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Functional expression and purification of *Anabaena* PCC 7120 XisA protein

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ABSTRACT

Anabaena PCC 7120 *xisA* gene product mediates the site-specific excision of 11,278 bp *nif*D element in heterocysts formed under nitrogen starvation conditions. Although XisA protein possesses both site-specific recombinase and endonuclease activities, till date neither *xisA* transcript nor XisA protein has been detected. Gene encoding XisA protein was isolated from plasmid pMX25 and overexpressed in *Escherichia coli* BL21 DE3 yielding 7.7 mg enzyme per L of growth culture in soluble fraction. His-tagged XisA was purified using Ni-NTA affinity chromatography with 95% recovery. The purified XisA showed a single band on SDS–PAGE with molecular mass of 52 kDa. Identity of XisA was confirmed by MALDI-TOF analysis and functionality of enzyme was confirmed using restriction digestion. A PCR based method was developed to monitor excision by XisA, which displayed near 100% activity in *E. coli* within 1 h at 37 °C on LB under static condition.

1. Introduction

Anabaena PCC 7120 is a filamentous, heterocytous cyanobacterium capable of oxygenic photosynthesis and nitrogen fixation. In the absence of a combined nitrogen source, a few vegetative cells differentiate in to a specialized nitrogen fixing cells, called heterocysts, at semi-regular interval of 10–20 vegetative cells in a filament [1]. Heterocyst differentiation involves morphological, metabolic and genetic adaptations leading to the development of micro-oxic environment suitable for the activity of the oxygen-sensitive nitrogenase complex [2,3]. *Anabaena* PCC 7120 genome is interrupted by three genetic elements; *nifD*, *fdxN* and *hupL*, named according to genes which each one disrupts. These elements are of 11,278 kb, 59,428 kb and 9419 kb size, respectively and get excised by site-specific recombination mediated by three different excisases (XisA, XisF, XisC, respectively) each are encoded within the specific element, during late stages of heterocyst differentiation resulting in the formation of functional genes facilitating nitrogen fixation [4]. The *nifD* element is flanked by 11 bp CGGAGTAATCC direct repeats targeted by the product of *xisA* gene located near one target site of *nifD* element [5]. XisA protein belongs to lambda integrase family of recombinase characterized by the presence of **R-(X)$_{70-139}$-H/Y-XX-R-(X)$_{29-38}$-Y** tetrad as conserved active site residues near the carboxyl terminal [6]. Excision of *nifD* element of *Anabaena* PCC 7120 does not occur in a *xisA* mutant strain [7,8]. Expression of *xisA* gene from P_{tac} promoter has been shown to result in the excision of the *nifD* element even in the vegetative cells [8]. Excision of *nifD* element has been well-studied in heterologous system of *Escherichia coli* wherein rearrangement occurs under nitrogen sufficient conditions at a frequency of about 0.3–11% depending on the growth medium [5,9]. Excision of *nifD* element in *E. coli* is enhanced by about 5 fold in M9 minimal medium compared to Luria Bertani (LB) broth when the strain is wild-type for *recA* [9]. In contrast to *Anabaena* PCC 7120, excision of *nifD* element in *E. coli* DH5α was not influenced by iron-deficient conditions. These results imply the involvement of additional accessory factors, such as RecA and physiological status in the excision of *nifD* element. An

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important feature of XisA protein that was demonstrated *in vivo* in *E. coli* was its endonuclease activity at 11 bp (**CGGAGTAATCC**) target site [10]. The presence of both recombinase and endonuclease activities is indicative of XisA being an evolutionary intermediate between endonucleases and recombinases.

Even though the *xisA* gene was cloned and sequenced long ago [5], neither the protein nor transcript has been detected so far in *Anabaena* nor in *E. coli* strains expressing *xisA*. It is interesting to note that in most of the studies on the rearrangement of *nifD* element in *E coli*, the *xisA* expression was indirectly monitored by *in vivo* excision of the *nifD* element by loss of *lacZ* gene inserted within the *nifD* element. In this paper, we report for the first time the detection and purification of XisA protein which was purified using T7 expression system and identified by MALDI-TOF analysis. We also report a PCR based robust assay to monitor the recombinase activity of XisA *in vivo* in *E. coli*. This paper also provides the first computational insight of secondary and 3D structure of XisA protein, an important enzyme of *Anabaena* PCC 7120 for nitrogen fixation.

2. Materials and methods

2.1. Bacterial strains and plasmids used in the study

Table 1 shows the *E. coli* strains and plasmids used in this study. Plasmids were maintained with appropriate antibiotic combinations.

2.2. Culture conditions

All microbiological media were purchased from HIMedia Laboratories, Mumbai, India. *E. coli* strains were routinely grown in Luria Bertani (LB) medium at 37 °C at 200 rpm, supplemented with appropriate antibiotics as follows: 100 μg ml^{-1} Ampicillin, 50 μg ml^{-1} Kanamycin, and 34 μg ml^{-1} Chloramphenicol. Bacterial growth was measured as OD_{600}.

2.3. DNA manipulation in vitro

Routine DNA manipulations were carried out as described in Sambrook and Russell (2006). All enzymes for DNA manipulation were purchased from Thermo Fisher Scientific Waltham, Massachusetts, USA. Oligonucleotides were purchased from Integrated DNA Technologies, Coralville, Iowa, USA. Routine chemicals were purchased from Sigma-Aldrich, St. Louis, Missouri, USA.

2.4. Cloning and overexpression of xisA gene

xisA gene was PCR amplified from pMX25 by Pfu polymerase (Thermo Scientific) using XisA(F) forward primer (5′-GGATCCATGCAAAATCAGGGTCAA-3′) and XisA(R) reverse primer (5′-GAGCTCTCAACTATTCTTATAAGCTATTTCCA-3′). *Bam*HI (in forward primer) and *Sac*I (in reverse primer) sites are underlined. The PCR amplified 1.4 kb *xisA* gene product was cloned in to pET28a (+) at *Bam*HI and *Sac*I sites resulting in the plasmid pNU1 in which *xisA* is under T7 promoter. *E. coli* BL21 (DE3) bearing pNU1 (named as NA1) was grown overnight in 100 ml LB medium containing 50 μg ml^{-1} kanamycin as described in section **2.2**. On the following day, 1% inoculum of overnight grown NA1 was transferred to fresh LB medium supplemented with 50 μg ml^{-1} kanamycin and was allowed to grow until the OD_{600} reached 0.6–0.7. Expression of *xisA* gene was induced by the addition of 0.8 mM IPTG. XisA protein was detected using SDS-PAGE followed by Coomassie blue R250 staining [14].

2.5. In vivo detection of pNU1-encoded XisA activity

In vivo detection of XisA recombinase activity was carried out according to Brusca et al. (1990) using pAM461, a pUC18 based *xisA* deficient plasmid which contains distal and proximal borders of *nifD* element, as a substrate plasmid. Excision of minimal *nifD* element was brought about by providing XisA *in trans* using pNU1. *E. coli* strain NA1 bearing pNU1 was co transformed with pAM461 and transformants were selected on LB agar plates with 50 μg ml^{-1} ampicillin and 25 μg ml^{-1} kanamycin. A single colony of dual transformant was inoculated in 15 ml LB medium with same antibiotics and was allowed to grow overnight in uninduced condition. On the following day, plasmids were isolated and digested with *Hind*III and *Eco*RI. Digestion mixture was analysed on 0.8% agarose gel. The appearance of 715 bp band indicates rearrangement of pAM461 and XisA activity.

2.6. PCR based method to monitor XisA excisase activity

A novel PCR based approach was developed monitoring XisA excisase activity using a substrate plasmid pAM461 containing only the distal and proximal borders of *nifD* element. XisA was provided in trans from pNU1. To suppress basal expression of *xisA* in pNU1, *E. coli* BL21 (DE3) pLysE was used as the expression host [13]. The strain was co-transformed with substrate plasmid pAM461 to obtain *E. coli* BL21 (DE3) pLysE (pAM461+pNU1) and named as UN2. After overnight growth on LB agar containing appropriate antibiotics, bacterial biomass was scraped and resuspended in LB broth to give a suspension of $OD_{600} = 1$. *xisA* expression was induced by adding 0.8 mM IPTG, after which the cells were incubated at 37 °C in static condition and an aliquot of 10 μl was collected every 5 min. 5 μl of which was utilized for each substrate specific and product specific PCR. Sequence of antisense (XR) reverse primer used for substrate specific PCR was 5′-TGCCGTCGGTAGATGAAAG TGGC-3′ and that for product specific PCR (CR) was 5′- ACCACCCACTACATCGATAACGCC-3′. Sequence of the forward sense primer (CF) used was 5′-GTATCTCTCTACGCTTGCTGGTTGG-3′ and was same for both the reactions. The intensity of the respective amplicons on agarose gel was quantified by measuring the integrated density values (IDV). Excision frequency was calculated using the formula:

Table 1
Plasmids and *E. coli* strains used in the study.

Name	Features/genotype	Reference
Plasmids		
pAM461	Contains distal and proximal borders of *nifD* element, Amp^R	[8]
pMX25	*nifD* element cloned in pBR322, Amp^R, Kan^R	[5]
pET28a (+)	T7 based expression vector, Kan^R	Novagen
pNU1	*xisA* cloned in BamHI – SacI sites in pET28a (+), Kan^R	This study
***E. coli* strains**		
DH5α	*F′endA1hsdR17(r_k^- m_k^+)supE44 thi-1recA1gyrA96(nal^r) relA1(lacZYA-argF)U169deoR (⌀80dlacΔ (lacZ)M15*	[11]
BL21 DE3	*F$^-$ ompT gal dcm lon hsdS$_B$(r_B^- m_B^-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])*	[12]
BL21 DE3 PlysE	*F$^-$ ompT gal dcm lon hsdS$_B$(r_B^- m_B^-) λ(DE3) pLysS(cm^R)*	[13]
UN2	*E.coli* BL21DE3 PLysE (pNU1+pAM461)	This study
NA1	*E. coli* BL21 (DE3) (pNU1)	This study

$$\text{Excision}(\%) = (\text{IDV}_{\text{Product}}) \times 100/\text{IDV}_{\text{Substrate}} + \text{IDV}_{\text{Product}}$$

2.7. Purification of XisA protein and identification by MALDI-TOF

IPTG induced NA1 cells were sonicated in 10 ml of lysis buffer (20 mM Na-phosphate, 0.5 M NaCl, 20 mM Imidazole). Cell debris were removed by centrifugation at 12,000 g for 10 min at 4 °C. Histidine-tagged protein was purified from the culture free supernatant by Nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography using AKTA Prime system (GE Healthcare Life Sciences, Buckinghamshire, UK). Binding of protein to the column was carried out in lysis buffer. Elution was carried out using 0.5 M Imidazole in lysis buffer. Eluted fractions were analysed by SDS PAGE. Purified XisA protein band was excised from SDS PAGE gel and the gel piece was analysed by tryptic digestion and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI/TOF MS) at Molecular Biophysics Unit, Indian Institute of Science, Banglore. Peptide masses generated from the MALDI/TOF MS analysis were used for protein identification by peptide mass fingerprinting (PMF) using the search program MASCOT (Matrix Science). Trypsin was given as the digestion enzyme, one missed cleavage site was allowed. All peptide mass values were set monoisotopic and the mass tolerance was set at 100 ppm.

2.8. Theoretical modelling of XisA protein

Secondary structure prediction and 3D modelling of XisA protein was performed using online bioinformatics tools. To predict the secondary structure of XisA, the amino acid sequence was submitted to PSIPERD tool [15,16]. The domain architecture of XisA was analysed by submitting *xisA* gene nucleotide sequence at Conserved Domain Database tool of National Centre for Biotechnology Information (NCBI) [17]. The amino acid sequence of XisA was submitted to I-TASSER server to determine 3D structure [18–20]. The output of I-TASSER was analysed using Pymol program [21]. Confidence score (C-score) and Template Modelling score (TM-score) are the parameters to rank the quality of the modelled structure. A C-score value typically rages from −0.2 to 5 and model with higher C-score describes model with higher confidence. A model with TM score >0.5 is considered to show significant topology with template homolog. XisA protein model with highest C-score and TM-score was selected for structural analysis.

3. Results

3.1. Purification and confirmation of XisA protein

E. coli JM101 (pMX25, pMC71A) did not show high *xisA* expression for the purpose of purification. Hence, *xisA* was cloned in pET28a to yield pNU1 plasmid where *xisA* is present under strong inducible P_{T7} promoter and the expressed protein would have N-terminal histidine tag. Fig. 1A is the schematic representation of *xisA* gene cloned in pET 28a and positions of major restriction enzyme sites. As seen in Fig. 1B (**lane 3**), strong expression of *xisA* gene was achieved using pNU1 plasmid and 52 kDa XisA protein

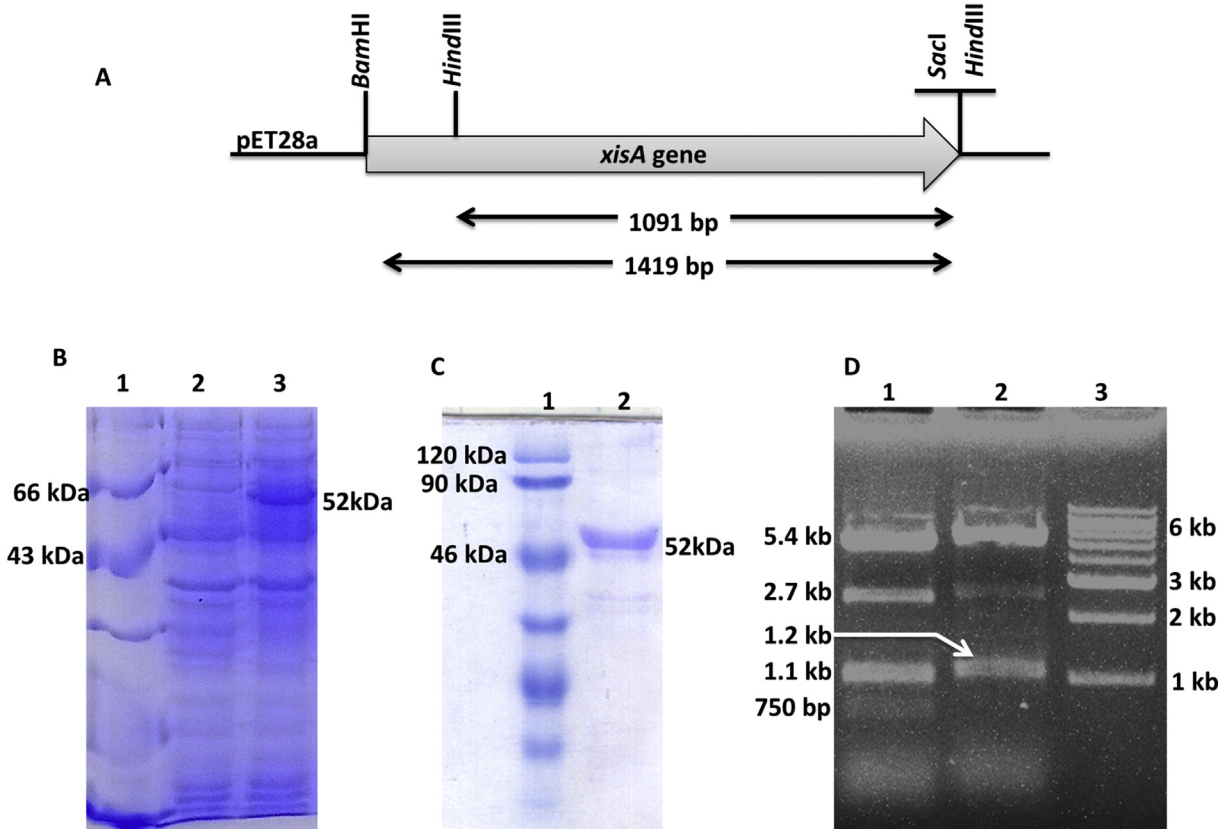

Fig. 1. Overexpression, purification and functional validation of XisA expressed using *E. coli* strain NA1 overexpression system. (A) Schematic representation of *xisA* gene expression cassette of pNU1 with recognition sites for major restriction endonucleases. (B) SDS PAGE gel representing overexpression XisA. Lane 1: Protein molecular weight marker; Lane 2 represents crude cell free extract of uninduced *E. coli* strain NA1; Lane 3 displays overexpressed 52 kDa XisA protein after 3 h of induction with 0.8 mM IPTG. (C) SDS gel picture displaying purification of XisA protein. Lane 1: protein Molecular weight marker; Lane 2: purified 52 kDa XisA protein facilitated through Ni-NTA affinity chromatography purification. (D) Agarose gel showing *Eco*R1 and *Hind*III digestion patterns for validation of XisA functionality. Lane 1: plasmid from *E. coli* strain NA1 (pAM461); Lane 2: plasmid from *E. coli* BL21 (DE3) (pET28a, pAM461); Lane 3: 1 kb DNA ladder. 715 bp band found exclusively in Lane 1 indicates rearrangement of pAM461.

was detected after 3 h of induction with 0.8 mM IPTG in NA1 grown on LB medium as analysed by SDS PAGE. Ni-NTA affinity chromatography with AKTA prime FPLC system allowed one step purification of the His-tagged 52 kDa XisA protein (Fig. 1C, **lane 2**). In order to determine functionality of XisA protein expressed from pNU1, *E. coli* strain NA1 was co-transformed with pAM461 substrate plasmid as described in methods. Rearrangement of pAM461 was detected by 715 bp fragment released upon *Hind*III and *Eco*RI double digestion of plasmid mixture isolated from NA1 (pAM461) (Fig. 1D, **lane 1**) which was not seen in *E. coli* BL21 (DE3) (pET28a, pAM461) which lacked *xisA* (Fig. 1D, **lane 2**). It was found that leaky expression of *xisA* gene from pNU1 was sufficient to carryout complete rearrangement of pAM461. Purified band was confirmed to be XisA by Protein Mass Finger Printing facilitated by MALDI TOF mass spectrometry. Analysis of m/z values analysis by MASCOT showed a match score of 84 and a query coverage of 36% with XisA protein corresponding to the genome sequence of *Anabaena* PCC 7120 *xisA* gene product (Fig. S1) (Fig. 2).

3.2. PCR based monitoring of excision

A PCR based method was developed to monitor excision frequency of XisA substrate plasmid (pAM461) using substrate and product specific primers (Fig. 2A). Near 100% excision was observed in *E. coli* UN2 strain within 1 h at 37 °C on LB under static condition (Fig. 2B and C).

3.3. XisA structure prediction

Secondary structure prediction by PSIPERD tool revealed *Anabaena* PCC 7120 XisA protein is predominantly α-helical in nature. Active site residues (R287, Y381, R384 and Y416) are located in or near close proximity to α-helices (Fig. S2). Conserved Domain Database analysis of XisA suggested that the region of 268–416 residues bears sequence homology to Integrase region of tyrosine recombinase family (Fig. S3). Additionally, the region of 435–472 showed homology with basic region of leucine zipper family (Fig. S2 and 3A). 3D modelling of XisA protein was performed by I-Tasser tool while model displaying a C-score value of 0.17 and a TM-score of 0.838 was chosen for structural and ligand binding analysis in Pymol (Fig. 3B). Closest structural homolog of XisA was found to be *Pyrococcus abyssi* Xer recombinase, which is also a member of tyrosine recombinase family. As seen in the XisA-DNA complex model, the active site residues are present in near vicinity of DNA (Fig. 3B **inset**).

4. Discussion

XisA protein is essential for excision of *nifD* element in heterocyst during nitrogen starvation condition to reconstitute functional *nifD* gene in *Anabaena* PCC 7120 [8]. Sequence homology studies have revealed that XisA protein belongs to the class of the lambda Integrase family of recombinases which is characterized by presence of RHRY tetrad as conserved active site residues situated near the carboxyl terminal of the protein [6]. Functional characterization of XisA has been largely carried out using heterologous model

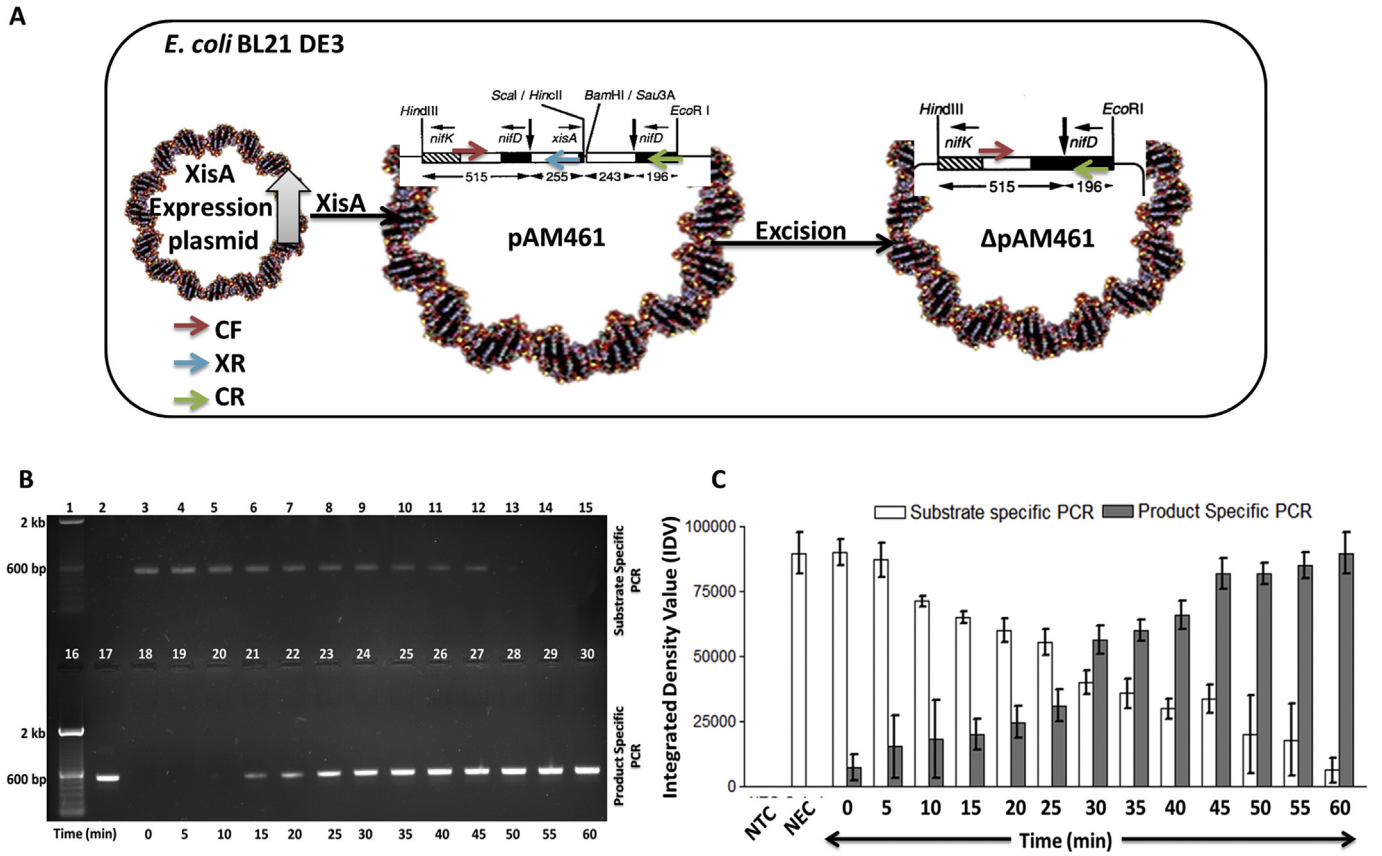

Fig. 2. PCR based monitoring of excision. (A) Strategy for PCR based *in vivo* monitoring of excision. (B) Agarose gel profile of substrate (using primers CF and XR) and product (using primers CF and CR) specific PCR amplicons obtained at different time points. Lane1 and Lane 16: 100 bp ladder; Lane 2: No template control (NTC); Lane 3–15: substrate specific PCR; Lane 17: No enzyme control (NEC); Lane 18–30: product specific PCR. (C) Quantification of substrate and product specific amplicons by measuring IDV. Values are expressed as Mean ± SEM (N = 3).

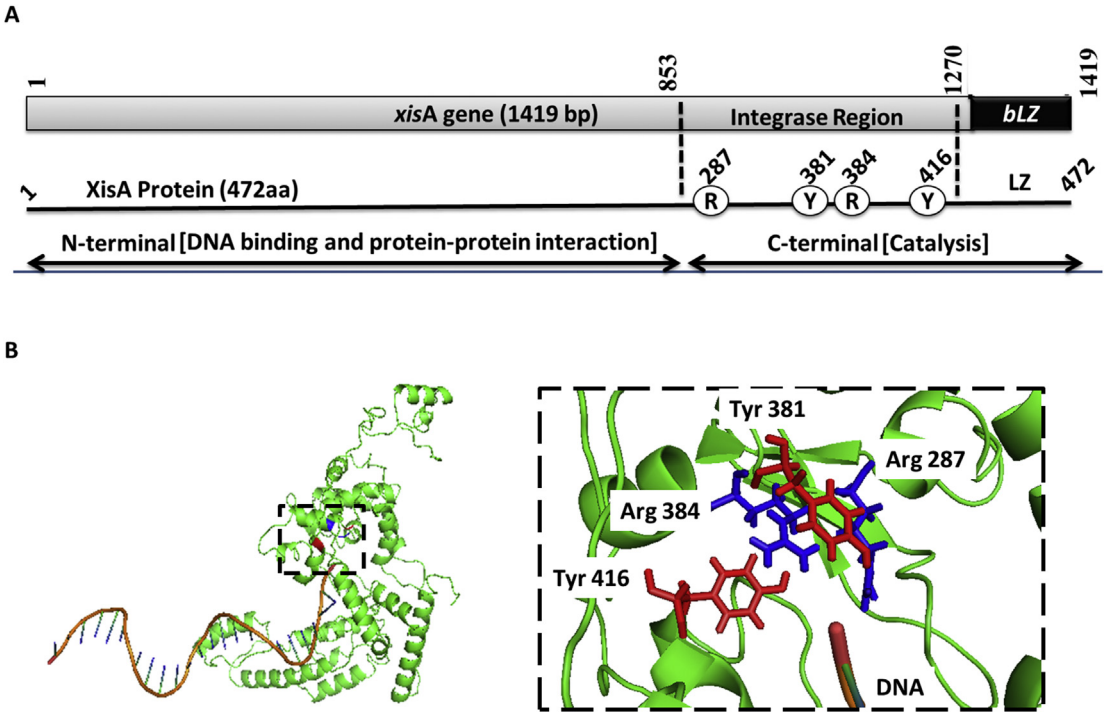

Fig. 3. Theoretical modelling of XisA protein. (A) Schematic representation of the protein and open reading frame of XisA. (B) Theoretical modelling of XisA protein bound to DNA using I-Tasser tool. Dotted box represents XisA active site residues.

system of *E. coli* wherein *in vivo* XisA activity has been demonstrated by monitoring plasmid rearrangements [5,8,9,22]. Several unique properties of XisA were understood using *E. coli* model, such as its target site preference, its endonuclease activity and its promoter region identification [5,23]. Presence of endonuclaese activity of XisA (Shah et al., 2007) suggests that this protein could be an evolutionary intermediate between site-specific recombinases and endonucleases. In spite of these interesting features, XisA protein has not been purified so far neither it is detected on protein gels and hence it's *in vitro* characterization has been elusive. The present study was aimed at understanding the structural architecture of XisA protein and to report first purification and identification of XisA protein. Site specific recombinases have a stringent requirement of accessory factors to carryout recombination. However, none of the accessory proteins have been identified for XisA, which hindered *in vitro* characterization of XisA protein. To overcome this problem, a sensitive and accurate PCR based method was developed to monitor the recombination frequency of XisA in *E. coli.*

Expression of *xisA* gene under native promoter seems to be highly restrictive owing to opposite orientation of putative promoters P1 and P2 (Lammers et al. 1986). This limitation was addressed by expressing *xisA* gene under strong P_{T7} promoter (pNU1). Overexpression of *xisA* gene was observed at 3 h after induction with IPTG. Expressed XisA protein was His-tagged that facilitated one step purification by Ni-NTA affinity chromatography which was confirmed by MALDI-TOF mass spectrometry. That the overexpressed protein in *E. coli* NA1 was functionally active was shown by rearrangement of a mini substrate plasmid pAM461 [8] by obtaining the restriction enzyme digestion pattern of the rearranged plasmid as reported by Brusca et al. (1990). Recombination efficiency of XisA was first monitored *in vivo* in *E. coli* by cloning entire the *nifD* element in pBR322 to generate pMX25 plasmid (Lammers et al., 1986). Assay for recombination was based on transformation assay and plating assay, which consist of screening blue-white colony formation in which blue colonies possess pMX25 plasmid while excised plasmid generated white colony. Since pMX25 is a medium copy plasmid, this method may not precisely estimate excision events, as the number of excised plasmids required for white colony phenotype is unknown. We report here a convenient PCR based assay to estimate excision events accurately. This method was used to demonstrate the functionality of XisA protein *in vivo*.

Sequence analysis and modelling studies revealed 3D geometry of XisA is identical to *Pyrococcus abyssi* Xer recombinase, which is also a member of tyrosine recombinase family and bears predominantly α-helical structure. Position of active site residues near DNA ligand is ideal for recombination reaction. Involvement of terminal tyrosine (Tyr416) appears to participate in the formation of phosphor-tyrosine intermediate, a conserved mechanism for the members of Tyrosine recombinase family. Presence of predicted basic region of leucine zipper (bZIP) at extreme C-terminal region of XisA suggests it could play an important role in the XisA dimer formation whereas N-terminal is known to be associated with DNA binding and dimer–dimer interaction for other members of tyrosine recombinase family.

In conclusion, the purified XisA protein can facilitate elucidation of XisA protein structure, which is critical in understanding the excision of *Anabaena* PCC *nifD* element and may also be helpful in unravelling its relation to other recombinases and endonucleases.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pep.2015.09.027.

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