized as the +1 nucleophile are cystine, serine, or threonine (Oeemig *et al.*, 2012). Though other factors can affect the speed and accuracy of splicing, the presence of a+1 nucleophile in the extein is thought to be the only requirement of the extein to enable intein splicing (Topilina et al. 2015). In the case of wild-type (WT) *Pho* RadA, threonine is used as the +1 nucleophile. To test the theory that *Pho* RadA coevolved with the intein as a means of post-translational protein regulation and gain insight on site selection of this intein, we moved it from its native location to 10 other threonines in the host DNA sequence to observe how the intein's placement affects its splicing behavior. In addition to these 10 mutants and the WT which served as a control, we also created a *Pho* RadA with no intein and a *Pho* RadA with a deactivated intein for a total of 13 different *Pho* RadA proteins.

Also included on each pET vector was antibiotic resistance gene Beta-Lactamase which ensured only cells containing the *Pho* RadA protein were cultured for the following steps of the experiment. The cells containing these vectors were grown on an Ampicillin infused auger that inhibited growth of cells lacking the Beta-Lactamase. Additionally, the pET vector coded for a string of 6 histidines preceding the N-extein. This His tag allowed the protein to be purified via Ni^{2+} chromatography later in the experiment.

METHODS

TRANSFORMATION

The first step in the experiment was to heat shock competent *E. coli* cells so they take up the plasmids. To do this, we first prepared several starter plates with LB agar and streaked for isolated colonies of *E. coli* to minimize the genetic variability of the cells. These plates were incubated overnight at 37 degrees Celsius. We then prepared microcentrifuge tubes with 50 μl of $CaCl_2$ competent cells for each of the plasmids. These tubes were kept on ice unless otherwise noted for the remainder of the procedure. We collected an isolated colony from the starter plate and inoculated the solution with the bacteria. Next, we added 1 μl of each plasmid via micropipette to a tube and allowed the solution to sit on ice for 10 minutes. We transferred the tube into a water bath set at 42 degrees Celsius for 50 seconds before returning it to the ice for another two minutes.

Now that the cells had taken up the plasmid, we added 1 mL of LB broth into the tubes, then incubate at room temperature for 10 minutes. We then pipetted a small amount of this solution onto agar plates we had previously prepared with LB and ampicillin and streaked for isolated colonies of the transformed E. *coli* before placing them in the incubator at 37 degrees Celsius overnight. The ampicillin in the agar acted as a selector to kill all the cells that failed to take up or retain the plasmid. This was made possible by the antibiotic resistance gene Beta-Lactamase that was built into the plasmid. These steps were repeated for the control group and each of the mutants.

CELL GROWTH AND INDUCTION

The next step was to take the transformed cells and grow them to a high concentration before we could overexpress the RadA protein. We inoculated 5 mLs of starter culture with

isolated colonies from the plate and incubated at 37 degrees Celsius overnight. We then sterilely pipetted the starter culture into growth media at a 1:100 volume ratio totaling 400 mLs and placed the beaker in the shaking incubator at 37 degrees Celsius and 250 RPM for approximately 30 to 60 minutes.

Using a spectrophotometer, we took samples from each beaker and measured its optical density every 30 minutes until cell growth reached the log phase, as indicated by an optical density of around 0.5 OD. This was the point at which the cells had the most active ribosomes ideal for expressing our proteins at high concentrations. Once the log phase of cell growth was reached, we added IPTG (Isopropyl β- d-1-thiogalactopyranoside) to induce overexpression of the protein.

PROTEIN EXPRESSION AND PURIFICATION

Now that our protein had been expressed within the *E. coli*, we had to separate the cells from the spent media and extract the RadA proteins from the cells. We poured the growth media into centrifuge tubes and centrifuged at 3000xG to pellet the cells in order to remove them from the media. We then carefully decanted the media from the pellets, added Nickel Buffer A (20 mM Tris pH 8, 500 mM NaCl, 10 mM Imidazole) to the tubes, and resuspended the contents of the pellet before freezing the contents at -20 degrees Celsius overnight in order to prepare the cells for sonication.

We allowed the liquid containing the cells to thaw then placed the tubes in a large beaker of ice to prevent excess heat produced by sonication from denaturing the protein or causing premature splicing. We placed the sonication probe into the solution, set the machine to 30%, and ran it 30 seconds on and 30 seconds off for a total of 30 minutes. After the cells were lysed, we centrifuged the solution at 20,000xG for 20 minutes to separate the cell debris from our

protein of interest suspended in solution. The supernatant was then decanted and ready for purification.

We purified the protein using a column of stationary phase Ni^{2+} that binds only to proteins containing the His-tag encoded on the plasmids. These proteins were able to bind to the nickel in the column strongly enough to remain in place while the column was rinsed with a washing solution. This purified the protein and ensured only the tagged RadA proteins were left for the next step. The column is then washed with an elution buffer. Because the stationary charged nickel had a higher affinity for the elution buffer that for the His-tags, the buffer dislodged the proteins which were rinsed from the column and collected. These steps were repeated for each of the protein variants.

SPLICING ASSAYS

Once the mutant proteins were expressed and purified, we performed splicing assays to determine under what conditions if any, the inteins were able to splice. We did this by changing two variables: temperature and the addition of single-stranded DNA. We heated each sample to 63 degrees Celsius, 75 degrees Celsius, and 87 degrees Celsius for 15 minutes without the addition of single-stranded DNA, before repeating with the addition of 188ng/μl single-stranded DNA.

SDS PAGE ELECTROPHORESIS

We then added 1x Tris-Glycine-SDS buffer into the chamber of the electrophoresis unit and loaded 20 μl of the latter into the first well of the gel cassette which served as a size reference for the protein segments in the samples. We then loaded 20 μl of each sample into a well using a micropipette. We connected the unit to a power supply set to 200 volts and allowed the unit to run for 30 minutes. When the electrophoresis was complete, we opened the cassette

using a metal spatula and placed the gel in a shallow dish containing stain on an oscillator overnight. We then repeated this step with a rinsing solution.

RESULTS

Splicing competence was evaluated for 12 mutant *Pho* RadA inteins. In order to study how splicing behavior is affected by the intein's location within the native host protein, 10 mutants were artificially created with the intein relocated to precede the T138, T142, T330, T353, T356, T408, T421, T462, T477, and T490 residues. Two additional mutants were created for comparison; one with an inactivated intein, another with the intein removed, and a WT *Pho* RadA was used as a control. Each mutant was initially tested for splicing ability without heating or the addition of single stranded DNA.

Several characteristics of each mutant were noted including the -1 residue, the predicted splicing efficiency of the -1 residue, and the location of the intein within the three-dimensional context of the protein (Table 1). Upon analysis of this data, mutant T353 stood out as being particularly favored to splice due to the intein's position within a loop and its asparagine -1 residue having a 95% predicted splicing efficiency. This protein was expressed using *E. coli* cells, purified using Ni^{2+} chromatography, and tested under two addition treatment groups. One was heated with the addition of single-stranded DNA, and the other was heated without the addition of single-stranded DNA. Samples from both treatment groups were heated to 63 degrees Celsius, 75 degrees Celsius, and 87 degrees Celsius for 15 minutes each.

Analysis of the mature proteins by SDS PAGE Electrophoresis revealed that though our control WT *Pho* RadA intein spliced in all conditions, none of the 12 mutants displayed splicing competence in the initial assay. Furthermore, mutant T353 was splicing incompetent under all condition tested (Fig. 3). Though splicing assays of the remaining mutants with single-stranded DNA and heating continue, each mutant tested thus far has yielded the same result. The mutants

failed to splice in conditions mimicking those that effectively induce splicing in WT *Pho* RadA confirming splicing incomitance.

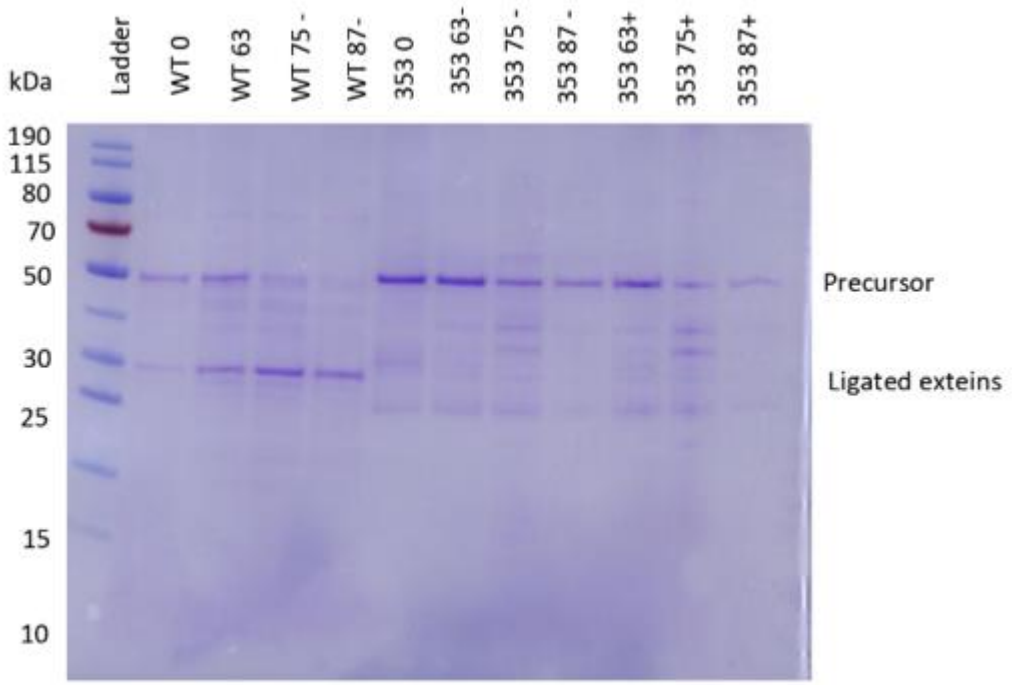

Figure 3. Splicing assays for T353 mutant. This gel compares the splicing capability of Wild Type RadA and the 353 T+1 mutant in various conditions. Samples of both proteins were heated to 63 degrees Celsius, 75 degrees Celsius, and 87 degrees Celsius for 15 minutes without the presence of single stranded DNA. In these conditions, Wild Type RadA spliced at each of the three temperatures, as indicated by the two bars in the WT columns, and the mutant did not, as indicated by the presence of only one bar in the 353 columns. This was repeated for 353 T+1 with single stranded DNA present in each of the columns with a + at the top. Again, the single bar in each of these columns indicated the mutant was not splicing competent. The ladder in the far-left column serves as a point of reference for the size amino acid chain.

Threonine Position Used as +1 Nucleophile	-1 Residue	Predicted -1 Residue Splicing Efficiency	Secondary Structure of Intein
138	E	40%	Loop
142	I	39%	B sheet
325*	K	97%	Loop
330	H	96%	Helix
353	N	95%	Loop - Native site
356	D	35%	Loop
408	N	95%	Loop
421	L	94%	Helix end
462	V	53%	B sheet
477	P	23%	Unstructured
490	A	45%	Loop/sheet end

Table 1. Details for each mutant. This table lists each of the different locations of the threonines used as the +1 nucleophile as well as the -1 residue, the splicing efficiency of that -1 residue, and the secondary structure surrounding the intein. T325 is the native +1 nucleophile

DISCUSSION

The splicing incompetence of inteins at alternate locations within the native extein context was surprising given that conditions were favorable for splicing and that the RadA intein is studied specifically for its impressive splicing efficiency in non-native contexts (Topilina et al. 2015). Not only did each intein have a +1 nucleophile, as required for splicing, but the inteins in each of the mutants also had the same +1 nucleophile as WT *Pho* RadA intein. In addition to the favorable +1 residue, some of the mutants also had residues in the -1 position, the last residue of the N-extein, shown to be successful in allowing splicing to occur in similar studies. A previous study in a non-native extein reporter changed the residue in the -1 position (Oeemig *et al.*, 2012). From this, the predicted splicing efficiency for each of the -1 residues tested was determined (Table 1).

A possible factor in preventing intein splicing in non-native locations is the surrounding secondary structure of the precursor protein (Table. 1). However, it would seem that inteins located within loops or unstructured segments of the protein would be unhindered by this variable. It is for this reason the mutant with the T353 +1 nucleophile caught our attention. This intein has asparagine as its -1 residue which has a roughly 95% predicted splicing efficiency with the WT intein location (Oeemig *et al.*, 2012) and is located within a loop (Fig. 4). There is no clear explanation at this time as to why this particular mutant is splicing incompetent.

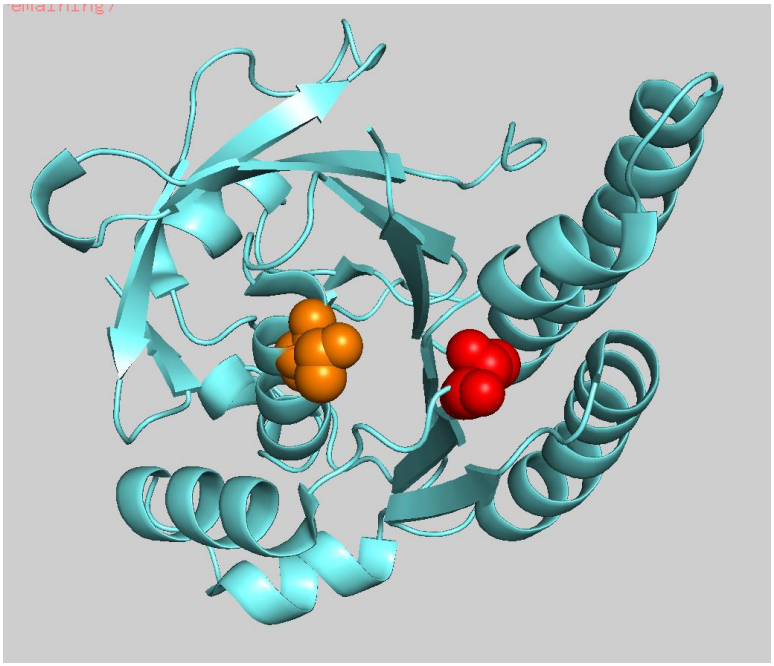

Figure 4. Locations of Wild Type and mutant T353 inteins. The orange residue represents the native location of the +1 nucleophile within the tertiary structure of RadA: T325. The red residue represents mutant intein location at T353.

These results raise exciting questions as to what factors are inhibiting splicing, or what unknown requirements for splicing are not met at these specific intein locations. Though it was originally assumed that only residues immediately flanking the intein directly affected splicing competence, studies have confirmed temperature dependent inhibition of intein splicing is mediated by interactions between the intein and remote residues in the extein (Lennon et al. 2016, Topilina et al. 2015). Further analysis of the three-dimensional structure of each mutant should be evaluated to determine if similar interactions to the WT *Pho* RadA protein are inhibiting splicing in a more permanent way in the mutants. Another possible explanation could be that though remote residues do not seem to interact with the WT intein, they may interact with our relocated inteins. The results were unexpected but contributed to our understanding of the *Pho* RadA system and intein site selection as a whole.

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