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The role of *Caulobacter crescentus* XerC and XerD recombinases in site-specific recombination

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The role of *Caulobacter crescentus* XerC and XerD recombinases in site-specific recombination

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Résumé

XerC et XerD, deux recombinases impliquées dans la recombinaison site spécifique, résolvent les multimères d'ADN en monomères. Cette réaction se produit au niveau du site *dif* du chromosome, et nécessite le domaine C-terminale de la protéine de division cellulaire FtsK. *Caulobacter crescentus* est une bactérie aquatique de type Gram-négative qui se retrouve dans plusieurs environnements. Elle présente un cycle cellulaire asymétrique avec deux types de cellules distinctes. Cette propriété peut être utilisée pour synchroniser la croissance d'une population bactérienne pour permettre l'étude de l'expression de gènes à travers le temps et les liens entre le cycle cellulaire et le développement de la bactérie. La liaison à l'ADN et la capacité de former des complexes covalents (phosphotyrosyl) avec le site *dif* de *C. crescentus* (*ccdif*) ont été testé pour les recombinases de *C. crescentus* (*cc*XerC et *cc*XerD). Les deux recombinases ont eu une meilleure liaison au demi-site gauche de *ccdif* et sont incapable d'effectuer une liaison coopérative, contrairement à ce qui se produit au niveau du site *dif* de *E. coli*. La formation de complexes covalents a été testé en utilisant des «substrats suicides avec bris» marqués à la fluorescence ainsi que des protéines de fusion (marquées ou non à la fluorescence). Des complexes ADN-protéines résistants à la chaleur et au SDS ont été observé lors de la réaction de *cc*XerC et *cc*XerD de type sauvage avec cc*dif*, mais pas lors de la réaction de mutants avec le même ADN. Des complexes covalents phosphotyrosine sont formés de façon plus efficace sur les substrats suicides avec un bris au niveau du brin supérieur que ceux ayant un bris au niveau du brin inférieur. Dans les deux cas, c'est *cc*XerC qui est resté lié de façon covalente à l'ADN de *ccdif*.

Mots-clés : Recombinasion spécifique de site /tyrosine recombinase/XerC/XerD/*dif* */ Caulobacter crescentus*

Summary

In most bacteria, the chromosomal dimer resolution process is mediated by two tyrosine recombinases, XerC and XerD, which bind cooperatively and perform the recombination reaction at the *dif* site near the terminus of replication. This reaction also requires the C-terminal domain of the cell division protein FtsK. *Caulobacter crescentus* is an aquatic Gram-negative bacterium found in various environments. This bacterium has an asymmetric cell cycle which can be used to synchronize cell growth in order to study the temporal expression of a gene and the interconnection between the cell cycle and development. The binding activity and the formation of phosphotyrosyl complex of the *C. crescentus* recombinases, *cc*XerC and *cc*XerD, were tested on the *C. crescentus dif* (*ccdif*) site. Both *cc*XerC and *cc*XerD bound preferentially to the left half-site of *ccdif* and showed reduced cooperative binding, unlike what was found with the *E. coli dif* site. Covalent complex formation activity was tested by using fluorescently labelled linear "nicked suicide substrates" and labelled proteins. Heat and SDS-resistant protein-DNA complexes were formed when both wild-type *cc*XerC and *cc*XerD reacted with *ccdif* but not in the presence of active-site tyrosine mutant proteins. Phosphotyrosine complexes formed on the top-nicked suicide substrate were found to be more efficient than on the bottom-nicked suicide substrates and surprisingly *cc*XerC remained bound to both top and bottom-nicked *ccdif* suicide substrates.

Keywords: Site-specific recombination/tyrosine recombinase/XerC/XerD/*dif/Caulobacter crescentus*

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List of Symbols and Abreviations

AMINO ACIDS

A: alanine

C: cysteine

D: aspartic acid

E: glutamic acid

F: phenylalanine

G: glycine

H: histidine

I: isoleucine

K: lysine

L: leucine

M: methionine

N : asparagine

P: proline

Q: glutamine

R: arginine

S: serine

T: threonine

V: valine

MEASUREMENT UNITS

Å: angstrom unit

bp: base pair

cm: centimetre

Da: Dalton

g: gram

h: hour

kb: kilobase

kDa: kilodalton

µg: microgram

µl: microlitre

µM: micromolar

min: minute

ml : millilitre

mM : millimolar

ng: nanogram

s: second

×g: centrifugation speed

°C: Degree Celsius

W: tryptophan v/cm: volt per centimeter

OTHERS

Ap: ampicillin ATP: adenosine triphosphate

ATPase: adenosine triphosphatase BSA: bovine serum albumin

ccdif: *Caulobacter dif* site *cc*XerC: XerC of *Caulobacter crescentue*

*cc*XerD: XerD of *Caulobacter crescentue* C-terminal: carboxyl-terminal

DAPI: 4', 6-diamidino-2-phenylindole *ecdif*: *Escherichia coli dif* site

*ec*XerC: XerC of *Escherichia coli* *ec*XerD: XerD of *Escherichia coli*

DIG: digoxygenin DNA: deoxyribonucleic acid

EDTA:ethylenedinitrolotetraacetic acid HJ: Holliday junction

IPTG: isopropyl B-D thiogalactopyranoside LB: Luria-Bertani

MBP: maltose binding protein NaCl: sodium chloride

NEB: New England Biolabs PBS: phosphate bufferred saline

PCR: polymerase chain reaction SDS: sodium dodecyl sulfate

TBE: tris-borate EDTA buffer THA: Todd-Hewitt broth with agar

Ts: thermosensitive α: alpha β: beta λ: lambda

THY: Todd-Hewitt broth with 1% yeast extract

UV: ultraviolet

λ Int: λ phage integrase

3’PO_4: three prime phosphate

Y: tyrosine

3’OH: three prime hydroxyl

ChapterI

INTRODUCTION

1. Site-specific recombination

1.1. Generalities

Genetic recombination is central to DNA metabolism. It promotes sequence diversity and maintains genome integrity in all organisms. Recombination is the breaking and rejoining of DNA in new combinations. This genetic exchange occurs between DNA molecules from the two parents or between two DNA segments within the same molecule. Such recombination may be general, occurring between two DNA substrates with extensive homology, which is called general homologous recombination, or site-specific, occurring between two specific, relatively short DNA targets, which is designated site-specific recombination.

The process of site-specific recombination can be divided into a series of conceptually simple steps. Firstly, the recombinase binds to the two recombination sites. The two recombinase-bound sites pair, forming a synaptic complex with crossover sites juxtaposed. The recombinase then catalyzes cleavage, strand exchange, and the rejoining of the DNA within the synaptic complex. Finally, the synaptic complex breaks down, releasing the recombinant products. From this description, it follows that the minimal components of a site-specific recombination system are a recombinase and a pair of recombination sites. In site-specific recombination, DNA strand exchange takes place between segments possessing only a limited degree of sequence homology (Kolb, 2002;

Coates *et al.,* 2005; Landy, 1989). The recombination sites are typically between 30 and 200 nucleotides in length and consist of two motifs with a partial inverted-repeat symmetry, to which the recombinase binds, and which flank a central crossover sequence at which the recombination takes place. The pairs of sites between which the recombination occurs are usually identical, but there are exceptions e.g. *attP* and *attB* of λ integrase (Landy, 1989). In the site-specific recombination reaction, recombinases perform rearrangements of DNA segments by recognising and binding to short DNA sequences (sites), at which they: (1) cleave the DNA backbone, (2) exchange the two DNA helices involved and (3) rejoin the DNA strands (Stark *et al*., 1992). While in some site-specific recombination systems having just a single recombinase enzyme together with the recombination sites is perfectly adequate to be able to perform all these reactions (Bourgeois *et al*., 2007), in some other systems a number of accessory proteins and accessory sites are also needed.

The reaction catalyzed by the recombinase may lead to different outcomes which are dictated mainly by the relative location and the orientation of sites that are to be recombined, but also by the innate specificity of the site-specific system in question. Intramolecular recombination between inverted or directly repeated sites will invert or excise respectively the intervening DNA segment. Recombination between sites on separate DNA molecules will integrate one molecule into the other (Fig. 1).

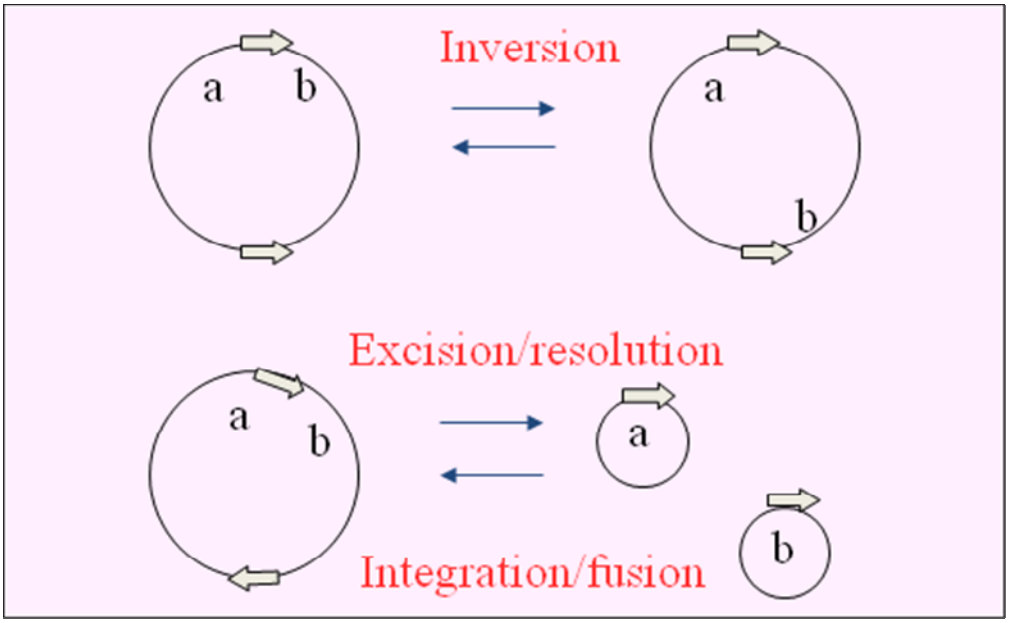

Figure 1. Outcomes from site-specific recombination. Arrows show the orientation of the recombination sites. a and b indicate the position of distinct genetic markers and the recombination loci. 'Excision' and 'integration' refer to recombination events involving genetic entities of different size and /or function (e.g., the bacterial chromosome and a phage genome), whereas 'resolution' and 'fusion' apply to equivalent DNA molecules, (e.g., two plasmids) (Hallet and Sherratt, 1997; with permission).

Most site-specific recombination systems are highly specialised catalyzing only one of these different types of reactions and have evolved to ignore the sites that are in the 'wrong' orientation. The natural site-specific recombination systems are highly specific, fast and efficient, even when faced with complex eukaryotic genomes (Sauer, 1998). As such, site-specific recombination systems are employed in a number of programmed DNA-rearrangement reactions in both prokaryotes and eukaryotes. The different structural consequences of site-specific recombination lead to various biological functions. It includes helping to specify developmental pathways in bacteria and bacteriophages (Sato *et al.*, 1990; Landy, 1993; Carrsco, 1994); determing cell type and virus host range (Zieg and Simon, 1980; Klippel, 1988; Tominaga *et al.,* 1991); processing the products of genetic transposition (Arthur and Sherratt, 1979); and

controlling circular replicon copy number and inheritance (Summers and Sherratt, 1984; Blakely *et al.*, 1991; Stark *et al.,* 1992).

Based on amino acid sequence homology and mechanistic relatedness most site-specific recombinases are grouped into two distinct families: the tyrosine recombinase family or the serine recombinase family. The names stem from the conserved nucleophilic amino acid residue that is used to attack the DNA and which becomes covalently linked to it during strand exchange. The serine recombinase family is also sometimes known as resolvase/invertase family, named after the cointegrate-resolving protein encoded by the transposons *γδ* and Tn*3*. Tyrosine recombinases are also known as the integrase family, named after the prototypical phage *λ* integrase (Argos *et al.,* 1986; Hatfull and Grindley, 1988; Sadowski, 1986; Stark, 1992). The integrase family includes *λ* and many other phage integrases, phage P1 Cre, the bacterial proteins XerC and XerD, and the FLP protein encoded by the yeast 2 *μ*m plasmid. The resolvase family includes most transposon-encoded resolvases and the DNA-invertases such as Hin and Gin. Enzymes of both families catalyze conservative DNA break-join reactions that proceed by two-step transesterifications in which protein phosphodiesters act as reaction intermediates. These two families are unrelated in protein sequence or structure, and employ different recombinational mechanisms, as illustrated in Fig. 2.

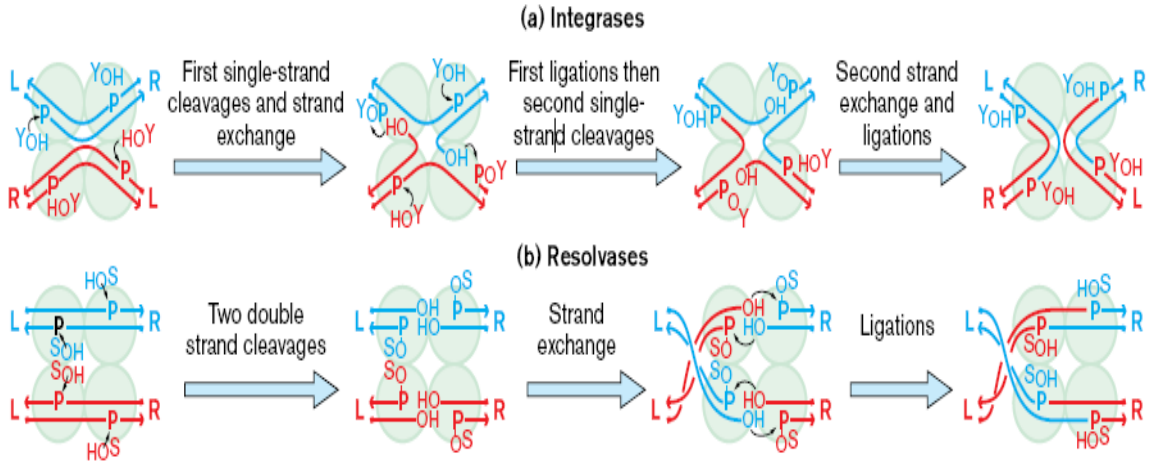

Figure 2. (a) *λ* integrase and its relatives make ordered and sequential pairs of single strand exchanges between the two recombinational partners; the first pair of exchanges form a four-way Holiday junction, the second pair resolves the junction to complete the recombination. The nucleophile used for cleavage and formation of the covalent recombinase–DNA intermediate is a conserved tyrosine (Y_{OH}). The cleavage sites on each DNA duplex are separated by 6–8 base pairs with a 5′ stagger, and the tyrosine joins to the 3′ phosphate. **(b)** *γδ* resolvase and its relatives make double strand breaks in both recombinational partners, then exchange ends and rejoin them. The resolvase nucleophile is a serine (S_{OH}) and it cleaves the DNA at sites that are separated by 2 base pairs with a 3′ stagger, attaching to the 5′ phosphate (Grindley, 1997; with permission).

1.2. <u>The resolvase/invertase family</u>

The resolvase/invertase family forms a rather homogenous group of related proteins in which a conserved serine residue plays a key catalytic role (Hatfull and Grindley, 1988; Leschziner *et al.*, 1995). There are currently approximately 40 different members, ranging in size from 180 to nearly 800 amino acid (aa) residues, and with unexpected variations in domain organization (Smith and Thorpe, 2002). The best-characterized recombinases of this family are the invertases Gin from bacteriophage Mu and Hin from *Salmonella sp*. and the resolvases of Tn*3* and γδ transposons (Stark *et al.,* 1992; Van de Putte and Goosen, 1992; Grindley, 1994; Arciszewska and Sherratt, 1995; Johnson, 1991). Some information regarding serine recombinase domain structure and

function given here has come from the prototypical recombinase, γδ resolvase. This 183-residue protein has an N-terminal catalytic domain of 100 residues, linked by a long (36aa) α-helix (the E-helix) and an unstructured segment (10aa) to a typical helix-turn-helix DNA-binding domain at the C terminus (Yang and Steitz, 1995). The serine nucleophile is close to the N terminus at position 10. γδ resolvase is a dimer in solution, with the N-terminal portion of the E-helix forming the bulk of the dimer interface. DNA binding (at least to the crossover site) involves not only the H-T-H domain but also the C-terminal portion of the E-helix and the intervening segment. The dimer's H-T-H domains bind symmetrically to the DNA, making sequence-specific major groove contacts ~10bp from the central cleavage point; E-helix residues (particularly the conserved Arg-125) hold the DNA (via phosphate and minor groove contacts) close to the cleavage site and the 3′ end of the DNA after cleavage (Li *et al*., 2005); and the unstructured segment snakes along the minor groove between the two (Yang and Steitz, 1995). The H-T-H domain appears to play no important roles outside of DNA binding because it could be replaced by a zinc finger DNA recognition domain in Tn3 resolvase without loss of recombination activity (Akopian *et al*., 2003).

In a recombination catalyzed by serine recombinases, double strand breaks staggered by 2bp occur at the middle of the two paired core sites, giving rise to recessed 5’ ends and 3’-OH overhangs (Fig. 3). One recombinase subunit is linked to each of the 5’ ends through the conserved serine residue of the family (Reed and Moser, 1984; Klippel *et al*., 1988). This serine presumably provides the primary nucleophile hydroxyl group in the cleavage reaction (Leschziner *et al*., 1995). The ligation step that follows strand exchange can be viewed as the converse of the cleavage: the protein-DNA

phosphoseryl bond of one strand is attacked by the 3'-OH end of the partner to release the enzyme and reseal the DNA backbone in the recombinant configuration (Fig. 3). Thus, recombination by a resolvase/invertase family occurs by a mechanism in which four DNA strands are broken and rejoined in a concerted manner. This mechanism is quite distinct from that of the tyrosine recombinases that proceed through the formation and resolution of a Holliday junction (HJ) intermediate, during which the DNA strands are transiently attached to recombiase subunits through phospho-tyrosine linkages (Landy, 1989; Stark *et a*l., 1992; Gopaul and Duyne, 1999; Chen *et al*., 2000). In the serine recombinase reaction, all catalytic processes usually occur within a synaptic complex with two crossover sites and four recombinase subunits (although the Sin recombinase appears to be at least one exception to this, Rowland *et al*., 2002). It is now clear that, in synaptic complexes formed by the serine recombinases, the crossover sites are located on the outside, separated by the catalytic domains (see review Grindley *et al*., 2006). The recently solved crystal structure of a minimal synaptic complex formed by γδ resolvase has elegantly confirmed the "DNA-out" configuration of the crossover site synapse and has thrown new light on the processes of synapsis and strand exchange (Li *et al*., 2005).

Sin is a resolvase of the serine recombinase family that is encoded by various *S. aureus* multiresistance plasmids (Paulsen *et al*., 1994; Rowland and Dyke, 1989). Sin is only distantly related to known resolvases and DNA invertases (e.g. Hin, Gin), although sequence alignment implies that it has a structural fold essentially the same as that of γδ resolvase (31% identical to pI9789 Sin) (Yang and Steitz,1995). The Sin recombination system differs from that of Tn3 and γδ resolvase (Rowland *et al*., 2002; 2005). First is its *res* site, although complex is only 86bp long and binds just two dimers of Sin, and site II

consists of direct (head-to-tail) repeats of the 12bp binding sequence. Second, recombination requires an architectural, DNA-bending protein such as *E. coli* HU or *Bacillus subtilis* Hbsu. Nevertheless, like the transposon-encoded cointegrate resolvases, Sin is specific for an excision reaction (its biological role is likely to be reducing plasmid dimers to monomers to ensure their stability; another possible role, related to the dimer to monomer conversion role, is to generate monomer plasmids after conjugal transfer, where the DNA is transferred by a rolling-circle type mechanism. This could potentially generate multimeric forms which must be converted into monomers.), and the product of recombination *in vitro* is a pair of singly linked, catenated circles. Furthermore, another difference between Sin and Tn3/γδ resolvase is that Sin is catalytically active in the absence of synapsis (presumably as a dimer) and is able to cleave and rejoin isolated crossover sites (without site II or Hbsu) (Rowland *et al*., 2002). Thus, for Sin, synapsis, which is essential for the resolution reaction, may simply be a way of bringing together a pair of recombination sites in a controlled (that is excision-specific) manner. By contrast, for Tn3/γδ resolvase, synapsis not only brings the crossover sites together but also activates the recombinase.

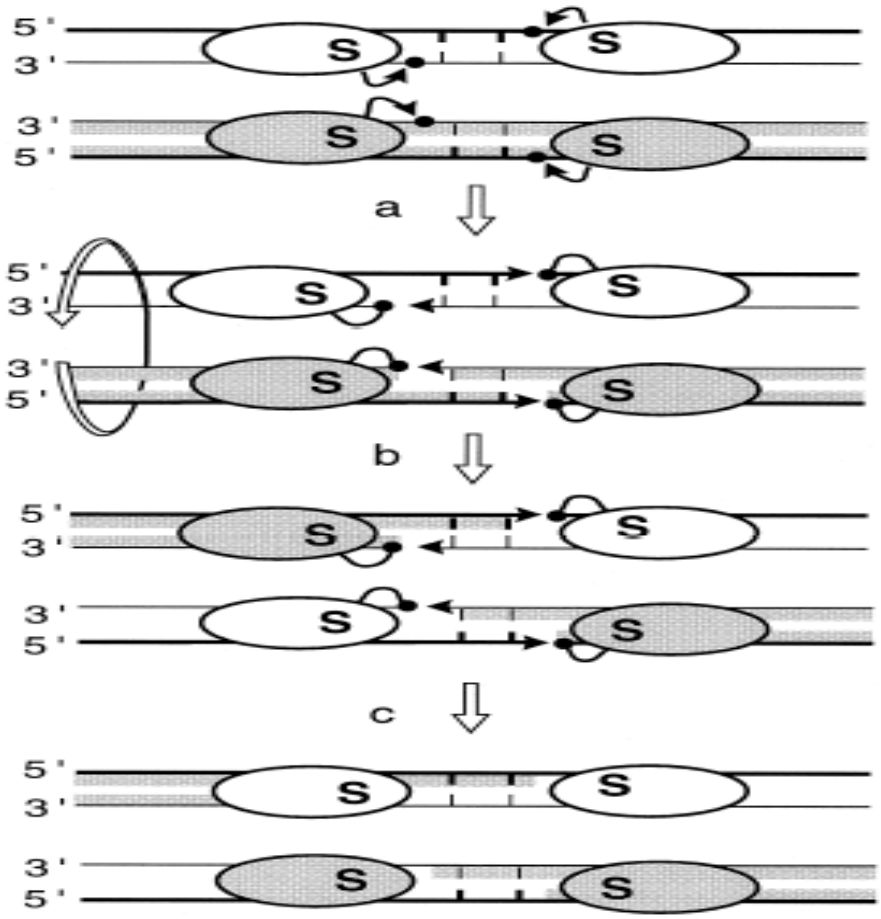

Figure 3. Model of the action of the serine recombianses. The subunit rotation model is shown. The ovals represent recombinase subunits with the conserved catalytic serine'S'. Thick and thin lines are the top and bottom strands of the recombination sites, respectively. The short vertical bars are the 2bp of the overlap region between the two cleavage points. Black arrows represent the nucleophilic attacks of phosphates (black dots) by hydroxyl groups (arrowheads). The four DNA strands are cleaved (a), exchanged by 180 º rotations of the half-site bound subunits (b) and religated in the recombinant configuration (c) (Hallet and Sherratt, 1997; with permission).

1.3. Lambda Integrase Family

1.3.1. Generalities

The lambda integrase or 'tyrosine recombinase' family includes over 100 members identified based on sequence similarity (Nunes-Düby *et al.,* 1998). Tyrosine recombinases are most widespread among prokarytoes but are also found in archaea and

even eukarytoes, where examples have been described in fungi, ciliates, and, most recently certain families of retrotransposons (Nunes-Düby *et al*., 1998; Poulter and Goodwin, 2005). The most well-studied examples include, in addition to the integrase protein from bacteriophage λ (Int) (Landy, 1989), the bacterial XerC and XerD recombinases (Sherratt *et al.,* 1995), Cre recombinase from bacteriophage P1 (Hoess *et al.,* 1985), and the Flp recombinase from the *Saccharomyces cerevisiae* 2μ circle (Sadowski, 1995). These recombinases carry out site-specific recombination in a stepwise manner, exchanging one pair of DNA strands to form a HJ intermediate (Craig, 1988) and then resolving the HJ to products by exchange of the second pair of strands. So, unlike the recombinases of the resolvase/invertase family, tyrosine recombinases exchange the two pairs of DNA strands separately and sequentially (Fig. 2).

Tyrosine recombinases share only limited sequence similarity and are much more divergent, with only four completely invariant residues intimately involved in catalysis: the RHRY tetrad (Argos *et al*., 1986; Abremski *et al.,* 1992; Blakely *et al.,* 1996). Alignments of this integrase family of proteins identified some conserved motifs, which are related to their catalytic function (Nunes-Düby *et al*., 1998; Esposito *et al*., 1997). Although some family members, such as FimB and FimE, contain only this domain, in most the catalytic domains are preceded by a variable N-terminal domain that helps bind DNA. All proteins harbor two conserved regions, Box I and Box II, with marked sequence similarity, originally identified from the alignment of only eight recombinases (Argos *et al.*, 1986). Box I includes the fourth conserved residue R, and Box II contains other three conserved residues, the triad H-R-Y, which includes the active site tyrosine (Abremski and Hoess, 1992; Nunes-Düby *et al*., 1998). The

conservation of Box I is striking in prokaryotic recombinases and it extends with some variation to eukaryotic recombinases. Box II is also relatively strongly conserved among the prokaryotic recombinases, but less so between prokaryotic and eukaryotic proteins. Whereas the active tyrosine is absolutely conserved, the surrounding residues are rather divergent, allowing for quite different secondary structures.

1.3.2. The recombination reaction

To initiate the first strand exchange, the tyrosine residue of the conserved catalytic motif RHRY attacks the phosphate of the scissile phosphodiester in one strand (defined here after as the top strand) of each recombination core sites, thereby forming a 3'phosphotyrosyl-linked- recombinase-DNA complex and generating a free 5'-OH end (Fig. 4). The polarity of this cleavage reaction is thus reversed when compared to that of the resolvase/invertase-mediated cleavages. In the second step, the recombinase-DNA phosphotyrosyl bond is attacked by the 5'-OH end from the partner duplex to generate a four-way branched structure, or HJ intermediate, in which only two DNA strands have recombined. To resolve this intermediate and complete the recombination reaction, the two other (bottom) strands are exchanged by repeating the cleavage/religation process 6-8bp downstream of the first strand cleavage position (Hallet and Sherratt, 1997).

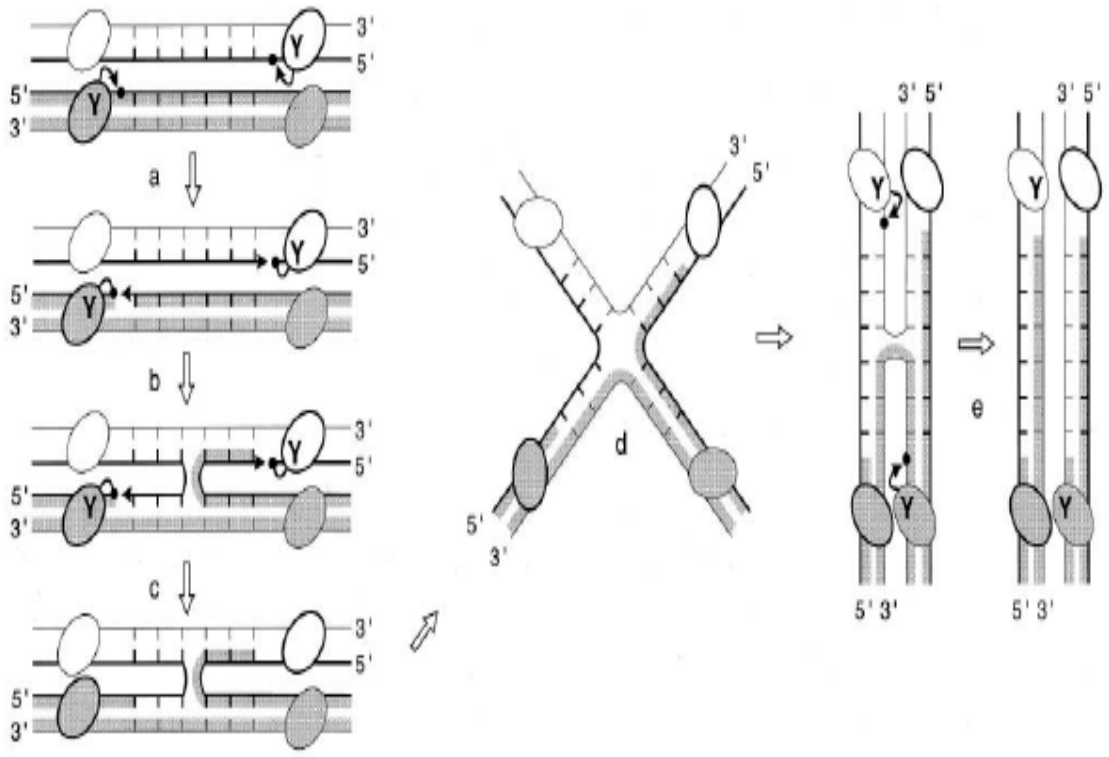

Figure 4. Sequential strand exchange by the tyrosine recombinases. The DNA strand swapping /isomerisation model is presented. The letter 'Y' refers to the conserved catalytic tyrosine. The ovals represent recombinase subunits. Thick and thin lines are the top and bottom strands of the recombination sites, respectively. Black arrows represent the nucleophilic attacks of phosphates (black dots) by hydroxyl groups (arrowheads). The top strands (thick lines) are cleaved first (a), swapped between the two partners (b), and then religated (c). The branch point of the generated HJ intermediate is positioned at the middle of the (6bp) overlap region and the top strands are crossed. Isomerisation of the HJ to a recombination configuration in which the bottom strands are crossed requires the reorganization of the DNA helices and the four half-sites-bound recombinase subunits within the complex (d). The resulting HJ isoform is resolved by repeating steps a to c in order to exchange the bottom strands (e) (Hallet and Sherratt, 1997; with permission).

2. Xer Site-Specific Recombination

2.1 Generalities

The physical state of circular chromosomes, unlike linear chromosomes, can be altered by homologous recombination. Odd numbers of homologous recombination

events between circular replicons during or after replication, produce dimers that need to be converted to monomers before they can be segregated normally at cell division (Austin *et al.*, 1981; Blakely *et al*., 1991; Kuempel *et al*., 1991). Plasmid dimers can also arise as a consequence of rolling circle replication during conjugal transfer and sometimes during vegetative replication (Warren and Clark, 1980; Erickson and Meyer, 1993). The Xer site-specific recombination system was initially discovered in 1984 through its role in converting multimers of ColE1-related multicopy plasmids to monomers and hence ensuring their stable inheritance within *E. coli* (Summers and Sherratt 1984). A model for the coordination of chromosome dimer resolution and cell division has been elaborated in *E. coli* based on a substantial accumulation of *in vivo* and *in vitro* data. In *E. coli*, the Xer site-specific recombination system is composed of two paralogous tyrosine recombinases, XerC and XerD, which cooperately catalyze strand exchanges at a 28bp DNA sequence, the *dif* site (*d*eletion-*i*nduced-*f*ilamentation), which must be located at the junction of the two replichores to be functional (Pérals *et al*., 2000; Blakely *et al*., 1993; Hallet *et al*., 1999). In addition, it has been demonstrated that FtsK protein is required for chromosome dimer resolution *in vivo* (Boyle *et al*., 2000; Steiner *et al.,* 1999) and site-specific recombination at other ectopic *dif* sites (Sciochetti *et al*., 2001). Deletion of the *E. coli dif* site or mutations in *xerC* or *xerD* result in the development of a subpopulation of filamentous cells containing abnormally partitioned nucleoids. Homologues of XerCD and FtsK are found in most eubacterial phyla and some archeal lineages (Recchia *et al.,* 1999) as well as the canonical *dif* site (Hendrickson *et al.,* 2007). Moreover, interactions between the *E. coli dif* site and the XerCD recombinases of *Haemophilus influenza* (Neilson *et al*., 1999), *Pseudomonas aeruginosa*

(Blakely *et al*., 2000), *Bacillus subtilis* (Sciochetti *et al*., 2001), *Proteus mirabilis* (Villion and Szatmari, 2003), and *Caulobacter crescentus* (Jouan and Szatmari, 2003) have been experimentally demonstrated *in vitro*. These observations lead to the general view that Xer recombination is a function conserved among bacteria harboring circular chromosome(s).

The classical Xer site-specific recombination system is an atypical member of the integrase family because, instead of using a single recombinase, it uses two related recombinases, XerC and XerD, each of which catalyses the exchange of one specific pair of strands (Blakely *et al*., 1993, 1997; Colloms *et al*., 1996; reviewed in Sherratt, 1993; Sherratt *et al*., 1995). The use of two recombinases, XerC and XerD, by the classical Xer site-specific recombination system is unusual but not unique. For example, FimB and FimE of *E. coli* mediate an inversion gene switch that regulates expression of type I fimbriae (Klemm, 1986), whereas in S*taphylococcis aureus*, two related tyrosine recombinases of Tn*554* mediate promiscuous site-specific recombination (Murphy, 1989). The use of two recombinases that bind to related yet different half-sites had provided a powerful tool for determining the precise role of different molecules as the recombination reaction proceeds. This system has evolved to ensure that a complete recombination reaction is complete only when very special conditions are met (Colloms *et al*., 1996). Besides the classical *dif*/Xer system, studies with *Streptococci* and *Lactococci* (Le Bougeois *et al*., 2007) indicate that these bacteria carry alternative Xer recombination machinery; an atypical 31bp *dif* recombination site associated with a single dedicated tyrosine recombinase (XerS). In these cases, either one Xer protein has been lost or, assuming that *xerC* and *xerD* genes arose from a single ancestral gene, these

organisms diverged from other bacterial lineages prior to this duplication (Le Bougeois *et al*., 2007). Recently, Carnoy and Roten (2009) analyzed 234 chromosomes from 156 proteobacterial species and showed that a subgroup of ε-proteobacteria display a sequence (*difH*) which is homologous to *dif*SL from *Streptococci* and *Lactococci* and harbor a single Xer-like recombinase (XerH) (Carnoy and Roten, 2009). However, no phylogenic association between XerS and XerH could be found, which strongly suggests the existence of two unrelated *dif*/Xer systems: the classical machinery found in most species and an atypical system present in a sub-group of ε-proteobacteria.

Xer recombination is also distinguished from most other site-specific recombination systems by its different requirements and outcomes, depending on whether it is recombining natural plasmid-borne recombination sites (for example, *cer* and *psi* located on plasmids ColE1 and pSC101 respectively) or the chromosomal site *dif*. Recombination *in vivo* at plasmid-borne *dif* sites occurs intermolecularly and intramolecularly, and is not known to require proteins in addition to the two recombinases. In contrast, recombination *in vivo* at *cer* and *psi* is preferentially intramolecular and requires, in addition to the approximately 30bp recombination core site, about 200bp of adjacent accessory sequences, with which accessory proteins interact in order to assemble a synaptic complex that has a precise architecture and entraps three or four negative supercoils (Alén *et al*., 1997; Colloms *et al*., 1997). Xer recombination on synaptic complexes of precise topology restricts recombination to intermolcular events between directly repeated recombination sites, and therefore acts to convert dimers to monomers (Colloms *et al*., 1996, 1997).

2.2 XerC and XerD

The XerC recombinase was initially identified by its role in resolution of ColE1 plasmid multimers (generated by homologous recombination) to monomers. This recombination is necessary for the stable inheritance of this naturally occurring high copy number plasmid and its relatives (Summers and Sherratt, 1984; Colloms *et al*., 1990). A second recombinase, XerD, was identified by sequence homology to XerC and is encoded in an operon with *recJ* and *dsbC* (Blakely *et al*., 1993; Lovett and Kolodner 1991; Missiakas *et al*., 1994). Both XerC and XerD are identified as members of the tyrosine recombinase family because of the characteristic four strictly conserved amino acids required for catalysis: the Arg-His-Arg triad and the tyrosine nucleophile (Esposito *et al*., 1997; Sherratt and Wigley, 1998). All four residues were found to be in the C-terminal halves of the recombinase proteins, which showed more sequence similarity than the N-terminal halves. XerC and XerD are encoded at 4024kb and 3050kb on the *E. coli* chromosome respectively. Each recombinase is expressed with at least two other proteins that don't appear to have a role in Xer recombination (Colloms *et al*., 1990; Blakely *et al*., 1993). Although *E. coli* XerC and XerD share only 37% identity, they are the closest relatives to each other among the highly diverged tyrosine recombinase family. Similarly, in other eubacteria, XerC-XerD homologues are readily identified by their homology to each other and to their *E. coli* recombinases (Hayes *et al.,* 1997; Neilson *et al.,* 1999; Recchia *et al.,* 1999; and Sciochetti *et al.,* 1999). The genes encoding XerC and XerD are invariably located in different regions of the chromosome, where they often lie adjacent to and may be coexpressed with genes involved in recombination, repair, and cellular response to stress (Recchia *et al.,* 1999).

XerC and XerD proteins can be divided into two domains that make a C-shaped clamp into which the DNA is bound (Gopaul *et al*., 1999). Based on analogy with Cre, α-helices B and D of the N-terminal domain are expected to interact with the major groove formed by the inner palindromic nucleotides of the recombinase binding sites. The N-terminal domain is also expected to contain determinants for protein-protein interaction, whereas the larger, more conserved C-terminal domain contains the catalytic residues and determinants for specific major groove DNA binding and protein-protein interactions. The residues proposed to be involved in specific major groove DNA binding are located within α-helices G and J, with R221 and Q222 being implicated in providing specificity (Subramanya *et al*., 1997). XerD shows a stronger affinity for its binding site than XerC, each of them binding more tightly to its site in the presence of the partner (Blakely *et al*., 1993, 1997; Spiers *et al*., 1999).

In site-specific recombination mediated by tyrosine recombinases, two pairs of DNA strand exchanges are separated in space and time with HJ being a recombination intermediate. In Xer recombination (Fig. 5), one pair of strand exchanges is catalyzed by XerC, while the other pair of strand exchanges is mediated by XerD (Neilson *et al*., 1999; Colloms *et al*., 1996; Arciszewska *et al*., 1995). Recombination between *psi* sites is initiated by XerC-mediated strand exchange to give an HJ intermediate that is resolved to recombination products by XerD. Strand exchange by XerC initiates recombination at *cer*, although the resulting HJ intermediate is resolved by Xer-independent celluar processes rather than by XerD (Colloms *et al*., 1996; McCulloch *et al*., 1994). In the absence of FtsK, XerC can catalyse the formation of HJ intermediates between *dif* sites and their conversion back to substrate (Barre *et al*., 2000). The presence of FtsK and ATP

leads to a remodeling of the nucleoprotein synaptic complex, so that reaction between two duplex *dif* sites is now initiated by XerD, forming an HJ intermediate that can be acted on by XerC, thereby completing the dimer resolution reaction (Aussel *et al*., 2002; Recchia *et al*., 1999). A switch in the preferred order of strand exchange at *psi* occurs when the core site is inverted with respect to the accessory sequences (Bregu *et al*., 2002).

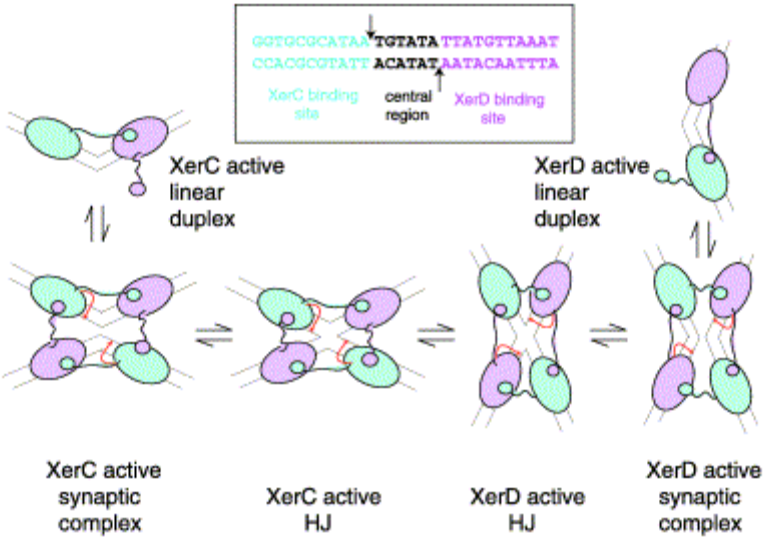

Figure 5. Schematic of a model for the control of catalytic activity within the XerCD–DNA complex. On binding to recombination site DNA, the XerCD–DNA complex preferentially adopts a conformation in which XerC (blue ovals) has its C-terminal end region extended across the wide angle of DNA arms to the receptor region of XerD (purple ovals). Recombinase-mediated synapsis of two such duplexes forms a complex, which is primed for nucleophilic attack (red arrows) by XerC. In this conformation, monomers of XerD, whose C-terminal regions span the acute angle between the DNA arms, have their C-terminal regions compacted and are inactive. A pair of coordinated strand exchanges gives HJ intermediate, which XerC can reconvert to substrate. Alternatively, the HJ complex can undergo a conformational change to form HJ that is primed for catalysis by XerD. A pair of strand exchanges by XerD can then generate complete recombinant products. The conversion of a XerCD–DNA synaptic complex, or a XerCD HJ, to a conformation that allows catalysis by XerD is facilitated by accessory proteins like FtsK and PepA. It is proposed that one role of the accessory

factors is to allow the recombining complex to adopt the energetically less favourable conformation required for catalysis by XerD. The inset shows a *dif* site with the arrows indicating the cleavage sites for catalysis by XerC (top) and XerD (bottom) (Ferreira *et al*., 2003; with permission).

Structural, biochemical, and genetic experiments have contributed to our understanding of the ways in which the activities of the four recombinase molecules of a recombining complex are coordinated so that the two pairs of strand exchanges occur at the correct time and in the correct place. Crystal structures of the Cre–*loxP* synaptic and HJ complexes reveal a tetramer of recombinase molecules assembled on two, antiparallel DNA duplexes in a four-way, almost planar conformation (Guo *et al*., 1997,1999; Gopaul *et al*.,1998, 1999). The structures of both the synaptic and HJ complexes are similar and indicate the existence of two conformationally different forms of the Cre–DNA complex, each appropriate for exchange of one specific pair of DNA strands. Only a subtle change in the overall architecture of the complex is required to make a switch between the two conformational states. Genetic and biochemical experiments with XerCD support this general model. Special mutations in either the presumptive donor or acceptor regions of XerCD, and indeed at other specific positions, lead to mutant phenotypes that exhibit either reciprocal stimulation of catalysis by partner and impairment of catalysis by self (the SPIS phenotype), or reciprocal impairment of partner catalysis and stimulation of catalysis by self (the IPSS phenotype) on synthetic HJ substrates (Arciszewska *et al*., 2000; Hallet *et al*., 1999; Spiers *et al*., 1999). In the model of Xer recombination, the C-terminal arms of the XerC monomers are preferentially in an extended conformation that allows them to span the greater angle between adjacent DNA arms in the HJ intermediate, and position the tyrosine nucleophiles for attack at the scissile phosphate groups on the more acute “crossing” strands. The C-terminal regions of XerD are in the

non-extended conformation, but likely make interactions important for duplex synapsis. Small movements in DNA and protein allow the change in conformation that reciprocally activates the second pair of recombinases, XerD, while inactivating the other recombinase pair, XerC.

A range of experimental data have supported the view that the XerCD-DNA complex preferentially assumes that conformation for XerC strand exchange, irrespective of whether the DNA substrate is two synapsed duplexes or a HJ intermediate(Colloms *et al*., 1996; McCulloch *et al*., 1994). This appears to be an intrinsic consequence of the XerC and XerD structures and the way they interact with each other and their substrate DNA. The nucleotide sequence of the central region can influence this preference, at least on HJ intermediates. In particular, the purine richness of a strand in the central region predisposes it to be a preferential substrate for the particular recombinase that acts on that strand (subject to the natural bias resulting from the two recombinases and their interactions) (Arciszewska *et al*., 1997; Azaro *et al*., 1997).

A major conclusion from these experiments is that the relative concentrations of the two HJ intermediate forms determine the relative levels of catalysis mediated by XerC and XerD. The use of two recombinases in the Xer system not only insures that XerC will initiate catalysis, but provides an editing mechanism; catalysis by XerD is only favored on substrates that can adopt a conformation in which the XerD substrate strands are crossed. Even then, additional factors are required to overcome the thermodynamic and/or kinetic barriers required to form the appropriate substrate for catalysis by XerD.

2.2.1 XerC

2.2.1.1 Generalities

The *xerC* gene maps close to the *E. coli* origin of replication, *oriC*, at 85 min (3700kb). This gene encodes a protein with a calculated molecular mass of 33.8kDa. The translated protein sequence of XerC contains two regions, which are homologous to the two conserved domains of the lambda integrase family of site-specific recombinases (Argos *et al.,* 1986; Colloms *et al*. 1990). Domain 2 of the XerC sequence has three totally conserved amino acids, histidine (H), arginine (R), and tyrosine (Y), as well as other less conserved amino acids. The XerC sequence has 32% amino acid identity to the *E. coli* proteins FimB and FimE in an alignment covering about 160 amino acids. These two proteins are involved in inverting a segment of the *E. coli* chromosome to switch fimbrial antigen (Klemm 1986). Within conserved domain 2, the XerC sequence shows considerable similarity (66% identity) to an integrase-like inferred protein sequence from plasmid R46 (Hall *et al.*, 1987). Given this similarity to the lambda integrase family, XerC presumably catalyzes recombination by a mechanism similar to that of these other recombinases.

2.2.1.2 Function

The stable inheritance of natural muticopy plasmids related to ColE1 requires the function of the Xer site-specific recombination system that convert multimers to monomers, thus increasing the number of segregating units (for example, *cer* in ColE1; Summers and Sherratt, 1984). Two *E. coli* chromosomal genes, *argR* and *pepA*, which were absolutely required for site-specific recombination at *cer* were found (Stirling *et al.,*

1988, 1989). The *E. coli xerC* gene was the third unlinked chromosomal gene to be identified as necessary for site-specific recombination at ColE1 *cer* (Colloms *et al.,* 1990). Together, ArgR (originally XerA), PepA (originally XerB), XerC (and XerD) are required to maintain ColE1 and related plasmids in a monomeric state, thus ensuring their stable inheritance (Stirling *et al.,* 1988, 1989; Summers, 1989).

In addition to its role in converting multimers of plasmid ColE1 to monomers, XerC also has a role in the segregation of replicated chromosome at cell division. *xerC* mutants form filaments with aberrant nucleoids that appear unable to partition properly. A DNA segment (*dif*) from the replication terminus region of *E. coli* binds XerC and acts as a substrate for Xer-mediated site-specific recombination when inserted into multicopy plasmids. This *dif* segment contains a region of 28bp with sequence similarity to the crossover region of ColE1 *cer* (Blakely *et al*., 1991). Therefore, XerC not only functions in maintaining ColE1-like plasmids in the monomeric state, but also has a role in normal *E. coli* chromosomal metabolism, which resolves chromosome dimers to monomers prior to cell division.

2.2.2 XerD

2.2.2.1 Generalities

During the characterization of the RecJ exonuclease of *E. coli*, an open reading frame was reported and showed sequence similarity to the integrase family of site-specific recombination (Lovett and Kolodner, 1991). This gene, designated *xerD* (originally designated *xprB*) (Blakely *et al.,* 1993), appeared to be part of the same transcriptional unit as another open reading frame (designatd *xprA*) and *recJ*. The

predicted amino acid sequence of the XerD protein showed a 37% amino acid identity to XerC. Both XerC and XerD are predicted to have 298 amino acids. A high degree of sequence conservation between XerC and XerD is present in domain I and II, regions highly conserved in all integrase family recombinases (Blakely *et al.,* 1993).

2.2.2.2 Function

The fact that XerD contain the same conserved residues as in XerC implies that both proteins are required for catalysis in Xer site-specific recombination. Experiments showed that both *xerD* and *xerC* mutants failed to support Xer-mediated site-specific recombination, as judged by their failure to recombine either *cer*- or *dif*-contianing reporter plasmids. Furthermore, a plasmid containing a functional *xerD* gene complemented the defect of *xerD* mutant strains, but not that of the *xerC* mutant. Conversely, a plasmid containing a functional *xerC* gene complemented the *xerC* mutant but not the *xerD* mutant. The *xerD* gene was cotranscribed with two other genes, *xprA* and *recJ*. Insertion of Tn*10-9* into *xprA* and *recJ* did not generate a Xer^{-} phenotype. In contrast, an insertion in the *xerD* gene gave a Xer^{-} phenotype suggesting *xerD* is transcribed from its own promoter. A plasmid containing a deletion that removes most of the *xerD* gene fails to complement the *xerD2* mutation (insertion of a transposon named Tn*10-9* in the *xerD* gene after nucleotide 846), whereas a plasmid deleted for more than half of the *xprA* gene complemented the *xerD2* defect. Therefore, it was concluded that, not only XerC, but also XerD were required for site-specific recombination at *cer* and *dif* (Blakely *et al.,* 1993). The two genes that were coexpressed with *xerD (xprA* and *recJ*) have no apparent role in Xer site-specific recombination. The putative catalytic sites of both XerC and XerD are required for normal Xer site-specific recombination *in vivo*.

XerC and XerD bind separately and cooperatively to the *dif* and *cer* sites *in vitro*. The cooperativity could occur as a consequence of specific interactions between XerC and XerD once they have bound to their respective half sites, or by changes in DNA structure that arise as a consequence of binding either recombinase. XerC has a higher affinity for the left-half site of *dif* than the right half-site. XerD binds preferentially to the *dif* right-half site (Sherratt *et al.,* 1997). Efficient recombination at the *dif* site requires the presence of both recombinases, XerC and XerD, though only one is needed to be catalytically active during each pair of strand exchanges, as judged by experiments using mutants defective in the active site tyrosine or the domain II arginine (Arcizewska and Sherratt, 1995; Colloms *et al*., 1996). Nevertheless, a given recombinase can influence the catalytic activity of its partner. A requirement for both recombinases to be bound at a recombination site to render either XerC or XerD competent for strand cleavage provides a means of controlling recombination by preventing initiation of strand exchange when only a single recombinase molecule is bound to a duplex recombination site.

2.2.3 The Catalytic Mechanism of XerC and XerD/Structure and function

Site-specific recombination mediated by the *E. coli* Xer system requires two related proteins, XerC and XerD, each of which is responsible for the exchange of one pair of strands in Xer recombination. Both recombinases encode functions necessary for sequence-specific DNA-binding, co-operative XerC/XerD interactions, synapsis and catalysis. In Xer site-specific recombination, DNA strands are cleaved and rejoined through the formation of a transient DNA–protein covalent intermediate involving a conserved tyrosine as the catalytic nucleophile. The same mechanism is used by the related type IB topoisomerases (reviewed in Sherratt and Wigley, 1998). However, type

IB topoisomerases break and reseal the same phosphodiester bond to remove supercoils in DNA, whereas XerC and XerD catalyze two consecutive pairs of strand exchanges, with the formation of a HJ as a recombination intermediate. Each reciprocal strand exchange reaction is a concerted two-step process in which the 3' phosphotyrosyl DNA–protein bonds generated by cleavage of one DNA strand in each recombination site are subsequently attacked by the free 5' OH ends of the partner sites. DNA strands are exchanged by swapping of a few central region nucleotides (Nunes-Düby *et al*., 1995; reviewed in Guo *et al.*, 1999). This mechanism implies that specific pairs of active sites are sequentially switched on and off in the recombinase tetramer to ensure that appropriate DNA strands will be exchanged at both reaction steps.

How do XerC and XerD interact with their recombination site DNA and then mediate recombination? The convergence of sustained biochemical efforts over many years (Landy, 1993) and the more explosive advances in structural studies (Guo *et al*., 1997; Hickman *et al*., 1997; Kwon *et al*., 1997; Subramanya *et al*., 1997; Gopaul *et al*., 1998) now offer a very detailed view of tyrosine recombinases. The structure of XerD has been solved at 2.5Å resolution and reveals that the protein comprises two domains (Subramanya *et al.,* 1997). Domain 1 consists of residues 1-107, while domain 2 comprises residues 108-298. Domain 1 contains four α-helices. Domain 2 is also mainly α-helical, but with a three–stranded antiparalled β-sheet along one edge (Fig. 6). The fold of this domain is similar to that determined for λ and HP1 integrase (Hickman *et al*., 1997; Kwon *et al.,* 1997). Domain 1 and Domain 2 of XerD correspond to domains of λ Int, HP1 Int and FLP identified by limited proteolysis (Moitoso de Vargas *et al.,* 1988;

Evans *et al.,* 1990; Chen *et al.,* 1991; Pan and Sadowski, 1993; Sadowski, 1995; Hickman *et al.,* 1997; Kwon *et al.,* 1997).

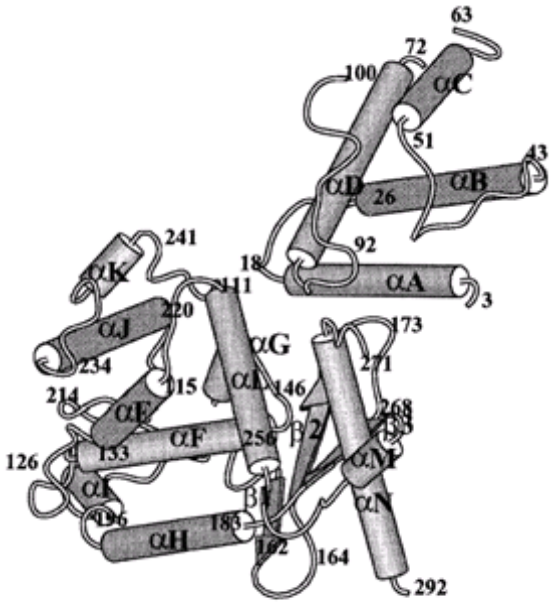

Figure 6. Overall structure of the XerD protein. The numbering refers to the beginning and end of secondary structural elements. Residues that are not defined are located at the N- and C-termini and in three disordered loops (residues 64–70, 101–110 and 269-270). (Subramanya *et al*., 1997; with permission).

The region of structural homology within the C-terminal regions of XerD, λ Int and HP1 Int spans ~170 residues (Fig. 7). Two conserved sequence motifs are located in domain 2 of XerD. The locations of motif I and the N-terminal portion of motif II are similar in the structure of XerD (residues 145–159 and 244–281, respectively) and those of λ and HP1 integrases (Hickman *et al.*, 1997; Kwon *et al.*, 1997). However, the extreme C-terminal portions of these proteins, which include the C-terminal portion of motif II, are quite different (Fig. 7). In λ Int, these C-terminal residues (334–356) form a flexible loop that is disordered in one of the two molecules in the asymmetric unit, but is more ordered in the other, where the final 15 residues form two additional β-strands

along one edge of the antiparallel sheet. In HP1 Int, this region (residues 307-337) forms an extended structure which protrudes from the surface of the protein molecule and contains two short helices. This region is involved in crystal contacts which are proposed to be representative of one of the protein dimer interfaces during the recombination reaction. By contrast, in XerD this region (residues 271–298) forms a turn followed by a long alpha-helix, containing the active site tyrosine, which extends almost to the C-terminus (Subramanya *et al.,* 1997).

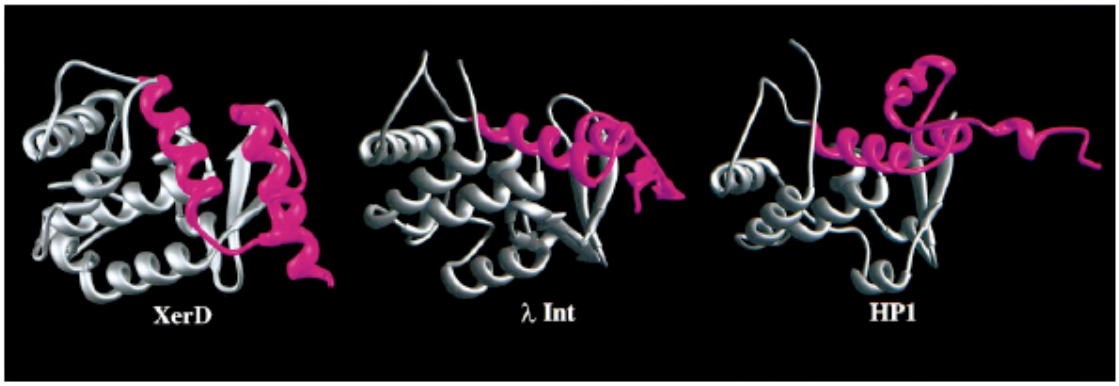

Figure 7. Comparison of the structures of the C-terminal domains of XerD, λ Int and HP1 Int. Regions of the C-terminal domains of the proteins that show the greatest structural similarity are shown in grey. The major structural differences (shown in magenta) are located in the polypeptide segments that extend from conserved motif II (Argos *et al.*, 1986) to the C-terminus of the proteins. (Subramanya *et al*., 1997; with permission).

The structures of the catalytic domains of λ and HP1 integrases suggested how DNA might interact with the C-terminal region of these proteins (Hickman *et al*., 1997; Kwon *et al*., 1997). Combined with the observation of electrostatic surface potential of domains of XerD and biochemical footprinting data, it supports the view that both domain 1 and domain 2 contribute to the DNA-binding surface of the protein. Hence, in order for XerD to bind to DNA, there has to be a large conformational change to allow

access of the DNA to the active site region (Subramanya *et al*., 1997). The proposed base-specific contacts between the XerD recognition helix, αJ and recombination site DNA are very similar to the comparable CAP-DNA contacts (Schultz *et al*., 1991), with both the CAP and the XerD recognition helices being oriented in the same way. In the model showed in Fig. 8, XerD residues 220R and 221Q could make base-specific contacts at precisely the position that have been identified as being important for XerD binding and XerD-XerC binding specificity. Moreover, examination of known XerD and XerC recombinases shows that all XerD recombinases have the equivalent of position 220R and 221Q, whereas the XerC recombinase has a conserved R in place of Q at the equivalent of position 221 and a non-conserved residue in the preceding position (Subramanya *et al*., 1997). Other putative Xer recombinase sequences present in the databases have either RQ at the positions corresponding to 220 and 221, respectively, or a conserved R at the position corresponding to 221, preceded by a non-conserved residue. This indicates that these presumptive recombinases can be classified as either XerC or XerD proteins on the basis of the amino acid sequence at positions corresponding to 220 and 221, and that these amino acids may provide much of the discrimination that directs XerC and XerD to their specific DNA-binding sites. The weaker binding of XerC, and the reduced bending it appears to induce, may be a consequence of fewer base-specific contacts. Furthermore, the high conservation of amino acid residues at these two positions in XerD recombinases from different bacteria (and at the one position in different XerC enzymes) suggests a very strong functional selection for the maintenance of specific recombinase-DNA contacts in these enzymes.

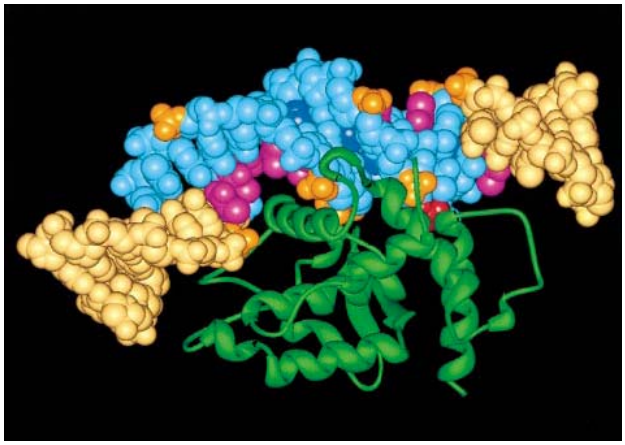

Figure 8. Model of XerD bound to DNA. Model of XerD domain 2 bound at its recognition sequence, derived from the CAP–DNA complex. The protein is shown in green as a ribbon, while the DNA is shown as a space-filling representation. Residues of the DNA within the OP-Cu footprint are shown in cyan, and those outside of the footprint in beige. Specific contacts, as shown by interference binding analysis, are overlaid in orange (phosphates), blue (adenine minor groove contacts) and magenta (thymine, major groove). The scissile phosphate is shown in red. (Subramanya *et al*., 1997; with permission).

The DNA-binding properties of deletion and pentapeptide insertion mutants also agree well with the model above (Fig. 8) in which helix αJ of domain 2 is the recognition helixes that interact with DNA. A truncated XerD derivative containing residues 1-233 is proficient in DNA binding and retains all but the last residue of helix αJ. In contrast, an even shorter XerD derivative, that is deleted for the six C-terminual residues of helix αJ, is binding-deficient (Spiers and Sherratt, 1997). The 11bp XerD- and XerC-binding sites can be subdivided into two regions; the inner four nucleotides, that are dyad-symmetrical in the XerC- and XerD-binding sites, and the outer seven nucleotides, at least four of which contribute to specific XerD binding (Blakely and Sherratt, 1994; Blake *et al*., 1997; Sherratt *et al*., 1997., Hayes and Sherratt, 1997). 1, 10-Phenanthroline-Copper (OP-Cu) intercalates into DNAthrough the minor groove, from where it can cleave the DNA backbone. The OP-Cu "footprint" made by XerD covers the whole of the XerD DNA-

binding site as indicated in Fig. 8. The resistance of the outer part of the site to cleavage by OP-Cu could be the consequence of a widening of the minor groove because of the proposed link in this region, thus preventing intercalation of the footprinting reagent. The resistance of the minor groove, on the inner part of the site and the proximal part of the central region, to OP-Cu cleavage could result from the interactions of residues 236-245 with the minor groove on the "front" face of the DNA viewed in Fig. 8, as well as interactions from "behind" the DNA by the antiparallel strands β2 and β3.

The model for DNA binding to XerD presented in Fig. 8 may also have important implications for the cooperative interactions that occur between XerC and XerD on DNA binding (Blakely *et al*., 1993; Blakely and Sherratt, 1996b; Spiers and Sherratt, 1997). Fig. 9 shows how XerC and XerD might interact when bound together at a *dif* site (Subramanya *et al*., 1997). A truncated XerD protein containing residues 1-268 is able to bind to DNA and to interact cooperatively with XerC, while a protein containing residues 1-262 lacks cooperativity, although it binds DNA normally (Spiers and Sherratt, 1997). Pentapeptide insertions into XerD also define this region as being important for cooperative interaction with XerC (Cao, *et al*., 1997). A second region likely to be involved in XerC-XerD interactions is defined by a XerD mutant containing a tripeptide substitution at residues 256-258(Hallet *et al*., 1999). The mutant protein is proficient in XerD cleavage and strand exchange, and can undergo cooperative interactions with XerC. Nevertheless, it is unable to promote efficient catalysis by XerC, thus identifying a region of XerD involved in activation of XerC catalysis. Moreover, XerC appears to induce a smaller bend than XerD (Blakely and Sherratt, 1996b). It is possible that the

XerD-XerC interactions utilize amino acid differences between the recombinases in the interface region.

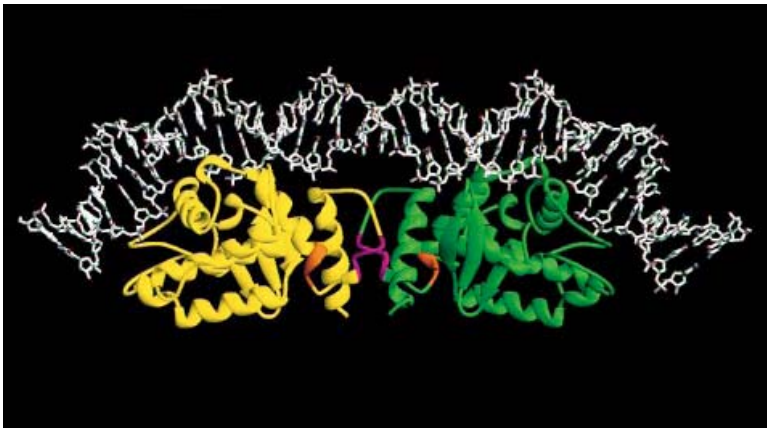

Figure 9. A model of the complex between XerC, XerD, and DNA. Ribbon representation of the catalytic domains of the two recombinase proteins at a *dif* site. Regions of the proteins implicated in XerC-XerD interactions are coloured in orange (residues 256-258) and magenta (residues 263-267) (Subramanya *et al*., 1997; with permission).

It is evident from this model indicated in Fig. 9 that the C-terminal helix, which contains the active-site tyrosine at one end, forms a major part of the interaction of XerD with XerC. The C-terminal domains of XerC and XerD interact with one face of their DNA substrate and carry the determinants for sequence-specific DNA binding, for catalysis, and for recombinase-recombinase interactions. Alteration of the interaction in which XerC donates its C-terminus to XerD impairs the activation of XerC and stimulates XerD catalytic activity, whereas alteration of the reciprocal interaction between XerD C-terminus and XerC acceptor region inhibits XerD and stimulates XerC (Ferreira *et al*., 2003). Therefore, the catalytic activity of XerC and XerD is controlled by allosteric-type interactions in which donor–acceptor region interactions between adjacent recombinase molecules act as molecular springs in the switch that leads to sequential and synchronized activation/inactivation of pairs of recombinase subunits during

recombination (Fig. 10) (Hallet *et al*., 1999). These results support the hypothesis that the "normal" state in the heterotetrameric complex, in which XerC is catalytically active and XerD is inactive, depends on the interactions between the C-terminal end of XerC and its receptor region within the C-terminal domain of XerD; Interference with these interactions leads to a switch in the catalytic state, so that XerD is now preferentially active (Ferreira *et al*., 2003).

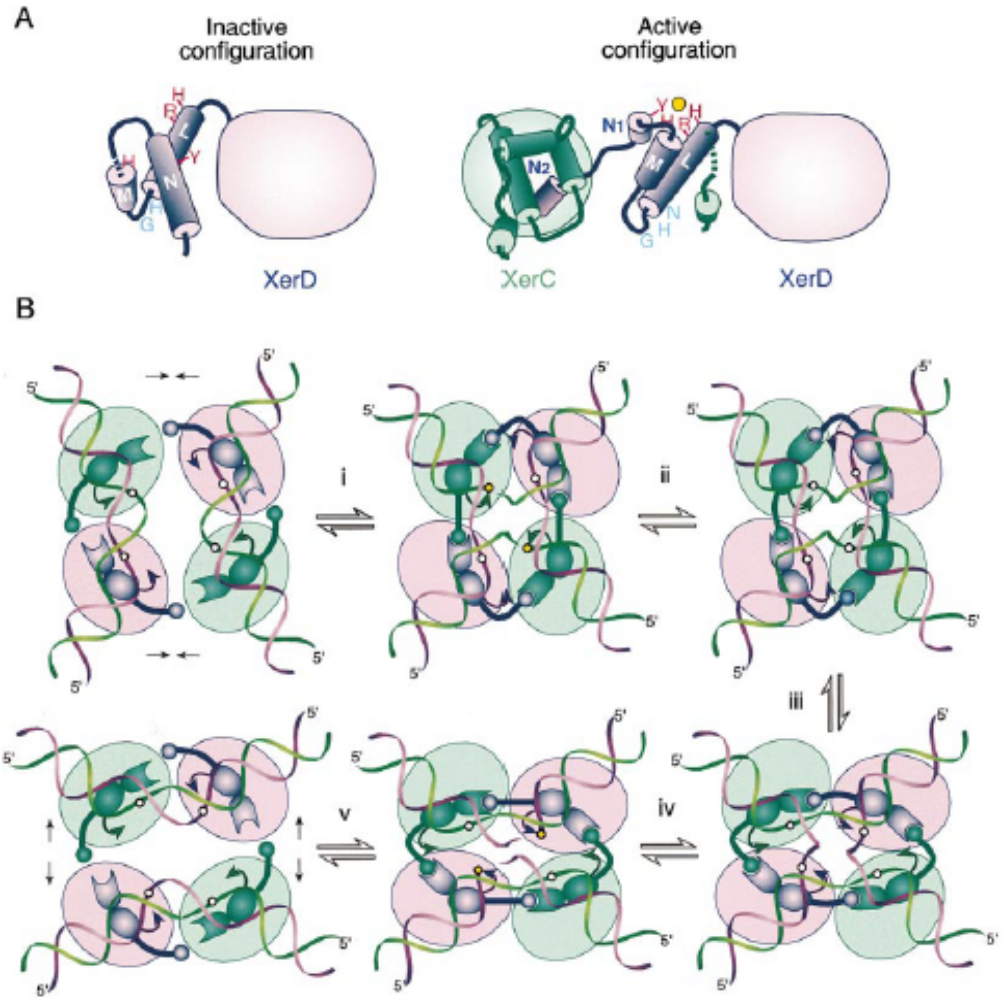

Figure 10. Control of Catalysis in Xer Recombination. (A) Proposed reconfiguration of XerD C-terminus upon assembly of the recombination complex on DNA. (B) A model for the reciprocal control of catalysis by XerC and XerD Color code is as in (A). The ball-and-socket joint depicts the interaction between the donor and

acceptor regions of adjacent subunits. Step i to step v is the recombination pathway in which XerC strand exchange occurs first. (i) Interactions between XerC and XerD molecules bound on a same duplex, possibly coupled with additional interprotomer interactions across the synapse, force the DNA to bend in a configuration where the top (green) strand of the recombination site central region is exposed toward the outside of the duplex. The torsion energy stored in the bent DNA may act on the XerC–XerD donor–acceptor interaction so as to activate XerC catalysis by repositioning of the tyrosine nucleophile (arrowhead), and possibly other catalytic residues with respect to the DNA target phosphate (circle). DNA torsion strains released upon cleavage may also promote the unwinding and extrusion of the cleaved strands in order to orient the 5′ OH ends for the rejoining step. (ii) Completion of the strand exchange reaction generates a 2-fold symmetric HJ intermediate in which the top strands are crossing. (iii) Coupled protein and DNA conformation changes convert the complex into a configuration in which the bottom strands (purple) are crossing. (iv)This leads to synchronized inactivation of the XerC subunits and concomitant activation of the XerD subunits. (v) The recombinant duplexes are bent in the opposite direction to that of the initial recombination sites. This inversion of the DNA bending strains may promote the restacking of the DNA helices and the dissociation of the resealed molecules from the complex (Hallet *et al*., 1999; with permission).

2.3 The Site of Action of the Xer Recombinases

Xer recombinase-mediated recombination occurs in two different recombination sites that have different biological functions. One is at chromosome recombination sites called *dif*, originally found in *E. coli*. Another is at plasmid sites such as ColE1 *cer* and pSC101 *psi.* The Xer site-specific recombination is conserved in most eubacteria (Recchia and Sherratt, 1999). The alignment of 19 naturally occurring plasmids and some eubacterial chromosomes revealed that the wide existence of the homologues of Xer recombination core site (Table 1) (Hayes *et al.,* 1997; Lesterlin *et al*., 2004). One difference between the sites is that *cer* is flanked by accessory sequences, which bind additional proteins and enhance recombination between sites in dimers. This provides directionality so that resolution of dimers to monomers is highly favoured (Blakely and Sherratt, 1996). Flanking sequences are not involved in resolution at *dif* as the phenotype of a 173kb deletion can be suppressed by insertion of a 33bp *dif* sequence (Tecklenburg

et al., 1995). A comparision between the binding sites shows that XerC binding sites are more variable whereas XerD binding sites are well conserved. Neither half-site can be used to replace the other half-site for recombination *in vivo*. The central region of the Xer sites, which displays no consensus and separates XerCD binding sites by a 6 (chromosome site) or 8bp (plasmid site) spacer, is a key determinant of the Xer recombination pathway. It determines the requirements for accessory proteins and accessory sequences on the plasmid recombination site (e.g. ColE1 *cer* site or pSC101 *psi* site). It also determines the presence of FtsK in chromosome dimer resolution (Barre *et al.,* 2001). Several sets of data, obtained on Xer systems and other tyrosine recombinase systems, indicated that this region is an important determinant of the comformation of the recombinase-core sequence complexes (Azaro and Landy, 1997; Gopaul *et al*., 1998; Arciszewska *et al*., 2000; Lee and Sadowski, 2001; Capiaux *et al.,* 2002). Based on the dyad symmetry of the half-sites and by anology with the cleavage positions from other recombinases (Hoess *et al*., 1986; Bruckner *et al*., 1986), the boundaries of the central region and recombinase binding sites have been proposed to contain the bases involved in strand nicking and exchange (Summers, 1989).

Table 1. Alignment of *dif* sites from different bacteria and core sequences of plasmid-borne Xer sites (Hayes and Sherratt, 1997; Lesterlin *et al*., 2004; with permission)

Origin	XerC binding site	Central region	XerD binding site
Plasmids Sites			
ColE1 *cer*	GGTGCGTACAA	TTAAGGGA	TTATGGTAAAT
ColA *car*	GGTGCGTACAA	--CGGATG	TTATGGTAAAT
CloDF13 *parB*	GGTACCGATAA	--GGGATG	TTATGGTAAAT
ColK *ckr*	GGTGCGTACAA	TTAAGGGA	TTATGGTAAAT
NPT16	GGTGCGCGTAA	-TGAGACG	TTATGGTAAAT
pMB1	GGTGCGTACAA	TTAAGGGA	TTATGGTAAAT
pSC101 *psi*	GGTGCGCGCAA	--GATCCA	TTATGTTAAAT
ColE2	GGGGCGTACAA	--CGGGAG	TTATGGTAAAT
ColE3	GGTGCGTACAA	--CGGGAG	TTATGGTAAAT
ColE4-CT9	GGTGCGTACAA	--CGGGAA	TTATGGTAAAT
ColE5-099	GGTACGTACAA	--CGGGAG	TTATGGTAAAT
ColE6-CT14	GGTGCGTACAA	--CGGGAG	TTATGGTAAAT
ColE7-K317	GGTGCGTACAA	--CGGGAG	TTATGGTAAAT
ColE9-J	GGTACGTACAA	--CGGGAG	TTATGGTAAAT
Chromosome Sites(*dif*)			
E. coli	GGTGCGCATAA	--TGTATA	TTATGTTAAAT
S. typhimurium	GGTGCGCATAA	--TGTATA	TTATGGTAAAT
V. cholerae chrI	GGTGCGCATAA	--TGTATA	TTATGGTAAAT
V. cholerae chrII	ATGGCGCATTA	--TGTATG	TTATGGTAAAT
H. influenzae	AATGCGCATTA	--CGTGCG	TTATGGTAAAT
B. subtilis	ATTTCGCATAA	--TATAAA	TTATGGTAAAT
C. crescentus	ACTTCCTAGAA	--TATATA	TTATGTAAACT
P.mirabilis	GGTTCGCATAA	--TGTATA	TTATGTTAAAT

2.3.1 Chromosome Recombination Sites

2.3.1.1 *Escherichia coli dif*

2.3.1.1.1 Position and Polarity

Termination of chromosome replication is a key event in the bacterial cell cycle because it is a time when chromosome structure and cell division must interact properly or cells will fail to enter the next cell cycle correctly. Two main sites of the circular *E. coli* chromosome are implicated in the cell cycle: *oriC*, where replisomes are assembled for bidirectional replication (Messer *et al*., 1996), and the diametrically opposite *dif* site, where chromosome dimers are resolved (Blakely *et al.,* 1991; De Massy *et al*., 1987; Steiner *et al*., 1998a). In *E. coli*, as in most bacteria, *dif* is located in the replication terminus region shown in Fig. 11(Hendrickson and Lawrence, 2007), directly opposite to *oriC*, which is at min 34.2 of the genetic map, 1600kb of the physical map, between the innermost terminators *terA* and *terC* (Kuempel *et al*., 1991). The *dif* locus is a RecA-independent resolvase site in the middle of the terminus. It has been discovered that this position is crucial for dimer resolution (Leslie and Sherratt, 1995; Tecklenburg *et al*., 1995; Cornet *et al*., 1996; Kuempel *et al*., 1996). Dimers arise from sister chromatid exchange (SCE). Dimers can also result from a break at or near a replication fork, followed by invasion of the intact circle by the double-stranded end (Kuzminov, 1995). Resolution of dimers is important for ensuring proper segregation of newly replicated chromosomes to daughter cells. In strains deleted for *dif*, failure to resolve dimer chromosomes leads to the Dif⁻ phenotype, which includes filamentation in a fraction of the cells, abnormal nucleoid morphology, SOS induction and decreased growth rate and

plating efficiency compared with wild-type cells (Kuempel *et al*., 1991; Cornet *et al*., 1996). To be active, *dif* must be inserted within a narrow zone, 15-20kb region, around its natural position, the DAZ (*dif* activity zone), even though it is able to recombine with a plasmid-borne *dif* site wherever its chromosome location. Although replication terminates normally near *dif* (Louarn *et al.,* 1994), compelling evidence indicates that *dif* resolution activity is not controlled by either termination of replication or timing of *dif* replication (Cornet *et al*., 1996; Kuempel *et al*., 1996). The DAZ is the scene of specific recombination between *dif* sites that occurs only in cells that are able to form chromosome dimers (i.e. proficient for homologous recombination) (Pérals *et al*., 2000; 2001).The location of the *dif* site on the chromosome seems critical for its activity. *dif* activity decreased progressively as *dif* was moved in either direction from its normal position; that was apparent for both the growth defect and the cassette segregation assays (Pérals *et al*., 2000). A Dif$^-$ phenotype results from translocations of *dif* to the *lac* operon at 8 min on the chromosome map, to a site near *oriC* (Leslie and Sherratt, 1995), and even to a site within the terminus region, 118kb to the left of is natural position (Tecklenburg *et al*., 1995). Nevertheless, these translocated sites were still able to recombine with a plasmid-born *dif* site. Conversely replacing *dif* at its natural position by either *psi*, the resolution site of plasmid pSC101, or *loxP*, the resolution site of bacteriophage P1 (in the presence of Cre resolvase), does not disrupt the Dif$^+$ phenotype (Cornet *et al*., 1994; Leslie and Sherratt, 1995). Therefore, the location of the recombination site seems more important for the chromosome than the nature of the site itself.

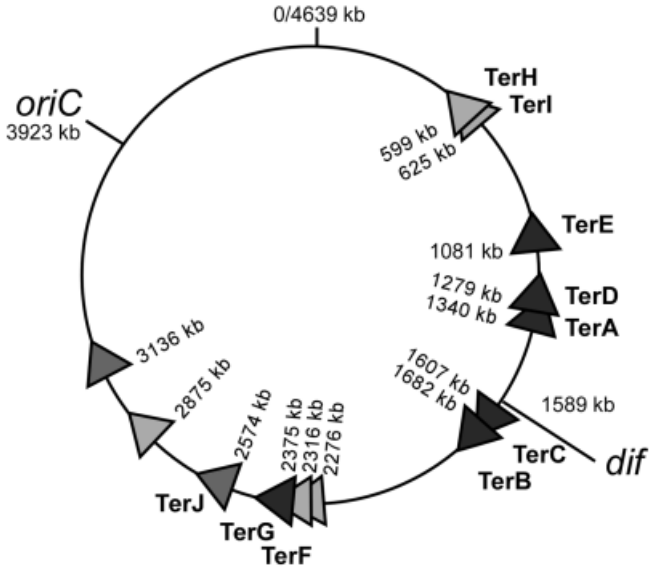

Figure 11. Positions of Ter sites in *E. coli*. Genome positions correspond to the *E. coli* K12 sequence. Ter sites are depicted as triangles; dark triangles are perfect matches to the consensus, medium and light grey triangles show one or two mismatches, respectively, at allowed variable positions (Hendrickson and Lawrence, 2007; with permission).

The existence of DAZ in *dif* recombination suggests that cells with a dimer must position the nucleoid in a specific way. The DAZ is contained within a larger structural entity called the Ter macrodomain. A structural peculiarity of this region was first suggested by the fact that it contains two 'non-divisible zones' that are regions refractory to inversion (Rebollo *et al*., 1988; Guijo *et al*., 2001). This Ter domain was defined following the observation that sequences belonging to a large part of the chromosome around the terminus, display a similar intracellular location, suggesting that these sequences behave as a structural unit during the cell cycle (Niki *et al*., 2000). It has been noticed that no deletion adjacent to *dif* or including *dif* has removed DAZ; Chromosomes with deletions of more than 150kb around *dif* (Cornet *et al*., 1996; Kuempel *et al*., 1996) or 59kb on one side of *dif* and 155kb on the other side still possess a DAZ; Each deletion

resulted in the formation of a new DAZ at the position that juxtaposed sequences from the left and right terminus arms (the DNA sequences from the left and the right of *dif* on a linear map of the terminus region). All this suggests that a variety of sequences from each terminus arm can combine to dictate formation of the DAZ. Inversion studies done by Pérals *et al*., (2000) suggest that each terminus arm must contain more than one polar element to generate the DAZ, as each contains at least two non-overlapping segments whose inversion inactivates *dif*. The following observations by Pérals strongly support the hypothesis that the regions flanking *dif* must display opposite polarity if *dif* is to function normally. I). Inversion of just the central region of the DAZ (the *zdc338–zdc346* segment adjacent to *dif*) has no effect on *dif*, suggesting that the DAZ itself is devoid of polar elements. II). Inversions that include *dif* are also harmless, indicating that sequences from both terminus arms may be exchanged without damage to *dif* activity. Thus, the active elements are not specific for either terminus arm but, rather, are interchangeable, provided that their orientation with respect to *dif* is conserved. All these data reveal that *dif* activity requires the proper orientation of the immediate flanking sequences.

A search for the determination of DAZ position revealed an unexpected phenomenon, chromosome polarization. Polarization may be caused by a number of short and/or degenerate sequences. These polarized elements of the chromosome may control the dynamics of nucleoid movement at division (Capiaux *et al*., 2001). It was observed that each of the two *oriC-dif* arms displays a polarization in opposite direction which is imprinted at the sequence level. Furthermore, the sequences surrounding *dif* also appear to be intrinsically polarized along the *oriC-dif* axis and their relative orientation is the main determinant of DAZ positioning. Notably the finding that the deletion of sequences

surrounding *dif* is harmless, whereas inversion of the same sequences inhibits dimer resolution (Tecklenburg *et al*., 1995; Cornet *et al*., 1996; Pérals *et al*., 2000). All together, these data suggest that the polarization determinants are present throughout a large terminal domain (more than 200kb around *dif*) and are highly repeated. Chromosome sequences are oriented following the *oriC/ter* axis, defining the two replichores (Blattner *et al*., 1997). Several sequence motifs have been identified with a skewed distribution in each replichore (Salzberg *et al*., 1998). For instance, the RRRAGGGY motif (R = purine; Y = pyrimidine) is distributed with an average skew of about 70% in favour of the leading strand of each replichore. Interestingly, this skew reaches 90% within 100kb on either side of *dif*, and the polarity switch point coincides with *dif* (Pérals *et al*., 2000). The same sequences are polarized in the λ DNA sequence in a way that could explain its ability to inhibit *dif* activity (Corre *et al*., 2000). This results from the intrinsic biased orientation of chromosome sequences that define its replichore organization: strongly expressed genes, G/C skew, Chi sites and numerous other oligomers (Salzberg *et al*., 1998; Lobry and Louarn, 2003). Among these, short degenerate motifs, termed RAG, have been proposed as good candidates based on their highly biased orientation (Lobry and Louarn, 2003). However, previous attempts to show that the RAG motif controls another FtsK activity or colocalized other active elements were unfruitful (Perals *et al*, 2000; Massey *et al*., 2004; Saleh *et al*., 2004). Moreover, another DNA motif, named FtsK orienting polar sequences (KOPS), have been identified which direct the movement of the *E. coli* FtsK translocase (Levy *et al*., 2005; Bigot *et al*., 2005). The Levy group (2005) identified the GNGNAGGG motif, its complement, or both as the best candidate to specify FtsK directionality. They found that a GNGNAGGG sequence efficiently

reverses FtsK translocation. The Bigot group (2005) used a functional approach and also identified this motif, displaying a high biased orientation and over-representation on the whole chromosome. *In vitro*, these motifs display KOPS activity: they inhibit Xer recombination activation by FtsK in an orientation-dependent manner; they also stop FtsK from dissociating branched DNA structures depending on their orientation; additionally, single molecule data suggest that they block FtsK translocation.

2.3.1.1.2 Structure

A DNA fragment of 32bp which contains a functional *dif* site is sufficient to allow Xer-mediated plasmid multimerization and dimer resolution (Blakely *et al*., 1991). The structural organization of *dif* is similar to that of related tyrosine recombinase family site-specific recombination loci, e.g. P1 *lox*P, and *Saccharomyces cerevisiae* FRT (Hoess *et al*., 1986; Bruckner and Cox, 1986). The core recombination site contains two 11bp inverted recombinase binding half-sites separated by a 6bp central region at the outer boundaries of which recombination occurs. The half- sites show homology with the core sequence of *cer*. The XerC and XerD binding sites are partial palindromes at six of 11 positions, but other five positions are never palindromes which determine the specific binding of XerC and XerD (Hayes *et al*. 1997). Based on the dyad symmetry of the half-sites and by analogy with the cleavage positions from other recombinases (Hoess *et al*., 1986; Bruckner and Cox, 1986), the boundaries of the central region and recombinase binding sites have been proposed to contain the bases involved in strand nicking and exchange (Summers, 1989). In *dif* the central region contains 6bp, while in *cer* it consists of 8bp; this difference may constitute a major determinant of the outcome in the recombination reaction and the requirement for accessory sequences. In contrast to

plasmid-located Xer recombination sites, the *dif* site requires neither accessory sequences nor accessory proteins for function. The lack of accessory sequences eliminates the resolution selectivity exhibited by other Xer recombination sites so that multimerization as well as resolution are detected when *dif* is placed on a plasmid substrate (Blakely *et al*., 1991). The *dif* site in the chromosome can be replaced either by the Xer recombination site, *psi*, derived from pSC101 (Cornet *et al*., 1994) or by the l*oxP* resolution site of bacteriophage P1 (Leslie and Sherratt, 1995). As with *dif*, both of these sites recombine intermolecularly as well as intramolecularly. In contrast, the recombination sites *res* and *cer*, which exhibit resolution selectivity, do not functionally substitute for *dif* (Leslie and Sherratt, 1995).

The recombination sites contain limited dyad symmetry of 4-5bp, yet XerC only binds to the left-half site and XerD binds to the right half-site under standard *in vitro* binding assays (Blakely *et al*., 1993). Examination of core recombination sites derived from the *E. coli* chromosome and from naturally occurring plasmids shows that the sequences of XerD binding sites are highly conserved, while the XerC binding sites show much greater divergence (Table 1). A sequence comparison of recombinase binding sites suggests that only 3 positions within the left half-site contain base pairs which do not appear in equivalent positions in the right half-site. For example, position -10 is always a G-C pair but never an A-T pair, while position 10 is always A-T (Fig. 12). These base pairs must at least be part of the sequence recognition determinants for each of the recombinases. Fig. 13 shows the sequences which determine the specific binding of XerC and XerD (Hayes and Sherratt, 1997). They identified that the −10C nucleotide appears to be most important for XerC binding specificity. This position is conserved

completely among known Xer recombination sites and is never palindromic with the corresponding position in the XerD binding site. They found that mutation of this position to an A nucleotide abolished XerC binding *in vitro* and, when combined with additional substitutions, eliminated the formation of the XerC-XerD complex *in vitro* and reduced intermolecular recombination activity both in plasmid substrates and when introduced into the chromosome. Mutation of the −13G position to a T nucleotide also abolished detectable XerC binding *in vitro*. They also observed that positions −14, −11, and −9 appear to be of less significance for XerC binding. Hayes and Sherratt found that the +9T and +13A nucleotides contribute significantly to XerD binding on *dif* site. The T and G nucleotides at position +9 appear to be interchangeable with respect to *in vitro* binding and recombination activity (Blake *et al.*, 1997). The nucleotides that contribute most significantly to XerC and XerD binding specificity are not reciprocal. Whereas the −10C position is important for XerC binding, the equivalent position (+10T) may be less critical for XerD binding. Conversely, the +9T nucleotide is a strong XerD binding specificity element but the corresponding −9G nucleotide seems less significant for XerC binding.

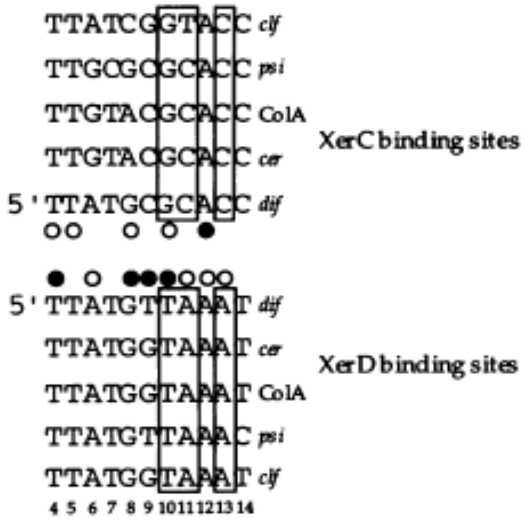

Figure 12. Alignment of half-sites from some of the known recombination loci (Sherratt *et al*., 1993) demonstrates that only three base pair positions (boxed) are unique to each half-site. The bases that interfere with protein binding when modified are indicated for top (●) and bottom (○) strands. Three of the seven interference positions determined for the XerD binding site map within the unique sequence. Central regions occur 5' to the shown sequences. Base pair coordinates are given below the last sequence (Blakely and Sherratt, 1994; with permission).

Figure 13. Hierarchy of specificity determinants in the XerC and XerD binding sites of *dif.* Shaded boxes below the sequence denote positions that are palindromic between the XerC and XerD binding sites. Bars above the sequence indicate the relative contributions of particular nucleotides to XerC or XerD binding specificity. The longest bars identify bases that are most significant for specificity and the shortest bars denote nucleotides whose contribution is least critical. Bars of intermediate length indicate positions of intermediate importance. Note that, while the T → C substitution at position +9 had a strong affect on XerD binding and recombination in *vivo*, all plasmid sites examined to date (except *psi)* have a G nucleotide at this position (Hayes and Sherratt, 1997; with permission).

2.3.1.1.3 Co-Location Model

Xer recombination between *dif* sites is subject to at least three different but interacting levels of control: the location of the *dif* site, homologous recombination and the presence of the division septum-associated protein FtsK. These controls interact with each other. Firstly, *dif* activity depends on formation of the division septum. Secondly, data show that *dif* recombination occurs most preferentially between sites carried on a chromosome dimer but not on chromosome monomers (Steiner and Kuempel, 1998a). The key observation that DAZ-specific induction is abolished in *recA*$^-$ strains, strongly supports that DAZ-specific induction only operates on dimeric chromosomes, restricting *dif* recombination to dimers and preventing recombination between monomers (Pérals *et al*., 2001). Taken together; these data suggest that *dif* recombination depends on homologous recombination, most certainly via chromosome dimer formation. Furthermore, FtsK protomers are septum associated in wild-type cells, thus restricting *dif* recombination to the septum proximity. Thus, dimer resolution appears to be achieved by an intergrated process. The conclusions are consistent with and reinforce the proposed "co-location" model, as depicted in Fig. 14 (Pérals *et al*., 2001). This model postulates that a mobilization mechanism, acting on polar sequence elements distributed in opposite orientations on either side of *dif*, drives the chromosomal DNA out of the division plane before cell division (Corre *et al*., 2000; Pérals *et al*., 2000). In the case of a dimer, the mobilization process acts to position the two DAZ (and *dif* sites) in close proximity to the septum, allowing the formation of an active XerCD-FtsK–*dif* complex and subsequent resolution of the dimer. Resolution may allow rapid separation of the chromosomes preventing additional rounds of *dif* recombination. When chromosomes are monomers,

the *dif* regions may be removed from the septum position before the time at which *dif* recombination is allowed to occur. Thus, the *dif* sites might be able to gain access to the septum only if located on a dimer.

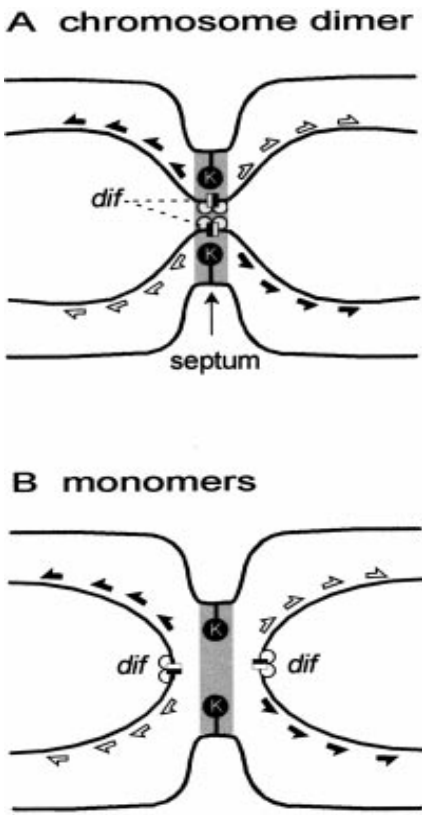

Figure 14. The co-location model. The drawings represent the central part of a dividing cell during septum constriction. Circles represent proteins: C, XerC; D, XerD; K, FtsK. The lines with arrows represent the terminal part of the chromosome carrying *dif* (the black and white square). The black arrows represent some oriented chromosome organizing elements involved in DAZ formation at their convergence point. (A). In the case of dimeric chromosomes, the *dif* region may stay entrapped at the septum position and the interaction of the XerCD±*dif* complex with factors such as FtsK results in activation of *dif* recombination and allows CDR to occur. The grey area represents the part of the cell in which dif sites must lie to access septum-associated activating factors. (B). If chromosomes are monomers or when resolution has occurred from a chromosome dimer, chromosome structure and segregation might prevent the *dif* sites acceding to the closing septum and thus to activating factors. (Pérals *et al*., 2001; with permission).

2.4 FtsK

Chromosome dimer resolution requires the XerC and XerD recombinases; it also requires FtsK, a large, multifunctional, integral membrane protein, which coordinates chromosome segregation and cell division (Liu *et al*., 1998; Capiaux *et al*., 2001). Such coordination is crucial in bacteria where DNA replication and segregation are not separated in time, and can occur as cells divide.

FtsK is a member of the FtsK/SpoIIIE/Tra family of DNA translocases. FtsK is a multifunctional and multidomain protein. *E. coli* FtsK contains three domains within its 1329aa residues (reviewed in Bigot *et al*., 2007): an approximately 200aa N-terminal domain ($FtsK_N$); a long linker region rich in proline and glutamine and an approximately 500aa C-terminal domain ($FtsK_C$). FtsK N-terminal domain serves to localize the protein to the division septum and is required for cell division (Begg *et al*., 1995; Draper *et al*., 1998; Yu *et al*., 1998a), while the C-terminal domain forms the translocation motor involved in chromosome segregation. The general structure and sequence conservation of FtsK is shown in Fig. 15.

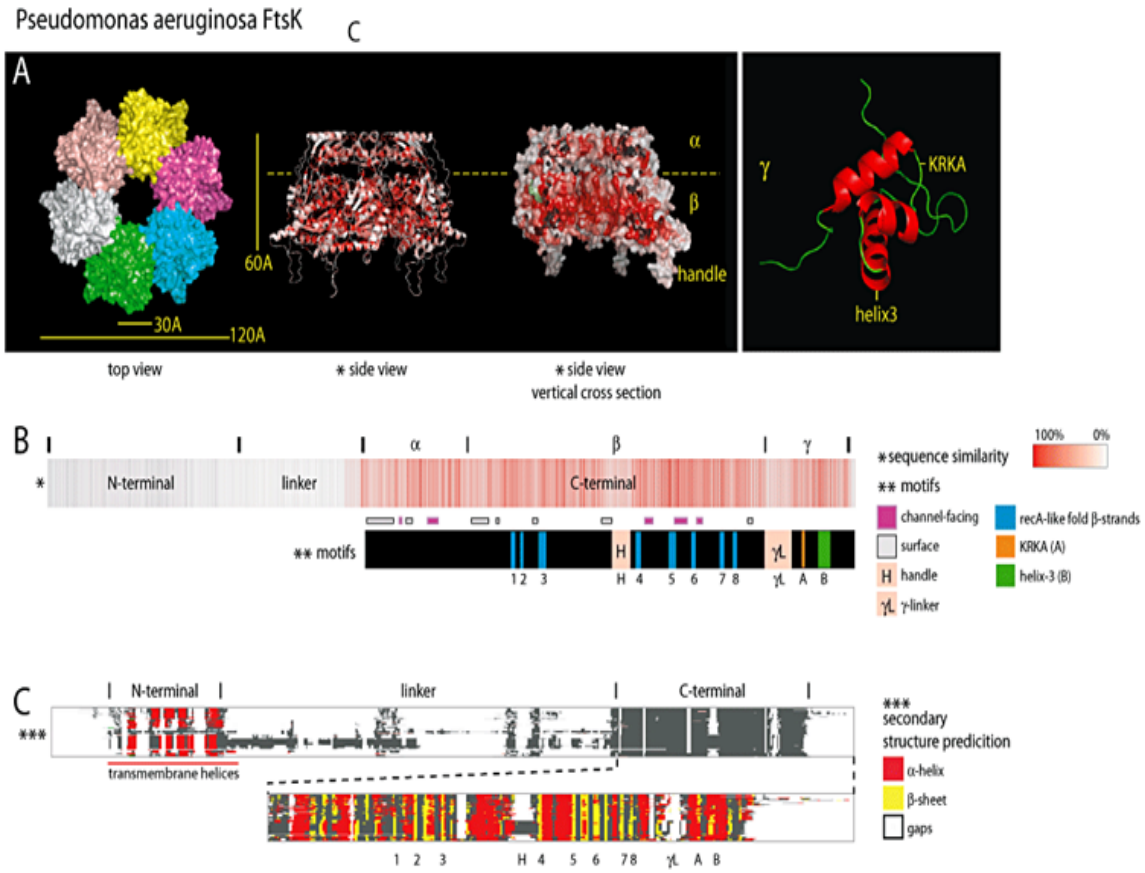

Figure 15. *Pseudomonas aeruginosa* FtsK domain organization and conservation (Bigot *et al*., 2007; with permision). A. Images of the crystal structure of hexameric FtsK from *P. aeruginosa* (α and β domains) (Massey *et al*., 2006) and of the NMR structure of the γ domain (Sivanathan *et al*., 2006). The top view shows the six subunits (in unique colours) that form the hexamer. The side views and (B) are colour-coded according to sequence similarity when comparing FtsK across eubacteria with deeper red indicating higher conservation [obtained using ProtSkin (http://www.mcgnmr.ca/ProtSkin)]. It clearly illustrates the cleft between the α and β domains and the conservation of residues lining the central channel (cross-section). The KRKA loop that interacts with XerD and helix-3 that is involved with KOPS recognition are indicated within the γ subdomain. B. A schematic of the general domain organization of FtsK using STRAP (http://www.charite.de/bioinf/strap) superimposed on annotated motifs for *P. aeruginosa*'s FtsK. Motifs within the C-terminal domain highlight the regions of high- and low-sequence conservation; the residues lining the central channel (upon hexamerization), the RecA fold β-strands and the regulatory domains within γ (motifs A and B) are among the highest conserved regions, while the residues on the outer surface of the hexamer, the handle and the γ-linker vary considerably. C. A schematic of an FtsK sequence alignment (Deprez *et al*., 2005) with secondary structure prediction using. FtsK homologue was selected from a blast in all sequenced bacterial genomes with a cut-off at $1.e^{-100}$. The three main domains are annotated for *P. aeruginosa* FtsK. Gaps litter the linker domains (linker and γL), highlighting their

variable length. The transmembrane helices of the N-terminal and the α-helices and β-strands of the C-terminal are strongly conserved. The handle region (H) within the C-terminal domain is prevalent only in proteobacteria. Predicted coiled coil structures in the linker domain are restricted to the long linkers and are thus not shown.

$FtsK_{N,}$ is ~200 residues long and poorly conserved at the sequence level. $FtsK_N$ invariably contains transmembrane helices that tether the protein to the cell membrane specifically at the division septum (Dorazi and Dewer, 2000), where it is proposed to interact with several other cell division proteins (Di *et al*., 2003). Unlike $FtsK_C$ which forms multimers (see below), the tertiary structure formed by $FtsK_N$ is unknown, rendering a general model for the structure of septum-borne FtsK difficult to draw. An attractive hypothesis is that $FtsK_N$ requires other division proteins and/or the process of septum closure itself to oligomerize, which may restrict the formation of active $FtsK_C$ multimers to a certain stage of septum closure, thus controlling FtsK activity temporally. The linker domain ($FtsK_L$) separates $FtsK_N$ from $FtsK_C$ and extends into the cytoplasm from the division septum. It shows high sequence and length variability. The longest linkers (~600aa) are found in proteobacteria, and in *E. coli*, it is required for proper function of $FtsK_C$ activities (Bigot *et al*., 2004). The longer linkers tend to be rich in proline and glutamine residues, and many adopt coiled coils as predicted secondary structures, suggesting they might participate in the formation of FtsK multimers and/or in interaction with other divisome proteins.

$FtsK_C$ is the signature domain of this protein and can be divided into three subdomains (Yates *et al*., 2003); α and β form the motor, while the following 85aa γ domain is a regulatory domain that binds DNA and interacts with the XerCD recombinase (Barre *et al*., 2000; Messey *et al*., 2006; Sivanathan *et al*., 2006; Yates *et al*., 2006). The recently solved crystal structure of *P. aeruginosa*'s FtsK (consisting of only

the α and β subdomains) shows a hexamer that assembles around double-stranded DNA (Massey *et al*., 2006). The fold of the α domain is unique to the FtsK/SpoIIIE/Tra family. The α subdomains form a smaller ring atop a larger β ring (Fig. 15). Note that the crystallized FtsK is a truncated form of $FtsK_N$, $FtsK_L$ and of the γ subdomain, and was solved as double head-to-head hexamers that interact via a 'handle' domain (Fig. 15A). However, a double hexamer is difficult to reconcile with functional data, in particular because γ, which is almost directly linked to β, must contact the DNA and thus be positioned in the vicinity of the central channel. Consistent with this view, the handle domain is not conserved (Fig. 15B and C). The highest homology is found in subdomain β. This subdomain contains the core RecA-like fold (with Walker P-loop and B motif) that is common to AAA+ proteins (ATPases Associated with various cellular Activities) and generates the force required for DNA translocation (Neuwald *et al*., 1999; Maurizi and Li, 2001). Based on an observed conformational change, a “rotary inchworm” mechanism has been proposed (Massey *et al*., 2006), that explains the very fast translocation of FtsK on DNA with very little supercoiling induction (1/150bp), as observed in “single-molecule” experiments (Saleh *et al*., 2005). In this mechanism, ATP hydrolysis by one of the six subunits of the FtsK ring translocates ~2bp of DNA, bringing the helical backbone of the DNA into position for translocation by the next subunit in the ring. Thus, very little net rotation of the protein ring against the DNA is required, and this may aid the very high translocation speeds of 5kbp/s observed in single-molecule experiments (Pease *et al*., 2005; Saleh *et al*., 2004).

At the tail end of $FtsK_C$, FtsKγ is a winged helix domain (WHD) (Fig. 16A). The WHD fold is comprised of helices H1 to H3 and a wing that is in β sheet-like

conformation. The γ subdomain forms a winged helix–turn–helix (wHTH) that is attached to the β domain via a flexible linker. The wHTH folds are commonly associated with DNA binding, while some participate in protein–protein interactions (Gajiwala and Burley, 2000). Estimation of the electrostatic potential on the surface of the domain, which can now be performed to a greater precision, identifies a patch of positive charge, mostly around the loop between helices H2 and H3 (Fig. 16B). Surprisingly, the crystals contained eight molecules per asymmetric unit (Fig. 16C). These are arranged in a tight octamer with 222 symmetry. The structure is very compact, with between ~1600 $Å^2$ and ~1300 $Å^2$ buried (26%–34% of the monomer surface) for the different positions in the octamer.

The γ domain utilizes both DNA binding and protein-protein interaction functions (Gajiwala and Burley, 2000) so it acts as a regulatory domain. It uses the loop1 forming an epitope that interacts with the recombinase XerD and helix3 recognizing specific DNA motifs, the KOPS (Ptacin *et al*., 2006; Sivanathan *et al*., 2006); KOPS has the consensus 5′-GGGNAGGG-3′, which is overrepresented on the chromosome and strongly biased for its orientation towards *dif*. A consequence of this guided translocation is that the chromosome terminus region (*ter*) is translocated towards the closing septum. The interaction between γ domain of FtsK with KOPS leads to the assembly of a FtsK hexamer on one side of KOPS, thereby imposing directional loading and translocation of FtsK along DNA, thus resulting in unidirectional translocation of each replichore arm.

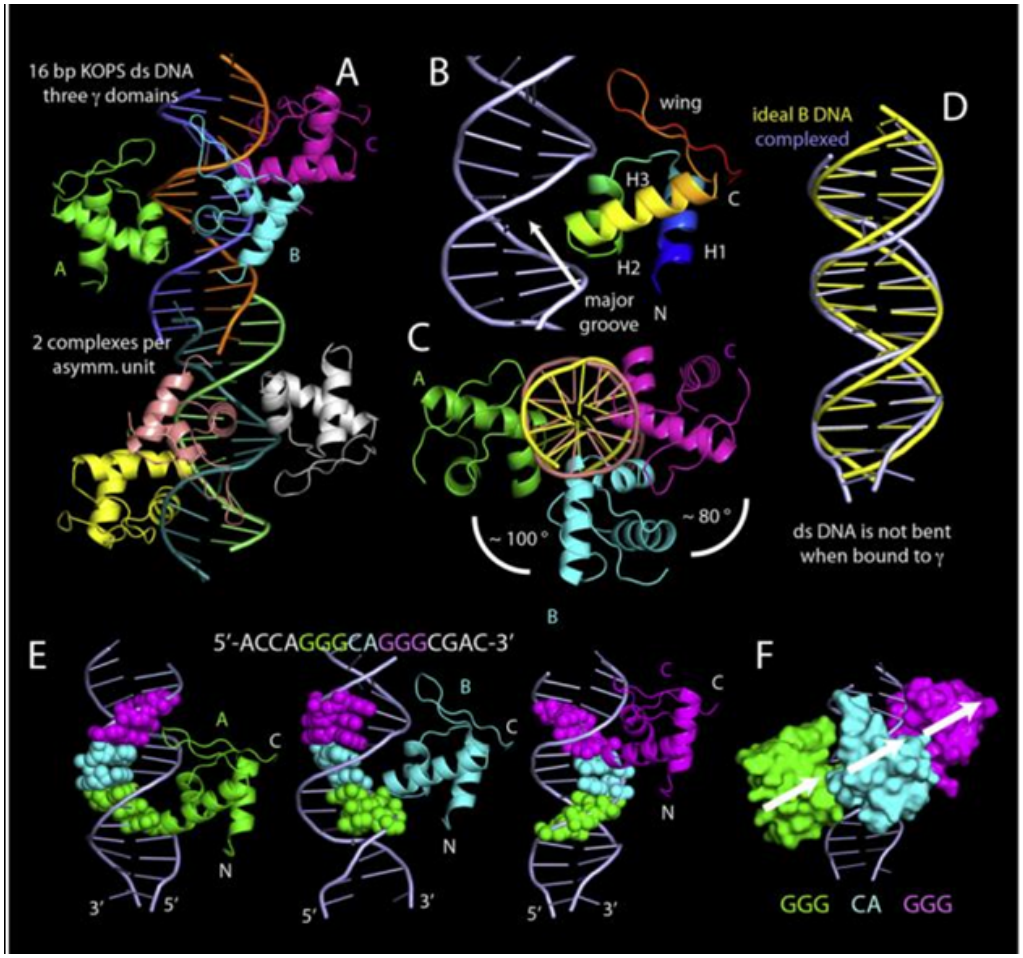

Figure 16. Crystal Structures of the FtsKγ Domain with and without DNA (Lowe *et al*., 2008; with permisssion).

(A) Cocrystallization of PaFtsKγ with the KOPS-containing DNA duplex 5′-ACCAGGGCAGGGCGAC-3′ (KOPS: GGGCAGGG) produced a structure containing three PaFtsKγ domains bound to double-stranded DNA. The asymmetric unit of these crystals contains two complete complexes. Protein chains A, B, and C are bound to the first duplex; chains D, E, and F are bound to the second. (B) In the complex, the winged helix domains insert with the loop between H2 and H3 into the major groove of the DNA. The wing, as is common for WHD domains, interacts with the minor groove of the DNA.(C) The three PaFtsKγ domains are arranged along the DNA to follow the major groove, leading to an arrangement in which they are ~90 degrees apart when looking along the axis of the DNA (A to B: ~100°, B to C: ~80°). (D) When superimposing the PaFtsKγ-complexed DNA duplex with ideal B DNA, it becomes clear that the DNA is not, or is only very slightly, bent from straight. (E) The three PaFtsKγ subunits together recognize the GGGCAGGG KOPS motif. Chains A and C recognize a GGG triplet, whereas chain B slots in between the two and recognizes mostly the CA duplet, although very few direct contacts exist, explaining the lack of conservation of the middle bases of the KOPS canonical sequence. The binding mode for chains A and C is very similar, with

a tight interaction of the wing with the minor groove. Chain B is tilted and binds in a slightly different way, as expected, because it recognizes a different DNA sequence despite being the same protein. (F) The recognition of different stretches of DNA by the same domain is possible because the three subunits also interact with themselves. They all bind in the same overall orientation to the DNA, though chain B is slightly tilted.

FtsK is an essential protein required for cell division (Begg *et al*., 1995). Two roles have been assigned to FtsK in chromosome dimer resolution (CDR). Firstly, FtsK has been implicated in positioning the terminus regions of chromosome dimers at mid-cell and synapsing their *dif* sites (Capiaux *et al*, 2002; Corre and Louarn, 2002). As mentioned before, a crucial element in chromosome dimer resolution is the position of the *dif* site on the chromosome (Leslie and Sherratt, 1995; Tecklenburg *et al*., 1995; Cornet *et al*., 1996; Kuempel *et al*., 1996). To be active, *dif* must be inserted within a narrow zone around its natural position, the DAZ. The DNA translocase activity of FtsK makes it a good candidate for positioning of the *dif* sites before recombination. There are some findings suggesting that septum-associated FtsK supports dimer resolution. For example : (i) Expression of the C-terminal domain of FtsK (lacking the N-terminal domain) in a strain lacking the C-terminal domain allows a high frequency of recombination between *dif* sites but does not support resolution of dimers (Barre *et al*., 2000 ; Perals *et al*., 2001). (ii) In strains harbouring displaced *dif* sites (but unable to resolve dimers because the sites are outside of the DAZ), overexpression of wild-type FtsK activates *dif* recombination but does not restore dimer resolution (Barre *et al*., 2000). These two findings also suggest that only septum-associated FtsK supports dimer resolution. FtsK would load onto chromosomes and mobilize DNA according to its intrinsic polarization. This process would stop when encountering XerCD-bound *dif* sites, thereby ensuring a proper sorting of chromosomal DNA in the sister cells and synapse of the *dif* sites (for review: Lesterlin *et al*., 2004). Most importantly, the outcome of terminal

recombination in *ftsK* mutants supports a role for FtsK in reading polarization (Corre *et al*., 2000). The finding that the C-terminal domain of *H. influenzae* FtsK can replace its *E. coli* counterpart for the *in vivo* processing of DNA polarity inside *E. coli,* indicates conservation of the mechanism of polarity reading (Bigot *et al*., 2004).

Secondly, FtsKc is directly involved in Xer recombination. In the absence of FtsK, HJ formed at *dif in vitro* are the result of catalysis by XerC (Barre *et al*., 2000). In contrast, in the presence of FtsK, XerD catalyzes HJ formation *in vitro* and *in vivo* (Aussel *et al*., 2002). Moreover, a low level of HJ formation *in vivo* by XerD is also reported when using a very sensitive detection assay in *E. coli* (Hallet *et al.,* 1999). Based on the above data, Aussel *et al.* (2002) proposed two alternative pathways (FtsK-dependent/independent pathway) of Xer recombination at *dif*, one initiated by XerC and the other by XerD. The role of FtsK in promoting chromosome dimer resolution is to switch the activity of the XerCD recombinases in the synaptic complex, so that Xer recombination follows one pathway in which XerD mediates the first pair of strand exchanges to form HJ intermediates that are resolved to products by XerC (FtsK-dependent pathway). On the contrary, the HJ intermediates that are formed by XerC-strand exchanges in the absence of FtsK are part of an abortive pathway as far as dimer resolution is concerned; the HJs are rapidly converted back to substrates in cycles of XerC-mediated strand exchanges (FtsK-independent pathway) (Fig. 17) Consistent with this pathway, synthetic *dif* HJs are resolved efficiently by XerC (Arciszewska and Sherratt, 1995), while synthetic *dif* HJs or plasmid HJs formed by XerC are not resolved by wildtype XerD under any of the conditions that have been tried, despite the presence of FtsK50C (Aussel *et al*., 2002). Also it is found that FtsKc ATPase activity is directly

involved in the local activation of the Xer recombination and activation only occurs with a DNA segment adjacent to the XerD-binding site. This suggests that FtsK needs to contact the XerD recombinase to switch its activity on using ATP hydrolysis (Massey *et al*., 2004). Furthermore, Yates *et al*. (2006) have shown that the γ domain in the C-terminus of FtsK interacts directly with the XerD C-terminus in order to stimulate the cleavage by XerD of bottom-nicked strand (BNS) in *E. coli.* Therefore, in *E. coli*, the requirement for FtsK to bring *dif* sites together and to activate the catalytic activity of XerD permits coordination of CDR with the last stage of cell division (Kennedy *et al*., 2008).

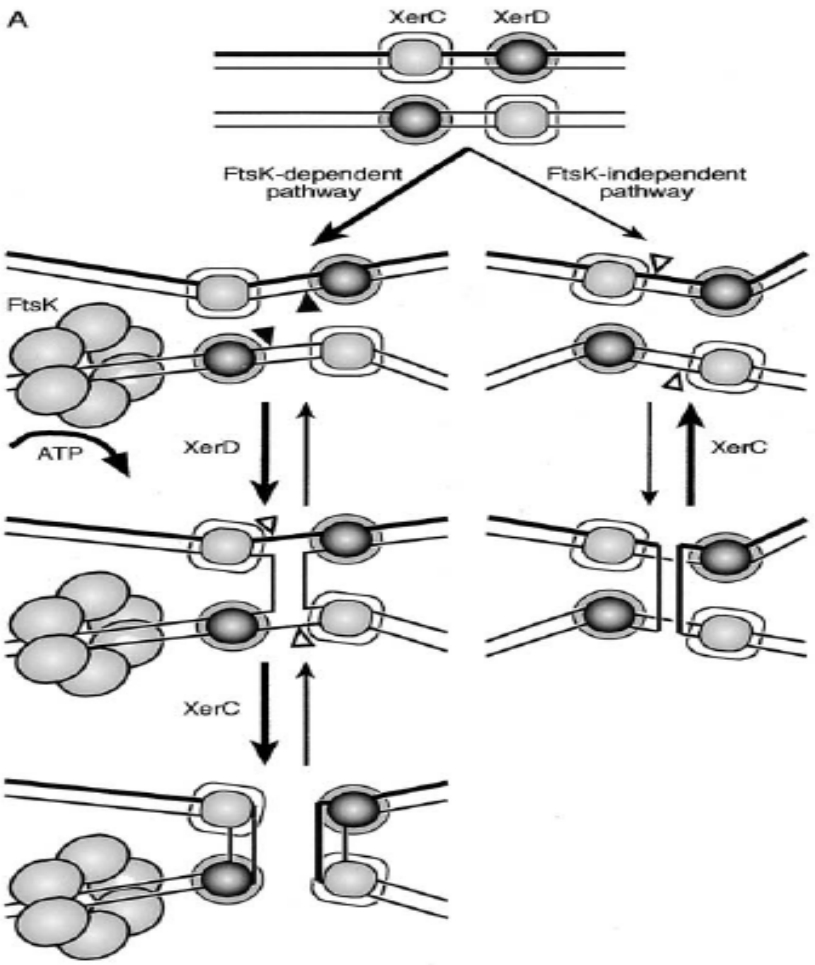

Figure 17. FtsK-Dependent and independent Pathways of Xer Recombination at *dif*. Dark triangles represent XerD recombinases and light triangles represent XerC recombinases. In the absence of FtsK, the Xer synaptic complex adopts a conformation suitable for XerC-mediated strand exchanges. FtsK can use the energy of ATP to switch the Xer synaptic complex to a conformation suitable for XerD-strand exchanges (Aussel *et al.,* 2002; with permission).

2.5 Regulation of Xer Recombination

Combining the involved factors mentioned before, Xer recombination is subject to at least three different but interacting levels of control: the location of the *dif* site, homologous recombination, and the presence of the division septum-associated protein FtsK. By their interacting control, *dif* recombination can only occur on dimer chromosomes but not on monomer chromosomes, which demonstrates the directionality of *dif* recombination. Therefore, Xer recombination is regulated temporally (i.e. by time, just before cell division) and spatially (i.e. at the *dif* site).

2.5.1 DAZ and FtsK Control

One of the crucial elements in chromosome dimer resolution is the position of the *dif* site on the chromosome. The *dif* site must be inserted within DAZ for its full activity (Cornet *et al*., 1996; Kuempel *et al*., 1996; Pérals et al., 2000; Tecklenburg *et al*., 1995). Specific DAZ induction only operates on dimeric chromosomes, restricting *dif* recombination to dimers and preventing recombination between monomers (Pérals *et al.,* 2001). A search for the determinants of DAZ positioning revealed an unexpected phenomenon, chromosome polarization. The sequences surrounding *dif* appear to be intrinsically polarized along the *oriC-dif* axis and precise positioning of the *dif* sites is then achieved by a polarization-depenent process. In summary, a septal location of DAZ

at cell division would seem to be optimal, because it allows Xer recombination to act only when the DNA has been almost completely segregated into the two daughter cells. If Xer recombination acted earlier, late homologous recombination events could still occur and create chromosome dimers that would not be resolved by Xer.

FtsK is another crucial factor for *dif* recombination (Lesterlin *et al*., 2004). FtsK has been assigned to two roles in the CDR. One role of FsK in the CDR is directly involved in Xer recombination catalysis by activating XerD; another is positioning the terminus regions of chromosome dimers at mid-cell and synapsing their *dif* sites (Capiaux *et al.,* 2002; Corre and Louarn, 2002; Aussel *et al.,* 2002; Yates *et al*., 2003). Septum-associated FtsK would load onto chromosomes and mobilize DNA according to its intrinsic polarization. This process would stop when encountering XerCD-bound *dif* sites, thereby ensuring a proper sorting of chromosomal DNA in the sister cells and synapse of the *dif* sites. Then a physical contact between XerCD/*dif* complexes and septum-borne FtsK allows resolution of dimers to occur. When a dimer is present, the XerCD/*dif* complexes and FtsK colocalize at the division septum at the time of septation. This restricts *dif* recombination to the septum region (see review: Bigot *et al*., 2007).

2.5.2 Homologous Recombination Control

Complete Xer recombination product at chromosomal or plasmid *dif* also depends on a functional homologous recombination system, which is necessary to generate dimeric chromosomes. Furthermore, the levels of Xer recombinational exchanges at *dif* seem to be proportional to the amount of ongoing homologous recombination (Recchia and Sherratt, 1999; Steiner and Kuempel, 1998a, 1998b).

The major role of homologous recombination is to allow the reassembly of functional replication forks that have broken or stalled, either as a consequence of DNA breaks or lesions (Cox *et al.,* 2000) or because of stalled transcription machinery (McGlynn *et al*., 2000). The recombination process can either exchange the flanking sequence (referred to as sister chromatid exchange (SCE) or 'crossing over') to produce a dimer, or noncrossover, leaving monomeric chromosomes.

Fig. 18 shows that reciprocal recombination between growing daughters will produce a dimer. Dimers can also result from a break or near a replication fork, followed by invasion of the intact circle by the double-stranded end (Kuzminov, 1995). The rate of dimer formation depends on the frequency of recombination between sister chromosomes and on the frequency at which recombination events lead to SCE. There are two major Rec-dependent recombination pathways in *E. coli,* the RecFOR and the RecBCD pathways. Both pathways produce HJ, which is normally resolved by the RuvABC complex, although it may be processed by other means in the absence of Ruv (Van Gool *et al*., 1999; Cromie and Leach, 2000; Michel *et al.,* 2000). On the basis of the assumption that all chromosome dimers are resolved at *dif*, chromosome dimer formation has been indirectly quantified by monitoring Xer recombinational exchanges at *dif* with a density label assay. Dimer formation reaches 15% in wild-type cells and depends on homologous recombination (Steiner and Kuempel, 1998a, 1998b). Mutations in either of these pathways lead to about a 50% decrease in the number of Xer recombinational exchanges at *dif*, whereas mutational ablation of both pathways almost abolishes Xer recombination at *dif*. This estimate of the frequency of SCEs that lead to dimers fits well

with the general phenotype of Xer mutants and is consistent with 15% of divisions giving no viable progeny (Pérals *et al*., 2000).

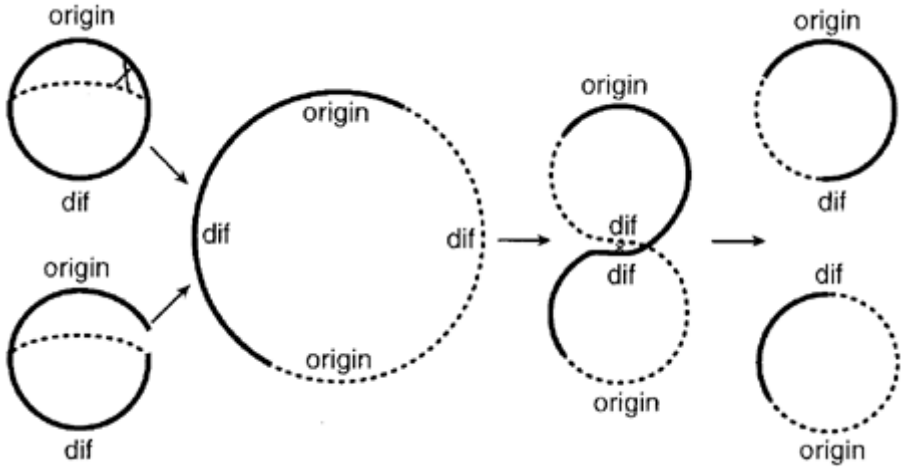

Figure 18. Sister chromatid exchange leads to circular dimer chromosomes. SCE can occur by reciprocal recombination between growing daughter chromosomes, or by breakage of one daughter, possibly at a replication fork, and subsequent invasion of the circular DNA (Kuzminov, 1995). Both situations produce Holliday junctions in which the appropriate resolution joins the newly synthesized strands to the template strands. These template strands will consequently be twice the normal length of a chromosome at the end of the replication cycle, which produces a circular dimer. Site-specific recombination at *dif* resolves dimers to monomers, and results in exchange of chromosomal segments (Steiner and Kuempel, 1998a; with permission).

3. *Caulobacter crescentus.*

3.1. *Caulobacter crescentus*—A Dimorphic Polarized Bacterium

C. crescentus is a Gram-negative, oligotrophic bacterium widely distributed in fresh water lakes and streams. It plays an important role in the global carbon cyclie by mineralization of dissolved organic material (Poindexter, 1981). One of the most spectacular features of these bacteria is dimorphism. In *C. crescentus,* dimorphism is maintained by obligate asymmetric cell division at each reproductive cycle (Fig. 19),

giving rise to two genetically identical but morphologically different daughter cells: a sessile cell equipped with an adhesive stalk and a motile flagellated swarmer cell (Brun and Janakiraman, 2000). The two daughter cells also inherit a different developmental program. The progeny stalked cell starts a new replicative cycle immediately after cell division. In contrast, the progeny swarmer cell has first an obligate motile life phase, during which the DNA replication and cell division programs are inhibited. After this motile period the swarmer cell undergoes cellular differentiation, which involves ejection of the flagellum, retraction of the pili, and generation of a stalk at the pole previously occupied by the flagellum and pili. Coincidentally with these developmental events the new stalked cell becomes reproduction competent and initiates a new round of DNA replication. This motile G1 phase in the swarmer cell cycle presumably gives the cell an opportunity to search for nutrients in new areas away from the sessile stalked mother cell. Thereby, the population is kept disperse to minimize competition for resources. This kind of dimorphism is believed to have evolved to cope with life in dilute nutrient-poor environments (Poindexter, 1981). The stalk in *C. crescentus* is a cellular appendix, which consists mostly of cell envelope material and cytoplasm with no DNA or ribosomes (Brun and Janakiraman, 2000). The stalk mediates attachment to surfaces through a holdfast organelle at its tip. It also constitutes an important means of adaptation to constant famine. Its elongation can considerably increase the total cell surface area, thereby increasing nutrient uptake (Ireland *et al*., 2000). This is illustrated by phosphate starvation, which results in elongation of the stalk several times the length of the cell body (Gonin *et al*., 2000; Schmidt and Stanier, 1966).

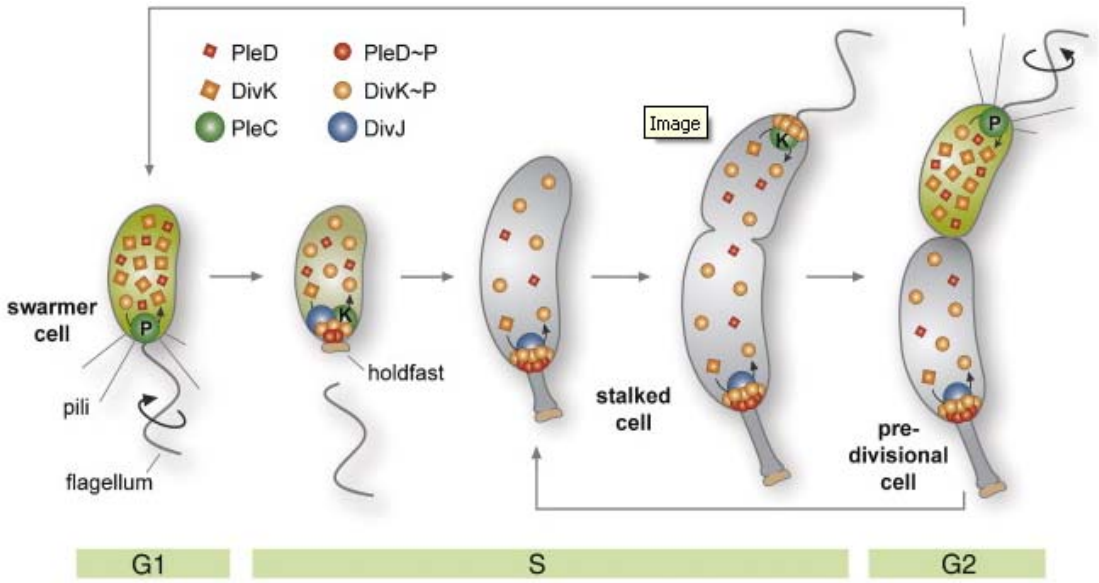

Figure 19. *C. crescentus* cell cycle. Each *C. crescentus* cell division yields a swarmer cell and a stalked cell. Upon differentiating into a stalked cell, the swarmer cell sheds its flagellum, builds a stalk and initiates DNA replication. Just as DNA replication and segregation are concluding, the predivisional cell begins to constrict at the nascent division site. A flagellum is constructed at the pole opposite the stalk, and the completion of cytokinesis generates a new stalked cell and a new swarmer cell. The different colors and shapes distrubated inside of the cells stand for the PleC–DivJ–DivK signaling network that control of cell cycle progression (Thanbichler, 2009; with permission).

C. crescentus has become the pre-eminent model system for understanding the cell cycle in bacteria for several reasons: Firstly, the organism is genetically tractable; For example, completion of the genome sequence has enabled the use of microarrays and proteomics for the comprehensive analysis of gene expression and protein stability during the cell cycle. At the same time, advances in fluorescence imaging are revealing remarkably dynamic subcellular localization patterns for many proteins, and even DNA sequences, reminding us that cell cycle and morphogenetic control mechanisms necessarily operate in three-dimensional space. Owing to *C. crescentus'* built-in differentiation program that occurs invariably at each cell cycle, cell cycle progression

can easily be followed in the laboratory by monitoring simple phenotypic traits, such as motility, chemotaxis, or susceptibility to a pilus-specific bacteriophage, which are characteristic of each cell cycle phase. So *C. crescentus* is easily amenable to genetic, biochemical, and cell biological dissection. Its genome has been completely sequenced and annotated (Nierman *et al*., 2001). It encodes approximately 3700 predicted genes that can be monitored for their expression in a systematic fashion with DNA microarrays (Jacobs *et al*., 2003; Laub *et al*., 2000; and 2003). Secondly, using the *C. crescentus* system, one can obtain synchronized cell populations using density gradient centrifugation that separates swarmer cells from stalked cells (Evinger and Agabian, 1977), which has the advantage that one can easily synchronize cells without perturbing their normal physiology. This allows for the examination of changes in morphology, mRNA levels, protein levels, protein modification, and protein localization during the course of the cell cycle. Additionally, morphological changes that occur over the course of the *C. crescentus* life cycle and are intimately coupled to other cell cycle events serve as faithful visual identifiers of the cell cycle status of any given cell. Furthermore, another unique aspect of *C. crescentus* is its inherent cell polarity. *C. crescentus* offers an opportunity to unravel, in its most basic and primitive form, the mechanisms governing cell polarity-one of the most basic principles in biology. All together, the small size of the *C. crescentus* genome and the ease of obtaining synchronized cell populations have opened the door to genome- and proteome-wide studies with the long-term goal to attain a global and integrated picture of the differentiation and cell cycle processes. Therefore, *C. crescentus* has now taken a place beside *E. coli* and *Bacillus subtilis* as an important

model system to study the genetic and regulatory network that controls the bacterial cell cycle in both temporal and spatial dimensions.

3.2. The *C. crescentus* cell cycle and regulation

Cell duplication, whether in a mammal with complex organ systems or in a single-celled bacterium, must use rigorous cell cycle regulation to ensure that the cell is ready before proceeding from one step to the next. Premature entry into DNA synthesis (S) phase or exit from mitosis (M) could have drastic consequences, notably fatal damage to the genome. Once initiated, improper execution of physical aspects of the cell cycle such as chromosome segregation and cytokinesis is equally dangerous. Thus, the cell has robust mechanisms to assure fidelity of every step of the cell duplication process.

C. crescentus has a life cycle characterized by precise developmental transitions and asymmetric cell division. Unlike many prokaryotes, *C. crescentus* replicates its chromosome only once during the cell division cycle such that the G1, S, and G2 phases are readily distinguishable. The ability to synchronize the cells and monitor changes in the transcriptome and proteome as cells proceed through the cell cycle has led to the identification of additional genes and proteins that are important for developmental regulation in *C. crescentus*. Transcription profiling of about 90% of the 3767 genes identified 553 genes (about 19% of the annotated open reading frames) whose mRNA levels varied as a function of the cell cycle (Laub *et al*., 2000). A parallel study revealed that about 15% of all *C. crescentus* proteins are synthesized in a cell-cycle-dependent manner (Grünenfelder *et al*., 2001). The finding that the cell cycle synthesis pattern of more than 80% of the identified proteins matched the observed fluctuations of the

corresponding mRNAs suggested a strong correlation between cell-cycle-dependent transcription and protein synthesis (Grünenfelder *et al*., 2001). Genes required for a given function are activated at the time in the cycle when these functions are needed. For instance, factors required for replication initiation were expressed early in the swarmer cell, paving the way for initiation at the swarmer→stalked-cell transition (i.e. entry into S phase). During S phase, expression of genes promoting nucleotide synthesis, DNA replication and DNA repair peaked. Finally, genes required for chromosome segregation, cytokinesis and DNA methylation were induced in the pre-divisional cell (Laub *et al*., 2000). Global analysis of cell cycle regulation has established the outline of a complex regulatory circuitry with the need to define the regulatory molecules and pathways responsible for the temporal transcription patterns (Stephens, 2001). Central to this genetic circuitry is a set of three master regulators (DnaA, CtrA, and GcrA) that together affect expression of ~200 cell-cycle-regulated genes (Laub *et al*., 2002; Holtzendorff *et al*., 2004; Hottes *et al*., 2005). The protein levels of these master regulators oscillate out of phase with one another (Collier *et al*., 2006). CtrA upregulates the expression of many genes involved in cell division: DNA methylation, flagella, stalk, and septal Z-ring biogenesis. In addition, CtrA binds to five DNA sites that overlap with the binding sites of the replication initiation protein, DnaA, and thereby precludes a new round of DNA replication. Furthermore, CtrA inhibits the expression of GcrA, which functions as an activator of components of the replisome and the segregation machinery.

3.2.1. CtrA

CtrA is an essential response regulator that controls the transcription of genes involved in cell division and in other important events (Kelly *et al*., 1998; Laub *et al*., 2002; Quon *et al*., 1996; Reisenauer *et al*., 1999; Skerker and Shapiro, 2000). CtrA acts as a repressor of replication initiation by binding to five sites in *Cori* (Quon *et al*. 1998). CtrA is only active in its phosphorylated form (CtrA~P) (Domian *et al*., 1997). Its phosphorylation state is controlled by a phosphorelay from the CckA histidine kinase through the ChpT phosphotransferase (Jacobs *et al*., 1999; Biondi *et al*., 2006). CtrA~P is present in swarmer cells and in predivisional cells, but it is redundantly inactivated by both dephosphorylation and proteolysis at the swarmer-to-stalked cell transition and in the stalked compartment of late-predivisional cells (Domian *et al*., 1997). Inactivation of CtrA is critical for licensing DNA replication initiation in newborn stalked cells; expression of a constitutively active, stable mutant of CtrA causes cell cycle arrest in G1 (Domian *et al*., 1997). To enter S phase, CtrA must be cleared from the cell. It is degraded by the essential ATP-dependent ClpXP protease complex (Jenal and Fuchs, 1998), which proteolyzes CtrA in vitro in the absence of accessory factors (Chien *et al*., 2007). In the cell, however, the levels of ClpXP remain constant throughout the cell cycle, indicating that the mere presence of protease and substrate is not sufficient for degradation in vivo (Domian *et al*., 1997; Jenal and Fuchs, 1998). Instead, the proteolysis of CtrA requires a specific spatial arrangement of the substrate and its regulators within the cell (Iniesta *et al*., 2006; McGrath *et al*., 2006; Ryan *et al*., 2004; Ryan *et al*., 2002).

Fluorescence microscopy has revealed that CtrA is located at the future stalked pole during the swarmer-to-stalked cell transition and at the stalked pole of the stalked

compartment in predivisional cells immediately prior to CtrA degradation (Ryan *et al*., 2004; Ryan *et al*., 2002). At the onset of DNA replication (the G1–S cell cycle transition), CtrA and the AAA+ protease ClpXP colocalize at one cell pole along with three accessory proteins, RcdA, CpdR, and PopA, and CtrA is rapidly degraded. Spatially constrained CtrA degradation is integrated with the timing of the cell cycle by multiple mechanisms. Firstly, as we known that RcdA is required for polar sequestration and regulated proteolysis of CtrA *in vivo*. *rcdA* transcript levels vary in time over the course of the cell cycle. Microarray and chromatin immunoprecipitation experiments suggest that this cell cycle variance stems, at least in part, from direct positive regulation of *rcdA* transcription by CtrA-*P* (Laub *et al*., 2002; McGrath *et al*., 2007). This constitutes a negative-feedback mechanism in which CtrA-*P* causes the accumulation of RcdA, which then targets CtrA-*P* for degradation. Secondly, polar targeting of ClpXP is controlled by the single-domain response regulator CpdR. The unphosphorylated version of CpdR localizes to the stalked pole and enables the localization and activity of ClpXP. In the absence of CpdR or in the presence of phosphorylated CpdR (CpdR-*P*), the ClpXP-RcdA-CtrA complex does not localize to the pole and CtrA is not degraded (Iniesta *et al*., 2006). Thus, the localization of the components required for inactivation of CtrA-*P* by proteolysis depends on the phosphorylation state of CpdR, which changes at specified points throughout the cell cycle. CpdR is in its phosphorylated state in the swarmer cell and is dephosphorylated and activated at the swarmer-to-stalked cell transition. Later, CpdR is phosphorylated and inactivated by the same phospho-signaling cascade that activates CtrA in predivisional cells (Biondi *et al*., 2006; Iniesta *et al*., 2006). Together, these integrated spatial and temporal mechanisms leave a small window of opportunity in

each cell cycle to initiate DNA replication, in which little CtrA-*P* exists in the cell and DnaA levels are high: this occurs only in new stalked cells, either after the swarmer-to-stalked cell transition or in stalked progeny upon cytokinesis.

3.2.2. DnaA

DnaA, an AAA+ ATPase, is a broadly conserved replication initiator in bacteria that binds to the replication origin (*Cori*) and locally unwinds the DNA to allow loading of the replication machinery (Mott and Berger, 2007). As an autoregulatory protein, it represses its own transcription; it further functions as a transcriptional regulator for other genes (Messer and Weigel, 2003) In *E. coli*, the activated form of DnaA (DnaA-ATP) starts replication by binding to 9 bp long AT-rich sequences (DnaA boxes) with the consensus sequence TT(A/T)TNCACA at ori (Messer, 2002). Opening of the double helix at *ori* further requires the histone-like proteins HU or IHF (Hwang and Kornberg, 1992). Then, DnaB helicase interacts with DnaA and is inserted into the open site to unwind the helix and allow the formation of the replisome complex.

The DnaA protein is essential for the initiation of DNA replication in *C. crescentus* (Gorbatyuk and Marczynski, 2001). Although *E. coli* DnaA is stable for more than 24h (Torheim *et al*., 2000), *C. crescentus* DnaA is actively degraded (Gorbatyuk and Marczynski, 2005) so that levels of DnaA can rapidly change as a function of the cell cycle (Collier *et al*., 2006). In *C. crescentus*, the DnaA protein also binds to the origin of replication, which, in addition to five CtrA-binding sites, contains fives DnaA boxes and an exceptionally AT-rich region (Marczynski and Shapiro, 1992) in an organism with a GC content of ~67%. The *dnaA* gene is located 2kb from the origin of replication and is

transcribed throughout the cell cycle. Interestingly, the rate of expression doubles just prior to the initiation of replication at the G1–S transition (Gorbatyuk and Marczynski, 2005; Zweiger and Shapiro, 1994). The presence of GANTC sites in the *dnaA* promoter region suggests expression may be affected by the DNA methylation state. For example, it is plausible that, immediately after initiation of replication, the hemimethylated *dnaA* promoter might be rendered inactive and is only able to fire when it is in the fully methylated state. Like the DnaA of *E. coli*, that of *C. crescentus* probably possesses autoregulatory properties, since *dnaA* transcription seems to be DnaA dependent. The absence of DnaA boxes in the *dnaA* promoter suggests that autoregulation may be an indirect effect (Hottes *et al*., 2005).

The transcription of *dnaA* is also cell cycle regulated, peaking in swarmer cells prior to the initiation of DNA replication (Zweiger and Shapiro, 1994; Laub *et al*., 2000). The transcription of *dnaA* is regulated by the methylation state of the *dnaA* promoter: the *dnaA* promoter is preferentially transcribed when it is in the fully methylated state prior to the initiation of replication (Collier *et al*., 2007). This DNA methylation-dependent regulation of *dnaA* transcription contributes to the transient high levels of DnaA observed at the swarmer-to-stalked cell transition. *C. crescentus* DnaA is posttranscriptionally regulated. At least two mechanisms are likely to be involved in DnaA inactivation to prevent early entry into S phase or overinitiation of replication. First, the DnaA protein is relatively unstable, with a half-life of about one third of the cell cycle (Gorbatyuk and Marczynski, 2005). Its short half-life combined with cell-cycle-regulated transcription results in high DnaA levels in stalked cells and early-predivisional cells and low levels of DnaA in swarmer and late-predivisional cells (Gorbatyuk and Marczynski, 2005; Collier

et al., 2006). The second mode of inactivation of DnaA uses the HdaA protein, which binds to the replisome upon replication initiation and inactivates DnaA, rendering any remaining DnaA protein incapable of reinitiating replication (Collier and Shapiro, 2009). This multilayered and tightly regulated control of the initiation of DNA replication ensures that *C. crescentus* replicates its chromosome only once per cell cycle. This is in contrast to *E. coli*, where overlapping rounds of replication can take place in each cell cycle when grown in rich media.

3.2.3. GcrA

Very little is known about the properties of the GcrA regulator. The protein was found in the same temperature sensitive genetic screen used to identify CtrA and the histidine kinase CckA (Collier *et al*., 2006). The 174aa GcrA protein is highly conserved among alpha proteobacteria but lacks known functional motifs.

Transcription of *gcrA* is very low in swarmer cells and increases during the swarmer-to-stalked cell transition. It reaches a maximum in the stalked cells, before it decreases to a low level again in predivisional cells. At the end of the cell cycle, transcription of *gcrA* rapidly resumes in the stalked progeny but remains very low in the swarmer progeny (Reisenauer and Shapiro, 2002; Holtzendorff *et al*., 2004). GcrA accumulation is regulated by multiple pathways (Collier *et al*., 2006). First, the temporally regulated transcription of *gcrA* is controlled by the combined effects of CtrA and DnaA, integrating the negative control by CtrA and the positive control by DnaA. In the SW cell, CtrA~P represses the *gcrA* promoter (P_{gcrA}) as well as one (*ctrA*P1) of its own two promoters (Collier *et al*., 2006). With the proteolytic removal of CtrA at the

G1–S transition, repression from P_{gcrA} and *ctrA*P1 is relieved. At the same time, DnaA reaches critical concentrations, initiates DNA replication and binds to the DnaA boxes at P_{gcrA} to activate *gcrA* transcription. This coupling mechanism ensures that expression of GcrA is linked to the initiation phase of DNA replication. Second, the cell cycle accumulation of GcrA is controlled both by transcriptional regulation and by regulated proteolysis. One study has shown that GcrA accumulation still exhibits cell cycle control when *gcrA* is transcribed constitutively (Collier *et al*., 2006). Overall, the DnaA/GcrA/CtrA cascade during the *C. crescentus* cell cycle defines the timing of expression of multiple genes encoding proteins with diverse functions needed for progress through the cell cycle. Recently, Collier and Shapiro (2009) proposed the following multistep control system (Fig. 20) that limits the initiation of DNA replication to only once per cell cycle: (i) the initiation process is inhibited in swarmer cells by CtrA bound to five sites within the origin region (Quon *et al*., 1998) (ii) CtrA is eliminated by targeted proteolysis (Domian *et al*., 1997) and DnaA accumulates during the swarmer-to-stalked cell transition (Collier *et al*., 2007), allowing the initiation of DNA replication; (iii) a second round of replication initiation is inhibited by an HdaA/DnaN complex bound to the chromosome once DNA replication has initiated in stalked cells and by the directed proteolysis of DnaA (Gorbatyuk and Marczynski, 2005); and (iv) CtrA accumulates again to strengthen the inhibition of replication initiation in predivisional cells.

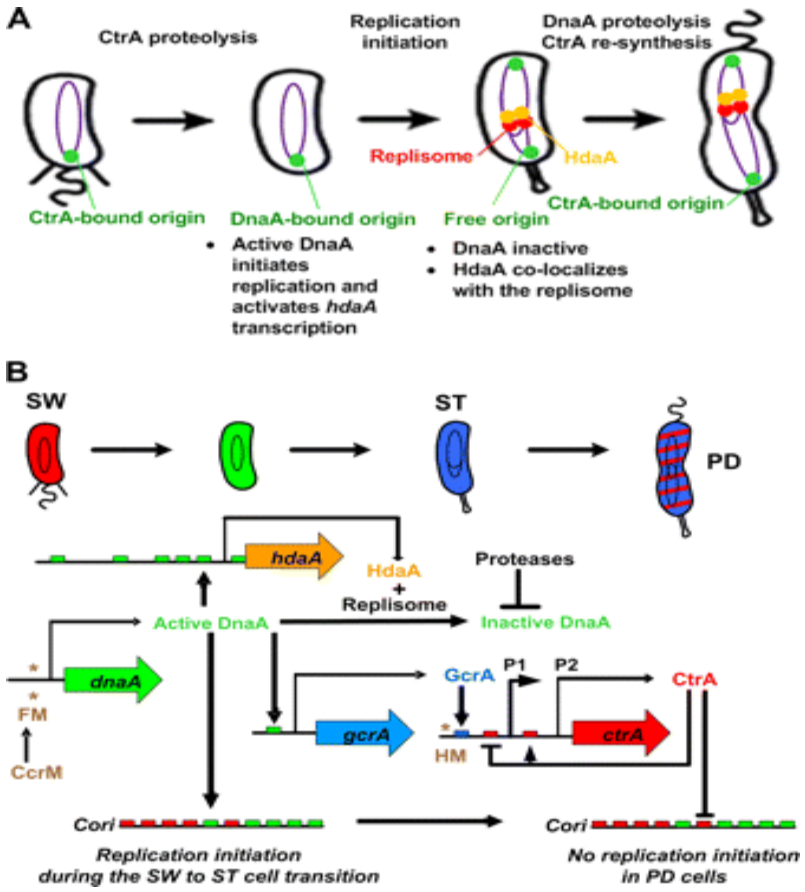

Figure 20. Model for the temporal control of DNA replication initiation in *C. crescentus* (Collier and Shapiro, 2009; with permission). (A). A schematic of the beginning of the *C. crescentus* cell cycle is shown. Purple theta structures inside the cells indicate replicating DNA. The single origin of replication (green focus) in swarmer cells is bound to CtrA, which represses the initiation of DNA replication. During the swarmer-to-stalked cell transition, CtrA is rapidly degraded by the ClpXP protease, and active DnaA binds to the origin to initiate DNA replication. The replisome (red foci), associated with HdaA (orange foci), replicates the chromosome and inactivates DnaA once DNA replication is ongoing. CtrA reaccumulates in predivisional cells and binds to the origin to prevent more replication initiation events. (B). A schematic of the beginning of the *C. crescentus* cell cycle is shown. Red indicates CtrA accumulation, green indicates DnaA accumulation, and blue indicates GcrA accumulation. SW, swarmer cell; ST, stalked cell; PD, predivisional cell. DnaA is synthesized in swarmer cells, when the *dnaA* promoter is in the fully methylated state (FM, two asterisks). New molecules of DnaA initiate DNA replication and activate the transcription of *gcrA* and *hdaA* by directly binding to DnaA boxes (green boxes). Once the replisome is assembled, the replisome-HdaA complex inhibits the initiation of DNA replication, probably by a mechanism similar to the RIDA mechanism in *E. coli*, and the DnaA protein is degraded to prevent more initiation events in stalked cells. Soon after the initiation of DNA replication, the *dnaA* and the *ctrA* genes are duplicated by the passage of the replication fork and therefore hemimethylated (HM, asterisk). Transcription from the hemimethylated *dnaA* gene is shut down, while

transcription from the hemimethylated *ctrA* gene is turned on by the binding of GcrA to the *ctrA* P1 promoter (blue box). Accumulation of CtrA in early predivisional cells then contributes to the inactivation of replication initiation by directly binding to the CtrA sites in the *Cori* (red boxes), yielding a robust replication control system.

3.3 FtsK in *C. crescentus*

The final stage of DNA replication is a critical juncture in the cell cycle during which multiple events must occur in a defined temporal order and in a precise position in the cell. These events include the decatenation of newly replicated chromosomes by Topo IV so that chromosome segregation and subsequent cell division can occur (Adams *et al*., 1992; Peng and Marians, 1993). Since formation of the division septum is initiated while DNA replication is still in progress, chromosomal termini are frequently found in only one of the two daughter cell compartments, trapping DNA in the dividing septum (Lau *et al*., 2003). Bacterial cell division is a complex process that requires the coordination of many mechanisms that ensure proper partitioning of the sister chromosomes to the daughter cells. FtsK has been reported to be responsible for clearing the division site of chromosomal DNA prior to cell division (Begg *et al*., 1995; Diez *et al*., 1997; Liu *et al*., 1998; Yu *et al*., 1998b).

In *C. crescentus*, DNA replication occurs once and only once per cell division cycle and the replication origin is always positioned at a cell pole (Jensen *et al*., 2002). As soon as replication is initiated, a copy of the replicated origin moves rapidly to the pole opposite the stalk in what appears to be an active process mediated by the actin homologue, MreB (Gitai *et al*., 2005; Viollier *et al*., 2004), whereas the terminus is gradually displaced to the division plane during the S phase **(**Jensen and Shapiro, 1999). The *C. crescentus* FtsK protein was found to be dynamically localized to the division

plane during the cell cycle and to remain transiently positioned at the new cell poles following cell division. Cells depleted of FtsK formed long, smooth filaments prior to cell death, indicating that FtsK is required for cell division (Wang *et al*., 2006). The FtsKc is essential for viability. It was noted that, in cells depleted for the FtsKc, approximately 15 to 20% of the cells had defects in terminal segregation. Furthermore, the localization of the Topo IV ParC replisome component (Wang and Shapiro, 2004), which is responsible for decatenation at the division plane, is dependent on the presence of the FtsKc. The first 258aa of the N-terminus are necessary and sufficient for targeting the FtsK protein to the division plane, where it is required to either assemble or maintain FtsZ rings. Thus, the bifunctional FtsK protein mediates an interdependence between chromosome partitioning and cell division in *C. crescentus* (Wang *et al*., 2006).

3.4 Xer/*dif* system in *C. crescentus*

The Xer recombination system was originally described for *E. coli* plasmids (Stirling *et al*., 1988; Clerget, 1991). Also this system has been functionally characterized in some other bacterial species including *C. crescentus* (Jouan and Szatmari, 2003). *C. crescentus xerC* enodes a protein that shows 34.9% identity and 54.8% similarity with *E. coli xerC* gene, whereas the *xerD* gene encodes a protein that presents 41.1% identity and 60.5% similarity with the *E. coli xerD* gene (Jouan and Szatmari, 2003). The *C. crescentus* proteins display the highest similarity with the *Sinorhizobium meliloti* XerC (49.3% identity and 64.9% similarity), and XerD proteins (52.0% identity and 69.1% similarity). *C. crescentus'* Xer recombinases display 36.2% identity and 63.9% similarity with each other. Also the highest level of similarity is found in the C-terminal region of the proteins. In addition, motif II and III (involved in catalytic activity) and the motif I

involved in DNA binding also display strong similarities (Nunes-Duby *et al*., 1998; Hayes *et al*., 1997). *In vitro*, *C. crescentus* Xer recombinases (*cc*XerC and *cc*XerD) exhibited binding activity on *E. coli dif* (*ecdif*) sites indicating that *cc*XerD had a higher affinity for the *ecdif* site than *cc*XerC. Cooperative binding was also observed between these two recombinases on *ecdif* site. This cooperative binding was observed at the *ecdif* between *cc*XerD and *ec*XerC, but not *cc*XerC and *ec*XerD (Jouan and Szatmari, 2003).

C. crescentus, like most bacteria, possesses a single chromosome where DNA replication initiates at a unique origin of replication (*Cori*) and proceeds bidireactionally (Brassinga and Marezynski, 2001; Dingwall and Shapiro, 1989). However, the *C. crescentus* terminus region is unusual, since it contains many essential or highly expressed genes. The *C. crescentus* genome does not contain obvious homologues of the *E. coli* or the *B. subtilis* termination systems, which contain several determinants involved in sister chromosome separation, including the *dif* site which is the chromosomal target of the XerCD site-specific recombinase. To identify the *dif* site of *C. crescentus*, Jensen (2006) used a combined bioinformatics and experimental approach to search the *C. crescentus* genome sequence. The best match was located at position 1,946,376bp, in the region of the chromosome expected to be the *ter* region. This site was located 62kb from the position in the genome opposite *Cori*, where the two replication forks were expected to meet. The putative *dif* site was located in a 262bp intergenic region between CC1763, a putative transcriptional regulator of unknown function, and an operon encoding the glyoxalate cycle enzymes AceA (isocitrate lyase) and AceB (malate synthase). Deletion of this putative *C. crescentus dif* site at 1.95Mb gave the same chromosome segregation, *ter* region separation, and cell division defect phenotypes as

were given by the absence of XerC or XerD, suggesting that this site was *dif* (Jensen, 2006). The *C. crescentus dif* (*ccdif*) site has a significantly different sequence when it is compared with *dif* sites from other bacterial chromosomes. For example, an alignment of the *ccdif* with an *ecdif* site shows that the divergent sequence is especially in the XerC binding site (see below). Therefore, further characterization of the Xer site-specific recombination in *C. crescentus* may give us important information about this system that resolves chromosome dimers into monomers to allow chromosome segregation before completion of cell division.

	XerC binding site	XerD binding site
E.coli dif	GGTGCGCATAA--TGTATA--TTATGTTAAAT	
C.crescentus dif	AAGATCGACTT--TGTAAT--TTATGTAAAGT	

4. The Master's project

We have previously identified and cloned the *xerC* and *xerD* genes from *C. crescentus*, and overexpressed them as maltose-binding fusion proteins. *In vitro* DNA-binding assays indicated that these two proteins could bind to an *ecdif* and could also interact with the Xer proteins of *E. coli*. After this work was completed, the *ccdif* was identified in Rasmus Jensen's lab and had a significantly different with *dif* sites from other bacterial chromosomes. Deletion of this sequence caused filamentation to occur in 4% of cells harboring this defect. Despite this interesting observation, no direct evidence of a Xer-*dif* interaction in *C. crescentus* was reported. Therefore, it would be very interesting to study the Xer recombinases activity on its own *dif* site. Our main objective was to study the binding and the formation of protein-DNA covalent complexes of the *C. crescentus* recombinases, *cc*XerC and *cc*XerD recombinses on the *ccdif* site *in vitro*.

ChapterII

Article

Interactions of the *Caulobacter crescentus* XerC and XerD recombinases

with the *ccdif* site

Keywords: XerC, XerD, *dif*, site-specific recombination, *Caulobacter crescentus*

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Abbreviations: LB: Luria-Bertani; IPTG: isopropyl B-D thigalactoside; MBP: maltose binding protein; PBS: phosphate buffered saline; SDS: sodium dodecyl sulfate.

Abstract

In most bacteria, the chromosomal dimer resolution process is mediated by two tyrosine recombinases, XerC and XerD, which bind cooperatively and perform the recombination reaction at the *dif* site near the terminus of replication. This reaction also requires the C-terminal domain of the cell division protein FtsK. The binding activity and the formation of phosphotyrosyl complex of the *C. crescentus* recombinases, *cc*XerC and *cc*XerD, were tested on the *C. crescentus dif* (*ccdif*) site. Both *cc*XerC and *cc*XerD bound preferentially to the left half-site of *ccdif* and showed lower cooperative binding, unlike what was found with the *E. coli dif* (*ecdif*) site. Covalent complexes formation activity was tested by using fluorescently labelled linear "nicked suicide substrates" and labelled proteins. Heat and SDS-resistant protein-DNA complexes were formed when both wild-type *cc*XerC and *cc*XerD reacted with *ccdif* but not in the presence of active-site tyrosine mutant proteins. Phosphotyrosine complexes formed on the top-nicked suicide substrate were found to be more efficient than on the bottom-nicked suicide substrates and surprisingly *cc*XerC remained bound to both top and bottom-nicked *ccdif* suicide substrates.

1. Introduction

Safeguarding the equal distribution of genetic material is a fundamental property of any living cell. Cells with circular chromosomes and homologous recombination systems must be able to resolve chromosome dimers, or higher-order multimeric forms, that are generated by an odd number of recombination events between chromosomes during DNA replication. In the classical bacterial model *Escherichia coli*, chromosome dimers are resolved in monomers by XerCD site-specific recombination (for recent reviews see 45, 46).

In *E. coli*, two related site-specific recombinases, XerC and XerD, act in concert at a site near the terminus of chromosome replication known as *dif* to resolve chromosome dimers into monomers prior to cell division (1, 2, 3). These recombinases belong to the tyrosine recombinase family and show 37% amino acid identity for each other and have sequence similarity to the lambda integrase class of site-specific recombinases (44, 50). The members of this family all contain four invariant amino acids, the RHRY tetrad (4). The site-specific recombination reaction proceeds, after recombinase binding and synapsis of sites, by activation of the recombinase and subsequent cleavage of specific phosphodiester bonds (5, 6). In Xer site-specific recombination reaction, three of these residues (R, H and R) are thought to be involved in activation of phosphodiester linkages in the cleavage and ligation reactions (48, 49), while the conserved tyrosine (Y) is the catalytic nucleophile that becomes covalently linked to the DNA in the cleavage reaction (7, 47). The chromosomal *dif* site is a 28bp sequence with two 11bp arms surrounding a 6bp central region, which differs from the plasmid recombination sites that have an 8bp central region (1, 2). The *dif* site is an

imperfect palindrome whose left and right halves are bound by XerC and XerD, respectively. Cooperative protein-protein interactions between XerC and XerD ensure stable synapsis. The γ domain of the cell division protein FtsK activates XerD to cleave the first DNA strand at the end of each 6bp spacer region to generate a 3' phosphotyrosyl covalent complex. The free 5'hydroxyl on the cleaved strand acts as the nucleophile to enable religation following strand exchange to generate a Holliday junction (HJ). Isomerization of the HJ then allows XerC to perform the second strand exchange and resolve the HJ (8, 9). This model for the coordination of chromosome dimer resolution and cell division has been elaborated in *E. coli* based on a substantial accumulation of *in vivo* and *in vitro* data. For example, interactions between the *E. coli dif* (*ecdif*) site and the XerCD recombinases of *Haemophilus influenzae* (51), *Pseudomonas aeruginosa* (52), *Bacillus subtilis*(33), *Proteus mirabilis* (22), *C. crescentus* (11), and *Vibrio chloerae* (54) have been experimentally demonstrated *in vitro*. These observations lead to the general view that Xer recombination is a function conserved among bacteria harboring circular chromosome(s).

The *E. coli* XerCD system, found in most bacteria species, is not universal. Studies with *Streptococci* and *Lactococci* (53) indicate that these bacteria carry alternative Xer recombination machinery; an atypical 31bp *dif* recombination site associated with a single dedicated tyrosine recombinase (XerS). Recently, Carnoy and Roten (2009) analyzed 234 chromosomes from 156 proteobacterial species and showed that a subgroup of ε-proteobacteria display a sequence (dif_H) which is homologous to dif_{SL} from *Streptococci* and *Lactococci* and harbor a single Xer-like recombinase (XerH) (58). However, no phylogenic association between XerS and XerH could be found, which

strongly suggests the existence of two unrelated *dif*/Xer systems: the classical machinery found in most species and an atypical system present in a sub-group of ε-proteobacteria. Furthermore, plasmids, such as ColE1 (*cer*) and pSC101 (*psi*) using the XerCD recombination system, have also adopted a different way to resolve multimers. In this latter case, XerC-catalysis initiates recombination independently of FtsK, an ATP-dependent-DNA translocase(55), and recombination requires ~200bp of accessory sequences flanking the plasmid sites and which are bound by accessory proteins (56, 57). The recombinase and accessory proteins form a highly organized protein-DNA complex with the recombination site DNA, and together exert control over the efficiency and timing of the recombination reaction. All together, these discoveries in the classical XerCD systems and unconventional Xer recombination machinery, reinforce the idea that chromosome dimer resolution can be viewed as a housekeeping function conserved among bacteria with circular chromosome(s), but that some species can use different functional analogs to perform this task.

C. crescentus is an aquatic Gram-negative bacterium found in various environments (10). This bacterium has an asymmetric cell cycle which can be used to synchronize cell growth in order to study the temporal expression of a gene and the interconnection between the cell cycle and development. A site-specific recombination system involved in chromosome partitioning has been described for *C. crescentus* (11). *C. crescentus* XerC(*cc*XerC) and XerD(*cc*XerD) show 34.9% and 41.1% identity with the *E. coli* XerC(*ec*XerC) and XerD(*ec*XerD) recombinases, respectively. *cc*XerC and *cc*XerD both possess the conserved amino acid residues indicative of the tyrosine family site-specific recombinases (4, 11). *In vitro*, *cc*XerC and *cc*XerD exhibited binding

activity on the *ecdif* site indicating that *cc*XerD had a higher affinity for the *ecdif* site than *cc*XerC. Cooperative binding was also observed between these two recombinases on *ecdif* site (11). Recently the *C. crescentus dif* site (*ccdif*) was characterized (12). Removal of *ccdif* or deletion of *ccxerC* and *ccxerD* resulted in chromosome segregation defects and an increase in the formation of cell filaments, largely as a consequence of failing to resolve chromosome dimers (1, 3, 12,).

In order to better understand how *cc*XerC and *cc*XerD recombinases recognize DNA, and how they perform their respective recombination reactions, we tested the binding activity and the formation of phosphotyrosyl complex of these two recombinases on the *ccdif* site.

2. Materials and methods

2.1 Bacterial strains and plasmids

The *C. crescentus* strain used was CB15N (NA1000) (13). *E. coli* DH5α (F^- *endA1 hsdR17* ($r_K^- m_K^+$) *supE44 thi-1* λ^- *recA1 gyrA96 relA1* Δ(*lacZYA-argF*)*U169* φ80d*lacZ*ΔM15) (14) was used for cloning and plasmid purification. For overexpression of maltose-binding protein (MBP)-fused genes, strain *E. coli* BL21 (F^-*ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm*) (Novagen) containing the *lacI*^{q} plasmid pREP4 (15) (Qiagen) was used. pMal-*ccxerC* and pMal-*ccxerD* both contain the *xerC* and *xerD* genes of *C. crescentus* respectively (11). The PCR-amplified *ccdif* site was cloned in plasmid pDrive (Qiagen). Plasmids expressing active-site tyrosine mutants of *ccxerC* and *ccxerD* were introduced into *E. coli* T7 Express *lysY* (miniF-*lysY* (Cam^R) */ fhuA2 lacZ::T7 gene1 [lon] ompT gal sulA11 R(mcr-73::miniTn10--*Tet^S*)2 [dcm] R(zgb-210::Tn10--*Tet^S*) endA1 Δ(mcrC-mrr)114::IS10 [miniF-lysY (*Cam^R*)])* (New England Biolabs) and were overexpressed and purified as MBP fusion proteins.

2.2 Growth conditions and DNA manipulations

Bacteria were grown in Luria–Bertani (LB) broth (Difco), autoinducible medium (4.76% Terrific broth, 0.4% Glycerol, 0.2% Lactose monohydrate, 0.05% Glucose, and 2mM $MgSO_4$) (34) or plated on LB agar containing the appropriate antibiotics when required. *C. crescentus* was grown at 30°C in peptone–yeast extract (PYE) medium. Ampicillin was used at 100 μg ml^{-1}, kanamycin at 50 μg ml^{-1}. Restriction enzymes, T4 polynucleotide kinase and DNA ligase were obtained from New England Biolabs and used according to the supplier's conditions. All routine DNA manipulations were

performed as described in (18, 19). DNA fragments were extracted from agarose gels using the QIAquick gel extraction kit (Qiagen) according to manufacturer's conditions. Plasmids were extracted and purified by using the QIAprep Spin Miniprep kit (Qiagen) according to manufacturer's conditions.

2.3 Polymerase chain reaction (PCR) conditions

PCR reactions were performed using a CyclePro Thermocycler (Bio-Can) with Taq DNA polymerase (New England Biolabs) or High-Fidelity Phusion DNA polymerase (New England Biolabs) according to the supplier's conditions. For the amplification of the *ccdif* sequence, the cycling conditions were: 95°C/15s, 51.5°C/30s and 72°C/45s for 30 cycles, with a final extension at 72°C/5 min. Reactions were carried out in 25 µl using Taq DNA polymerase, with the following primers: CCdifF (5'CGTCATGGTCGTTCGATCCCAC) and CCdifR (5'CGCCCAGGAACAGCTTCTTGTC). Site-directed mutant *ccxerC*^{Y297F} and *ccxerD*^{Y277F} genes were amplified by PCR with High-Fidelity Phusion DNA polymerase under the following conditions: 98°C/15 s, 53°C/30 s and 72°C/4min for 30 cycles, with a final extension at 72°C/5 min. Reactions were done in 50µl with the following primers: ccXerCYF-F (5'ACGCAACGTTTCACCCAGGTG) and ccXerCYF-R (5'GGTCGAGAGCGAGGCGTG) for the *xerC*^{Y297F} gene, and the following primers: ccXerDCYF-F (5'ACCCAGATCTTCACCCACGTG) and ccXerDCYF-R (5'GGTGGCGATGTCGGCGTG) for the *xerD*^{Y277F} gene. The PCR products were then treated with T4 polynucleotide kinase, purified and religated prior to introduction into *E. coli* DH5α. Plasmid clones were verified by DNA sequencing.

For DNA-binding assays, *ccdif* and *ecdif* were 5' end-labelled with 6-Hex using PCR amplification with the following Primers: M13F-40HEX5' (5'CGCCAGGGTTTTCCCAGTCACGAC) and M13R-48HEX5' (5'AGCGGATAACAATTTCACACAGGA), under the following conditions: 95°C/15 s, 51.5°C/30 s and 72°C/45 s for 30 cycles, with a final extension at 72°C/5 min. Reactions were carried out in 50µl volumes using Taq DNA polymerase.

2.4 Oligonucleotides

Synthetic oligonucleotides containing *ccdif* were based on the following sequences, top strand: 5'TCAAAGATCGACTT**TGTAAT**TTATGTAAAGTTGT; bottom strand: 5'ACAACTTTACATAA**ATTACA**AAGTCGATCTTTGA. The *ccdif* core site is underlined, with the central region sequence given in bold type. Oligonucleotides were 5' 6-Hex or Cy5 labelled, and were obtained from Alpha DNA, Montreal QC. Unlabelled oligonucleotides were obtained from BIOcorp, Montreal QC.

2.5 Purification of Xer–MBP fusion proteins

Clones expressing wild-type *ccxerC and ccxerD* genes or active-site tyrosine mutants were introduced into *E. coli* T7 Express. 1.2 ml of an overnight culture was used to inoculate 100 ml autoinducible medium containing ampicillin for overnight induction. Once harvested, pellets were resuspended in column buffer (20 mM Tris–HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA) followed by the addition of 1 mM phenylmethylsulfonylfluoride or complete Mini protease inhibitor cocktail tablet, EDTA-free (Roche Diagnostics). The cells were then freeze–thawed and sonicated followed by centrifugation at 2800×*g* for 15 min at 4°C. Supernatants were passed through an

amylose column prepared according to (18). Elutions were done according to the same protocol, except that one additional step of washing in 20 mM Tris–HCl, pH 7.4, 0.5 M NaCl, 1 mM EDTA was added before washing with column buffer and elution with 10 mM maltose in column buffer. Most of the protein eluted in the first fraction and was more than 80% pure, as visualized on a 12.5% SDS–PAGE gel stained with Coomassie blue.

2.6 Protein labelling

Purified proteins, at a concentration of 1mg/ml in PBS buffer, were labelled with 200µM fluorescein-5-EX, succinimidyl ester (Invitrogen) in 100mM sodium bicarbonate buffer. The reaction mixture was incubated in the dark for 15 minutes at room temperature and kept at 4°C.

2.7 DNA-binding assays

Specific DNA binding was determined by a gel retardation assay (21) using fluorescently-labelled specific DNA fragments that were purified from an agarose gel. Reaction conditions consisted of 2ng labelled DNA, 125ng polydIdC (Roche) in TENg-binding buffer (20 mM Tris–HCl pH 7.5, 1 mM EDTA, 50 mM NaCl and 10% glycerol) and increasing *cc*XerC and *cc*XerD concentrations. Reactions were incubated for 60 min at 30°C before undergoing electrophoresis on a 6% polyacrylamide gel in 0.25× TBE buffer at 65 V at 4°C. Wet gels were scanned with a Typhoon Trio Imager and processed using ImageQuant software. The experiments were replicated more than five times.

2.8 *In vitro* cleavage assays

Suicide substrates were constructed by annealing two oligonucleotides (17nt, one of which was 5'-labelled with 6-Hex or Cy5), corresponding to left and right halves of core sites, to an oligonucleotide corresponding to the appropriate complete top or bottom strand (34nt). The resulting double stranded DNA thus contained a nick either in the top or bottom strand of the central region. The substrates were then incubated with *cc*XerCD-MBP at concentrations of 1230nM for both proteins (labelled or unlabelled) in the presence of 125ng polydIdC. After a 90min incubation at 30^{o}C followed by 10min heat treatment at 95^{o}C, reactions were electrophoresed in a 6% polyacrylamide TBE gel in the presence of 0.1% SDS in 0.25× TBE buffer containing 0.1% SDS at 4°C. The experiments were replicated more than five times.

3. Results

3.1 *cc*XerC and *cc*XerD bind to the *ccdif* site

The *ccdif* site is defined by two 11bp half sites that share partial dyad symmetry separated by a 6bp central region. An alignment of *ccdif* with *dif* sites from *E. coli, Haemophilus influenzea,* and *Bacillus subtilis* (Fig. 1A) demonstrated that the right half-site and central region sequence are highly conserved (9 of 11bp and 4 of 6bp matches to *ecdif* respectively), while the left half-site is more divergent (1 of 11bp matches to *ecdif*). The sequence similarity of the right half-site probably explains the observed high affinity binding of *cc*XerD to the *ecdif* site (11).

To ascertain if *cc*XerC and *cc*XerD could bind specifically to the *ccdif* site, we used gel retardation analysis of Hex-labelled DNA with purified MBP fusion proteins *cc*XerC and *cc*XerD (Fig. 1B). Previous work with the *E. coli* and *Proteus mirabilis* Xer proteins has demonstrated that these N-terminal fusions do not hamper the catalytic activity of these proteins (20, 22). Addition of *cc*XerC to *ccdif* gave rise to protein-DNA complexes that migrated with mobility consistent with a single recombinase monomer (Fig. 1B, lane 4-7) binding to the recombination site or mobilities consistent with binding of two XerC protomers (Fig. 1B, Lane 2, 3). Similar binding activity was observed in the addition of *cc*XerD to the *ccdif* site (Fig. 1B, Lane 9, 10). *cc*XerD bound to the *ccdif* with a higher apparent affinity than *cc*XerC, as similar retardations were obtained with 70nM *cc*XerD and 309nM *cc*XerC, both in the presence of 125ng polydIdC. This higher apparent *cc*XerD affinity was also found when the *ecdif* site was used in the binding reaction (11). A control non-specific labelled DNA fragment did not display any

significant retardation at concentrations of both proteins, *cc*XerC and *cc*XerD, that were able to retain the *ccdif* site respectively (data not shown). After the addition of increasing amounts of polydIdC to a constant amount of *cc*XerC or *cc*XerD, specific protein-DNA complexes were observed in added polydIdC up to 2000ng or 100-fold excess to the DNA used in the reactions (data not shown). All together, these results strongly suggest that the retardation observed in Fig. 1B is due to specific binding.

Our previous *in vitro* experiments demonstrated that *cc*XerC and *cc*XerD were capable of binding the *ecdif* site cooperatively which showed that one recombinase can stimulate the other recombinase binding to the DNA site. This cooperative binding was also seen when increasing amounts of *cc*XerD were added to a constant amount of *ec*XerC, but not observed when the proteins were reversed (11). The binding of *cc*XerC and *cc*XerD to the *ccdif* showed significant differences to what has been observed when these proteins bind to the *ecdif*. If *cc*XerCD recombinases on the *ccdif* reaction are cooperative, a much stronger shifted recombinases-DNA complex band should be observed when two recombinases added together. However, little evidence of cooperative binding with these proteins to the *ccdif* was observed under these conditions when comparing Fig. 2 lane 3, and lane10, in which two recombinases were incubated with *ccdif*, to lane 5, 6 and lane12, 13, in which one of the recombinase in the same concentration was added in each reaction (Fig. 2). There is also a slight production of higher-order protein-DNA complexes, (Fig. 2, lane3, 4 and lane 10, 11), which may be tetramers of *cc*XerC and *cc*XerD. Since the binding activity of the *cc*Xer proteins on *ecdif* was previously shown (11), we further tested the binding of *ec*XerC and *ec*XerD to

ccdif. *E. coli* Xer proteins bound very poorly to *ccdif* and no interaction between *ec*Xer recombinases and their partner *cc*Xer proteins with *ccdif* was observed (data not shown).

To further characterize the binding activity of *cc*XerC and *cc*XerD to the *ccdif*, we used 17bp Hex-labelled double stranded oligonucleotides, corresponding to the left and right halves of the *ccdif* site in gel shift assays (Fig. 3). Firstly, we observed that *cc*XerC and *cc*XerD could bind to both left and right half-sites respectively and that *cc*XerD bound better to both sites than *cc*XerC (data not shown). Therefore, *cc*XerD had a higher affinity to both double stranded *ccdif* oligonucleotides than *cc*XerC, which is consistent with the observation of binding of *cc*XerC and *cc*XerD with the full *ccdif* site shown in Fig. 1B. Furthermore, *cc*Xer recombinase binding to the half-site of *ccdif* appears to be much weaker than to the full *ccdif* site when the shifted bands and the amount of DNA used in the reactions are compared. This suggests that *ccdif* half-site substrate may not be an ideal substrate for DNA binding, or perhaps that the central region of *ccdif* may contribute to XerC binding activity at the half-site. Secondly, we also observed that both *cc*XerC and *cc*XerD bound preferentially to the left half-site of *ccdif* (Fig. 3), which is different from what was observed with *E. coli.* In the case of *E. coli*, it was shown that *ec*XerC could bind to both the left and right half-site of *ecdif* with a higher affinity to the left half-site, whereas *ec*XerD could only bind to the right half-site (2, 23). *cc*Xer recombinases binding to the both half-sites of *ccdif* may explain the presence of the faint complex representing two monomers of *cc*Xer proteins binding to the full *ccdif* site as shown in Fig. 1B lane2, 3 and lane 9, 10.

3.2 *In vitro* cleavage of *ccdif* suicide substrates

The catalytic function of a tyrosine recombinase is the cleavage and subsequent exchange of one DNA strand between two synapsed recombination sites. After strand cleavage and prior to strand exchange, a phosphotyrosyl bond is formed between the recombinase and its DNA target. Recombinase-mediated strand cleavage can be assayed *in vitro* by the accumulation of recombinase/DNA covalent complexes using linear suicide substrates that contain a nick at the central position of the spacer, three nucleotides from the 3' side of the recombinase cleavage site. Cleavage of the substrate generates a three-nucleotide fragment that is free to diffuse from the complex, thus preventing religation because there is no 5'-OH to act as the nucleophile for the reverse reaction (42). The convention for the terms "top" and "bottom" strands that is used here, relates to the first (top) pair of strands and the second (bottom) pair of strands which are exchanged in *cer* and *psi in vivo* and *in vitro* reactions (24, 25).

The abilities of these proteins to form stable phosphotyrosyl linkages with DNA were tested by using a nicked 'suicide substrate' DNA which was labelled with 6-Hex. Covalent recombinase-DNA complexes were generated and detected after heat treatment by polyacrylamide gel electrophoresis in the presence of SDS. These covalent complexes were formed when both *cc*XerC and *cc*XerD were incubated with *ccdif* suicide substrates, which represent covalently-linked recombinase-DNA complexes formed during the initial steps in tyrosine recombinase site-specific recombination (Fig. 4, lane 4). We did not observe any covalent complex when only one of the recombinases was present in these conditions in *C. crescentus* although low levels of *ec*XerC-mediated top-strand cleavage at *ecdif* in the absence of *ec*XerD have been reported previously (23). Cleavage of the top-nicked suicide substrate was more efficient than the bottom nicked suicide substrate

as seen in Fig. 4 lane 4 and 8 in which the same amount of *cc*XerC and *cc*XerD were incubated with top-nicked and bottom-nicked suicide substrates respectively. The different rates of cleavage detected for top and bottom-nicked strands might be at least partly a consequence of a difference in catalytic activity between XerC and XerD on these substrates. The requirement for both proteins to be present in order to detect cleavage activity demonstrates the importance of interactions between the partner recombinases for controlling catalytic activity in a bound complex.

In the recombination reaction, the tyrosine of the RHRY tetrad represents the catalytic residue, whereas the first arginine is involved in specific binding to DNA. Catalysis is initiated by the nucleophilic attack of the active-site tyrosine on the scissile phosphate generating a 3' phosphotyrosyl DNA-protein intermediate and a free 5'-OH. Mutant recombinases in which the active-site tyrosine had been replaced by phenylalanine (creating *cc*XerC^{Y297F} and *cc*XerD^{Y277F}, respectively) were used to investigate the catalytic role of each recombinase during recombination at *ccdif*. The binding affinity of *cc*XerC^{Y297F} or *cc*XerD^{Y277F} was similar to wild-type *cc*XerC and *cc*XerD respectively (data not shown). The covalent complex formed after strand cleavage was not detected when wild type *cc*XerC was combined with mutant *cc*XerD^{Y277F} and/or wild- type *cc*XerD with mutant *cc*XerC^{Y297F} (Fig. 5). Similar observations with bottom-nicked strand were also obtained (data not shown). These results indicated that both active-site tyrosines are important for the catalytic reaction but not for binding activity.

3.3 *cc*XerC forms phosphotyrosyl complexes on both top and bottom nick strand of *ccdif*

In order to determine which recombinase was covalently linked to DNA, Cy5-labelled suicide substrates, top-nicked *ccdif* and bottom-nicked *ccdif*, were incubated with FITC-labelled recombinases. The different color signal from Cy5 and FITC and the slight size difference (0.5kDa) between *cc*XerC and *cc*XerD enables us to detect which protein made a covalent complex. *cc*XerC migrated slightly slower than *cc*XerD (Fig. 6A middle image lane 4 vs 5). The left image shows that all DNA species (covalent complex) migrated at the same position (Fig. 6A, lane 3, 6, and 7). The right image shows the merged DNA and protein signals. In Fig. 6A lane 6 of the right image, the labelled *ccdif* in the covalent complex migrated a little slower than the *cc*XerD. This non-comigration with labelled *cc*XerD suggested that it was *cc*XerC which formed the covalent complex. This was confirmed by the evidence in Fig. 6A lane7 of the right image, where labelled *ccdif* and labelled *cc*XerC comigrate in the same position in which the red signal from DNA and green signal from protein merged to yellow. Similar evidence was observed in the bottom-nicked strand although a lower level of cleavage was mediated by *cc*XerC when compared to top-nicked strand (Fig. 6B, and Fig. 4). Together these results indicated that it was *cc*XerC that mediated the top-nicked strand and bottom-nicked strand cleavage on *ccdif*.

4. Discussion

Xer site-specific recombination functions in the stable inheritance of circular replicons by converting chromosome dimers to monomers prior to cell division (1, 2, 3). Here we report three prominent features of Xer recombination in *C. crescentus*. First, *cc*XerD binds to the *ccdif* site with a higher affinity than *cc*XerC; both *cc*XerC and *cc*XerD bind preferentially to the left half-site of *ccdif*. Second, there is very little cooperative binding between these two recombinases on *ccdif*. Third, the cleavage activity of these two *cc*Xer recombinases on *ccdif* requires both wild type recombinases to be present; cleavage of the top-nicked suicide substrate is more efficient than cleavage of the bottom-nicked suicide substrate; after cleavage, *cc*XerC remains bound to both the top and bottom strand.

In *E. coli*, XerC has a higher affinity for the left half-site of *ecdif* and XerD has a higher affinity for the right half-site (2, 21). It was proposed that the nucleotides of the outer ends of the binding site contribute most significantly to XerC and XerD binding specificity and substitution of specific nucleotides alters Xer binding activity (40). At the outer ends of the *E. coli dif* site (Fig. 1A, positions -14 to -9 and +9 to +14), nucleotides -10C and -13G appear to be very important for XerC binding, while +9T and +13A contribute significantly to XerD binding (40). When compared to the outer ends of *ecdif* sequence, nucleotides -14 to -10 of the *ccdif* are divergent from *ecdif,* while the right half-site is more conserved in which only the +13 nucleotide of *ccdif* is changed to G and +9T is conserved (Fig. 1A). Consistent with the conclusion that +9T is a major specificity determinant in XerD binding, *cc*XerD showed a higher binding affinity to right half-site of *ccdif* than *cc*XerC. Furthermore, similar to *ec*XerC binding on *ecdif*, *cc*XerC bound

preferentially to the left half-site of *ccdif* although the left half-site of *ccdif* is much more divergent. However, *cc*XerD did not show a higher affinity to the right half-site but showed a higher affinity to the left half-site of *ccdif* in our assay. It is possible that the left *ccdif* half-site provides the sequence(s) recognition specificity for both recombinases' binding. Another possibility is that *cc*XerD might require accessory sequences for successful intermolecular recombination which is different from what is known in *E. coli.* This possibility was first reported by Mcleod and Waldor (16), who showed that the recombination of XerC and XerD between the CTXφ *attP* site and the *V. cholera* chromosomal *dif* site requires additional recombinase binding sites. Not only did XerC and XerD bind to the DNA sequence where strand exchanges were predicted to occur, they were also able to bind to a related sequence approximately 84bp downstream from the positions of strand exchange (16). This was also seen with the topological filters of γδ and Tn3 resolvases bound to *res* sites, although the resolvase/*res* synapsis was intramolecular (17, 36). Considering that the *ccdif* sequence is quite divergent from *ecdif*, additional architectural support might be needed for successful *cc*XerD recombination activity. A third possibility is that *cc*XerD may require some sort of modification, for example, like FtsK activation, in order to bind specifically and to cleave DNA.

In *E. coli*, binding of XerC and XerD to *dif* is highly cooperative (2, 21). This cooperativity could occur as a consequence of specific interactions between XerC and XerD, or by changes in DNA structure that arise as a consequence of binding either recombinase. The findings, for example, of the ability to cross-link one recombinase to the other when bound to DNA and the region involved in the interaction between XerC and XerD (21, 37, 38), favour the former explanation. However, the observation that the

presumptive XerC-XerC interactions are only detected on a substrate containing a bottom-nicked strand and indicate that spatial flexibility in the DNA is required to enable the interactions to occur (23). Although observations such as the loss of cooperativity due to the insertion of 2bp in the phage HK022 cI repressor binding site resulted in a lack of flexibly in the protein structure (39), the differences in XerC and XerD-induced *dif6* and *dif8* bending suggest that distortion in the DNA induced by recombinase binding, rather than a protein's flexibility, could explain the relatively small loss in cooperative interactions between *dif*6 at *dif*8 (21). The gain in chemical sensitivity in the *dif*8 central region and the left half-site are consistent with this idea (21). Therefore, it is possible that the conformation of a recombination site bound by one recombinase may allow better binding of the second recombinase, which would also result in cooperativity. The reduced cooperativity between *cc*XerC and *cc*XerD on *ccdif* could be the consequence of the higher occupancy of left half-site of *ccdif* by both recombinases since this could limit the conformational change of *ccdif* site and thus prevent the contact of these two recombinases. The different binding affinities of *cc*XerC and *cc*XerD to *ccdif* might also explain the observed difference of the cleavage activities on *ccdif*.

Previous studies indicated that efficient recombination required both recombinases to be present (25, 41); also a given recombinase could influence the catalytic activity of its partner (23). These observations are also confirmed in *cc*Xer protein interaction with *ccdif*. The failure of singly-bound *cc*XerC or *cc*XerD molecule to catalyze *ccdif* cleavage suggests that XerC and XerD each activate catalysis of its partner recombinase when bound to DNA. The observation that mutant *cc*XerC or *cc*XerD proteins can influence the catalytic activity of its wild-type partner recombinase

strengthens this view. Moreover, a requirement for both recombinases to be bound at a recombination site to render *cc*XerC competent for strand cleavage *in vitro* provides a means of controlling recombination by preventing initiation of the following strand exchange when only a single recombinase molecule is bound to a duplex *ccdif* site. It was previously concluded that the ability of XerC to cleave DNA is strongly influenced by the interactions between XerC and XerD and the geometry of the recombinase-DNA complex (21). Therefore, the observations of similar binding activity but different cleavage activities of the mutant proteins suggest that the active-site tyrosine in a wild-type recombinase molecule directly or indirectly contacts with the DNA backbone of the half-site to which it is bound. Furthermore, results that covalent stable SDS-resistant complexes were formed when both wild-type *cc*XerC and *cc*XerD were present but not in the presence of active-site tyrosine mutant proteins indicate that both active-site tyrosines of *cc*XerC and *cc*XerD are crucial for catalysis. A substitution of amino acid residues within *cc*XerC and *cc*XerD could result in the disruption of intermolecular interaction between them, as these interactions are important to maintain the DNA substrate in a conformation appropriate for recombinase catalysis (23).

In *E. coli*, *ec*XerC preferentially cleaves the top strand of linear *ecdif* efficiently, while *ec*XerD cleaves the bottom strand inefficiently (21, 23, 35). In *C. crescentus*, *cc*XerC mediated both top-nicked strand and bottom-nicked strand cleavage in the presence of *cc*XerD. This could be as a consequence of the different binding activities of *cc*XerC and *cc*XerD with *ccdif* since *cc*XerC could bind to both left and right half-sites of *ccdif* in our *in vitro* binding studies as discussed in previous part. This also suggests that the recombinase-DNA complex adopts a conformation that is suitable for XerC but not

for XerD cleavage. The failure to observe *cc*XerD cleavage activity in our conditions could be explained by the requirement for specific cofactor(s) or conditions to form an "XerD-active" state, or the specific substrate DNA structure or conformation.

In *E. coli* it has been established that, in addition to the Xer recombinases, at least one other protein, FtsK, is required for chromosome dimer resolution *in vivo* (28, 29) and site-specific recombination at other ectopic *dif* sites (33). In site-specific recombination, FtsK serves to activate recombination at the *dif* site via a direct interaction with XerD, thus stimulating XerD–mediated cleavage of bottom-nicked strand DNA in the presence of XerC (9). The interaction between XerD and FtsK promotes the formation of heterotetrameric synaptic complexes that have a conformation appropriate for catalysis by XerD and allows XerD to perform a first pair of strand exchanges, resulting in the formation of a HJ. This HJ is converted to a crossover by a second pair of strand exchanges, which is catalyzed by XerC independently of FtsK. Studies have shown that the γ domain in the C-terminus of FtsK interacts directly with the XerD C-terminus in order to stimulate the cleavage by XerD of bottom-nicked strand (BNS) in *E. coli* (27) . Mutational impairment of the XerD-FtsK C-terminus interaction leads to a reduction in the *in vitro* stimulation of BNS cleavage by XerD and a concomitant decrease in the resolution of chromosomal dimers at *dif in vivo* (27). Thus, in *E. coli*, the requirement for FtsK to activate the catalytic activity of XerD permits coordination of chromosome dimer resolution (32). Recchia and Sherratt have since proposed that all eubacteria with circular chromosomes and Xer homologues also have FtsK homologues and suggest that this demonstrates a functional interaction (30). Furthermore, previous work has demonstrated that FtsK is present in *C. crescentus* and its C-terminus is essential and involved in

maintaining accurate chromosome partitioning (31). It has also been established that the C-terminual region of XerD carries major determinants for interaction with the FtsK C-terminal region and is important for the mediation of normal recombination at the chromosomal *dif* site (27, 43). As revealed by the alignment of *C. crescentus* XerD and *E. coli* XerD proteins, maximum identity is found in the C-terminal region of the proteins (11). Also, the γ domain of C-terminal region of *C. crescentus* FtsK protein shows 45% identity and 77.5% similarity with γ domain of C-terminal region of *E. coli* FtsK protein (data not shown). Therefore, all these observations support the idea that chromosome dimer resolution in *C. crescentus* is controlled possibly, in part, by the FtsK protein by activating the initial chemical step of the recombination reaction, cleavage of DNA by XerCD to form a recombinase–DNA covalent complex. We propose that FtsK could promote the formation of an 'XerD-active" state and thus control the catalytic active of *cc*XerD on *C. crescentus*. However we only observed partial complementation of filamentation in *E. coli xerD* mutants with a cloned copy of the *C. crescentus xerD* gene (data not shown). This may be due to the species specificity in the direct interaction between XerD and FtsK C-terminal residues (26). Further studies on the role of *C. crescentus* FtsK in site-specific recombination may help to understand how *cc*XerC and *cc*XerD perform in the catalytic reaction at the *ccdif* site.

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7. Figure Legends

Fig. 1. Alignment of *dif* recombination sites and gel retardation analysis of *cc*XerC and *cc*XerD binding to *ccdif*.

A, *dif* site alignments. Central region sequences are in boldface type. B, Binding activity of *cc*XerC and *cc*XerD to *ccdif*. 2ng of *ccdif* (490bp) was 5'-labelled by PCR using 6-Hex labelled primers. Proteins at the indicated concentration in nM were incubated with *ccdif* for 60min at 30^{o}C as described in Materials and Methods. The asterisk indicates the position of the 6-Hex fluorescent label.

Fig. 2. Reduced cooperative binding of *cc*XerC and *cc*XerD with *ccdif*.

Reactions were incubated for 60min as described in Materials and Methods. The concentration of *cc*XerC and *cc*XerD are indicated below each lane in nM. All reactions contained 2ng of Hex-labelled *ccdif* fragments and 125ng polydIdC. The asterisk indicates the position of the 6-Hex fluorescent label.

Fig. 3. *cc*XerC and *cc*XerD bind preferentially to the left half-site of *ccdif*.

5' 6-Hex labelled double stranded oligonucleotides (17bp) corresponding to the left and right half-site of *ccdif* were synthesized and annealed. The asterisk indicates the position of the 6-Hex fluorescent label. The *ccdif* core site is underlined, with the central region sequence given in bold type. 10ng of *ccdif* was used in reactions containing *cc*XerC or *cc*XerD as described in Materials and Methods. The concentration of *cc*XerC and *cc*XerD are indicated below each lane in nM.

Fig. 4. Phosphotyrosyl complex formation on *ccdif* DNA using suicide substrates.

Top-nicked (lanes 1-4) or bottom-nicked (lanes 5-8) suicide substrates were prepared as described in Materials and Methods. The nick is positioned after the 3rd nucleotide of the spacer region. The asterisk indicates the position of the 6-Hex fluorescent label. 10ng suicide substrates were incubated in the presence of *cc*XerCD-MBP at concentrations of 1230 nM for both proteins in the presence of 125ng polydIdC.

Fig. 5. Y-F recombinase mutants do not form phosphotyrosyl complexes with suicide substrates.

Top-nicked suicide substrate was incubated with either *cc*XerC, *cc*XerD, *cc*XerC^{Y297F} or *cc*XerD^{Y277F} as described in Materials and Methods. Cleavage activity to the top-nicked suicide substrate was tested by migration on 6% 0.25X TBE gel in the presence of 0.1% SDS. The indicated protein(s) and top-nicked strand were incubated for 90 minutes at 30^{o}C and heated for 10min at 95^{o}C.

Fig. 6. XerC, but not XerD forms phosphotyrosyl complexes on suicide substrate.

10ng Cy5-labelled top-nicked (Fig. 6A) and bottom-nicked (Fig. 6B) suicide substrates were incubated with *cc*XerC and/or *cc*XerD (FITC-labelled protein indicated by asterisk) as described in Materials and Methods. Cy5-labelled *ccdif* DNA is indicated in red and FITC-labelled proteins are indicated in green. The recombinases present in each reaction are indicated below the appropriate lane. The left panel indicates the position of labelled DNA, the middle panel shows the labelled protein, and the right panel shows the merged protein and DNA signal.

Fig. 1.

A

dif B. subtilis	ACTTCCTAGAA--**TATATA**--TTATGTAAACT
dif H. influenzae	ATTTCGCATAA--**TATAAA**--TTATGTTAAAT
dif E. coli	GGTGCGCATAA--**TGTATA**--TTATGTTAAAT
dif C. crescentus	AAGATCGACTT--**TGTAAT**--TTATGTAAAGT

B.

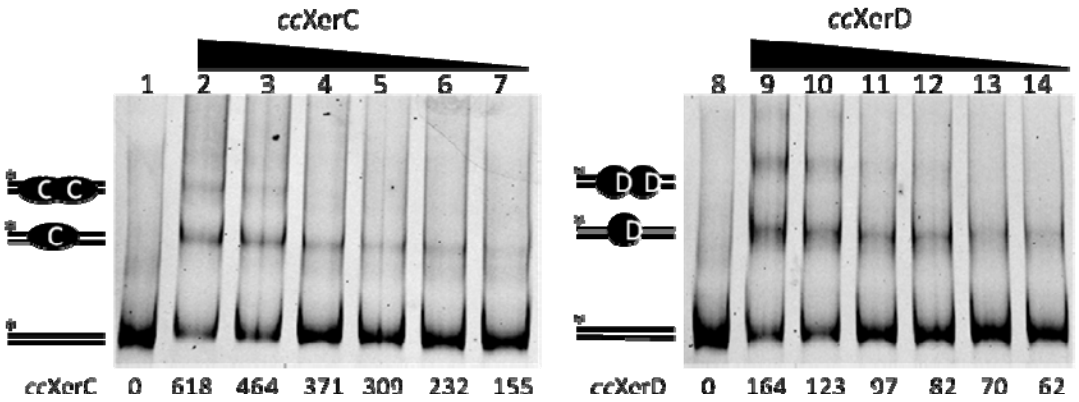


Fig. 2

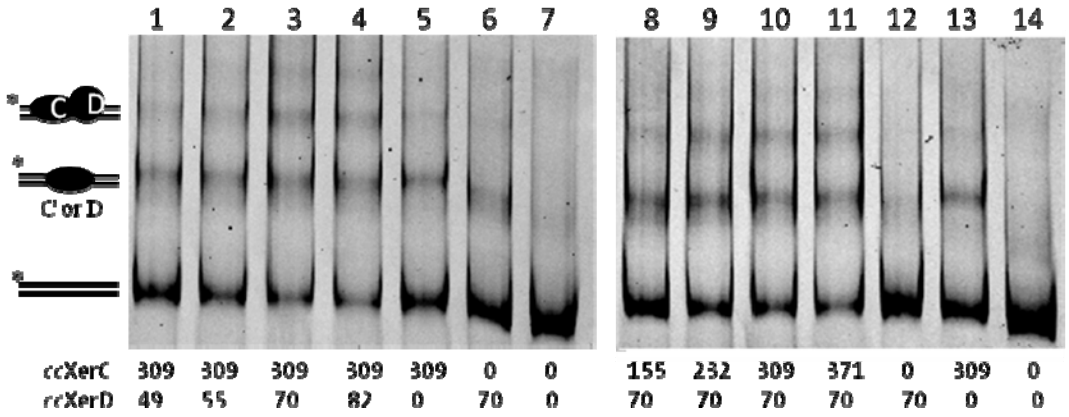

1 2 3 4 5 6 7 8 9 10 11 12 13 14
C D
C or D
ccXerC 309 309 309 309 309 0 0 155 232 309 371 0 309 0
ccXerD 49 55 70 82 0 70 0 70 70 70 70 70 0 0

Fig. 3.

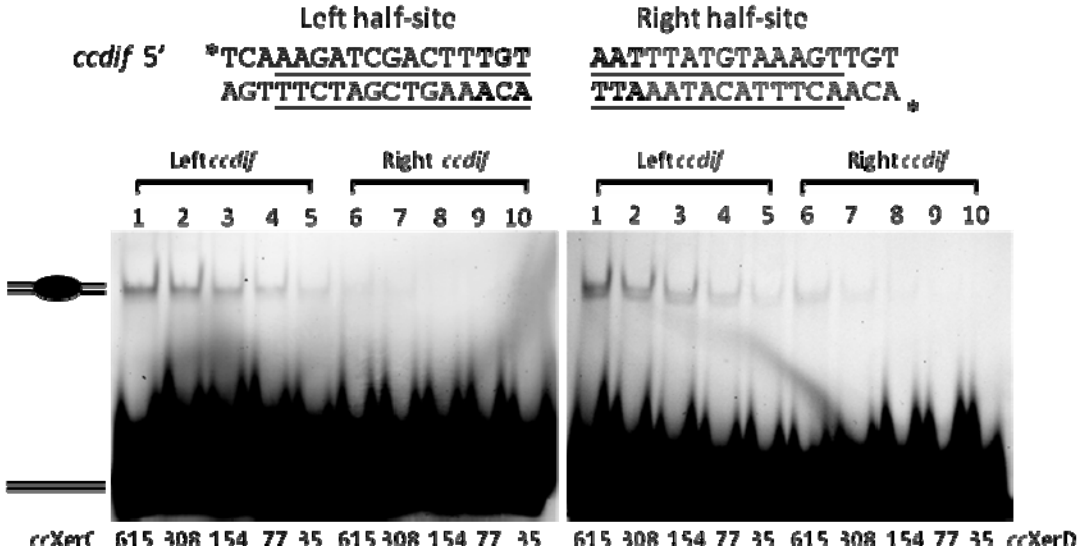
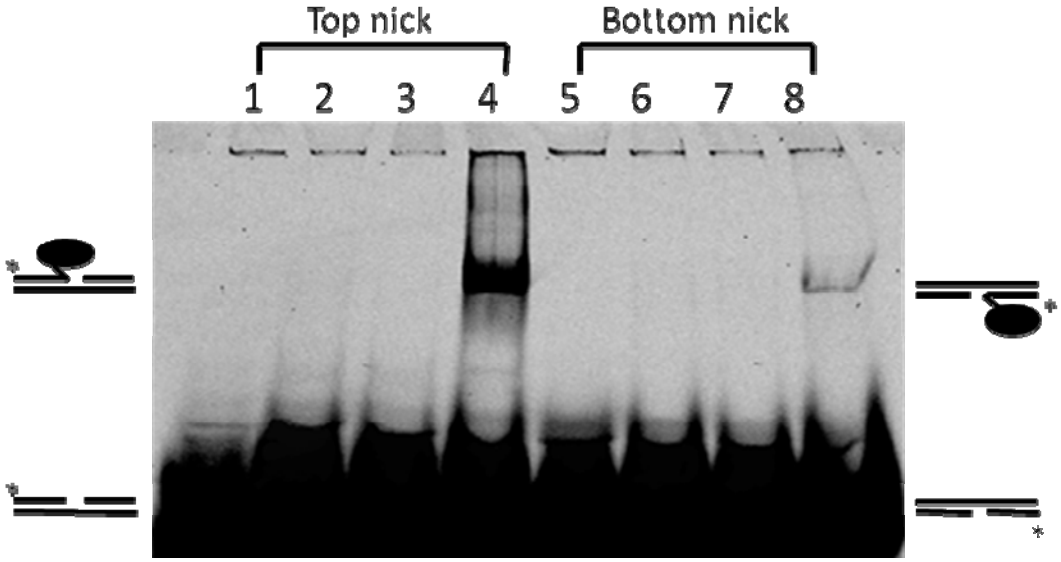
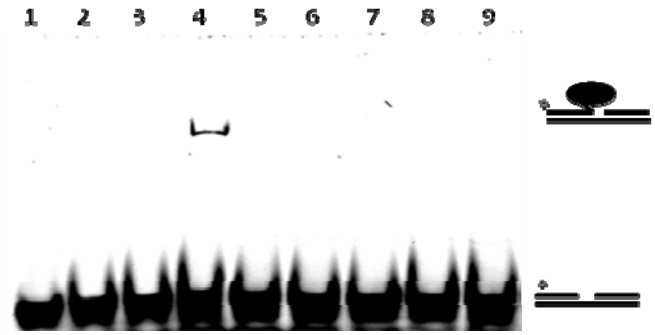

Left half-site
Right half-site
ccdif 5'
*TCAAAGATCGACTTTGT
AGTTTCTAGCTGAAACA
AATTTATGTAAAGTTGT
TTAAATACATTTCAACA
*
Left ccdif
Right ccdif
Left ccdif
Right ccdif
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10
ccXerC 615 308 154 77 35 615 308 154 77 35
615 308 154 77 35 615 308 154 77 35 ccXerD

Fig. 4.



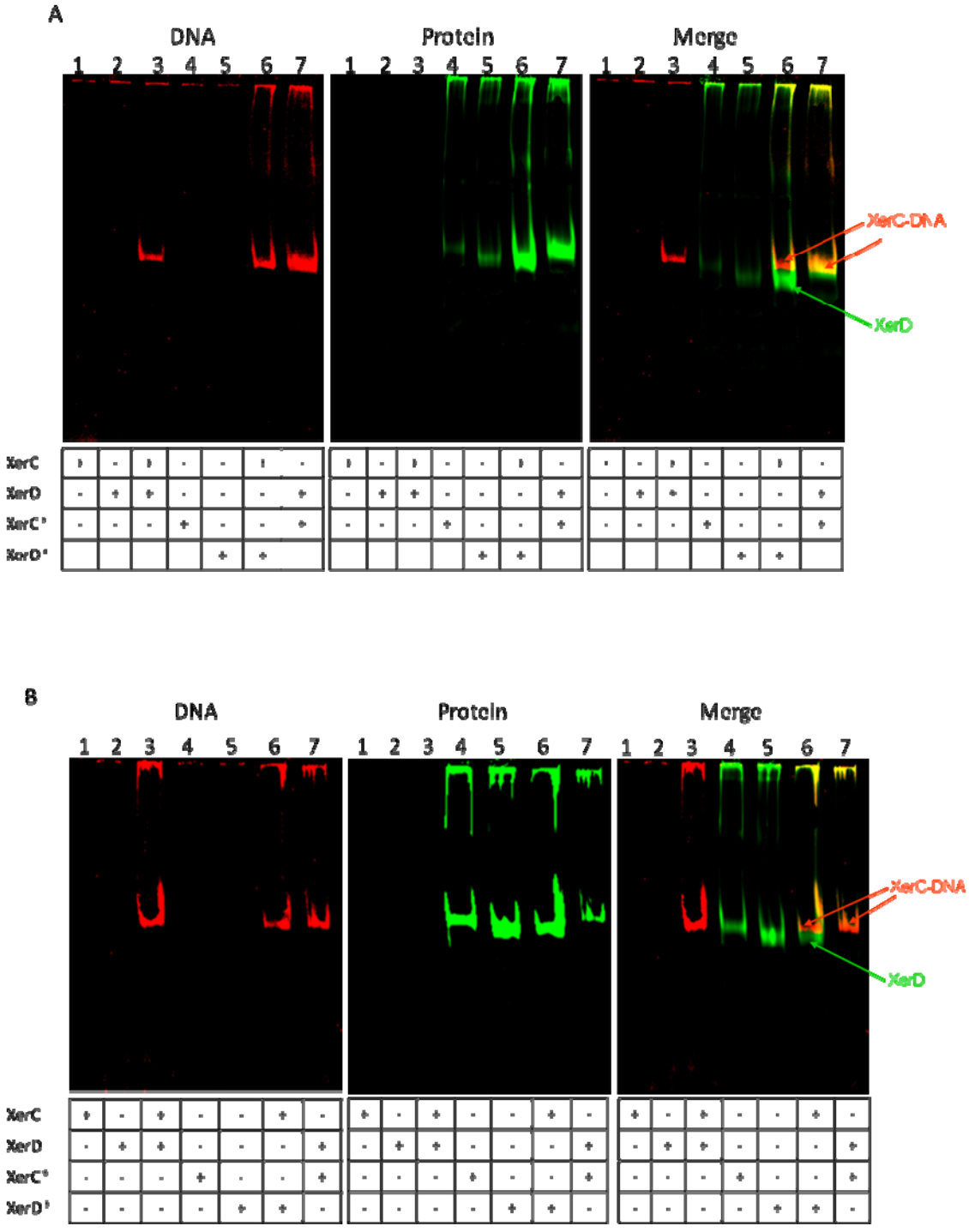
XerC	-	+	-	+	-	+	-	+
XerD	-	-	+	+	-	-	+	+

Fig. 5.



-	+	-	+	-	-	-	+	-	XerC
-	-	+	+	-	-	-	-	+	XerD
-	-	-	-	+	-	+	-	+	$XerC^{Tf}$
-	-	-	-	-	+	+	+	-	$XerD^{Tf}$

Fig. 6.


A
DNA
Protein
Merge
1 2 3 4 5 6 7
XerC-DNA
XerD
XerC
XerD
XerC'
XerD'
B
DNA
Protein
Merge
1 2 3 4 5 6 7
XerC-DNA
XerD
XerC
XerD
XerC'
XerD'

Chapter III

DISCUSSION

1. Binding activity of *cc*XerC and *cc*XerD with *C. crescentus dif* (*ccdif*) site

As reported by Jouan and Szatmari (2003), the *C. crescentus xerC* gene encodes a protein (*cc*XerC) that shows 34.9% identity and 54.8% similarity with the *E. coli xerC* gene, whereas the *xerD* gene encodes a protein (*cc*XerD) that displays 41.1% identity and 60.5% similarity with the *E. coli xerD* gene. *C. crescentus'* Xer recombinases display 36.2% identity and 63.9% similarity with each other. Also the highest level of similarity is found in the C-terminal region of the proteins. In addition, motif II and III (involved in catalytic activity) and the motif I involved in DNA binding also display strong similarities (Nunes-Duby *et al*., 1998; Hayes *et al*., 1997). *In vitro*, *cc*XerC and *cc*XerD exhibited binding activity to the *ecdif* site (Jouan and Szatmari, 2003). However, the *C. crescentus* genome does not contain obvious homologues of the *E. coli* termination systems, which contain several determinants involved in sister chromosome separation, including the *dif* site which is the chromosomal target of the XerCD site-specific recombinases. The *C. crescentus dif* (*ccdif*) site has a significantly different sequence when the *ccdif* site is compared with *dif* sites from other bacterial chromosomes (Jensen, 2006). All these previous results prompted us to investigate how these two recombinases, *cc*XerC and *cc*XerD, function in resolving chromosome dimers and hence facilitate chromosome partitioning in *C. crescentus*.

In the XerCD site-specific recombination system, XerC and XerD bind cooperatively to the chromosomal *dif* site and then execute cleavage and recombination

reactions that resolve multimeric replicons (Blakely *et al*., 1991; 1993). So firstly, we used gel retardation analysis to test the binding activity of *cc*XerC and *cc*XerD to the *ccdif* site. These two recombinases were cloned and expressed as N-terminal MBP fusions, which optimized the solubility of these proteins when overexpressed in *E. coli*. Previous work on Xer proteins in the *E. coli, Proteus mirabilis* and currently studied *Streptococcus suis* in our lab has demonstrated that these fusions do not hamper the catalytic activity of these proteins (Villion and Szatmari, 2003; Leroux, Jia and Szatmari in preparation). Our results showed that *cc*XerC and *cc*XerD were able to bind to *ccdif*, and that *cc*XerD bound to *ccdif* with a higher apparent affinity than *cc*XerC, as similar levels of retardation were obtained with 70nM *cc*XerD and 309nM *cc*XerC (Fig. 1B. in chapter II). This higher apparent affinity was also found when the *ecdif* site was used in the binding reaction (Fig. 7), as similar level of retardation were observed with 10nM *cc*XerD and 155nM *cc*XerC with the *ecdif* site. As the alignment of *ccdif* and *ecdif* (Fig1A. in Chapter II) shows, the right half-sites are highly conserved. This explains the observed higher affinity binding activity of *cc*XerD to the *ecdif* site. Besides this observation, there is another interesting point when comparing *cc*XerCD binding to *ccdif* and *ecdif* (Fig. 1B in chapter II, and Fig7). Both *cc*Xer proteins bound better to the *ecdif* than to the *ccdif* site, since similar retardations were obtained when 155nM *cc*XerC was added to the *ecdif* sites but 309nM *cc*XerC was added to the *ccdif* sites, also similar retardations were observed when 10nM *cc*XerD was added to the *ecdif* site but 70nM *cc*XerD was added to the *ccdif* site. We offer four possible explanations for this lower binding activity of *cc*Xer recombinase to the *ccdif* site. (1) Binding to the *ccdif* site occurred, but the proteins dissociated more readily from their substrate. Some smears

were observed in the reaction with the *ccdif* (Fig. 1B in chapter II) but not with the *ecdif* (Fig. 7). The appearance of these smears is indicative of proteins dissociating from their DNA substrate. This could be due to the same reaction conditions used which may allow for better binding to the *ecdif* site since these reactions were *in vitro*. (2) Another possibility is the size of the substrate used in the reaction as *ccdif* is 409bp and *ecdif* is 280bp. It is possible that the longer size of the *ccdif* DNA fragment may promote distortion during the reaction by the recombinase binding, leading to dissociation. One way to test this possibility in the future would be to use *ccdif* sites and *ecdif* sites of equivalent lengths. (3) It was proposed that the nucleotides of the binding site contribute significantly to XerCD binding specificity and substitution of specific nucleotides alters Xer binding activity (Hayes and Sherratt, 1997). Considering the divergence between *ccdif* and *ecdif*, it is likely that *ecdif* provides a better recognition sequence than the *ccdif* site. Since these results were observed in *in vitro* binding reactions, it also possible that other accessory sequences or proteins might play a role to overcome these inefficient DNA binding activities observed in *C. crescentus*. (4) The Xer site-specific recombination proceeds, after recombinase binding and synapsis of sites, by activation and subsequent cleavage of specific phosphodiester bonds. However, the binding affinity is not relevant to cleavage affinity. This was reported in *E. coli* where XerD had a higher binding affinity but showed a lower cleavage activity (Blakely *et al*., 1997). So it is possible that the lower binding of the *cc*Xer recombinase to the *ccdif* site is enough to process the next cleavage step in *C. crescentus*. Further tests on cleavage activity using the *ecdif* site with *cc*Xer recombinases may allow us to test this possibility. All together,

these explanations are not mutually exclusive and we cannot distinguish between them on the basis of these *in vitro* experiments.

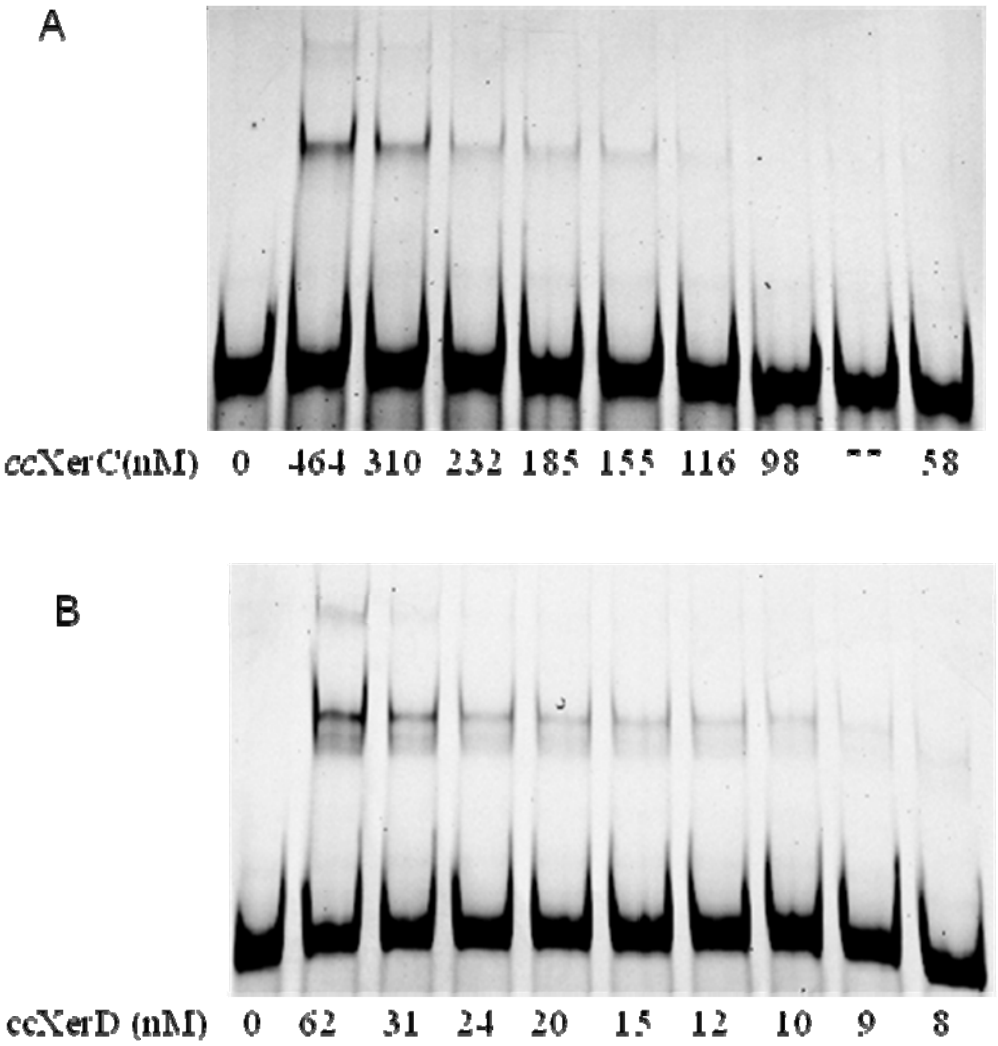
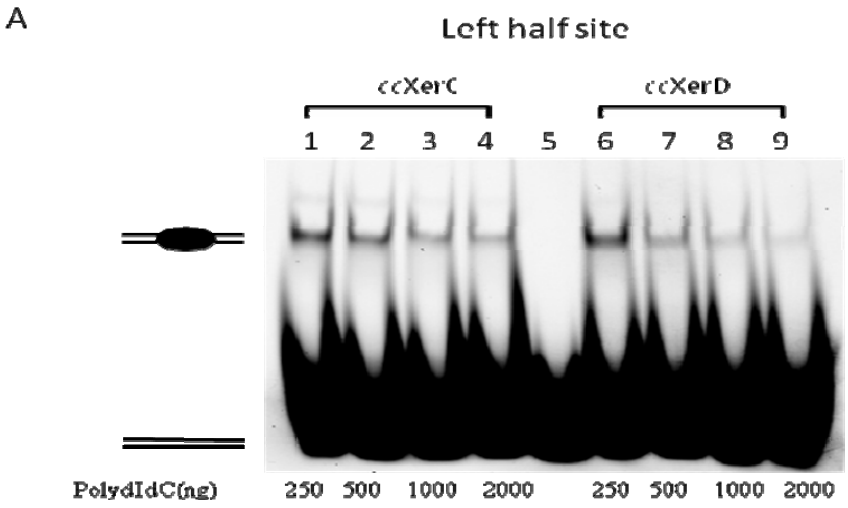


Figure 7. Binding activity of *cc*XerC and *cc*XerD with *ecdif*. The concentration of *cc*XerC (A) and *cc*XerD (B) are indicated below each lane in nM. All reactions contained 2ng of Hex-labelled *ecdif* fragments and 125ng polydIdC.

2. *cc*XerC and *cc*XerD bind to both half-sites of *ccdif* with different affinities

To further characterize the binding activity of *cc*XerC and *cc*XerD to *ccdif*, we used 17bp Hex-labelled double stranded oligonucleotides, corresponding to the left and right halves of the *ccdif* site in gel shift assays (Fig. 3 in Chapter II). The 17bp oligonucleotides consist of three nucleotides of the central region and the rest of the 14bp XerC or XerD binding sites as the similar constructions were also used in the XerCD studies done on the *dif* site of *Bacillus subtilis* (Sciochetti *et al*., 2001). Altough these half sites are not very ideal structure, they are still useful for comparison studies on left versus right site in binding activity. We also positioned the 'nick' in the middle of the spacer region in suicide substrates stuied in cleavage test. Firstly, we observed that each protein could bind to the left and right half-sites respectively and this specific DNA-protein binding activity was still observed with a 200-fold excess of polydIdC in the reactions (Fig. 8). Secondly, *cc*XerD displayed stronger binding to half-sites than *cc*XerC (Fig. 8A and B) which is consistent with the observation of binding to the full *ccdif* site shown in Fig. 1B in Chapter II. Thirdly, we also observed that *cc*XerC bound the left *ccdif* site better and *cc*XerD bound also well to left than right half-sites (Fig. 3 in chapter II), which is different from what was observed with *E. coli* where it was found that *ec*XerD could only bind to the right half-site of *ecdif* (Blakely *et al*., 1993; 1997). *cc*Xer proteins binding to the both half-sites of *ccdif* may explain the presence of the faint complex representing two monomers of *cc*Xer proteins binding to the full *ccdif* site as shown in Fig. 1B lane2, 3 and lane 9, 10. Finally, we found that *cc*Xer recombinases binding to the half-sites of *ccdif* was much weaker than to the full *ccdif* site when comparing the concentration of proteins and the amount of DNA used in the reactions (Fig. 1B and Fig.

3. Chapter II). Although it is hard to estimate the percentage of the retarded bands in Fig. 1B and Fig. 3 because of the small amount of DNA used in the reactions, we can still observe the difference of the unbound substrates from these two figures. It suggests that the half-site of *ccdif* substrate may not be an ideal substrate for DNA binding, or perhaps the central region of *ccdif* may contribute to XerCD binding activity since the footprinting data for *ec*XerC and *ec*XerD binding shows that the protected region overlaps with the spacer (Blakely *et al*., 1993).



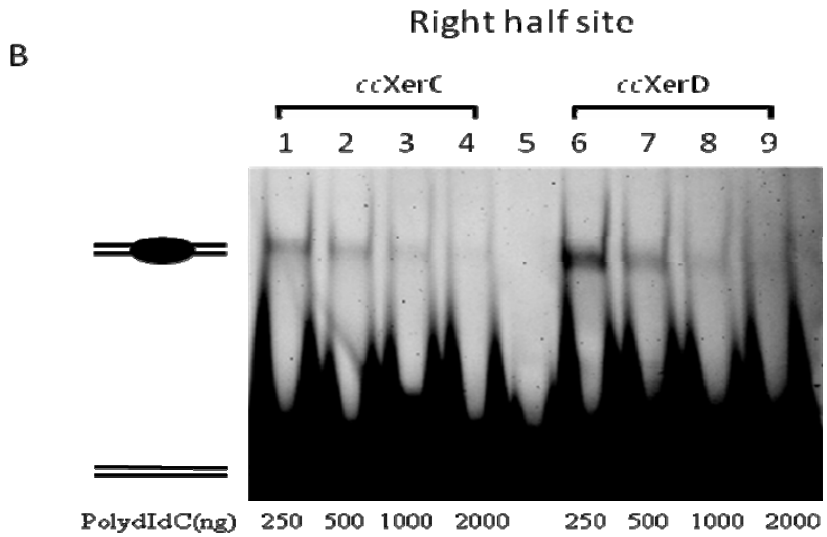


Figure 8. Binding activity of *cc*XerC and *cc*XerD to the left and right half-site of *ccdif*. All reactions contained proteins in the concentration of 615nM and 10ng of Hex-labelled half *ccdif* fragments (17bp). Reactions were incubated for 60min at 30^{o}C and the amount of polydIdC added to each reaction is indicated below each lane in ng.

In *E. coli*, XerC has a higher affinity to the left half-site of *ecdif* and XerD has a higher affinity to the right half-site (Blakely *et al*, 1993; Blakely and Sherratt, 1996). It was proposed that the nucleotides at the outer ends of the binding site contribute most significantly to XerC and XerD binding specificity and substitution of specific nucleotides alters Xer binding activity (Hayes and Sherratt, 1997). At the outer ends of the *ecdif* site (Fig. 1A, positions -14 to -9 and +9 to +14), nucleotides -10C and -13G appear to be very important for XerC binding, while +9T and +13A contribute significantly to XerD binding (Hayes and Sherratt, 1997). When compared to the outer ends of *ecdif* sequence, nucleotides -14 to -10 of *ccdif* are divergent from *ecdif,* while the right half-site is more conserved in which only the +13 nucleotide of *ccdif* is changed to G and +9T is conserved (Fig. 1A in chapter II). Consistent with the conclusion that +9T is a major specificity determinant in XerD binding, *cc*XerD showed a higher binding affinity to right half-site of *ccdif* than *cc*XerC. Furthermore, similar to *ec*XerC binding on *ecdif*, *cc*XerC bound preferentially to the left half-site of *ccdif*. However, *cc*XerD bound both half-sites of *ccdif* quite well although better to the right half-site. It is possible that the divergent left *ccdif* half-site provides the sequence(s) recognition specificity for both recombinases' binding. Therefore, to further test the exact sequence to which *cc*XerC and *cc*XerD are binding, DNAse footprinting assays should be done in the future. We can also try competition experiments by adding increasing amount of unlabelled left or right half-sites to the binding reaction of XerC/D with complete *ccdif* site to see what effect

these unlabelled half-sites have on XerC/D binding to the complete *ccdif* site. In addition, mutant *ccdif* sites may be introduced into the normal location of *ccdif* site to test determinants of binding specificity. Another possibility is that *cc*XerD might require accessory sequences for successful intermolecular recombination which is different from what was known with *E. coli.* This possibility was first reported by Mcleod and Waldor (Mcleod and Waldor, 2004). The authors show that not only did XerC and XerD bind to the DNA sequence where strand exchanges were predicted to occur; they were also able to bind to a related sequence approximately 84bp downstream from the positions of strand exchange (Mcleod and Waldor, 2004). The presence of additional recombinase binding sites might play a structural role, or may be required to stabilize the recombinase-DNA complex. Considering that the *ccdif* sequence is quite divergent from *ecdif*, additional architectural support might be needed for successful *cc*XerD recombination activity. Therefore, longer oligonucleotides of the *ccdif* half-site may be constructed and tested for the binding activity in the future studies. A third possibility is that *cc*XerD may require some sort of modification, for example, like FtsK activation, in order to bind specifically and to cleave DNA. In *E. coli*, XerD has to be activited by *E. coli* FtsK (Aussi *et al*., 2002; Yates *et al*., 2006). This possibility of FtsK mediated control needs to be analyzed in the future.

3. Reduced cooperative binding between *cc*Xer proteins and the *ccdif* site

Previous *in vitro* experiments demonstrated that *cc*XerC and *cc*XerD were capable of binding the *ecdif* site cooperatively (Jouan and Szatmari, 2003). We wanted to see if this was also the case for binding to *ccdif* by utilizing a constant amount of *cc*XerC (309nM) and increasing the *cc*XerD concentrations from 49nM to 70nM. In addition, the

reverse experiment was also performed where a constant amount of *cc*XerD (70nM) was added with increasing the *cc*XerC concentations. However, little evidence of cooperative binding with these proteins to the *ccdif* was observed under the conditions used. Comparing Fig. 2 (in chapter II) lane 3, and lane10, in which two recombinases were incubated with *ccdif*, to lane5, 6 and lane 12, 13, in which one of the recombinase in the same concentration was added in each reaction (Fig. 2 in chapter II), there is little evidence of increased binding when XerC and XerD are together compared to when the proteins are added alone. This reduced cooperative binding activity could be also observed in Fig. 10 (chapter III) comparing lane 3 in which both *cc*XerC and *cc*XerD were added in the reaction with the lane 1 and lane 2 in which only one of the recombinase was incubated with the top-nicked *ccdif* site. However, cooperative binding was observed with *cc*XerC and *cc*XerD and the *ecdif*, in which we kept one protein, either *cc*XerC or *cc*XeD, at a constant concentration and the other one with variable concentrations (Fig. 9A , in chapter III lanes 5, 7 and 8 and Fig. 9B, in chapter III, lanes 5, 7 and 8).

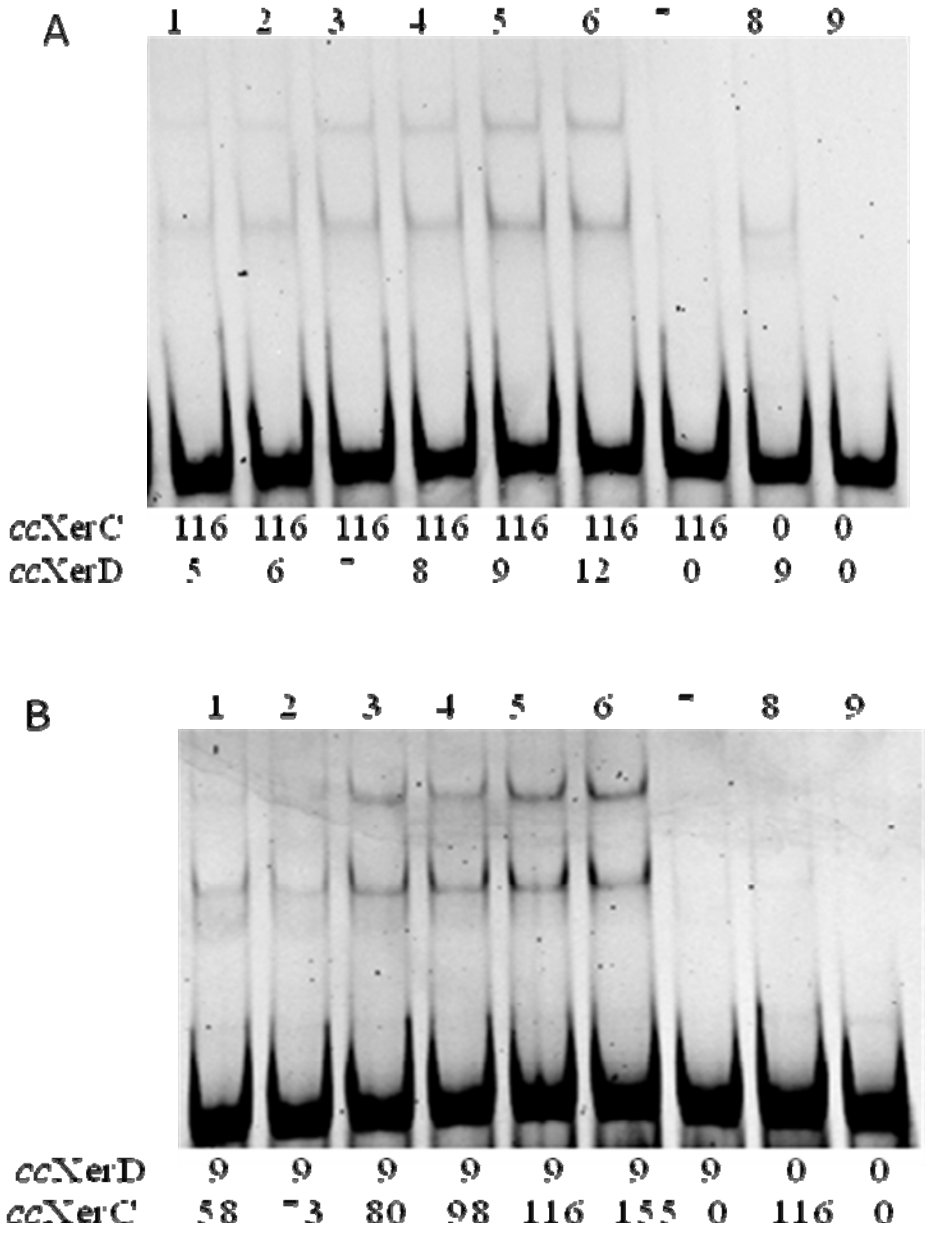


Figure 9. Cooperative binding between *cc*XerC and *cc*XerD with *ecdif*. Reactions were incubated for 60min at 30°C. The concentration of *cc*XerC and *cc*XerD are indicated below each lane in nM. All reaction contained 2ng of Hex-labelled *ecdif* fragments and 125ng polydIdC.

In *E. coli*, binding of XerC and XerD to *dif* is highly cooperative (Blakely *et al*., 1993; Blakely and Sherratt, 1996b). This cooperativity could occur as a consequence of

specific interactions between XerC and XerD, or by changes in DNA structure that arise as a consequence of binding either recombinase. The first explanation was supported by the work of Blakely and Sherratt (1996b) using footprinting on *dif6* and *dif8* sites in which the spacing between XerC-and XerD-binding sites varied from 6 to 8bp. In *dif6*, XerC and XerD together protect the entire central region from chemical enzymatic cleavage. The footprinting results demonstrated that each recombinase spans the central region strand that they cleave, which suggested that XerC and XerD may be in close contact with each other in this region. Such contacts may in part be responsible for the cooperative activity. Moreover, the *dif8* central region is sensitive to cleavage by both reagents when XerC and XerD are bound, again demonstrating that specific domains of the recombinases have moved away from the central region DNA possibly to facilitate formation of cooperative protein/protein interactions. In addition, some other findings, for example, the ability to cross-link one recombinase to the other when bound to DNA and the region involved in the interaction between XerC and XerD (Blakely and Sherratt, 1996b; Hallet *et al*., 1999; Subramanya *et al*., 1997), support the former explanation of Xer recombinase cooperativity that is the consequence of interaction between XerC and XerD. However, Blakely *et al.,* (1997) observed that the presumptive XerC-XerD interactions are only detected on a substrate containing a bottom-nicked strand which indicated that a spatial flexibility in the DNA is required to enable the interactions to occur (Blakely *et al*., 1997). Furthermore, the finding that XerC and XerD induced different bending in *dif6* and *dif8* also suggested that distortion in the DNA induced by recombinase binding, rather than protein's flexibility, could explain the relatively small loss in cooperative interactions between *dif*6 at *dif*8 (Blakely and Sherratt, 1996b).

Therefore, it is possible that the conformation of a recombination site bound by one recombinase may allow better binding of the second recombinase, which would also result in cooperativity. This possibility might also explain our observation of the cooperative binding between *cc*XerC and *cc*XerD to the *ecdif* while the reduced cooperativity to *ccdif* when one considers that only the binding site was different in these two cases. The different binding sites may provide a different geometry of the core site-recombinase complex and thus influence the efficiency of these two recombinases cooperative reaction. Moreover, the higher occupancy of the *ccdif* left half-site by both recombinases may also limit or alter the conformational change of *ccdif* site and then prevent the contact of these two recombinases thus reduce the cooperativity. Alternatively, this cooperativity between *cc*XerC and *cc*XerD to the *ccdif* may not be necessary for Xer recombination to proceed in *C. crescentus in vivo*. Summarized the binding activity of *cc*XerCD on *ccdif* site, we can see that *cc*XerCD recombinases bound poorly to *ccdif* site than *ec*XerCD to *ccdif* site and showed lower cooperative binding; Both *cc*XerC and *cc*XerD bound preferentially to the left half-site of *ccdif* although *cc*XerCD could bind to both half-sites, while *ec*XerC had a higher affinity to the left half-site of *ecdif* and XerD had a higher affinity to the right half-site.

4. The ability of *cc*XerC and *cc*XerD to form phosphotyrosine covalent complexes on *ccdif* suicide substrate

Recombination proceeds, after recombinase binding and synapsis of sites, by activation and subsequent cleavage of specific phosphodiester bonds. The active site tyrosine of the recombinase acts as a nucleophile and cleaves the DNA to form a covalent protein-DNA intermediate. Free 5' hydroxyl ends, generated by the initial DNA cleavage,

then act as attacking nucleophiles to religate the DNA. A total of four strand cleavages and religations are required to generate recombinant products (Stark *et al*., 1992). We used linear suicide substrates that contain a nick at the central position of the spacer to test the recombinase/DNA covalent complexes. We labelled the constructed suicide substrates at 5' end with 6-Hex or Cy5, Covalent recombinase-DNA complexes were generated and detected after heat treatment by electrophoresis through polyacrylamide gel containing 0.1% SDS. Reactions were performed at defined protein concentrations that ensured saturation of binding sites (confirmed by gel retardation); 1230nM each for *cc*XerC and *cc*XerD were added to top or bottom-nicked strand. This concentration is similar to the concentration used in the test on *E. coli* and *Bacillus subtilis* (Blakely and Sherratt, 1994; Sciochetti *et al*., 2001)

Firstly, we found that these covalent complexes were formed when both *cc*XerC and *cc*XerD were incubated with the *ccdif* suicide substrates, which represent covalently-linked recombinase-DNA complexes formed after the initial steps in tyrosine recombinase site-specific recombination (Fig. 4, lane 4 in chapter II). We did not observe any covalent complex when only one of the recombinases was present in these conditions in *C. crescentus*. The requirement for two different recombinases appears to provide the asymmetry for ensuring correct alignment of recombining sites before the first strand exchanges occur. This model may enable each pair of strand exchange to be under separate genetic control, as well as facilitating site alignment immediately after site replication in the chromosome. In addition, the need for both proteins to be present in order to detect phosphotyrosyl complex formation again demonstrates the importance of

interactions between the XerC and XerD recombinases for controlling catalytic activity in a bound complex.

Secondly, formation of covalent phosphotyrosyl complexes was more efficient using the top-nicked suicide substrate than the bottom-nicked substrate, as shown in Fig. 4 in chapter II. It is worth noting that Xer recombination is catalyzed inside a tetramer of recombinases bound to a pair of core sequences arranged in antiparallel configuration. During this period, the DNA sites are able to synapse and then subjected to a cleavage event which leads to strand exchanges. Exchange of a first pair of strands is catalyzed by a pair of recombinases (either XerC or XerD) and leads to an intermediate containing an HJ. This complex then isomerizes to allow exchange of the second pair of strands by the second pair of recombinases (Barre *et al*., 2000). The outcomes and the modalities of Xer recombination are influenced by (1) the sequence of the recombination sites; (2) by intrinsic properties of the recombinases; and also (3) by modification of the nucleoprotein structure that imposed by additional factors, such as FtsK, which activate XerD monomers to initiate the first cleavage in the chromosome dimer resolution reaction. Since each pair of strand exchanges requires the catalytic activity of a different recombinase, differential biochemical control of the two pairs of strand exchanges is possible and also important in the *in vivo* role of this recombination system. Therefore, the differential rates of cleavage detected in the top and bottom-nicked strands might be at least partly a consequence of a difference in catalytic activity between XerC and XerD on these substrates.

5. Active-site tyrosine mutant proteins do not form phosphotyrosyl covalent complexes with *ccdif*

Four completely conserved amino acids have been implicated in catalysis by tyrosine recombinases (Argos *et al*., 1986; Abremski and Hoess, 1992; Lee *et al*., 1992). In the recombination reaction, the tyrosine of the RHRY tetrad represents the catalytic residue in activation of the scissile DNA phosphodiester and phosphotyrosyl linkage prior to strand cleavage, whereas the first arginine is involved in specific binding to DNA. The fact that both XerC and XerD contain these conserved residues implies that both proteins are required for catalysis in Xer site-specific recombination. To verify this experimentally, site-directed mutagenesis was used to convert the putative active site tyrosine to phenylalanine in both proteins (creating *cc*XerC^{Y297F} and *cc*XerD^{Y277F}, respectively). The mutants were able to bind their respective DNA binding sites in an *in vitro* gel shift assay. As shown in Fig. 10, the binding affinity of *cc*XerC^{Y297F} or *cc*XerD^{Y277F} was similar to wild-type *cc*XerC and *cc*XerD respectively. However, the covalent complex formed after strand cleavage was not detected when wild-type *cc*XerC was combined with mutant *cc*XerD^{Y277F} and/or wild-type *cc*XerD with mutant *cc*XerC^{Y297F} (Fig. 5 in chapter II). Similar observations with the bottom-nicked suicide substrate were also obtained. These results indicated that both active-site tyrosines in *cc*XerC and *cc*XerD are important for the catalytic reaction but not for binding activity. Efficient Xer recombination requires that both wild-type recombinases are present because the interactions between them are important for controlling catalytic activity in a bound complex. The observations that the mutant *cc*XerC or *cc*XerD proteins can influence the catalytic activity of its wild-type partner recombinase strengthen this view. The failure to obtain cleavage with XerC or XerD in the presence of their mutant partner also suggests that XerC-XerD interactions can modulate the disposition of key catalytic

residues with respect to the scissile phosphate and /or with each other. Moreover, it was previously concluded that the ability of XerC to cleave DNA is strongly influenced by: (i) the interactions between XerC and XerD, and (ii) the geometry of the recombinase-DNA complex (Blakely and Sherratt, 1996). Therefore, the observations of similar binding activity but different cleavage activities of the mutant proteins suggest that the active-site tyrosine in a wild-type recombinase molecule directly or indirectly contacts with the DNA backbone of the half-site to which it is bound. An exchange of amino acid residues within *cc*XerC and *cc*XerD could result in the disruption of intermolecular interaction between them, as these interactions are important to maintain the DNA substrate in a conformation appropriate for recombinase catalysis (Blakely *et al*., 1997). Future *in vivo* experiment such as a complementation assay with mutant strains may help to further confirm the ability of these mutant proteins to act in Xer site-specific recombination.

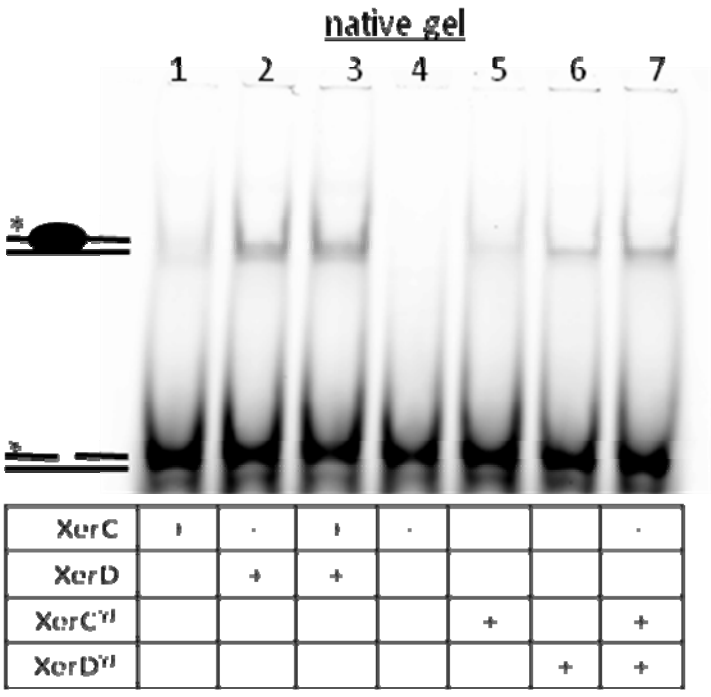


Figure 10. Comparison of wild-type recombinases, *cc*XerC, *cc*XerD, and mutant proteins, *cc*XerC^{Y297F} or *cc*XerD^{Y277F} binding to top-nicked suicide substrate by migrating on native 6% 0.25X TBE gel. The indicated protein(s) at the concentration of 410nM for each and 10ng of Hex-labelled top-nicked strand were incubated for 60 minutes at 30^{o}C. All reaction contained 125ng polydIdC.

6. *cc*XerC forms phosphotyrosyl complexes on both top and bottom-nicked strand of *ccdif*

In order to determine which recombinase was covalently linked to DNA, Cy5-labelled suicide substrates, top-nicked and bottom-nicked *ccdif*, were incubated with FITC-labelled proteins. As mentioned before, both *cc*XerC and *cc*XerD were expressed as N-terminal MBP fusions as to optimize the solubility of these proteins when overexpressed in *E. coli*. However, a small size difference in molecular mass (0.5KDa) between the two MBP fusions allowed us to distinguish which of the two recombinases have become covalently bound to DNA. Previously, we attempted to remove the MBP portion of these two recombinases by factor Xa cleavage in different steps during the purification. Unfortunately, a nonspecific protease activity of factor Xa did not allow us to recover sufficient quantities of wild-type recombinase. We therefore decided to label the proteins *in vitro* using amine-reactive fluorescence labelling to allow us to detect which protein is covalently linked to the DNA after cleavage. The different fluorescence spectra of Cy5-labelled DNA and FITC-labelled proteins allowed us to easily detect these molecules separately using the Typhoon Trio imager.

Cy5-labelled *ccdif* was incubated with FITC-labelled *cc*XerC and/or *cc*XerD to produce the covalent protein-DNA complexes. Better resolution was obtained by separating the samples in a large denaturing gel. If DNA and protein comigrated in the

same position, we observed yellow because the red from the *ccdif* and the green from recombinase merged to yellow. Otherwise, there were separate red and green signals which suggested that covalent complex migrated differently from the labelled recombinase. By using this method, we could determine which recombinase was covalently linked to DNA after cleavage reaction. We observed that *cc*XerC, but not *cc*XerD comigrated with the labelled suicide substrate (see Fig. 6A, 6B in chapter II). The results indicated that it was *cc*XerC that remained bound to both top and bottom-nicked *ccdif* suicide substrates although a lower level of recombinase-DNA covalent complex was observed in bottom-nicked strand when compared to top-nicked *ccdif*.

In *E. coli*, *ec*XerC preferentially cleaves the top strand of linear *ecdif* efficiently, while *ec*XerD cleaves the bottom strand inefficiently (Blakely and Sherratt, 1996; Blakely *et al*., 1997; Sherratt *et al*., 1995) In *C. crescentus*, *cc*XerC mediated both top-nicked strand and bottom-nicked strand cleavage in the presence of *cc*XerD. This could be a consequence of the different binding activities of *cc*XerC and *cc*XerD on the *ccdif* since *cc*XerC could bind to the both left and right half-sites of *ccdif* in our *in vitro* binding studies. This also suggests that the recombinase-DNA complex adopts a conformation that is suitable for *cc*XerC but not for *cc*XerD cleavage in our *in vitro* test. The failure to observe *cc*XerD-*ccdif* covalent complexes could be explained by inactivity of our XerD preparation, or the requirement for specific cofactor(s) or conditions for XerD cleavage, or the specific substrate DNA structure or conformation.

The first possibility can be ruled out, as we were able to clearly demonstrate that *cc*XerD certainly bound to the *ccdif* site and we observed partial complementation of filamentation in *E. coli xerD* mutants with the *ccxerD* gene which will be discussed later.

Therefore, we feel that the failure to obtain *cc*XerD-*ccdif* covalent complexes is more likely due to the absence of a required cofactor, or the possibility that *cc*XerC may perform all the strand cleavages in the recombination reaction. It has been established in *E. coli* that, in addition to the Xer recombinase, at least one other protein, FtsK, is required for chromosome dimer resolution *in vivo* (Boyl *et al*., 2000; Steiner *et al*., 1999). Aussel *et al*., (2002) found that no resolution product was detected in strains carrying either of the two catalytically inactive XerCD recombinases when $FtsK_{50C}$ was not expressed. However, when $FtsK_{50C}$ was overexpressed, resolution products could be detected in the strain carrying a wild-type allele of XerD and a catalytically inactive form of XerC. This result was confirmed by *in vitro* experiments that showed that in the presence of $FtsK_{50C}$, the $XerC^{YF}$ and XerD recombinases created a level of HJs similar to that obtained with wild-type XerC and XerD. Therefore, it is believed that the role of FtsK in promoting chromosome dimer resolution is to switch the activity of the XerCD recombinases in the synaptic complex (Aussel *et al*., 2002). This means that for *E. coli*, in the absence of FtsK, the Xer synaptic complex adopts a conformation suitable for XerC-mediated strand exchanges. FtsK can use the energy of ATP to switch the Xer synaptic complex to a conformation suitable for XerD-strand exchanges. Furthermore, Yates *et al*., (2006) found that γ domain in the C-terminus of FtsK directly interacts with XerD to stimulate the formation of an 'XerD-active', conformation, which mediates cleavage of the bottom strand of the *ecdif* site in the presence of XerC. All these results suggest that an efficient recombination reaction needs XerD to be stimulated into an active state. The failure to observe *cc*XerD-*ccdif* covalent complexes reflects the requirement for a control to activate XerD catalytic activity. It has also been established that the C-terminal region

of XerD carries major determinants for interaction with the FtsK C-terminal region and is important for the mediation of normal recombination at the chromosomal *dif* site (Spiers *et al*., 1999; Yates *et al*., 2006). As revealed by the alignment of *C. crescentus* XerD and *E. coli* XerD proteins, maximum identity is found in the C-terminal region of the protein (Jouan and Szatmari, 2003). Also, the γ domain of the C-terminal region of FtsK protein of *C. crescentus* shows 45% identity and 77.5% similarity with the γ domain of the C-terminal region of *E. coli* of FtsK protein (Fig. 11). It follows that the two homologues should have very similar biochemical activities. This view is confirmed by the previous work that FtsK is present in *C. crescentus* and its C-terminus is essential and involved in maintaining accurate chromosome partitioning (Wang *et al*., 2006). All together, it would be reasonable to propose that FtsK may have an important role in promoting the formation of an "XerD-active" state to allow XerD to perform the strand exchange on the *C. crescentus dif* site.

```
FtsK-Cc         ----LPELAMLAKSKPRSSEVDAAALRQNARLLESVLAEFGVKGQIDQIRPGPVVTMYEL 378
FtsK-EC         PTTPLPSLDLLTPPPSEVEPVDTFALEQMARLVEARLADFRIKADVVNYSPGPVITRFEL 895
                    **.* :*: . .. . **: **.* ***:*: **:* :*.:: :  ****:* :**

FtsK-Cc         VPAPGVKTARVVALADDIARSMSVISCR-VAVAQGRNAIGIEMPNQRRETVYLRDLLSSA 437
FtsK-EC         NLAPGVKAARISNLSRDLARSLSTVAVRVVEVIPGKPYVGLELPNKKRQTVYLREVLDNA 955
                  *****:**:  *: *:***:*.:: * * *  *:  :*:*:**::*:*****::*..*

FtsK-Cc         DYEKASQILPMALGETIGGEPYIADLAKMPHLLIAGTTGSGKSVGVNAMILSILYKLPPE 497
FtsK-EC         KFRDNPSPLTVVLGKDIAGEPVVADLAKMPHLLVAGTTGSGKSVGVNAMILSMLYKAQPE 1015
                .:.. .. *.:.**: *.*** :**********:******************:***  **

FtsK-Cc         KCRFIMVDPKMLELSVYDGIPHLLAPVVTDPKKAVVALKWTVREMEDRYRRMSKIGVRNI 557
FtsK-EC         DVRFIMIDPKMLELSVYEGIPHLLTEVVTDMKDAANALRWCVNEMERRYKLMSALGVRNL 1075
                . ****:**********:******: **** *.*. **:* *.*** **: ** :****:

FtsK-Cc         GGYNEKANEAAAKGEHFERTVQTGFDDAGRPIYETEQIRPEPMPYLVVVIDEVADLMMVA 617
FtsK-EC         AGYNEKIAEADR----MMRPIPDPYWKPGDSMDAQHPVLKK-EPYIVVLVDEFADLMMTV 1130
                .*****  **      : *.:   : ..* .:   . :  :  **:**::**.*****..

FtsK-Cc         GKDIEGAVQRLAQMARAAGIHLIMATQRPSVDVITGTIKANFPTRISFQVTSKIDARTIL 677
FtsK-EC         GKKVEELIARLAQKARAAGIHLVLATQRPSVDVITGLIKANIPTRIAFTVSSKIDSRTIL 1190
                **.:*  : **** ********::************ ****:****:* *:****:****

FtsK-Cc         GEQGAEQLLGQGDMLYMAGGGRI-TRLHGPFVSDGEVEAVARFLRDQGIPQYLDEVTAGG 736
FtsK-EC         DQAGAESLLGMGDMLYSGPNSTLPVRVHGAFVRDQEVHAVVQDWKARGRPQYVDGITS-- 1248
                .: ***.*** ***** . .. : .*:**.** * **.**.:  : :* ***:* :*:

FtsK-Cc         DEEQEEAIEGAFSGEGGANDLYDHAVAVVTRDRKASTSYIQRRLQIGYNRAASLMERMEK 796
FtsK-EC         -DSESEGGAGGFDGAEELDPLFDQAVQFVTEKRKASISGVQRQFRIGYNRAARIIEQMEA 1307
                 :.:.*.  *.*.*    : *:*:** .**..**** * :**:::******* ::*:**

FtsK-Cc         EGVVGAANHAGKREILAPPTPPL 819
FtsK-EC         QGIVSEQGHNGNREVLAPPPFD- 1329
                :*:*.  .* *:**:****.
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Figure. 11. Alignment of *C. crescentus* and *E. coli* FtsK protein C-terminal domains. The alignment was done using Clustalw2 (www.ebi.ac.uk/Tools/clustalw2/index). Vertical lines (*) respresent identity; (:) represent a high degree of similarity; and a single dots (.) represent less similarity between amino acids. According to the value given by the program, the proteins have 45% identity. FtsK-Cc, *C. crescentus* FtsK; FtsK-EC, *E. coli* FtsK.

7. Complementation assay

As mentioned previously, dimer resolution requires the *dif* site (Kuempel *et al*., 1991) and XerC and XerD resolvase proteins (Blakely *et al*., 1991; 1993), as well as cell division and the FtsK cell division protein (Steiner and Kuempel, 1998a; Steiner *et al*., 1999). Strains which fail to resolve dimer chromosomes lead to the Dif⁻ phenotype, which includes filamentation in a fraction of the cells, abnormal nucleoid morphology,

SOS induction and decreased growth rate and plating efficiency compared with wild-type cells (Kuempel *et al*., 1991; Cornet *et al*., 1996). As shown previously, 10-20% of cells filament if there is no XerCD recombination at the *dif* site (Kuempel *et al*., 1991; Jensen *et al*., 2006). To test the role of FtsK protein in the Xer site-specific recombination, *in vivo* complementation assays were done by testing recombination through visualizing cell shape. Filamentation indicated an *xer* mutant phenotype. The *C. crescentus xerC* and *xerD* plasmids (pMal*xerC* and pMal*xerD*) were isolated and then introduced into an *E. coli xerC*$^-$ strain (DS981) and *E. coli xerD*$^-$ strain (DS9008) respectively. *E. coli xerC* plasmid (pSDC105) (Colloms *et al*., 1990) and *E. coli xerD* plasmid (pRM130) (Blakely *et al*., 1993) were used as positive controls. Filamentation is readily observed in the *E. coli* XerC and XerD mutant strains. When either a cloned copy of *E. coli xerC* or *xerD* was introduced into the appropriate *xer* mutant strain, a reduction in filamentation is observed (95-98% of the cells appear normal). Filamentation was still observed when a cloned *C. crescentus xerC* was introduced into an *E. coli xerC* mutant. The amount of filamentation observed was equivalent to the amount of filamentation observed in *E. coli xerC* mutant cells lacking plasmid. This suggests that the XerC proteins of these two bacterial species are not interchangeable. However, partial complementation of filamentation was observed in *E. coli xerD* mutants with the *C. crescentus xerD* gene (Fig. 12). This inefficient complementation observed suggests that these two recombinases are partially interchangeable for recombination at *ecdif*. Since *cc*XerD can efficiently bind to *ecdif*, the partial complementation is not due to inefficient binding, but could be due to inefficient interactions between *cc*XerD and *ec*XerC or between *cc*XerD and *ec*FtsK. As previous *in vitro* binding tests showed the cooperative activity between

*cc*XerD and *ec*XerC on the *ecdif* site (Jouan and Szatmari, 2003), we think that this partial complementation may due to inefficient interactions between *cc*XerD and *ec*FtsK. Yates *et al*., (2003) reported that *Haemophilus influenzae* FtsK activated recombination by *H. influenzae* XerCD at *H. influenza dif*. However, it could not activate recombination by *E. coli* XerCD. Reciprocally, *E. coli* FtsK could not activate recombination by the *H. influenzae* recombinases at *H. influenzae dif* (Yates *et al*., 2003). These authors found that the C-terminal domain of FtsK dictated specificity. The result that γ domain in the C-terminal of FtsK directly interacts with XerD to stimulate the formation of an 'XerD-active' conformation strengthens the species specificity conclusion (Yates *et al*., 2006). All together, the inefficient complementation of filamentation in *E. coli xerD* mutants with the *C. crescentus xerD* gene reflects the imperfect interaction between *C. crescentus* XerD and *E. coli* FtsK. Therefore, further studies on the role of *C. crescentus* FtsK in site-specific recombination may help to understand how *cc*XerC and *cc*XerD perform in the catalytic reaction at the *ccdif* site.

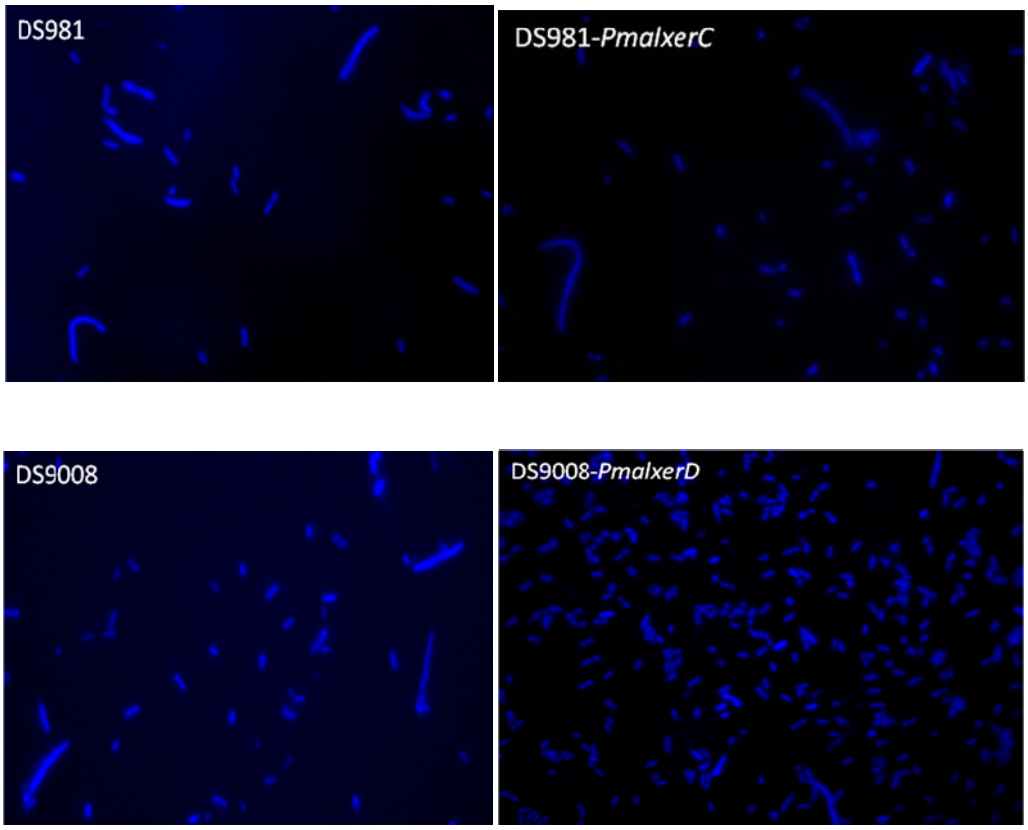

Figure.12. Complementation of filamentation in strains DS981 (*E. coli xerC* mutant), DS9008 (*E. coli xerD* mutant); *C. crescentus xerC* (Pmal*xerC*) and *xerD* (Pmal*xerD*) plasmids were introduced to each *E. coli* parent strain DS981 and/or DS9008 respectively. Cells were fixed with cold 77% EthOH. Fixed cell samples were spread onto 0.1% (wt/vol) poly-L-lysine-treated coverslips and dried. Bacterial DNA was then stained with 4'6-diamidino-2-phenylindole (DAPI). Fluorescence observations were made with NiKon Eclipe E600 fluorescent microscope with standard DAPI filter sets. Images were photographed with a DXM1200F Nikon Digital camera and acquired with NIS-Elements-F software.

8. Perspectives

Here we reported the binding and phosphotyrosine complex formation activities of the *C. crescentus* recombinases, *cc*XerC and *cc*XerD, on the *Caulobacter dif* (*ccdif*) site. One of the interesting results shows that both *cc*XerC and *cc*XerD prefer to bind to the divergent left-site of *ccdif*. Therefore, constructing mutant *ccdif* sites by site-directed mutagenesis may be used to test determinants of binding specificity. Secondly, one possibility for the lower affinity of *cc*XerD to the right half-site of *ccdif* is that

*cc*XerD might require accessory sequences for successful intermolecular recombination which is different from what was found for *E. coli*. The use of longer oligonucleotides containing the *dif* site and surrounding sequences to test this possibility or PCR fragments with left or right halves deleted may allow us to further dissect the binding specificities of these proteins. Finally, to further test the exact sequence to which *cc*XerC and *cc*XerD are binding, nuclease protection and modification assays such as Dnase hydroxyl radical footprinting, DMS protection, etc. may help to answer this question. Also the "competition" type experiments should be tried by binding recombinase to labelled *dif* site then adding excess unlabelled half *dif* sites to compete away the binding.

The catalytic function of a tyrosine recombinase is the cleavage and subsequent exchange of one DNA strand between two synapsed recombination sites. Recombinase-mediated strand cleavage in the absence of strand transfer can lead to (i) the accumulation of the DNA-protein covalent complexes between the attacking recombinase and the 5'-end fragment of the continuous strand and (ii) the accumulation of free 3'-end fragment of the continuous strand. Therefore, the exact position of cleavage by recombinase could be then determined by comparing the length of the free DNA fragments liberated by recombinase cleavage to a ladder. This can be done by using 3'-labelled nicked suicide substrate and measuring the length of the cleaved product. It should be noted that the small incised fragment that is released by nicked suicide substrates can re-attack the protein-DNA complex (Christiansen and Westergaard, 1994), and the ratio of cleavage cannot be easily controlled or measured. Another approach would be the use of DNA containing phosphorothioate linkages at the cleavage site to use as suicide substrates. Because this substrate contains no interruption in the polynucleotide backbone prior to

exposure to the recombinase under study, it offers several advantages over nicked suicide substrates, for example, no re-attack for the small cleaved fragment. This tool may be very useful to locate the cleavage site in the future studies.

Moreover, a Holliday junction-containing substrate would be another good candidate for *in vitro* recombination mediated by XerCD. The recovery of recombinant products covalently attached to protein provided the opportunity to determine the site of cleavage in the top or bottom strands (Arciszewska and Sherratt, 1995). To do this, Holliday junction-containing substrate would be 3' end-labelled either in all four strands or only in strand I or IV. After the incubation with the recombinases, the Holliday junction and linear duplex DNA bands would be recovered and analysed on a sequencing gel. Comparison of the mobility may allow us to identify the cleavage products and locate the cleavage position. Furthermore, these experiments will also provide a good way to visualize the presence of covalent protein-DNA complexes produced during the reaction.

For the exchange of one DNA strand between two synapsed recombination sites after cleavage, we incubated linear, nicked *ccdif* suicide substrates and *ccdif* sites present in supercoiled plasmid DNA with the two recombinases. We could not detect any recombination product. The failure of supercoiled plasmid-borne *dif* sites to undergo a complete recombination reaction *in vitro* could be because strand exchange is not initiated or because HJ intermediates formed by a first recombinase mediated strand exchange cannot adopt a conformation that supports the second recombinase mediated strand exchange and are efficiently converted back to initial substrate. This failed *in vitro* test also reflects an *in vivo* control process that limits Xer recombination to correctly

positioned chromosmal *dif* sites in cells that have initiated cell division and contain dimeric chromosomes. This control could be performed at least by FtsK. As we mentioned before, the failure to observe *cc*XerD phosphotyrosyl complex formation in our *in vitro* results could also be the requirement for specific cofactor(s), like FtsK. Therefore, future studies on the role of *C. crescentus* FtsK in site-specific recombination may help to understand how *cc*XerC and *cc*XerD perform in the catalytic reaction at the *ccdif* site.

Besides *in vitro* studies, *in vivo* studies on these two recombinases experiments will be very interesting. We have already constructed *xerC*$^-$ mutants of *C. crescentus*. Filaments were observed in this mutant phenotype. The other mutant strain is in the process of being prepared. We also expect that this filamentation could be reduced or eliminated by introducing plasmids containing the appropriate *xer* gene. This property could also be exploited to isolate mutants that allow recombination but do not interact with FtsK, for example. As mentioned before, the uniqueness of *C. crescentus* resides on its asymmetric cell cycle. Different stages of the cell cycle are associated with distinct morphologies, and cell cultures can be synchronized easily. Synchronous growth of swarmer cells can provide a cell population at any desired cell stage. We could study *xerC* and *xerD* gene expression by constructing a chromosomal *xer-lacZ* fusion, and then measure the expression level of these two genes during the cell cycle with β-galactosidase assays and/or western blots. We can also construct GFP fusion proteins to study the localization of these two proteins during the cell cycle using fluorescence microscopy. Thes studies should yield some important information as to how *C. crescentus* uses and regulates Xer recombinases during its cell cycle.

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