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Research article: Construction of Mycobacterium tuberculosis recD $^{K476A-D269A}$ double mutant using overlap PCR method

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ABSTRACT: Mycobacterium tuberculosis is a pathogenic bacterium which infect humans. Researchers have tried to target mycobacteria employing various approach such as drug screening, designing novel treatment regime etc. Site directed mutagenesis of gene involved in important metabolic pathway and cellular function remains a promising approach to target pathogenic bacteria. One of the important pathways is homologous recombination pathway, which regulate the proper functioning of bacterial genome. HR proteins are also involved in DNA damage repair pathway crucial for genomic maintenance. In this study site directed mutagenesis of an important gene recD involved in DNA repair pathway was constructed using overlap PCR method. This technique allows construction of mutant with a high degree of reliability.

Keywords: Mycobacterium tuberculosis, homologous recombination, RecD, PCR, DNA repair, Drug

I. INTRODUCTION

Tuberculosis caused by the bacterium Mycobacterium tuberculosis is a pandemic disease which already infecting one third of the human population. It primarily infects lungs but can also infect bone, kidney, brain etc. It has the ability to remain dormant over a long period of time without causing any detectable symptoms. However, due to compromised immunity, it manifests itself and causes tuberculosis with symptoms such as high fever, loss of weight, extreme coughing etc. Once the infection is diagnosed, the standard threaten includes the drugs such as isoniazid, rifampin, pyrazinamide and ethambutol over a duration of 24 to 30 months depending upon the prognosis and response, age of the patient. worldwide efforts are made to contain and treat tuberculosis but had proved challenging due to various reasons, one of the main reasons being the emergence of multiple drug resistant bacteria immune to current line of drugs and treatment. One of the important reasons of emergence MDR bacteria is the incomplete drug treatment regime (DOTS), as often patients discontinue the treatment midway. This allows the bacteria to become resistant to a particular drug. As and when during secondary infection tuberculosis happens, it become difficult to treat dur to antibiotic resistance displayed by the bacterium. Tuberculosis become even more difficult to treat in patients with AIDS due to severe compromised immunity [1]

Homologous recombination pathway is a very important pathway as it maintains genomic integrity by regulating crucial genes. These genes are critical for the proper functioning of the bacteria by regulating various aspect such as DNA segregation during replication, regulation of DNA damage and repair pathway etc. Some of the HR genes such as recBCD, recA, ssb are extremely important for the proper functioning and survival of bacteria. Deletion or mutation of these genes greatly impact the survival of bacteria. In E.coli RecBCD also known as exonuclease V is a very important multi subunit complex protein.. Deletion of recBCD causes the HR efficiency of bacteria to fall below 99%. It is involved in multiple pathways such as HR and DNA damage repair pathway and boast an impressive of biochemical activities. RecBCD display DNA dependent ATPase, ATP dependent DNA unwinding activity, ATP dependent endo and exonuclease activities. It also plays an important role in loading of RecA protein onto ssDNA thus playing an important role in recombination pathway. RecBCD is also involved in protecting bacterial genome, as it is involved in rapidly degrading foreign invading DNA, due to its endo and exonuclease activities. RecBCD is also involved in the rescue of stalled replication forks, which is crucial for genomic integrity.[2]

One of the subunits of RecBCD hetero trimeric complex is RecD protein. It displays important biochemical activities such as ATP dependent 5’ to 3’ DNA unwinding and DNA dependent ATPase activity. RecD also regulate the nuclease activities of RecBCD complex. Genetic level studies have revealed that deletion of recD

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gene in E.coli make the cell hyper recombination implying that it has role in maintenance of HR pathway. Indeed, we have shown previously that MtRecD interact with RecA and inhibits MtRecA mediated strand exchange, thus abrogating the HR mediated DNA transfer. In our previous studies, we have reported RecD ^{K179A} mutant which is walker A motif mutant and exhibited attenuated ATPase activity as compared to native RecD (ref). However, RecD also had another very important motif. i.e motif II which is implicated in ATP and DNA binding.[3]

In this study, double mutant of recD gene was constructed using PCR overlap method, as this technique although involved multiple steps, but offers advantage of introducing site directed mutagenesis in long genes, which sometimes causes problem in other method as single primer extension mutagenesis method. Construction of double mutant allow the study of biochemical activities of RecD protein in greater detail and potentially help in understanding the intricacies of homologous recombination pathway and potentially help in improved drug targeting.

II. II EXPERIMENTATION

Multiple sequence alignment:

The protein sequence of E. coli RecD, Mycobacterium tuberculosis RecD and Mycobacterium leprae RecD was downloaded in FASTA format from Uniprot data base (https://www.uniprot.org/). Sequence alignment of was performed in Clustal Omega (https://www.ebi.ac.uk/jdispatcher/msa/clustalo) [10]and the results were analysed and interpreted using Jalview 2.11.3.3.

Site-directed Mutagenesis of M. tuberculosis recD gene

To construct a double mutant, a point mutation at residue in Asp 269 was introduced by site-directed mutagenesis in pMtRD^{K179A} construct (39). Two primers were designed specifically incorporating the point mutation (Table 1). In this method two separate PCRs were performed using primers: In first set of PCR gene specific primer forward primer and reverse mutant primer was used and in second set gene specific reverse primer and forward mutant primer was used.[4] After confirming the PCR product formation in both sets of reaction, the fragments were gel purified. final extension was performed using gene specific forward and reverse primer in a single PCR reaction. NdeI and Xho I restriction sites present in forward and reverse primer respectively are double digested with both the restriction enzymes and then directionally cloned into pET21a(+) expression vector. The resulting construct was named pMtRD$^{K179A, D269A}$

S.No.	Primer Name	Sequence of primer (5'-3')
1	*recD* FP	AAGGCTGCACATATGAAGCTCACC
2	*recD* RP	ATCCCCCTCGAGTCAGCCACACCC-
3	D269A FP	ATCGTGGTCGCCGAGACCTCG
4	D269A RP	CGAGGTCTCGGCGACCACGAT

Table.1 Sequence of primer used in this study

Colony PCR and double digestion

The construct was transformed into DH5α using PEG mediated transformation and colony were visualized on LB plate containing antibiotic (Amp and Chl). The resulting colonies were used for colony PCR for confirmation of positive clone. In this method replica plate method was used to create a duplicate of colony and then colonies were picked and transferred to PCR microfuge tub containing PCR mix. PCR cycle reaction was followed as previously reported. The resulting product was analysed on agarose gel electrophoresis and compared with standard DNA marker. The mutant clone was double digestion using NdeI and Xho I restriction enzymes. The mutant clone was incubated with restriction enzymes at 16°C overnight and then product analysed on agarose gel electrophoresis.

III. RESULTS:

Multiple Sequence alignment:

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Multiple sequence alignment of EcRecD, MtRecD and MsRecD amino acid sequence sequences revealed 37.99 and 37.2 % sequence identity. Interestingly, the multiple sequence alignment shows that the protein shares highly conserved motifs such as walker A and walker B motif along with other motifs spanning the entire length of the protein (Fig. 1).

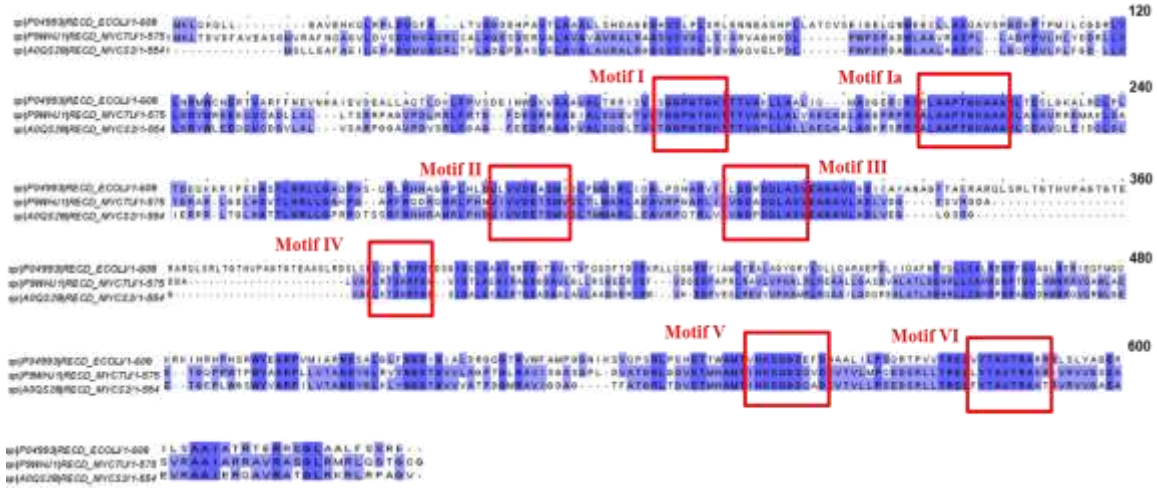

Figure 1. **Multiple Sequence alignment of RecD from different bacterial species**.

Amino acid in FASTA format were aligned using the Clustal Omega program and displayed by Jalview. Conserved motifs are enclosed in a box The details of the accession number of amino acid sequence used for the multiple sequence alignment are: P04993, E.coli RecD; P9WHJ1, Mycobacterium tuberculosis; A0QS28, Mycobacterium smegmatis

Amplification of PCR fragment:

PCR reaction condition was standardized for each fragment using variable conditions involving template DNA, annealing temperature and duration of PCR. However, addition of DMSO (5 to 15%) played a key role in PCR reaction (Fig 2.).

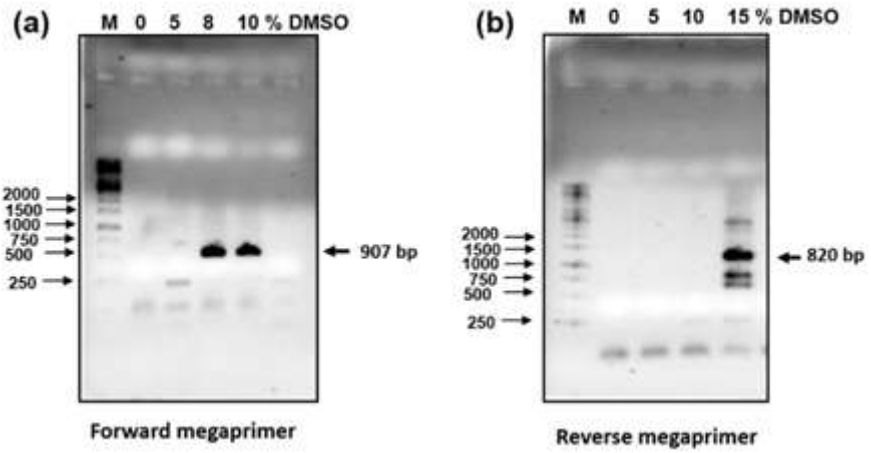

Fig. 2 **Effect of DMSO on the amplification of PCR products.** Increasing conc. of DMSO lead to the amplification of PCR products (a) forward megaprimer (b) Reverse megaprimer

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The products after gel purified were mixed and final full-length PCR was performed using gene specific primer. The product was confirmed using gel electrophoresis (fig 3)

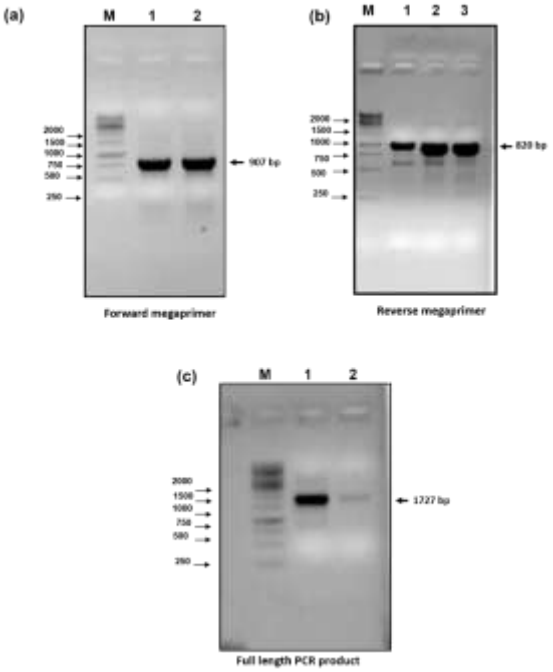

Fig 3: **Specific amplification of PCR fragments:** PCR reactions were performed for forward and reverse primer along with gene specific primer to yield the PCR fragments (a) Forward megaprimer (b) reverse megaprimer (c) Full length PCR product

Positive clone identification and confirmation

After transformation significant number of colonies appear on LB plates and it is very tedious to manually perform plasmid isolation even with kits. So, we have performed colony PCR to identify potential positive clones and simultaneously analyse large number of clones. Colony PCR results led to the identification of multiple positive clones (Fig; 4)

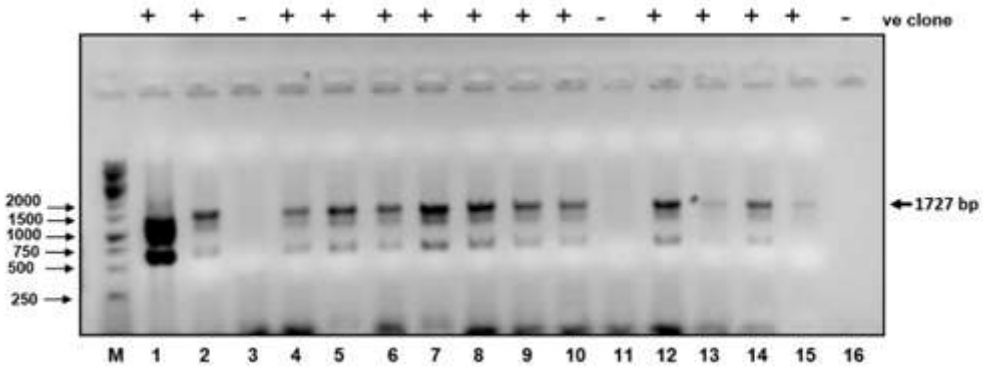

Fig 4: **Clone screening using colony PCR**: The colony PCR products were run on agarose gel to screen the putative positive clone

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Based on the result, the colony was picked and grown in LB media. The plasmid was isolated and double digestion with NdeI and XhoI restriction enzymes to confirm the clone (fig)

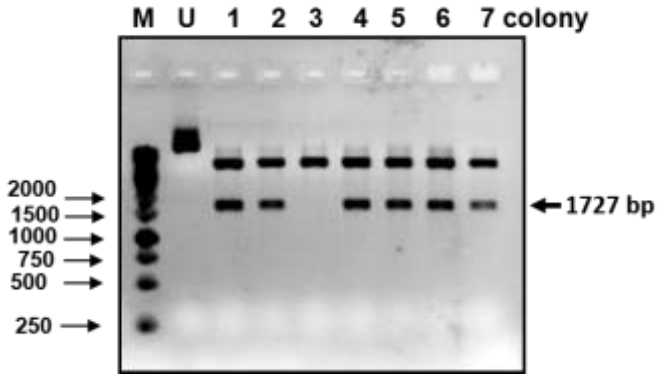

Fig 5: **Clone confirmation**: Putative positive clone was double digested using NdeI and XhoI and products analysed on agarose gel electrophoresis.

IV. DISCUSSION

In the present study, Mt recD gene double mutant using PCR overlap extension method was successfully created. Although his method involves multiple steps but it allows the incorporation of point mutation at a very precise location in the nucleotide sequence as compared to single primer extension method. PCR reaction and further analysis revealed the PCR product formation i.e fragment as well as full length gene product. Colony PCR and restriction enzyme double digestion has led to the confirmation of pMtRD$^{K179A,\ D269A}$ double mutant clone. Further studies will involve the transformation, purification and characterization of pMtRD$^{K179A,\ D269A}$ mutant

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