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Isolation of plasmid from a Gram negative bacteria *Aeromonas dhakensis* strain F2S2-1 and its partial characterization

Authors Info

M. Nadiga[1,2] V.V. Vaidyanathan[1] and T. Thayumanavan[2]*

[1]Syngene International Ltd Bangalore, India

[2]Department of Biotechnology, Dr. G. R Damodaran College of Science, Civil Aerodrome Post, Coimbatore – 641014, India

*Corresponding Author Email : thayumanavan@yahoo.com

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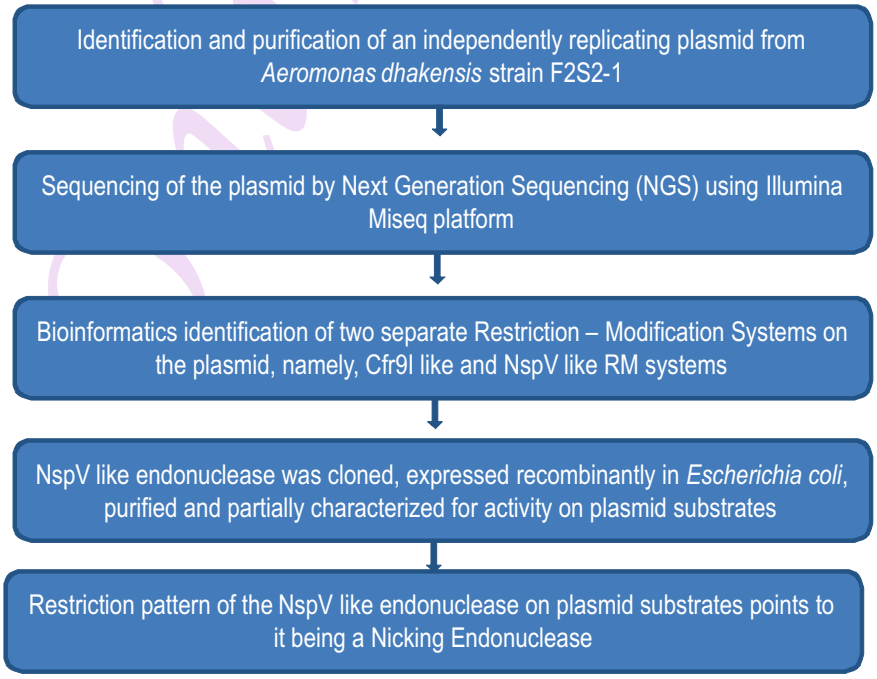
Abstract

Aim: A plasmid was isolated from *Aeromonas dhakensis* strain F2S2-1 to understand their function in the host. The main objective of the study was to isolate and sequence the plasmid, predict genes and proteins encoded and to experimentally prove their biochemical function.

Methodology: The *Aeromonas dhakensis* strain F2S2-1 plasmid was sequenced by Next Generation Sequencing (NGS) on Illumina Miseq platform. Bioinformatics analysis was carried out to predict the putative gene and encoded protein functions. The NspV like endonuclease was recombinantly expressed in *Escherichia coli*, partially purified and characterized for activity on plasmid substrates.

Results: Plasmid sequencing and bioinformatics analysis predicted the presence of NspV like endonuclease.Recombinant expression, purification and characterization of NspV like endonuclease revealed the Nicking endonuclease activity of the protein with plasmid substrates.

Interpretation: The NspV like endonuclease described in this study has DNA nicking activity. this study would help in understanding better survival of *Aeromonas* sp. in its ecosystem and the role of host plamids.



Introduction

Aeromonas species are Gram negative, predominantly motile bacteria distributed in aquatic ecosystems across the world. They are also found in varied physical and biological eco systems like aquatic, fish, food, domesticated pets, birds, ticks, insects and to lesser extent natural soils. The species is dominated by *Aeromonas hydrophila, Aeromonas salmonicida* and to a lesser extent by *Aeromonas caviae, Aeromonas sobria, Aeromonas tecta, Aeromonas aquorium, Aeromonas bivalvium, Aeromonas sharmana, Aeromonas simiae* etc (Janda and Abbott, 2010). *Aeromonas* sp. cause fin rot and tail rot in fish, red leg disease in frogs and wound infection in animals. In humans, *Aeromonas* sp. cause gastroenteritis in children and immune-compromised patients and wound / soft tissue infections (Janda and Abbott, 1996). Hence it is important to study this organism to understand its biology.

Nearly, 3500 Restriction-modification systems are reported in literature till date. These are classified into Type I, Type II, Type III, Type IV systems depending on the protein structure and function (Roberts *et al*., 2003). Advantage of R-M systems to the bacterial / viral host is less clear but they are described as selfish genetic elements (Kobayashi, 2001). Their presence is known to decrease the viral replication efficiencies in the bacterial host (Pigoud and Jeltsch, 1997; Luria and Human, 1952) and function as defense for bacterial host against bacteriophage infections. Pre-dominant class of Type II restriction enzymes recognize and cut within a typically a 6-bp recognition site producing either blunt ends or sticky, overhanging ends. Type II restriction enzymes are widely used in Biotechnology industry for cloning and DNA modification / engineering.

Nicking endonucleases (NEases) are similar to restriction endonucleases but cleave only one strand of the DNA, where as Type II restriction endonucleases cleave both strands (Higgins *et al*., 2001). NEases invariably recognize non-palindromes for their mode of action. NEases can be frequent cutters or rare cutters like homing endonucleases (HEases) (Chan *et al*., 2011). NEases have wide applications like isothermal DNA amplification, cloning of DNA, studying DNA repair etc. (Zheleznaya *et al*., 2009). Reported here is a study on the isolation and sequencing of a plasmid pAH-F2S2-1 from *Aeromonas dhakensis* strain F2S2-1, the cloning - expression and partial purification of NspV like endonuclease and characterization of its Nickase like function.

Materials and Methods

Isolation of strain and plasmids : *Aeromonas dhakensis* strain F2S2-1 was isolated from skin surface of Indian oil sardine *(Sardinella longiceps)* by *Aeromonas selection* media (Himedia). The strain was observed to host a plasmid during agarose gel analysis and was purified by plasmid mini prep kit (Qiagen). All the plasmids used in the present study were purified using plasmid mini prep kit (Qiagen)

Sequencing of the plasmid pAH-F2S2-1 and prediction of ORFs : The plasmid was sequenced by Next Generation Sequencing (NGS) on Miseq platform (Illumina) with 2 X 250 bp paired end reads. A total of 233424 reads were obtained, which were assembled by CLC Genomics workbench (Ver. 8.5). The final sequencing to fill the gaps in DNA sequence of the plasmid was carried out by Sanger sequencing on ABI3730XL (ABI). The sequenced plasmid was finally determined to be 10483 bp in length. The ORF prediction was carried out by VNTI (Ver.11), as well as PlasMapper (Ver. 2.0) (Xiaoli *et al*., 2004).

Cloning of NspV like RM genes : The NspV like methylase was cloned first into NdeI and KpnI sites of pACYC-Duet vector using the primers NspV-like-M-NdeI-FP - 5' TAA GAA GGA GAT ATA CAT ATG AGT AAA GAG AAA ATT GAG 3' and NspV-like-M-KpnI-RP 5' TTT ACC AGA CTC GAG GGT ACC TCA CTA TTA TGC CGT CCA ATC AAT AGA ATT C 3'. After cloning methylase, the NspV like endonuclease was cloned into BamHI and NotI sites of the same vector with the primers NspV-like-E-BamHI-FP 5' CAT CAT CAC AGC CAG GAT CCC ATG GTT TTA ACT CTG ATG 3'and NspV-like-E-NotI-RP – 5' GTT CGA CTT AAG CAT TAT GCG GCC GCC TAT CAT TAT CAT TAT AAA AAG GCA GCC CGA TA 3'. All the cloning work was carried out in *E. coli* host DH5alpha.

Expression and purification of NspV like endonuclease : The plasmid pACYC-Duet1 with NspV like methylase and endonuclease were transformed into E. coli BL21(DE3) electro-competent cells and the protein was induced with IPTG (Sigma). The induced protein was purified with batch processing by Ni-NTA resin (GE health care) affinity purification according to manufacturer's instruction. The purified protein was dialyzed against 50mM Tris-HCl, pH 8.0 to remove the Imidazole and stored in -80^0 for assays.

Protein staining and western blotting : The affinity purified protein was resolved on 4-12% Bis-Tris gel (Thermofisher) with 1X MES running buffer. The protein was stained with simply blue stain (Thermofisher). For western blot, the protein was transferred to nitrocellulose membrane (PALL life sciences) and developed with 1:1000 penta His mouse primary antibody (Qiagen) and 1:2000 goat anti-mouse secondary antibody (Santa Cruz Biotechnologies). The blot was developed with ECL chemi luminescence kit (GE health care) with Bio rad imaging system.

Endonuclease assays : Approximately, 1ug of affinity purified endonuclease was incubated with 2 ug of test DNA in 1X cut smart buffer (NEB-50mM potassium acetate, 20mM tris-acetate, 10mM magnesium acetate, 100ug^{-1}ml BSA, pH 7.9) for 2 hrs at 37 degree and the restriction profile was checked on 1% TAE agarose gel. The lambda DNA and phi x 174 circular DNA used in the assay were procured from Clontech, USA.

Results and Discussion

The sequencing of plasmid pAHF2S2-1 resulted in a final, circular plasmid of 10483 bp (Genbank Accession - KX863708 and KX863709). The annotation of the plasmid

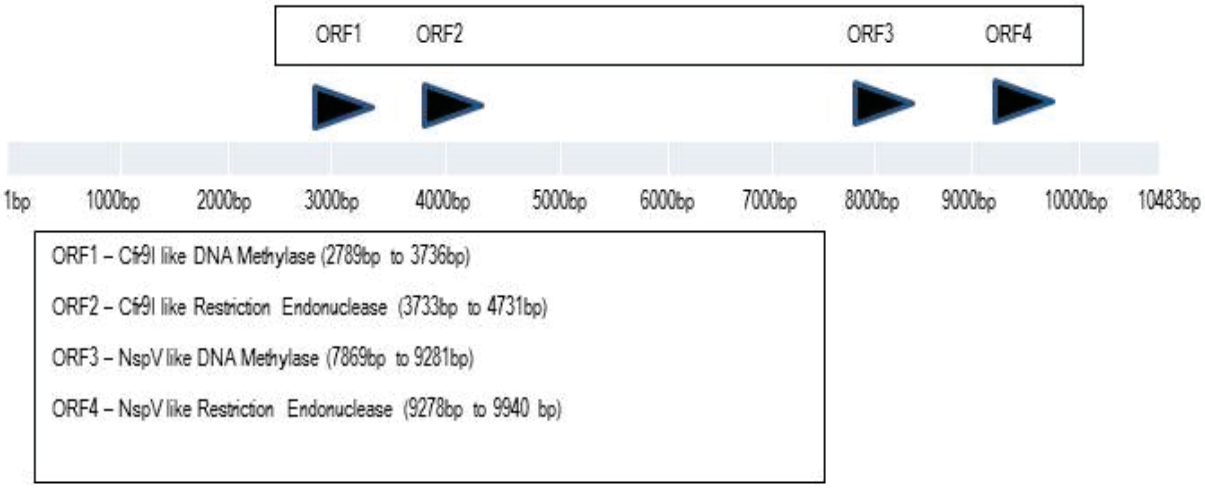


Fig.1 : Layout of the plasmid isolated from *Aeromonas dhakensis* strain F2S2-1 and the ORF annolation

A. Nucleotide sequence of NspV like DNA Methylase

ATGCCGTCCAATCAATAGAATTCAATATTGTTGCTTTAATCGGTCTTTTTTCATCCCAGAAAATCATTGATGATATAGCTCCCAAAACCGGTTTGGATTGAAGCTTATCAAGCGCATCCAAAGCATCCTGCTC
AAGATCAAAAGAGAGGAAATATACCGTATCATCAAAAACAACAGGTTTGTTATCAATTGGTTCAACAAGGGTAAAGGATAGCTTTTTATATAACCCGCAAATAGCTATTTTCCATGGTTTGAATGTATATTCAC
CGACCCCAAAAACTGAAAACCTAGGGTTCTTCTTATATATAACGCTCTTTCTTCCATCAAGCTTGTCAGCATGAGATATTAAATAGCGCCACGCTTTAGGGTATTTAGTTTCAATGTATGATGTATCTTCACC
AACACGTTCTTGCGTGACTATAACAAATCTATTATCCCACCCTTTCCCACTACCCACTCGAGAGCCTTTCAATAAAGGGTAAACTACCTCAACCTCAATATCTACAACATCACCATAACCGTTAACCAGCACA
CCATCATGTTGTTTTAGCTCCATAACAGATGATGCGTCGTGCTTCATTCCTGACCTCCATTTTTTTGGGGGGGCTGAAACAAATCGACTTGATAACTCAAATGTATCTATATCACTTACAAGAAACCCTTTCC
TATGACCACACAACCGCCCAGTCTTATCACTGAAAGATTCAAAAACTTTATAGTCATACGATGGCTTAACCTGTGAATCTATTCGCATTATAAAAAAGCAAGCATCCACAGATGCGCCAAACTCTTTTCTTGC
ATCAACGGTTATTATCTCTGAGTAATAACATCCTATTTTATGCTTCTCAACATAAGAAAGTATTTTCCTTGCCACTGCTGTCTTAAGCATGATTGCTATATCAATTTTTTTTCCTGGCGACCATTTGAGCATATC
AATCATCATCCACTCTGATATGTCAAAATTTGCCTTACCAGTTATCGCGTCCAACCCGCCAAGACCAACCACATTGTCTTTTTCTGGAACGTTATAACTATCAATTTTACTCTGGACGGTGTTGGTCACCCAT
GGCAAGTTCCCCAAAACAAGCAGATTACCATCCACATCAGACGACTTACCTGCCCAGTCATAACGAAAAAAGTCACCATGCTCGATAGATACTACATTTTGGCGAGGGTGACCACGCAACGCCCCCTCAA
GCACATCGAGATAATCACTGTTAATGTCAATACCATAAACTTTCTTCGTGGATGAGAAGTAATTCACTGATGCAATAACAAATGCCCCAACACCACAAGTTGGCTCAATAATAACATCAGGAGAAACCCCTA
AAGTAACCAAACGCTCACAGGCTATCTCTGCTAATTCTAGCGGCGTTTGGAAATCACCAAACTCAATTTTCTCTTTACTCATAA

B. Amino acid sequence of NspV like DNA Methylase

MSKEKIEFGDFQTPLELAEIACERLVTLGVSPDVIIEPTCGVGAFVIASVNYFSSTKKVYGIDINSDYLDVLEGALRGHPRQNVVSIEHGDFFRYDWAGKSSDVDGNLLVLGNLPWVTNTVQSKIDSYNVPEKDNVVG
LGGLDAITGKANFDISEWMMIDMLKWSPGKKIDIAIMLKTAVARKILSYVEKHKIGCYYSEIITVDARKEFGASVDACFFIMRIDSQVKPSYDYKVFESFSDKTGRLCGHRKGFLVSDIDTFELSSRFVSAPPKKWRSGM
KHDASSVMELKQHDGVLVNGYGDVVDIEVEVVYPLLKGSRVGSGKGWDNRFVIVTQERVGEDTSYIETKYPKAWRYLISHADKLDGRKSVIYKKNPRFSVFGVGEYTFKPWKIAICGLYKKLSFTLVEPIDNKPVVF
DDTVYFLSFDLEQDALDALDKLQSKPVLGAISSMIFWDEKRPIKATILNSIDWTA

C. Nucleotide sequence of NspV like endonuclease

ATGGTTTTAACTCTGATGAATTTATGTCGTGAGGCGCGGGACTTTTCTCGTCAGGAATCTGTTCATAATGAACCGACATTATTTGGTGTTACGGATGGAAAGGCAGTTGGAACATATTTAGAGCAGAAGTTT
AGAAATTACCTTTTAGCGAGGTATCAGTTTGAGGTTGGTAATTCAGCAAGAGGGATTGACTTTCCTAGTTTAAATGTTGACATGAAAGTTACAAGTGCGAGGCAGCCTCAGTCTTCATGCCCGTTCAGATCT
GCCAGACAAAAGGTGTATGGGTTGGGGTATGGTCTTATAATTTTTGTCTATGATAAATCAGATTCCCCTGAAAATAGAACGGCGAGATTGAATATATCTAGCACCATATATGTCGAACCGAATAAAACAGCT
GACTACCAGCTGACATCTAGTATTGCTCAGGTACTTGATAATAATGGTTCAGAAGCAGATCTTATTGCGTTATTTATGGATAAAAACCTTCCTGTTGATGATATAGGTGCTAGGGATTTAGCAAGGGAAATAT
TGAGAGATAGGCCTCAAATAGGCTGCCTTACAATATCAAATGCTTTGCAATGGCGACTTCAGTATACAAGGGCTATTGAGTGGGCTGGTAATTATGATGGTGTTCACGCTATTTATCGGGCTGCCTTTTTAT
AATGATAG

D. Amino acid sequence of NspV like endonuclease

MVLTLMNLCREARDFSRQESVHNEPTLFGVTDGKAVGTYLEQKFRNYLLARYQFEVGNSARGIDFPSLNVDMKVTSARQPQSSCPFRSARQKVYGLGYGLIIFVYDKSDSPENRTARLNISSTIYVEPNKTADYQL
TSSIAQVLDNNGSEADLIALFMDKNLPVDDIGARDLAREILRDRPQIGCLTISNALQWRLQYTRAIEWAGNYDGVHAIYRAAFL

Fig.2 : Nucleotide and amino acid sequence of putative NspV like methylase and endonuclease from *Aeromonas dhakensis* strain F2S2-1 plasmid

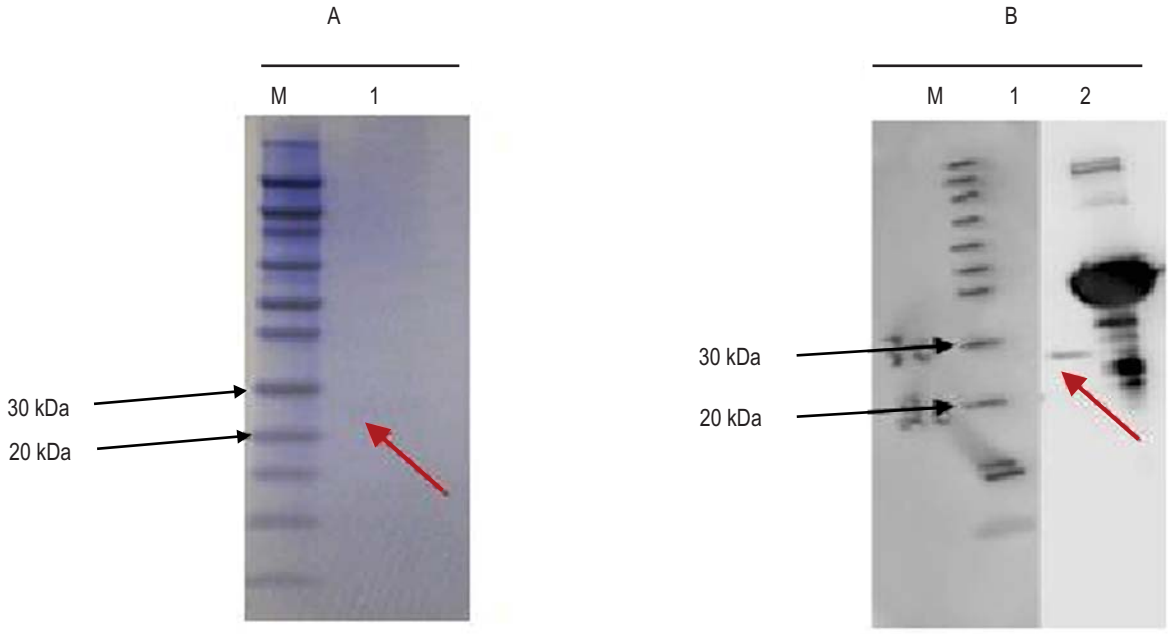


Fig.3 : Western blot of purifed NspV like endonucleas showing as expression in Ecoil lysare (lane M-) Themofsher Novex Protein marker (35, 10, 15, 20, 30, 40, 50, 60, 80, 110, 160, 260, kDa) .
Lane 1 - Restriction endonuclease expressed (predicted protein MW - 24 845 kda, shown by red arrow)
Lane 2 - His tag positive control protein A. Protein staining B. Anti His western blot

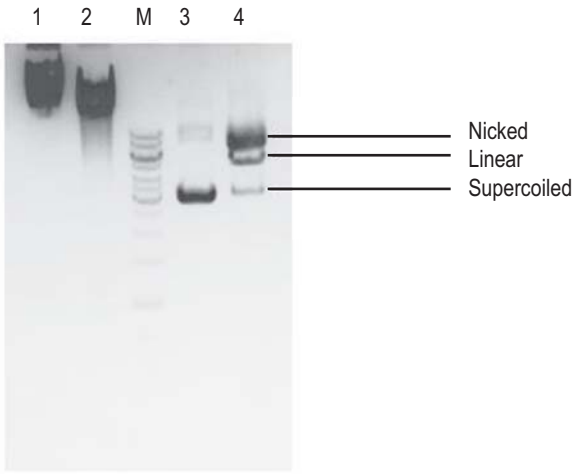


Fig.4 : Restriction profile of lamda DNA and phi x 174 DNA cut with NspV like endonuclease.
Lane 1 - Uncut lamda DNA Lane 2- Lamda DNA cut with NspV like endonuclease Lane M-Fermentas DNA ladder (0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, 10 kb)
Lane 3- Uncut phi x 174 DNA Lane 4-phi x 174 DNA cut with NspV like endonuclease

revealed the presence of two Restriction-Modification systems similar to Cfr9I and NspV type II restriction enzymes. The Cfr9I like DNA methylase was 1048 bp long and the corresponding endonuclease was 999bp. NspV like DNA methylase was 1413bp long and its endonuclease was 663 bp long. The structure of RM systems are given in Fig.1. and the nucleotide and amino acid sequence of NspV like R-M system is shown in Fig.2.

NspV like endonuclease expressed and purified from *E. coli* was confirmed for protein expression by protein staining and anti his Western Blot is shown in Fig.3.

Sequencing and annotation of the plasmid pAH-F2S2-1 from *Aeromonas dhakensis* revealed the presence of two R-M systems. The two R-M systems were Cfr9I like and NspV like respectively. NspV like DNA methylase revealed the presence of conserved motif PDXnEPT (where, n= 1 to 30 residues) present in many Type II restriction enzymes with the consensus motif being PDXn(D/E)XK (Pigoud and Jeltsch, 1997). The NspV like DNA endonuclease revealed the presence of conserved motif DXnEQK present in some of the Type I restriction endonucleases (Murray, 2000; Davies *et al.*, 1999). The secondary structure prediction with Jpred 4 (Drozdetskiy *et al.*, 2015) revealed that it was structurally close to NspV enzyme.

DNA cleavage of NspV like endonuclease from *Aeromonas dhakensis* revealed that it digests lambda DNA and Phi X 174 DNA. The cleavage of lambda DNA was less efficient than Phi X 174 (Fig. 4.). The NspV like endonuclease cleaves the Phi X 174 DNA, pACYC DUET and pTZ57R resulting in predominantly a nicked form (Fig. 4, 5, 6) with formation of a small quantity of linear form. The NspV like restriction endonuclease the present study failed to digest a fragment containing the NspV recognition site TTCGAA (unpublished results).

Fig.5 : Restriction profile of phi x 174 circular DNA incubated with NspV like endonuclease.
Lane M - Fermentas DNA ladder (0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, 10 kb) Lane 1-Uncut phi x 174 DNA, lane 2 - phi x 174 DNA cut with NspV like endonuclease

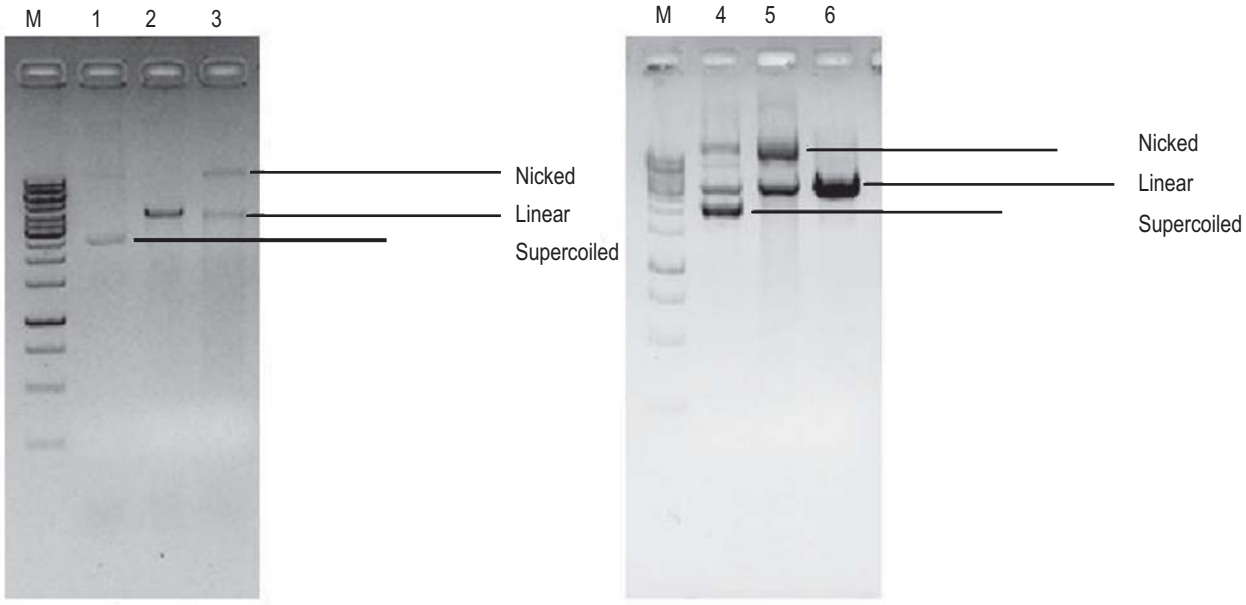


Fig.6 : pACYCDUET and pTZ57R plamid cut with NspV like Restriction Enzyme.
Lane M - Fermentas DNA MW marker (0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, 10 kb) Lane 1-Uncut pACYCDUET-1 plasmid
Lane 2-pACYCDUET-1vector cut with Sac I Lane 3- pACYC-DUET-1 vector cut with NspV like Restriction enzyme Lane 4-Uncut pTZ57R plasmid
Lane 5 - pTZ57R plasmid cut with NspV like Restriction Enzyme Lane 6 - pTZ57R cut with EcoRI

Nicking endonucleases recognize specific sequences and create nicks in DNA (Higgins *et al*., 2001; Xu *et al.*, 2001). The endonuclease characterized in this study, resulting in a mixture of nicked plasmid (major product observed) and linear plasmid , may be a nicking endonuclease. Double digest of *Aeromonas* NspV like endonuclease and Xho I or Sac II resulted in a single, linear form of plasmid DNA (Fig. 5) with nicked form dis-appearing completely. Similar nicking endonucleases profiles have been observed in other studies resulting in a mixture of nicked plasmid (major form) and linear plasmid as seen with our enzyme (Lee *at al.,* 2015; Somyoonsap *et al*., 2013; Higgins *et al.*, 2001). Incubation of various concentrations of *Helicobacter pylori* HP0268 nuclease, characterized to have nickase activity, with plamsids like pET15b(+), pET-21a(+) and pET28a(+) resulted in open circular (nicked) and linear forms of DNA (Lee et al., 2015). Similarly, incubation of *Streptomyces* DC13 nuclease or nickase, with plasmids pBluescript, pUC18, pET15b, pET26b resulted in accumulation of predominantly Open Circular form and low levels of linear form (Somyoonsap *et al.,* 2013). Another nicking endonuclease, N. BstBI was shown to produce major, open circular form and minor, linear form upon its activity on plasmid substrate (Higgins *et al.*, 2001). The NspV like endonuclease, in this study, on incubation with circular DNA like Phi X 174, pACYC DUET and pTZ57R showed a predominant, open circular form and minor, linear form indicating that it is a nickase.

The conserved domain and amino acid sequence data suggests that it is similar to restriction endonuclease and has been reviewed extensively, nicking enzymes are similar to restriction endonucleases (Zheleznaya *et al.*, 2009; Higgins *et al*., 2001; Xu *et al*., 2001). Nickases Nb.BsrD1 and Nb.BtsI have been enginnered from their restriction enzymes, BsrD1 and BtsI (Xu *et al*., 2007). Along similar lines, a nickase N.AlwI has been engineered from its restriction endonuclease, AlwI (Xu *et al*., 2001). The comparison of restriction pattern of restriction endonucleases PleI / MlyI and nickase, N. BstBI shows that they function in similar manner on plasmid substrates (Higgins et al., 2001). Most of the well characterized nicking endonucleases are larger in size (~38 or 56 or 70 kDa) while the NspV like endonuclease described in our study is smaller (~25kDa). The smaller sized nicking endonucleases could be of interest to the industry with novel applications involving better stability, increased specificity, easier access to site of action and immobilization. Further work needs to be carried out to characterize the cleavage site, the optimal cleavage conditions and potential applications.

Acknowledgements

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