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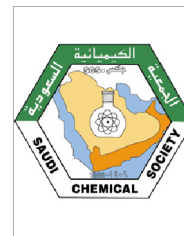
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## ORIGINAL ARTICLE

# Genetic characterisation of *Escherichia coli* RecN protein as a member of SMC family of proteins



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SMC;  
Tn10 excision;  
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Reverse genetic

**Abstract** The proteins of SMC family are characterised by having Walker A and B sites. The *Escherichia coli* RecN protein is a prokaryotic member of SMC family that involved in the induced excision of Tn10 and the repair of the DNA double strand breaks. In this work, the Walker A nucleotide binding site of the *E. coli* RecN protein was mutated by changing the highly conserved lysine residue 35 to the aspartic acid (D), designated as *recN*<sub>K35D</sub>. Reverse genetics was utilized to delete the entire *recN* gene ( $\Delta recN108$ ) or introduce the *recN*<sub>K35D</sub> gene into the *E. coli* chromosomal DNA. The *recN*<sub>K35D</sub> cells showed decreasing in the frequency of excision of Tn10 from *gal76::Tn10* after treatment with mitomycin C compared to *recN*<sup>+</sup> cells. The  $\Delta recN108$  cells showed an un-induced increase frequency of Tn10 excision from *gal76::Tn10* in *rec*<sup>+</sup> background. While, *recBC sbcBC*  $\Delta recN108$  cells are completely deficient in Tn10 excision. The recombination proficiency is reduced in cells carrying *recBC sbcBC* cells in addition *recN*<sub>K35D</sub> mutation. We observed that the Walker A nucleotide binding site is important for the RecN protein. Strains that deleted *recN* gene are recombination deficient and more sensitive to mitomycin C than strains carrying *recN*<sub>K35D</sub>.

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## 1. Introduction

The *Escherichia coli* *recN* gene was originally identified in *recBC sbcB* mutant by Lloyd et al. (1983). The *recN* gene of *E. coli* was cloned (Picksley et al., 1985) and sequenced (Rostas et al., 1987) and the gene product has a molecular weight of 63 kDa (Finch et al., 1985; Picksley et al., 1985; Rostas et al., 1987; Elie et al., 1997). The RecN protein has the predicted characterization for the proteins belonging to the SMC family of proteins (Hirano et al., 1995). This family of proteins plays an important role in chromosomal condensation and segregation, dosage compensation (Hirano et al., 1995, 1997) and DNA recombinational repair (Connelly et al.,

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1999; Cao et al., 1990). Members of the SMC family are characterized by having Walker A and Walker B nucleotide binding sites (Walker et al., 1982). The RecN protein belong to subclass of the SMC proteins implicated in DNA repair and recombination that show high sequence similarity in and around the Walker A and Walker B nucleotide binding sites (Hirano et al., 1995; Graumann and Knust, 2009). The *recN* gene product plays an important role in the RecF recombination pathway in *recBC sbcBC* (Lloyd et al., 1983; Picksley et al., 1984b). Picksley et al. (1984a) showed that the *recN* gene product has been involved in the repair of double strand breaks of DNA and that *recN* mutants strains are sensitive to ionisation radiation and mitomycin C. The product of *recN* gene is involved in the induced excision of Tn10 (Chan et al., 1994; Zhuravel and Boreiko, 2002).

Recombination is an important process that generates genetic variation and preserves the integrity of genome. In *E. coli* K12 the homologous recombination relies on the product of several genes such as *recA* (Clark and Margulies, 1965; Cassuto et al., 1980) *recB* (Howard-Flanders and Theriot, 1966) and *recC* (Emmersom and Howard-Flanders, 1967). Mutations in the *recA* gene cause a major deficiency in the recombination which demonstrate that RecA protein is essential for homologous recombination (Emmerson, 1968). The role of the *recBCD* genes in the recombination is illustrated by the fact that mutation in *recBC* genes was shown to reduce the homologous recombination proficiency of cells to 1% compared to the wild type (Willets and Mount, 1969). The RecBCD enzyme is an important component of the main pathway of homologous recombination. The RecBCD enzyme consists of three subunits RecB, RecC and RecD proteins encoded by *recB*, *recC* and *recD* genes, respectively (Hickson and Emmerson, 1981). The RecBCD enzyme is an ATP dependent dsDNA 3'–5' and 5'–3' exonuclease (Exonuclease V). Moreover, it has also been shown to have ATP dependent ssDNA endonuclease and exonuclease activity and DNA helicase properties (Goldmark and Linn, 1972). The recombination deficiency in *recBC* mutants is suppressed by additional mutation in *sbcBC* genes. The *sbcB* gene codes for DNA exonuclease I, an enzyme which degrade ssDNA from the 3' end (Kushner et al., 1972; Lloyd and Buckman, 1985). In *recBC sbcBC* cells the recombination process is catalysed by the RecF pathway. The RecF pathway requires a group of genes named *recA*, *recF*, *recO*, *recR*, *recQ*, *recJ*, *recN* and *ruvABC* (Clark and Margulies, 1965; Lloyd et al., 1983; Lovett and Clark, 1984; Sawitzke and Stahl, 1992). Bacterial strains carrying *recBC sbcBC* in addition to *recN* are recombination deficient (Wang and Maier, 2008). Moreover, *recN recJ* mutant reduce the recombination in *recBC sbcBC* cells (Picksley et al., 1984a; Clark et al., 1984; Nagashima et al., 2006). *recN* gene is expressed and regulated by LexA repressor as a part of the SOS response for recovering the damaged DNA (Picksley et al., 1984b). In *recBC sbcBC* a single additional mutation in *recF*, *recO*, *recR*, *recQ*, *recJ*, *recN* and *ruvABC* genes decrease the recombination proficiency (Lovett and Clark, 1984; Kosa et al., 2004).

In the present work we generated two bacterial strains by reverse genetics, one is carrying a *recN* gene mutated in the Walker A nucleotide binding site while the other the entire *recN* gene is deleted. We characterized the *recN* gene by utilizing these two generated bacterial strains in the precise excision of Tn10 and the recombination process.

## 2. Materials and methods

The *E. coli* K12 strains and plasmids DNA used in this work are listed in Tables 1 and 2.

### 2.1. Media

The Luria–Bertani (LB) and 56/2 buffer salts minimal media was made as described by Lloyd et al. (1974). For minimal agar plates 56/2, buffer salts were diluted with equal volume of 3% (w/v) agar solution. The minimal 56/2 agar plates were supplemented with 1 mg of thiamine, 3.3 g glucose and 0.1 g of the required L-amino acids. MacConkey lactose medium was made by dissolving 40 g MacConkey base (Difco) in 900 ml ddH<sub>2</sub>O, sterilised by autoclaving. The volume was completed to one litre with sterilised 10% (w/v) lactose. Induced excision of Tn10 work was performed as described by Chan et al. (1994).

### 2.2. Plasmids construction

All recombinant DNA procedures were performed as described by Sambrook et al. (1989). Plasmid pSP100 (Picksley et al., 1985) carries *recN* gene on 5.6 kb DNA *Hind* III fragment. pSM106 plasmid (Picksley et al., 1985) was constructed by subcloning 4.9 kb *Hind* III-*Bgl* II DNA fragment from pSP100 to pBR322 *Hind* III-*Bam*HI. pMMY106 is similar to pSM106 but carries *recN*<sub>K35D</sub> mutation. pMMY109 and pMMY110 were constructed from pSM106, pMMY106 respectively by inserting 1.3 kb blunt end *Hae* II DNA fragment containing the chloramphenicol gene from pHSG415 downstream the *recN* gene in *Psp*Omi blunt end site. pMMY107 was constructed by subcloning 5.6 kb *Hind* III DNA fragment from pSP100 to pBR322 *Hind* III site. pMMY108 was constructed by deleting 1.728 kb *Bsr*G I-*Psp*Omi I DNA fragment containing the entire *recN* gene from pMMY107 and inserting 1.3 kb blunt end *Hae* II DNA fragment containing the Cm<sup>r</sup> gene from pHSG415 instead of the *recN* gene.

### 2.3. Transformation with a linear DNA fragment

Plasmids pMMY108, pMMY109, and pMMY110 contain a unique restriction site for *Ata* II in the vector (pBR322) sequence. So, pMMY108, pMMY109, and pMMY110 were linearized by digestion with *Ata* II. Bacterial strain N2525 (*recD1009*) was made competent by treatment with cold 0.1 M CaCl<sub>2</sub> as described by Sambrook et al. (1989) and exposed to the transforming linear DNA in the same manner as if introducing a circular plasmid DNA. The chloramphenicol resistant transformants were selected and screened for Ampicillin resistance. Ampicillin resistance transformants were discarded while ampicillin sensitive transformants were screened for the mitomycin C sensitivity for  $\Delta$ *recN*108 and *recN*<sub>K35D</sub>. P1 transduction was performed as described by Miller (1972).

### 2.4. Unique site elimination (USE) mutagenesis

The procedure of USE mutagenesis was carried out according to the manufacturer (Amersham Pharmacia Biotech) instructions.

**Table 1** The strains described in the present study.

| Strains | Genotype (or relevant phenotype)  | Reference or source      |
|---------|---|--------------------------|
| AB1157  | F <sup>-</sup> <i>thi-1 thr-1 araC14 leuB6Δ(gpt-proA2)62 lacY1 tsx-33galK2 λ<sup>-</sup> Rac<sup>-</sup> his G4 rfbD1 rpsL31 str<sup>R</sup> kdgK51 xylA5 mlt-1 argE3</i> | Bachmann (1972)          |
| AB2463  | As AB1157 but <i>recA13</i>   | Bachmann (1972)          |
| JC7623  | As AB1157 but <i>recB21 recC22 sbcB15 sbcC201</i>   | Kushner et al. (1971)    |
| MMY096  | As AB1157 but Cm <sup>R</sup> to Mc <sup>R</sup> Cm <sup>R</sup>  | P1.MMY080 X AB1157       |
| MMY097  | As JC7623 but Cm <sup>R</sup> to Mc <sup>R</sup> Cm <sup>R</sup>  | P1.MMY080 X JC7623       |
| MMY100  | As AB1157 but <i>recN<sub>K35D</sub></i> to Mc <sup>S</sup> Cm <sup>R</sup>   | P1.MMY094 X AB1157       |
| MMY103  | As JC7623 but <i>recN<sub>K35D</sub></i> to Mc <sup>S</sup> Cm <sup>R</sup>   | P1.MMY094 X JC7623       |
| MMY104  | As AB1157 but <i>ΔrecN108</i> to Mc <sup>S</sup> Cm <sup>R</sup>  | P1.MMY074 X AB1157       |
| MMY108  | As JC7623 but <i>ΔrecN108</i> to Mc <sup>S</sup> Cm <sup>R</sup>  | P1.MMY074 X JC7623       |
| MMY114  | As AB1157 but <i>recN<sub>K35D</sub> gal-76::Tn10</i> to Tc <sup>R</sup> Mc <sup>S</sup> Cm <sup>R</sup>  | P1.MMY094 X NF471        |
| MMY116  | As AB1157 but <i>ΔrecN108 gal-76::Tn10</i> to Tc <sup>R</sup> Mc <sup>S</sup> Cm <sup>R</sup>   | P1.MMY074 X NF471        |
| MMY118  | As AB1157 but <i>gal-76::Tn10</i> to Tc <sup>R</sup> Mc <sup>R</sup> Cm <sup>R</sup>  | P1.MMY080 X NF471        |
| MMY123  | As JC7623 but <i>ΔrecN108, gal76::Tn10</i> to Tc <sup>R</sup> Mc <sup>S</sup> Cm <sup>R</sup>   | P1.NF471 X MMY108        |
| MMY124  | As JC7623 but <i>recN<sub>K35D</sub>, gal76::Tn10</i> to Tc <sup>R</sup> Mc <sup>S</sup> Cm <sup>R</sup>  | P1.NF471 X MMY103        |
| MMY126  | As JC7623 but <i>gal76::Tn10</i> to Tc <sup>R</sup> Mc <sup>R</sup> Cm <sup>R</sup>   | P1.NF471 X MMY097        |
| MMY130  | As JC7623 but <i>gal76::Tn10</i> to Tc <sup>R</sup> Mc <sup>R</sup>   | P1.NF471 X JC7623        |
| N2525   | As AB1157 but <i>recD1009</i>   | Lloyd and Buckman (1985) |
| NF471   | As AB1157 but <i>gal-76::Tn10</i>   | Chan et al. (1994)       |
| NF618   | As AB1157 but <i>recN618 gal-76::Tn10</i>   | Chan et al. (1994)       |
| SP226   | As JC7623 but <i>recN261</i>  | Picksley et al. (1984b)  |
| SP231   | As JC7623 but <i>recN262</i>  | Picksley et al. (1984b)  |
| SP253   | As AB1157 but <i>recN261</i>  | Picksley et al. (1984b)  |
| SP254   | As AB1157 but <i>recN262</i>  | Picksley et al. (1984b)  |
| SP261   | As AB1157 but <i>tyrA16::Tn10</i>   | Lloyd et al. (1974)      |
| KL226   | Hfr (Cavalli) <i>relA1tonA221</i>   | Lloyd and Buckman (1985) |
| KL584   | F128 <i>proAB<sup>+</sup> lacI13 lacZ813 Δ(gpt-lac)5 rpsE<sup>-</sup> xyl<sup>-</sup> mlf<sup>-</sup> recA1</i>   | Lloyd and Buckman (1985) |

**Table 2** Plasmids DNA used in the present study.

| Plasmid | Characterisation   | Reference or source          |
|---------|--|------------------------------|
| pBR322  | pBR322 high copy plasmid Ap <sup>R</sup> , Tc <sup>R</sup>   | Bolivar et al. (1977)        |
| pHSG415 | pHSG415 low copy plasmid Ap <sup>R</sup> , Cm <sup>R</sup> & Km <sup>R</sup>   | Hashimoto-Goth et al. (1981) |
| pMMY106 | As pSM106 but containing <i>recN<sub>K35D</sub></i> gene Ap <sup>R</sup> , Tc <sup>S</sup>   | This work                    |
| pMMY107 | pBR322 <i>Hind</i> III-5.6 kb <i>Hind</i> III DNA fragment of pSP100 containing <i>recN</i> gene Ap <sup>R</sup> , Tc <sup>S</sup>   | This work                    |
| pMMY108 | pBR322 <i>Hind</i> III-5.6 kb <i>Hind</i> III DNA fragment of pSP100 but <i>ΔrecN</i> gene (1.728 kb <i>Bsr</i> G I- <i>Psp</i> OM I) and Insertion of 1.33 kb <i>Hae</i> II blunt end DNA fragment of <i>Cm</i> gene from Ap <sup>r</sup> , Cm <sup>R</sup> , Tc <sup>R</sup> , Km <sup>S</sup>   | This work                    |
| pHSG415 | pSM106 digested with <i>Psp</i> OM I and inserted 1.3 kb blunt end <i>Cm</i> <i>Psp</i> OM I site down stream of the <i>recN</i> gene pMMY110 pMMY106 ( <i>recN<sub>K35D</sub></i> ) digested with <i>Psp</i> OM I This work and inserted 1.3 kb blunt end <i>Cm</i> gene in <i>Psp</i> OM I site down stream of the <i>recN<sub>K35D</sub></i> gene | This work                    |
| pMMY112 | As pSP100 but contain <i>recN<sub>K35D</sub></i> Ap <sup>R</sup> , Cm <sup>R</sup> & Km <sup>S</sup>   | This work                    |
| pSM106  | pBR322 <i>Hind</i> III/ <i>Bam</i> H I – 4.9 kb <i>Hind</i> III- <i>Bgl</i> II DNA fragment of pSP100 containing <i>recN</i> gene Ap <sup>R</sup> , Tc <sup>S</sup>  | Picksley et al. (1985)       |
| pSP100  | pSP100 low copy plasmid <i>recN<sup>+</sup></i> Ap <sup>R</sup> , Cm <sup>R</sup> & Km <sup>S</sup>  | Picksley et al. (1985)       |

The target mutagenic primer 5'GCGCGGGTGACTCT ATT-GCAAT3' was used to change the AAA codon of lysine 35 of *recN* gene in pSM106 DNA to GAC codon of aspartic acid. While, selection mutagenic primer 5'AAACATGAGAA-GTCTGAA GAC3' was used to eliminate the unimportant

unique restriction site of *Eco*R I in pSM106, by changing *Eco*R I recognition site from 5'GAATTC3' to 5'GAAATC3'. After completing the USE mutagenesis procedure, plasmid DNA was treated with *Eco*R I to identify the presumably mutated plasmids. Five plasmids of those which resist digestion

with *EcoR* I were sequenced (MWG Biotech, Germany) using the following primer 5'GTTGCGACAGCCAGATAGCAC TGCCG3'.

### 2.5. Sensitivity to mitomycin C

Strains were grown in LB broth to mid-log phase (O.D<sub>595</sub> ~0.4), washed twice with 56/2 minimal salt buffer and resuspended in the same buffer containing 2 µg/ml mitomycin C. The cells were incubated at 37 °C and samples taken at different times and diluted with 56/2 minimal salt buffer to establish the viable cell count using appropriate serial dilution with 56/2 salt buffer.

### 2.6. Recombination proficiency

Matings for measuring the recombination proficiency were carried out by growing both the donor and the recipient bacterial strains at 37 °C to OD<sub>595</sub> ~0.4. The bacterial strains were mixed in a ratio of 1:10 donor to recipient and incubated at 37 °C in static water bath for 30 min (for F' donor) or 40 min (for Hfr donor). The mating mixtures were vortexed vigorously and chilled on ice. Samples were diluted in 56/2 minimal salt buffer and plated on 56/2 agar plates lacking proline, supplemented with 100 µg/ml streptomycin. The number of colony forming unites were counted after 2–3 days.

## 3. Results

### 3.1. Mutation of the Walker A nucleotide binding site of *recN* gene

We are interested in knowing the importance of the Walker A nucleotide binding site to the *E. coli* RecN protein. The Walker A nucleotide binding site of the *E. coli recN* gene was mutated (see Section 2). The mutation was transferred to plasmid

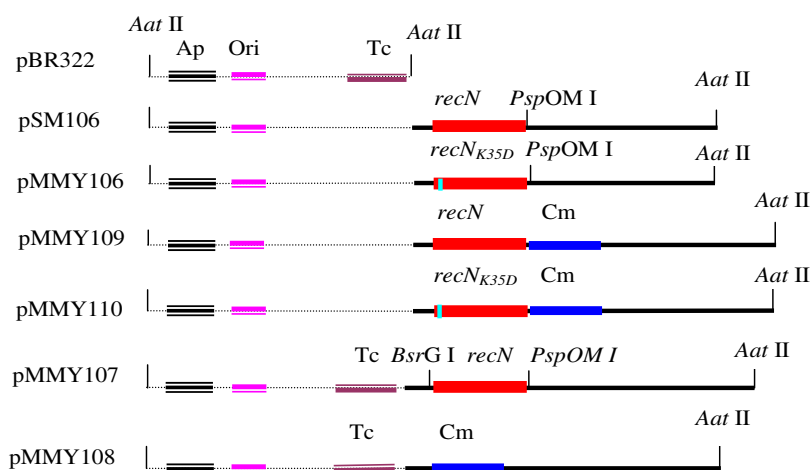
pSM106 yielding the plasmid DNA containing GAC codon of aspartic acid instead of AAA codon of lysine residue 35 of the *recN* gene and was designated as pMMY106 (Fig. 1). The mutation was confirmed by partial sequencing (the *recN* gene in plasmid pMMY106 data not shown) and the mutated *recN* gene was designated *recN*<sub>K35D</sub>.

### 3.2. Introduction of the *recN*<sub>K35D</sub> gene into *E. coli* chromosomal DNA

The plasmid pMMY110 carries *recN*<sub>K35D</sub> gene was linearized with *Aat* II restriction enzyme (Fig. 1), which cut the plasmid only in the vector DNA, and transformed to *E. coli* N2525 (*recD1009*) to Cm<sup>R</sup> (Table 3). The Ap<sup>R</sup> colonies may produced from undigested plasmid or relegated plasmid inside the bacte-

**Table 3** Conjugation recombination proficiency of bacterial strains carrying *recN*<sub>K35D</sub> mutation and *ΔrecN108*.

| Bacterial strain                                     | Hfr    | F'   |
|--|--------|------|
| <i>AB157</i> background                              |        |      |
| AB1157 ( <i>rec</i> <sup>+</sup> )                   | 1      | 1    |
| AB2463 ( <i>recA13</i> )                             | 0.0005 | 0.43 |
| MMY096 ( <i>rec</i> <sup>+</sup> , Cm <sup>R</sup> ) | 0.80   | 1.07 |
| SP253 ( <i>recN261</i> )                             | 0.30   | 0.54 |
| SP254 ( <i>recN262</i> )                             | 0.50   | 0.75 |
| MMY100 ( <i>recN</i> <sub>K35D</sub> )               | 0.24   | 0.60 |
| MMY104 ( <i>ΔrecN108</i> )                           | 0.26   | 0.95 |
| <i>JC7623</i> background                             |        |      |
| JC7623 ( <i>recBC sbcBC</i> )                        | 0.70   | 0.80 |
| MMY097 ( <i>recBC sbcBC</i> , Cm <sup>r</sup> )      | 0.40   | 0.63 |
| SP226 ( <i>recN261</i> )                             | 0.054  | 0.91 |
| SP231 ( <i>recN262</i> )                             | 0.014  | 0.40 |
| MMY103 ( <i>recN</i> <sub>K35D</sub> )               | 0.018  | 0.21 |
| MMY108 ( <i>ΔrecN108</i> )                           | 0.006  | 0.68 |



**Figure 1** Plasmids construction. All the plasmids constructed in this work are pBR322 derivatives. The *recN* gene in plasmid pSM106 (Picksley et al., 1985) was mutated by site directed mutagenesis to *recN*<sub>K35D</sub> (see below) and the new plasmid designated pMMY106. pMMY109 and pMMY110 plasmids were constructed from pSM106, pMMY106, respectively by inserting 1.3 kb blunt end *Hae* II DNA fragment containing the chloramphenicol gene from pHSG415 (Hashimoto-Goth et al., 1981) downstream the *recN* gene in *PspOM I* blunt end site. pMMY107 plasmid was constructed by subcloning 5.6 kb *Hind* III DNA fragment containing *recN* gene from pSP100 (Picksley et al., 1985) into pBR322 *Hind* III site. pMMY108 was constructed by deleting 1.7 kb *BsrG I*-*PspOM I* DNA fragment containing the entire *recN* gene from pMMY107 and inserting 1.3 kb blunt end *Hae* II DNA fragment containing the chloramphenicol gene from pHSG415 to 8.2 kb blunt end *BsrG I*-*PspOM I* DNA fragment of pMMY107 (instead of the *recN* gene).



rial cell while, Ap<sup>S</sup> colonies were screened for Mc<sup>S</sup>. The loss of Ap<sup>S</sup> and presence of Cm<sup>R</sup>, Mc<sup>S</sup> mean that the linear DNA has integrated into the chromosomal DNA and the Mc<sup>S</sup> presumably indicates the integration of *recN*<sub>K35D</sub> into the chromosomal DNA (Fig. 2). The Cm<sup>R</sup>, Mc<sup>S</sup>, Ap<sup>S</sup> strain was designated MMY080. The P1 lysate was made from bacterial strain MMY080 and was used to cross the *recN*<sub>K35D</sub> with Cm<sup>r</sup> to bacterial strains AB1157 (wt) and JC7623 (*recB21 recC22 sbcB15 sbcC201*). Similarly, pMMY109 was used as a control containing *recN*<sup>+</sup> gene and Cm<sup>R</sup>.

### 3.3. Deletion of the entire *recN* gene from *E. coli* chromosomal DNA

The plasmid pMMY108 carries 1.3 kb Cm<sup>R</sup> gene instead of *recN* gene and the entire *recN* gene was deleted (designated as *recN108*). The linear pMMY108 DNA was transformed to *E. coli* N2525 (*recD1009*) similar to pMMY110. The produced Cm<sup>R</sup>, Mc<sup>S</sup>, Ap<sup>S</sup> bacterial strain (*recD1009 recN108*) designated MMY074 (Fig. 3). The P1 lysate was made from bacterial strain MMY074 (*recD1009 recN108*). The P1 lysate of MMY074 was used to cross the *recN108* with Cm<sup>R</sup> to bacterial strains AB1157 (wt) and JC7623 (*recB21 recC22 sbcB15 sbcC201*).

### 3.4. Complementation analysis of *recN108*, and *recN*<sub>K35D</sub> mutant strains

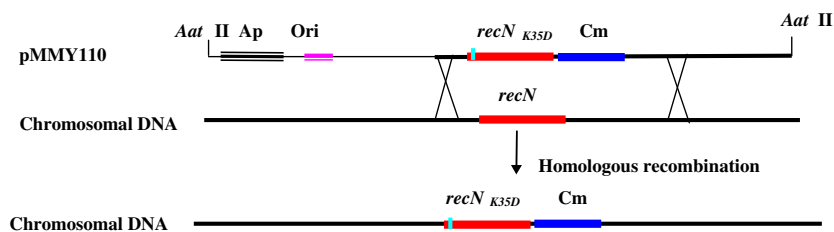
Having generated bacterial strains deleted the entire *recN* gene (*recN108*) and carrying *recN* gene mutated in the Walker A nucleotide binding site (*recN*<sub>K35D</sub>), we are interested in the

examination of mitomycin C sensitivity to these generated bacterial strains. Mitomycin C is a cross linking-agent which causes breaks in both DNA strands and it has been established that *recN* mutant strains are sensitive to mitomycin C (Picksley et al. 1984a). The results of mitomycin sensitivity to strains MMY100 (*recN*<sub>K35D</sub>), MMY104 (*recN108*) are illustrated in (Fig. 4). The strains having *recN108*, *recN*<sub>K35D</sub>, *recN261*, *recN262* are more sensitive to mitomycin C than strain AB1157 (*recN*<sup>+</sup>) and less sensitive than strain AB2463 (*recA13*). *recN*<sub>K35D</sub> cells exhibit sensitivity to mitomycin C as *recN261*, *recN262* cells, however *recN108* cells show *recN* more sensitivity to mitomycin C than *recN*<sub>K35D</sub>, *recN261* and *recN262* cells.

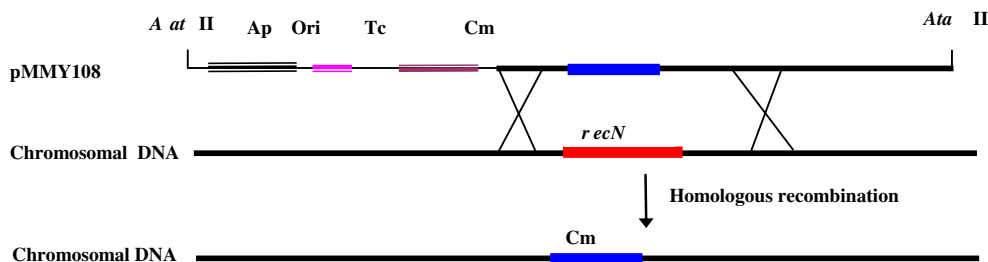
Bacterial cells *recN108*, *recN*<sub>K35D</sub>, *recN261*, *recN262* were transfected with plasmid pSP100 (*recN*<sup>+</sup>) and the mitomycin C sensitivity was examined (Fig. 5). It is clear that plasmid pSP100 restored the mitomycin resistance to *recN108*, *recN*<sub>K35D</sub>, *recN261*, *recN262* cells comparable to AB1157 (*recN*<sup>+</sup>), but not to the AB2463 (*recA13*).

### 3.5. Effect of *recN*<sub>K35D</sub> mutation and $\Delta recN108$ on genetic recombination in *rec*<sup>+</sup> and *recBC sbcBC* cells

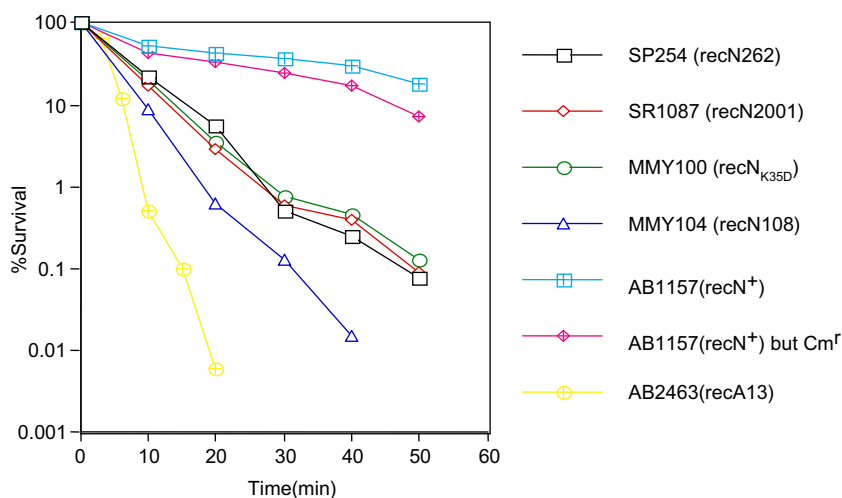
The *recN*<sub>K35D</sub> mutation and  $\Delta recN108$  were introduced into AB1157 (*rec*<sup>+</sup>) and JC7623 (*recBC sbcBC*) genetic background and the constructed strains were tested for homologous recombination using Hfr donor cells. The data (Table 3) indicate that *recN*<sub>K35D</sub> mutation and  $\Delta recN108$  reduce the homologous recombination in *recBC sbcBC* cells. A strain carrying  $\Delta recN108$  reduces the recombination proficiency more than a strain carrying *recN*<sub>K35D</sub> mutation. The effect of *recN*<sub>K35D</sub>



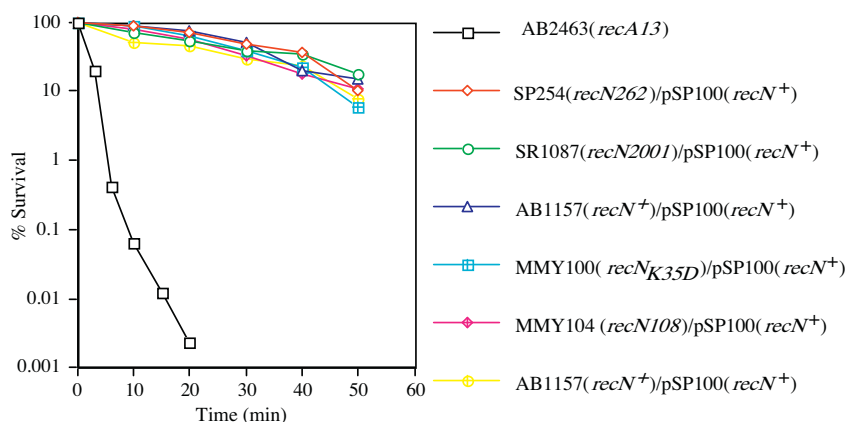
**Figure 2** Introduction of the *recN*<sub>K35D</sub> gene into *E. coli* chromosomal DNA by reverse genetics. The plasmid pMMY110 (containing *recN*<sub>K35D</sub>) was linearized by *Aat* II restriction enzyme and transformed into *E. coli* strain N2525 (*recD1009*). Selection was for Cm<sup>R</sup> clones and screened for Ap<sup>S</sup> and Mc<sup>S</sup>. Clones with Cm<sup>R</sup>, Ap<sup>S</sup> and Mc<sup>S</sup> were presumed to have integrated the DNA fragment containing *recN*<sub>K35D</sub> gene and Cm resistant gene into the chromosomal DNA by homologous recombination.



**Figure 3** Deletion of the entire *recN* gene from *E. coli* chromosomal DNA. A linear DNA fragment from pMMY108 DNA containing Cm resistant gene and deleted the entire *recN* gene was transformed into *E. coli* strain N2525 (*recD1009*). Selection was for Cm<sup>R</sup> clones and screened for Ap<sup>S</sup>, Tc<sup>S</sup> and Mc<sup>S</sup>. Clones with Cm<sup>R</sup>, Ap<sup>S</sup>, Tc<sup>S</sup>, and Mc<sup>S</sup> were presumed to have integrated the DNA fragment containing Cm resistant gene and deleted *recN* gene into the chromosomal DNA by homologous recombination.



**Figure 4** Mitomycin C sensitivity of *E. coli* strains SP254 (*recN262*), SR1087 (*recN2001*), MMY100 (*recN<sub>K35D</sub>*) & MMY104 (*recN108*). Bacterial strains AB1157 (*recN<sup>+</sup>*), SP254 (*recN262*), SR1087 (*recN2001*), MMY100 (*recN<sub>K35D</sub>*), MMY104 (*recN108*) & AB2463 (*recA13*) were exposed to mitomycin C 2 µg/ml for defined periods of time (min).



**Figure 5** Complementation of the mitomycin C sensitivity of bacterial strains SP254 (*recN262*), SR1087 (*recN2001*), MMY100 (*recN<sub>K35D</sub>*) & MMY104 (*recN108*) by the *recN<sup>+</sup>* plasmid pSP100. Bacterial strains AB1157 (*recN<sup>+</sup>*), SP254 (*recN262*), SR1087 (*recN2001*), MMY100 (*recN<sub>K35D</sub>*), MMY104 (*recN108*) & AB2463 (*recA13*) were transfected by the *recN<sup>+</sup>* plasmid pSP100 and exposed to mitomycin C 2 µg/ml for defined periods of time (min).

mutation on the homologous recombination is similar to that of *recN262* and *recN261* mutations. Also, we observed that both *recN<sub>K35D</sub>* mutation and  $\Delta$ *recN108* are not important as *recN262* and *recN261* mutations for homologous recombination in wild type (*rec<sup>+</sup>*) strains (Picksley et al. 1984a).

### 3.6. Effect of *recN* on the excision of Tn10

Chan et al. (1994) have observed that *recN* gene product is involved in the induced excision of Tn10. From this point of view, we are interested in the examination of the effect of the strains carrying *recN<sub>K35D</sub>* mutation and  $\Delta$ *recN108* on the induced excision of Tn10 from *gal76::Tn10* in AB1157 (*rec<sup>+</sup>*) and JC7623 (*recBC sbcBC*) backgrounds. Mitomycin C induces the precise excision of Tn10 from *gal76::Tn10* in an *E. coli recN<sup>+</sup>* strains. In, both AB1157 (*rec<sup>+</sup>*) and JC7623 (*recBC sbcBC*) backgrounds the *recN<sub>K35D</sub>* mutation affects in the induced excision of Tn10 from *gal76::Tn10* (Table 4). Our

studies show that mitomycin C induces the excision of Tn10 in *recN<sub>K35D</sub>* mutant cells. All the tested revertants were found to be Tc<sup>s</sup> and Cm<sup>r</sup>. The role of  $\Delta$ *recN108* in excision of Tn10 in AB1157 (*rec<sup>+</sup>*) background is uninducible by mitomycin C. On the other hand, the  $\Delta$ *recN108* in JC7623 (*recBC sbcBC*) background completely blocks the excision of Tn10.

## 4. Discussion

The members of the SMC family of proteins (Hirano et al., 1995, 1997) and various recombinational repair proteins as RecA (Knight and McEntee; 1985), RecB, RecD (Korangy and Julin; 1992) and SbcC (Connelly et al., 1999) have the Walker A nucleotide binding site (Gly-X-X-X-Lys-Ser/Thr where X is any amino acid) (Walker et al., 1982). In addition, the comparison of the RecN protein sequence of various organisms (Funayama et al., 1999) reveals that the RecN protein contains the Walker A nucleotide binding site. The basic

**Table 4** Effect of *recN* mutations on the excision of Tn10 from *gal76::Tn10*.

| Bacterial strain   | Mitomycin C concentration (ng/ml) |     |     |     |
|--|-----------------------------------|-----|-----|-----|
|  | 0                                 | 2.5 | 5   | 10  |
| <i>AB1157 background</i>   |                                   |     |     |     |
| MMY118 ( <i>rec</i> <sup>+</sup> , Cm <sup>r</sup> )               | 23                                | 236 | 357 | 509 |
| MMY114 ( <i>recN</i> <sub>K35D</sub> , Cm <sup>r</sup> )           | 21                                | 45  | 87  | 111 |
| MMY116 ( <i>recN108</i> , Cm <sup>r</sup> )                        | 413                               | 434 | 423 | 441 |
| NF471 ( <i>rec</i> <sup>+</sup> )                                  | 28                                | 296 | 468 | 626 |
| NF618 ( <i>recN618</i> )   | 20                                | 34  | 21  | 29  |
| <i>JC7623 background</i>   |                                   |     |     |     |
| MMY130 ( <i>recBC sbcBC</i> )                                      | 12                                | 38  | 52  | 68  |
| MMY126 ( <i>recBC sbcBC</i> , Cm <sup>r</sup> )                    | 10                                | 39  | 61  | 88  |
| MMY124 ( <i>recBC sbcBC recN</i> <sub>K35D</sub> Cm <sup>r</sup> ) | 9                                 | 25  | 28  | 35  |
| MMY123 ( <i>recBC sbcBC recN108</i> , Cm <sup>r</sup> )            | 0                                 | 0   | 0   | 0   |

NB: All bacterial strains used in the excision Tn10 experiment are *gal76::Tn10*.

amino acid lysine residue in the Walker A nucleotide binding site has been shown to be essential for the RecD protein (Korangy and Julin; 1992). To determine the importance of the Walker A nucleotide binding site to the RecN protein, the highly conserved basic amino acid lysine 35 in the Walker A nucleotide binding site of the *E. coli* RecN protein was mutated to an acidic amino acid aspartic acid (Fig. 1).

Many reports have used bacterial strains carrying *recD* mutation and transformed it with linear pieces of DNA containing homology to delete or replace chromosomal genes with genes that had been modified (Chauhudry and Smith, 1984; Russel et al., 1989). In this work, the linear pMMY108 (*recN108*) and pMMY110 (*recN*<sub>K35D</sub>) plasmids carrying the chloramphenicol and ampicillin resistant genes were introduced into *recD1009* competent cells. The transformants that integrate the linear plasmids DNA into the chromosomal DNA by homologous recombination were identified presumably for loss of the ampicillin resistant gene and accept of the chloramphenicol resistant gene. The deletion of *recN* or reintroduction of *recN*<sub>K35D</sub> into the chromosomal DNA identified in clones which shows sensitivity to mitomycin C (Figs. 2 and 3).

The product of *recN* gene is implicated in the repair of DNA double strand breaks (Picksley et al., 1984a; Meddows et al., 2005). Known *recN* mutations are sensitive to the mitomycin C a cross linking-agent (Picksley et al., 1984a,b; Chan et al., 1994; Funayama et al., 1999). The strains carrying *recN*<sub>K35D</sub> and *recN108* show sensitivity to mitomycin C (Fig. 4). It is clear that,  $\Delta$ *recN108* cells are more sensitive to the mitomycin C than *recN*<sub>K35D</sub> and the identified *recN* mutations. The pSPI100 (*recN*<sup>+</sup>) plasmid DNA restores the resistant to the mitomycin C of strains carrying *recN*<sub>K35D</sub> and  $\Delta$ *recN108* to the level of wild type strains (*recN*<sup>+</sup>).

The recombination in *rec*<sup>+</sup> strains is activated by RecBCD pathway and *recN* gene product not implicated in this pathway. In *recBC* strains the recombination proficiency is restored by additional mutation in *sbcA* or *sbcBC*. In *recBC sbcA* background the recombination is catalysed by the RecE pathway and *recN* gene product is not involved in this pathway (Robbins-Manke et al., 2005). On the other hand, in the *recBC sbcBC* cells the recombination is catalysed by the RecF pathway and *recN* gene product have an important role in the RecF pathway (Picksley et al., 1984a; Wang and Maier, 2008). In this

study we observed that *recN108* in *recBC sbcBC* background reduce the homologous recombination more than that any of the *recN* mutation in *recBC sbcBC* background (Table 3).

Many of the SOS genes were found to be implicated in the precise excision of Tn10 (Chan et al., 1994). *recN* gene is induced as one of the SOS genes under the regulation of LexA repressor to remind the damaged DNA (Picksley et al., 1984b; Kosa et al., 2004). The data (Table 4) indicate that *recN* gene product have a role in the excision of Tn10 in *rec*<sup>+</sup> and *recBC sbcBC* background. The frequency of revertants of *gal*<sup>+</sup> is decreased in *recN*<sub>K35D</sub> strains than that of the *recN*<sup>+</sup> in AB1157 and JC7623 background.

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