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RecBCD helicase/nuclease

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The RecBCD enzyme of *Escherichia coli* participates in several aspects of DNA recombination and repair. It is essential to the main pathway of homologous recombination in *Escherichia coli*, where it contributes to the exchange of genetic material between homologous DNA molecules (*i.e.* conjugal recombination), and to the recombinational repair of chromosomal double-stranded breaks, lesions which are potentially lethal in the absence of repair. The potent nuclease activity of RecBCD enzyme is also responsible for the degradation of invading viral genomes, thus preventing establishment of infection. The outcome of a DNA processing event by RecBCD enzyme, whether recombinogenic or nucleolytic, is subject to specific regulation by a DNA sequence known as χ .

Introduction

The *E. coli* RecBCD enzyme is a multifunctional protein complex (330 kDa) containing three subunits, the products of the *recB*, *recC*, and *recD* genes (Amundsen et al., 1986; Emmerson, 1968; Glickman, 1979). This enzyme displays four distinct activities: nuclease, helicase, ATPase, and site-specific recognition of the DNA regulatory sequence, Chi (Crossover Hotspot Instigator = χ). Originally identified as Exonuclease V, the RecBCD enzyme is responsible for the seemingly disparate functions of DNA degradation and repair of the bacterial chromosome. The former function is achieved

by the combined action of its helicase and nuclease activities, whereas a recombinationally activated form accomplishes the latter, after interaction of RecBCD enzyme with χ . The RecBCD enzyme is a principal component of the main pathway for homologous genetic recombination in *E. coli*, referred to as the RecBCD-pathway. Structural or functional analogs of the RecBCD enzyme are present in both Gram-negative and Gram-positive bacteria, and interspecies complementation studies suggest that the mechanism of homologous recombination via the RecBCD-pathway is conserved, at least among bacteria.

Genetics/effects of null mutations

Although the cellular level of RecBCD enzyme is low (~10 copies per cell), the absence of this enzyme has a tremendous effect on DNA metabolism in *E. coli*. Mutations which inactivate RecB or RecC proteins lower viability approximately four-fold, reduce conjugal recombination 100- to 1000-fold, and sensitise the bacterium to DNA damaging agents such as UV and gamma irradiation (Emmerson, 1968, Glickman, 1979). These cells also lack detectable levels of ATP-dependent nuclease and so are susceptible to infection by bacteriophages. These findings emphasise the many roles of RecBCD enzyme in *E. coli*, and underscore the importance of the RecB and RecC subunits to the most basic functions of the holoenzyme.

In striking contrast, however, strains containing null mutations in the *recD* gene display wild-type cell viability, resistance to UV and gamma irradiation, and are recombination proficient (Amundsen et al., 1986). Like the *recB* and *recC* strains, these cells do not display nuclease activity, making them sensitive to infection by bacteriophages. The biochemical basis for this phenotype will be discussed later.

Helicase Activity

Under optimal conditions, the RecBCD enzyme is a highly processive helicase, unwinding an average of 30 kilobases per binding event at a rate of 1000-1500 base pairs per second (Figure 1) (Roman et al., 1992). A blunt double-stranded DNA (dsDNA) end is the preferred substrate for initiation of unwinding, but single-stranded DNA (ssDNA) tails of less than 25 nucleotides can also serve as initiation sites. RecBCD enzyme displays a high affinity for dsDNA ends, about 50-fold higher than that for ssDNA ends, arguing that the relevant substrate *in vivo* is a blunt or nearly blunt dsDNA end. At the dsDNA end, the RecB subunit associates with the strand terminating with a 3'-hydroxyl, and the RecC and RecD subunits associate with the strand terminating with a 5'-phosphate, with translocation occurring in the 3' to 5' direction relative to the 3'-hydroxyl end. Thus, the requirement for a blunt end may be due to the need for all three subunits to simultaneously interact with the appropriate DNA termini.

The amino acid sequence of the RecB subunit displays homology to other known helicases, such as Rep and UvrD proteins. The purified RecB subunit itself possesses both DNA-dependent ATPase and weak 3' to 5' helicase activities. The RecB and RecC subunits can be reconstituted to produce the RecBC enzyme, which is an active helicase with a lower affinity for dsDNA ends than the RecBCD holoenzyme.

During unwinding, either ssDNA loop-tails, or twin-loops of ssDNA are formed which extend from the RecBCD enzyme complex (Taylor and Smith, 1980). At physiological temperature (37°C), the loops grow at a rate of about 100 nucleotides per second. The presence of the loop-tail or twin-loop structures implies that there are at least two translocating domains in the holoenzyme: a more rapidly moving domain(s) containing the helicase activity and a

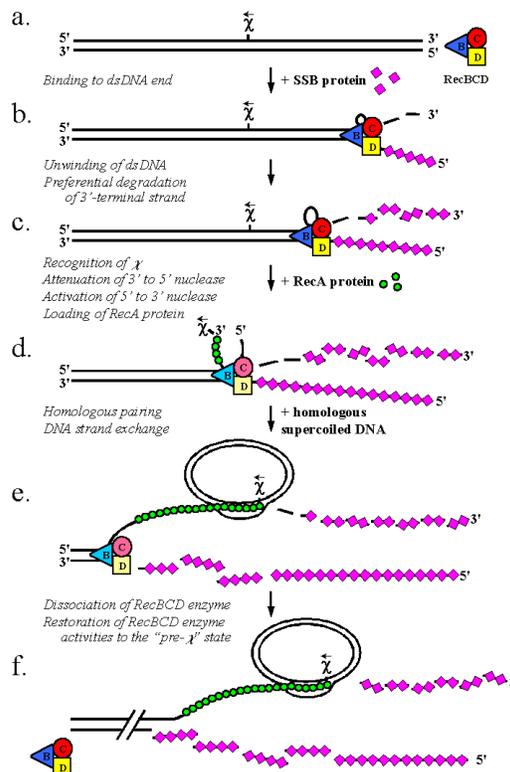


Figure 1: **Initiation of homologous recombination by the co-ordinated activities of RecBCD enzyme and RecA protein.** An *in vitro* model depicting recombination between a linear χ -containing dsDNA and a supercoiled plasmid is shown. a-c) First, RecBCD enzyme enters at a dsDNA end and unwinds the duplex while preferentially degrading the strand corresponding to the 3'-terminus at the point of entry; SSB protein binds the ssDNA produced. d-e) Upon recognition of χ , the 3' to 5' nuclease activity is attenuated and a weaker 5' to 3' nuclease activity is activated on the opposite strand. Following the interaction with χ , RecBCD enzyme facilitates the loading of RecA protein (to the exclusion of SSB protein) onto the ssDNA produced by continued translocation and unwinding of the DNA molecule. e-f) This RecA protein-ssDNA filament then invades a homologous duplex DNA molecule, producing a recombination intermediate known as a joint molecule.

slower domain(s) that translocates along the ssDNA produced. Thus, as the dsDNA substrate is processed, ssDNA loop(s) form between these domains. *E. coli* single-stranded DNA binding protein (SSB) favours production of the loop-tail structure with the loop being created from the DNA strand terminating with a 3'-hydroxyl at the point of entry of the RecBCD enzyme, presumably by binding to the complementary strand and disrupting its contact with the slower translocating domain(s) (Anderson and Kowalczykowski, 1998).

The DNA helicase activity requires the nucleotide cofactor ATP and magnesium ion, with 1.7 to 3 molecules of ATP being hydrolysed per base pair unwound. Helicase activity is inhibited by the ssDNA produced during processing of a dsDNA molecule. Binding of SSB protein to the ssDNA products alleviates

this inhibition (Figure 1) (Anderson and Kowalczykowski, 1998).

Nuclease Activity

In addition to being a helicase, the RecBCD enzyme is also a potent nuclease, functioning to protect the cell from invasion by infecting viral DNA. The RecBCD enzyme degrades both double-stranded DNA and single-stranded DNA, but the activity toward ssDNA is much lower than for dsDNA (Wright et al., 1971).

The dsDNA nuclease activity is coincident with translocation by RecBCD enzyme. Thus, although this degradation of dsDNA is formally defined as an “exonuclease” activity, the cleavage is actually endonucleolytic and the requirement for a dsDNA end pertains to helicase function. Degradation during unwinding is asymmetric, with the 3'-terminal strand (relative to the dsDNA entry site) being degraded much more vigorously than its complement (Figure 1a-c). Hence the enzyme degrades dsDNA primarily in a 3' to 5' direction (Dixon and Kowalczykowski, 1993). SSB protein, in addition to binding the potentially inhibitory ssDNA produced by the helicase activity as discussed above, also moderates the 5' to 3' nuclease activity, lowering the frequency of cutting by the enzyme (Anderson and Kowalczykowski, 1998).

In vitro, the ratio of magnesium ion concentration to ATP concentration greatly influences the observed dsDNA exonuclease activity. As the ratio of magnesium ion concentration to ATP concentration rises, the frequency of endonucleolytic cleavages increases, resulting in the production of increasingly smaller ssDNA fragments. When this ratio is very low, nuclease activity is barely detectable and the enzyme behaves essentially as a helicase. The seemingly complex effects of magnesium ion concentration are the result of competing requirements for this cofactor. Magnesium is required in the form of a complex with ATP for helicase activity, but in its free form for nuclease activity. Hence, when magnesium ion concentration exceeds the ATP concentration, the resultant excess free magnesium ion concentration increases the dsDNA nuclease activity, but not the helicase activity.

Bacteriophages that infect *E. coli* have developed strategies to circumvent the destructive nuclease activities of the RecBCD enzyme. For example, the λ phage produces the Gam protein early in its lytic cycle; this protein specifically binds to RecBCD enzyme, preventing degradation of the linear λ phage genome. A different defence is utilised by T4 phage; rather than binding to the enzyme, T4 gene 2 protein caps the ends of the T4 genome, blocking entry by RecBCD enzyme. The N15 phage does not produce an inhibitory protein; instead it forms hairpin loops at the ends of its linear genome, thereby blocking binding of the RecBCD enzyme (Malinin et al., 1992). As discussed below, even *E. coli* itself has developed a means to protect broken chromosomal DNA from degradation by RecBCD enzyme, a mechanism which also serves to initiate repair of these DNA lesions.

This voracious nuclease activity seems at odds with the fact that RecBCD enzyme plays a principal role in promoting DNA repair and homologous recombination, processes that, by their very nature, require the *preservation* of DNA. Resolution of this apparent inconsistency is found in the regulatory aspects of the recombination hotspot χ .

Modification of the nuclease activities of RecBCD enzyme by χ

χ is a DNA locus that stimulates the frequency of genetic recombination in its vicinity. This recombination hotspot was originally discovered as a mutation in λ phage that protected the phage genome from degradation by RecBCD enzyme (Lam et al., 1974). As shown in Figure 2, the sequence of χ is the octamer 5'-GCTGGTGG-3' (Bianco and Kowalczykowski, 1997), and most single base mutations within the octamer reduce χ -activity (Smith et al., 1981). Recombination in the vicinity of a χ site is stimulated by 5- to 10-fold over background levels. Key features need to be emphasised to understand the nature of this recombination hotspot. First, the stimulation is highly polar, with the region of enhanced recombination extending downstream of the 5' end of the χ sequence. Enhancement of recombination downstream of χ decreases by a factor of two for every 2.2 to 3.2 kilobases, returning to background levels 10 kilobases downstream when no heterologous regions intervene (Myers et al., 1995). Second, all recombination stimulated by this site requires the

activity of the RecBCD enzyme, whose nuclease activity would seemingly destroy its own substrate. Numerous genetic and biochemical studies have shown that the increase in recombination is due to a direct interaction between the χ sequence and the RecBCD enzyme, and that this stimulation only occurs if the enzyme approaches from the 3' side (Figure 2) (Ponticelli et al, 1985; Taylor et al, 1985). The interaction with χ elicits several changes in enzyme function that are manifest in an overall decrease in nuclease activity, which accounts for the protection of DNA observed *in vivo*. Thus, χ is a regulator of RecBCD enzyme and, hence, of genetic recombination.

The sequence of events preceding and succeeding interaction with χ are illustrated in Figure 1. As described above, the RecBCD enzyme translocates through a dsDNA molecule, simultaneously unwinding the dsDNA and degrading primarily the ssDNA strand that is 3'-terminated at the entry site. Upon reaching a χ site in the correct orientation, the enzyme pauses and several enzymatic changes occur. Although the helicase activity of the modified enzyme is unaffected by this encounter, degradation of the strand corresponding to the 3'-end is attenuated at least 500-fold (Dixon and Kowalczykowski, 1993).

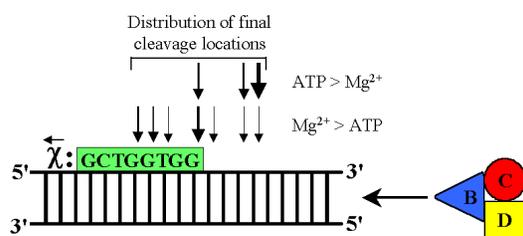


Figure 2: Orientation dependence of χ recognition. For recognition to occur, the RecBCD enzyme must approach the χ site from the 3' side, as depicted. Recognition results in attenuation of the 3' to 5' nuclease activity of the enzyme with the final cleavage event occurring near the 3' end of χ . Arrows denote the positions at which the final cut may occur, with corresponding thicknesses indicating the relative frequencies at each position. (Adapted from (Kowalczykowski et al., 1994) and (Taylor and Smith, 1995)).

The last non-specific cleavage event occurs with a high probability near χ , thereby generating a 3'-hydroxyl end within a few base pairs of the χ site (Figure 2). This attenuation of nuclease activity is manifest until the enzyme dissociates from the DNA, explaining the elevated recombination frequency downstream of χ sites. But, in addition to the attenuation of 3' to 5' nuclease activity, a weaker 5' to 3' nuclease activity is activated on the 5'-terminal strand

(Figure 1e) (Anderson and Kowalczykowski, 1997a). This switch in the polarity of nuclease activity produces a dsDNA molecule with a ssDNA tail retaining χ at the 3' terminus (Figure 1f). Further, the RecBCD enzyme facilitates the loading of the RecA DNA strand exchange protein onto this 3'-terminal ssDNA tail, which is the preferred substrate for RecA protein-dependent strand invasion of a homologous recipient dsDNA (Figure 1d-e) (see chapter on RecA protein) (Dixon and Kowalczykowski, 1991; Anderson and Kowalczykowski, 1997b). The co-operative 5' to 3' polymerisation of RecA protein onto this ssDNA substrate yields an invasive 3'-end that is completely coated with RecA protein (1f). Thus, χ co-ordinates the activities of RecBCD enzyme and RecA protein such that stimulation of homologous pairing initiates at the 3' end generated by the overall attenuation of nuclease activity at χ and propagates downstream from χ .

The precise biochemical basis for these changes is not known, but they are likely the result of a modification of the RecD subunit. Both genetic and biochemical evidence demonstrates that the RecBC enzyme, without the RecD subunit, mimics many, but not all, characteristics of the χ -modified RecBCD enzyme. This view explains why the level of conjugal recombination in *recD* strains is similar to that observed in wild-type strains, but is not stimulated by χ . The recombination proficiency of these strains can be largely attributed to the lack of RecBCD nuclease activity. However, unlike recombination in a wild-type strain, it is also dependent on a 5' to 3' ssDNA nuclease, the RecJ protein. Together, the nuclease-deficient RecBC enzyme and the RecJ protein produce a 3'-terminal ssDNA substrate appropriate for RecA-protein promoted strand invasion and, hence, comprise an effective substitute for the χ -modified RecBCD enzyme.

A special class of *recBCD* mutants, the *recC** class, specifically affects the ability of the enzyme to respond to χ . The *recC** strains display moderate to wild-type levels of recombination and resistance to DNA damaging agents, but they lack stimulation of recombination at χ . Unlike the *recD* mutants, however, these strains possess ATP-dependent nuclease activity. At least one member of this class, the RecBC¹⁰⁰⁴D enzyme, recognises a novel sequence variant of χ , known as χ^* (5'-

GCTGGTGCCTCG-3') (Handa et al., 1997; Arnold et al., 1998). These findings suggest that the RecC subunit is responsible for χ recognition.

Role in Conjugal Recombination

During conjugation, a piece of chromosomal DNA is delivered from a donor cell to a recipient cell. After transfer, genetic markers may be exchanged between the transferred DNA and the genome of the recipient cell. Since this exchange occurs by recombination, it is referred to as conjugal recombination. During conjugation, DNA enters the recipient cell as a single-stranded molecule and is largely converted to linear dsDNA by DNA replication. RecBCD enzyme can load at the ends of this dsDNA molecule, both unwinding and degrading the duplex DNA until it encounters a χ site. As described above, this encounter modifies the enzyme from its destructive form to its recombination-promoting form. Approximately 80% of conjugal recombination events are believed to occur at the ends of transferred DNA via the RecBCD pathway, and nearly all of these are χ -mediated, correlating well with biochemical studies.

Role in Recombinational DNA Repair

Alkylating agents, gamma radiation, and ultraviolet light cause damage to DNA; whether directly or indirectly, these lesions can result in the production of dsDNA breaks, which are potentially lethal to cells. Repair of this type of DNA damage is dependent on the RecBCD-pathway and on the availability of an additional intact homologous chromosome. The processing of such dsDNA breaks by RecBCD enzyme initiates the recombination-dependent repair of these breaks, and is termed recombinational DNA repair. This repair is facilitated by the abundant presence of χ sites, occurring approximately once every 4.6 kilobases, making it one of the most over-represented octamers in the *E. coli* genome (Blattner et al., 1997). Interestingly, the orientation of these sequence elements is not random in the chromosome: 75.5% of χ sites in *E. coli* are oriented toward the origin of replication, *oriC* (Figure 3a).

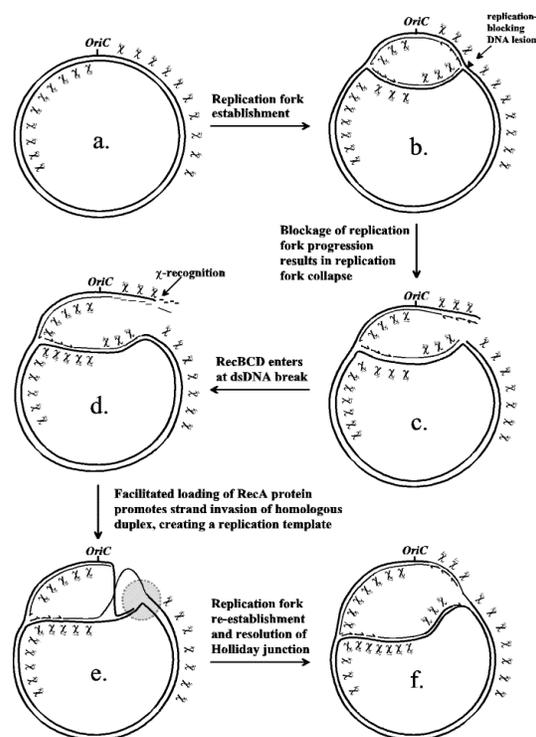


Figure 3: **Recombinational repair of a collapsed replication fork.** a) Unreplicated *E. coli* chromosome. b) Replication is bi-directional with two replication forks initiating at *oriC* and progressing in opposite directions around the chromosome; a lesion in the DNA template blocks progression of one replication fork. c) The blockage causes detachment of one arm and collapse of the replication fork resulting in a dsDNA break. d) RecBCD enzyme enters at the dsDNA end, degrading until reaching a χ site; enzymatic modification of RecBCD enzyme occurs and the facilitated loading of RecA protein follows. e) RecA protein promotes strand invasion of the ssDNA substrate into the homologous duplex, recreating a template for replication as shown in the shaded circle. f) The replication fork is re-assembled and replication of the chromosome resumes. The Holliday junction formed by the strand exchange event is resolved by the specific resolvases of *E. coli*.

This biased orientation of χ sites accommodates another aspect of cell survival: namely, the repair and resumption of detached arms of replication forks. Replication fork collapse is common in *E. coli*, contributing to the lowered viability of *recB* and *recC* null mutants. Collapse of a replication fork can be caused by a ssDNA nick in either template strand, or by proteins bound to the template DNA that block progression of the fork. Such an interruption is potentially lethal, ultimately resulting in a double-strand DNA break and creating an entry site for the RecBCD enzyme. Repair of the break by recombinational repair allows restoration of the replication fork (Figure 3). Thus, a major function of the *E. coli* dsDNA break repair pathway is the re-assembly of collapsed replication forks, and the preferential orientation of χ sites facilitates this repair through its interaction with the RecBCD enzyme (Kuzminov, 1995). As shown in Figure 3c, a double-stranded break resulting from the

collapse of a replication fork provides a dsDNA end at which RecBCD enzyme can initiate unwinding and degradation in the direction of the origin of replication, *oriC*. The preferential orientation of χ sites toward *oriC* favours recognition by, and χ -dependent modification of, RecBCD enzyme (3d), thereby promoting recombination between the broken and intact replication arms and restoring the replication fork (3e-f).

Summary

The multifunctional RecBCD enzyme serves many functions in *E. coli*. Its potent nuclease activities degrade foreign DNA, such as viral genomes, that might otherwise infect and destroy the cell. Subsequent to the occurrence of dsDNA breaks, it would likely do the same to the bacterial genome itself, were it not for the highly over-represented χ site. Acting as a “self-recognition” tactic, interaction with the χ site promotes a modification of RecBCD activities, effectively changing a destructive nuclease into a genome-protecting recombination enzyme. In addition to formation and preservation of the invasive 3'-terminal ssDNA with χ at its terminus, the χ -activated RecBCD enzyme stimulates loading of the RecA DNA strand-exchange protein onto this strand, prompting its invasion into a homologous dsDNA molecule. This ability of RecBCD enzyme to promote recombination provides a means to repair DNA damage to the *E. coli* chromosome, such as the dsDNA breaks formed by radiation, chemical mutagens, or the collapse of replication forks.

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Glossary

1. **Bacteriophage** – (phage) A virus that infects and replicates in bacteria.
2. **Conjugation** - The transfer of genetic material mediated by direct contact between a donor and a recipient bacterial cell.
3. **DNA strand exchange protein** - A protein that, upon binding to a ssDNA molecule, catalyses the transfer of that strand to a homologous region of a duplex DNA molecule, disrupting the original duplex to produce a new heteroduplex.
4. **Endonuclease** - A nuclease that cleaves at sites internal to a nucleic acid.
5. **Exonuclease** - A nuclease that cleaves only at the terminus of a nucleic acid.
6. **Helicase** - An enzyme that catalyses the ATP-dependent strand separation of double-stranded DNA.
7. **Homologous chromosomes** - Chromosomes that, except for allelic differences, are identical.
8. **Homologous genetic recombination** - (generalized recombination) Physical exchange of genetic information between two homologous chromosomes.
9. **Joint molecule** - An intermediate in the recombination process consisting of ssDNA paired to a homologous region within a dsDNA molecule.
10. **Nuclease** - An enzyme that degrades nucleic acids (DNA or RNA) by catalysing hydrolysis of phosphodiester bonds.