Dissecting the roles of XerC and XerD in Xer site-specific recombination

A thesis submitted for the degree of Doctor of Philosophy
at the University of Oxford

by

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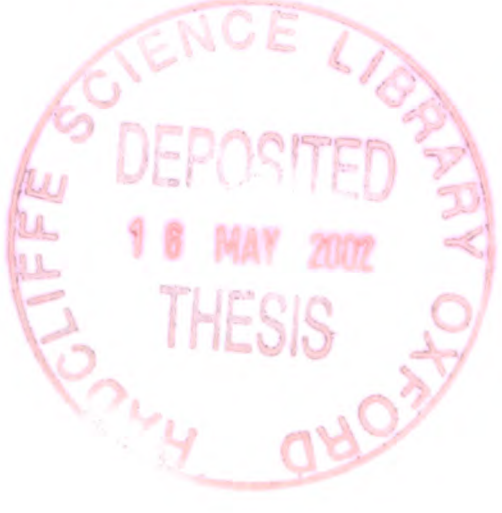
January 2002



The research reported in this thesis is my own

and original work except where otherwise stated

and has not been submitted for any other degree.

Dedicated to my mothers Dirce Maria Ferreira

and Maria Lúcia Ferreira

and to my wife Ivy Freitas Lau

ACKNOWLEDGEMENTS

Agradeço a Deus por minha vida e por mais esta oportunidade de aprendizado.

Many thanks to my Sponsor CNPq/Brazil, and everybody from the "Ação Induzida para Formação de Doutores no Exterior" who did an excellent job in coordinating this programme.

Thanks to my supervisors, Dr. L. Arciszewska and Professor D.J. Sherratt, for their kind welcome to the laboratory and for all their contributions to the work described in this Thesis.

Special thanks to my friend Sean Colloms, who was always around when I needed, even after going to Glasgow, and for his help with just everything during my DPhil.

Thanks to Migena Bregu, for the support, the friendship, and for the always-pleasant presence in the laboratory; and also for her gifts of PepA and many of the "magic" plasmid substrates from the "pMIG" series.

Thanks to Rachel Baker, for the friendship, for the extraordinary good humour, for her patience when showing me how to operate the FPLC and many other machines in the lab, and for all of those high quality wild type XerC and XerD recombinases.

Special thanks to Syretta West, for the friendship and the excellent technical support during my first year. Thanks to my "bay-mate" Yunhong Cao, for her friendship and for teaching me a lot about protein purification. Many thanks to Thomas Massey for helpful discussions and comments on initial drafts of the Thesis. Thanks to everybody else from A1 and also Microbiology that have contributed directly or indirectly for the completion of my work. Thanks to my friend Nicolas Buisine, for the personal support during the hardest moments of my work, for sometimes helping me to fix the Silicon Graphics, and for showing me the beauty of Linux; besides the good laughs.

Thanks to Ronald Chalmers and Georgi Muskhelishvili for the gifts of purified IHF and FIS, respectively.

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Hilary 2002

ABSTRACT

The tyrosine recombinases XerC and XerD function in the monomerisation of circular dimer replicons in many bacteria. The recombining complex contains two synapsed recombination sites and two molecules each of XerC and XerD. Recombination proceeds through two sequential steps of DNA strand exchanges separated in time and space. A specific pair of recombinases initiates the reaction forming a Holliday junction intermediate, which undergoes a conformational change to allow resolution to recombinant products by the other pair of enzymes. In an attempt to understand the molecular basis of recombination machine assembly and coordination of catalysis, chimeras of XerC and XerD were constructed and their properties studied in partial and complete recombination reactions. XerC and XerD are two-domain proteins, whose C-terminal regions contain all of the catalytic residues. It is demonstrated here that XerC or XerD variants lacking their N-terminal domains are active in recombination when combined with their wild type partners. However, the normal pattern of catalysis is dramatically altered: strand exchange by the recombinase variant is stimulated, while that by the wild type partner is impaired. The primary determinants for the mutant phenotype are shown to reside in the region of α-helix B of XerCD. It is also demonstrated that the exchange of the extreme C-termini of XerCD has a profound effect on the direction of HJ resolution. These observations confirm the importance of the cyclic C-terminal "donor-acceptor" interactions between XerC and XerD. Finally, the recombination reaction catalysed by ResD, a tyrosine recombinase encoded by the F-plasmid of *E. coli*, which is believed to function in the monomerisation of F-plasmid dimers, was reconstituted *in vitro*. Recombination is intramolecular and shows topological selectivity. ResD lacks a region corresponding to the N-terminal domains of XerCD, and hence its characterisation might supply further insights about the roles of the N-terminal domains of tyrosine recombinases.

CONTENTS

ABBREVIATIONS

International units of measurement and biology were used throughout. Other abbreviations are as follows:

ATP	adenosine triphosphate
bp	base pair
DNA	2'-deoxyribonucleic acid
EDTA	ethylene diamine tetra-acetic acid
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilo base pair
kDa	kilo-Dalton
OD	optical density
oligonucleotide	2'-deoxyribo-oligonucleotide
RNA	ribonucleic acid
SDS	Sodium dodecyl sulphate

CHAPTER 1

Introduction

1. INTRODUCTION

1.1. Recombination: a general overview

DNA recombination is an essential process that was first recognised as a means to introduce genetic diversity in all living cells. Recombination events can be mediated by general homologous recombination, and also by a variety of specialised processes that can be grouped into two categories: conservative site-specific recombination and transposition (Hallet & Sherratt, 1997).

Homologous recombination functions in the re-assortment of DNA segments within and between chromosomes, it facilitates DNA repair and supports DNA duplication (Cox, 1998; Sharples, 2001; Sharples et al., 1999). In the basic molecular model, homologous recombination is initiated at a double-strand break or single-strand gap in a DNA molecule, followed by the polymerisation of RecA-like proteins on regions of single-stranded DNA generated at the ends of the duplex DNA by nucleases. Recombination proceeds via a RecA-mediated single strand invasion at homologous regions, and the reciprocal exchange of strands between recombining partner molecules often leads to the generation of a Holliday junction (HJ) intermediate structure (Holliday, 1964). The HJ intermediate of recombination can be resolved to products in *Escherichia coli* (*E. coli*) by the action of the enzymes RuvABC, RecG and RusA. Recently, a new group of proteins named Mus81 has been identified in yeasts and humans as having HJ resolvase activity, and that reinforces the idea of a conserved mechanism of homologous recombination from bacteria to man (Boddy et al., 2001; Chen et al., 2001; Constantinou et al., 2001; Haber & Heyer, 2001; Kaliraman et al., 2001).

Conservative site-specific recombination is a process that produces precise DNA rearrangements at defined DNA sequences (Hallet & Sherratt, 1997; Nash,

1996; Sadowski, 1993; Stark et al., 1992). During site-specific recombination, two homologous DNA target sites are brought together by the action of DNA-binding proteins (synapsis), the two pairs of strands are cleaved at specific positions, and the ends are rejoined to new partners in a series of precise catalytic steps that do not involve any synthesis or degradation of DNA. On other hand, transposition is another form of DNA rearrangement which does not require homology between the target sites where cleavage and strand exchanges occur, and DNA repair is needed in order to complete the transposition reaction and to restore the DNA to a double stranded form (Hallet & Sherratt, 1997). Another difference between transposition and site-specific recombination is that tranposases catalyse hydrolysis of DNA phosphodiesters, while site-specific recombinases catalyse strand exchanges via protein-DNA covalent intermediates. A more detailed comparison of the mechanisms of transposition and site-specific recombination can be found in Hallet & Sherratt (1997).

1.2. Outcomes of site-specific recombination

In the best characterised site-specific recombination systems, the DNA substrates are usually circular supercoiled molecules (e.g. bacteriophages, bacteria and yeast plasmids), and the outcome of recombination between two sites will depend on whether they are located in the same molecule or not, and also on their relative orientation with respect to each other, if localised in the same DNA substrate (Stark et al., 1992).

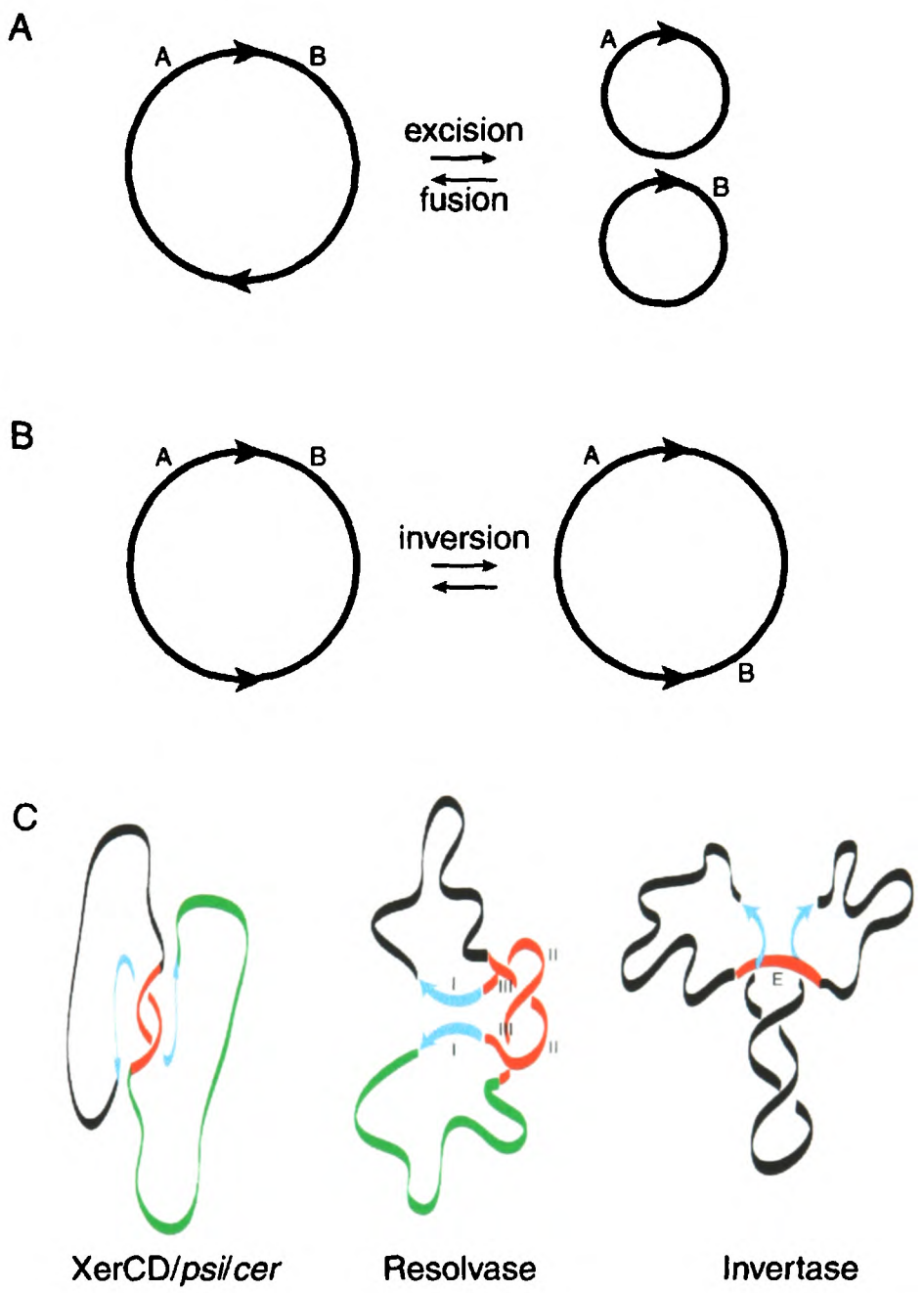


Figure 1.1. Outcomes of site-specific recombination at circular DNA molecules (**A** and **B**); (**C**) three models of highly ordered synaptic complexes assembled by the Xer system at *cer* or *psi* recombination sites, γδ and Tn3 resolvases at *res* sites (I, II and III), and Invertases (the recombination sites are represented by the red and blue lines, which correspond to accessory/enhancer sequences and core sites, respectively).

When the recombination sites are located in the same circle and organised in direct repeat (Figure 1.1-A, sites are represented by the directly repeated arrows), recombination between them will lead to the excision of a DNA segment in which each of the newly formed molecules will carry one of the initial recombination sites. If the sites are in different molecules, recombination can produce intermolecular fusion, which is the reverse of the excision reaction. And finally, recombination

between two sites that are inverted with respect to each other within the same molecule will invert the DNA segment between them (Figure 1.1-B).

Site-specific recombination at circular DNA molecules often requires the assembly of highly ordered synaptic complexes in order to bring the two recombination sites together (Colloms et al., 1997; Landy, 1993; Stark & Boocock, 1995). This process is accomplished by protein binding to DNA accessory sequences, which produces specific bends in the DNA substrate (Figure 1.1-C). In various systems, productive synapsis is only achieved upon the assembly of well defined protein-DNA complexes, where the topology of the recombinant products will reflect the topology adopted in the synaptic complex (Colloms et al., 1997; Stark & Boocock, 1995).

1.3. General mechanisms of site-specific recombination

The majority of the eukaryotic and prokaryotic site-specific recombinases identified so far can be assigned to two families of enzymes by using primary amino acid sequence comparison: the resolvase/invertase family and the phage λ-integrase (λ-Int) family (Stark et al., 1992). Since resolvases/invertases and λ-integrases utilise a serine and a tyrosine as catalytic residues, respectively, members of these families are also known as serine and tyrosine recombinases (Sherratt & Wigley, 1998). Some of the functions of these site-specific recombinases include phage integration and excision, resolution of transposition intermediates, control of gene expression by switching the orientation of promoter regions, DNA splicing, and monomerisation of circular replicons (Sadowski, 1986; Stark et al., 1992).

Recombination by serine and tyrosine recombinases takes place within a ~30 bp DNA segment called core or crossover site, which consists of two recombinase

DNA-binding elements organised as inverted repeats, and having a short spacer region separating them (Stark et al., 1992). Strand exchange involves a two steps transesterification reaction in which cleavage results in a covalent protein-DNA complex, and the rejoining reaction is just the reverse of the cleavage (Hallet & Sherratt, 1997; Stark et al., 1992). This mechanism is related to those utilised by topoisomerases (Cheng et al., 1998; Krogh & Shuman, 2000). The use of a covalent intermediate between the recombinase and the DNA conserves the bond energy and there is no requirement for high-energy cofactors such as ATP.

1.4. Serine recombinases

Serine recombinase members can be exemplified by the invertases Gin and Hin from phage Mu and *Salmonella* sp., respectively, and also by the resolvases of Tn3 and γδ transposons (Grindley, 1994; Johnson, 1991; Nash, 1996; Stark & Boocock, 1995; Stark et al., 1992). Resolvases act upon directly repeated recombination sites to resolve the fused donor and target replicons of a transpositional cointegrate into the final transposon products, while the invertases Gin and Hin switch gene expression between two alternative states by inverting DNA segments contained within two target/inverted recombination sites. Although the chemistry of strand exchange is performed by a pair of serine recombinase dimers bound to a pair of recombination sites, the process generally occurs in an elaborate synaptic complex that contains additional protein and DNA elements (Hallet & Sherratt, 1997; Sarkis et al., 2001). Formation of these complexes may be needed for synapsis of the crossover sites, as well as for the activation of the recombinases for catalysis, and to control the outcome of the recombination reaction. The organisation of the recombination sites and the synaptic complexes formed differ between invertases and resolvases (Figure 1.1-C).

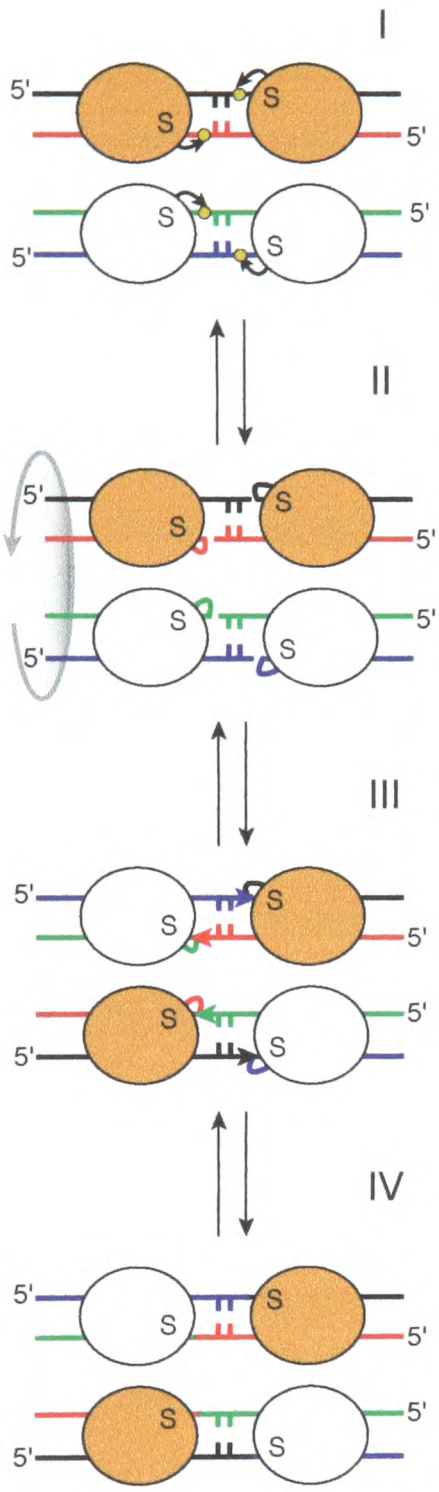
The Tn3/γδ resolvase recombination site *res* contains three subsites, each of which is bound by two resolvase subunits, with strand exchanges occurring at subsite I (Sarkis et al., 2001; Stark & Boocock, 1995; Stark et al., 1989a). On other hand, DNA invertases assemble a synaptic complex containing two minimal recombination sites plus an enhancer element, which is located between the two inverted repeat sites (Hallet & Sherratt, 1997; Johnson, 1991).

Strand exchange by invertases and resolvases follow a common pathway that is illustrated in Figure 1.2-A {adapted from Hallet & Sherratt (1997) and Stark et al. (1992)}. Four recombinases occupy two synapsed core sites and start the recombination reaction by producing double strand breaks staggered by 2 bp, leaving recessed 5'-ends and 3'-OH overhangs. One recombinase subunit is linked to each of the 5'-ends through the conserved serine residue. Exchange of strands is achieved by a 180^{O} rotation of two half-sites, followed by a re-ligation step in which the protein-DNA phosphoseryl bonds are attacked by the 3'-OH ends of the partners, and the DNA strands are resealed in the recombinant forms. Homology between the central regions of the two recombining sites is required, as well as supercoiling of the DNA substrate, which has been proposed to drive the process of strand exchanges (Benjamin et al., 1996; Stark et al., 1992).

Two main models have been proposed to explain the 180^{O} rotation of half-sites during the strand exchange step. In the "subunit rotation" model (Stark et al., 1989b), strand exchanges is coupled to a 180^{O} rotation of half-sites and bound recombinase subunits in a right-handed sense with respect to the other two pairs of strands (Figure 1.2-A).

Figure 1.2. Diagram illustrating the recombination reactions catalysed by serine and tyrosine recombinases. **A)** The subunit rotation model of serine recombinases. White and orange ovals represent recombinase subunits (with the conserved catalytic serine "S") bound to two synapsed core recombination sites. Black arrows in (I) represent the cleavage of the four scissile phosphates (yellow balls); strand exchanges are carried out by 180^{O} rotation of the half-site bound subunits (II and III), followed by religation of the DNA strands in the recombinant configuration (IV). **B)** Sequential strand exchanges by tyrosine recombinases. Two recombination sites, each bound by two recombinase subunits (purple and green ovals) are synapsed in anti-parallel configuration (I). One pair of recombinases becomes activated for catalysis (green pair) and mediates the cleavage of the red and green strands (II); strands are swapped between the recombining DNA duplexes, and then religated to produce the HJ intermediate (III). Inactivation of the green pair of recombinases and activation of the purple pair for catalysis is coupled to the isomerisation step of the HJ intermediate (III-IV and C). The resolution of the HJ intermediate to recombinant products is carried out by similar cleavage and religation reactions mediated by the purple pair of recombinases (IV to VI). **C)** Model of HJ isomerisation (Gopaul et al. 1998). Change of conformation of the HJ intermediate is accomplished by compressing arm I-III and II-IV angles, and opening arm I-II and III-IV angles.

A



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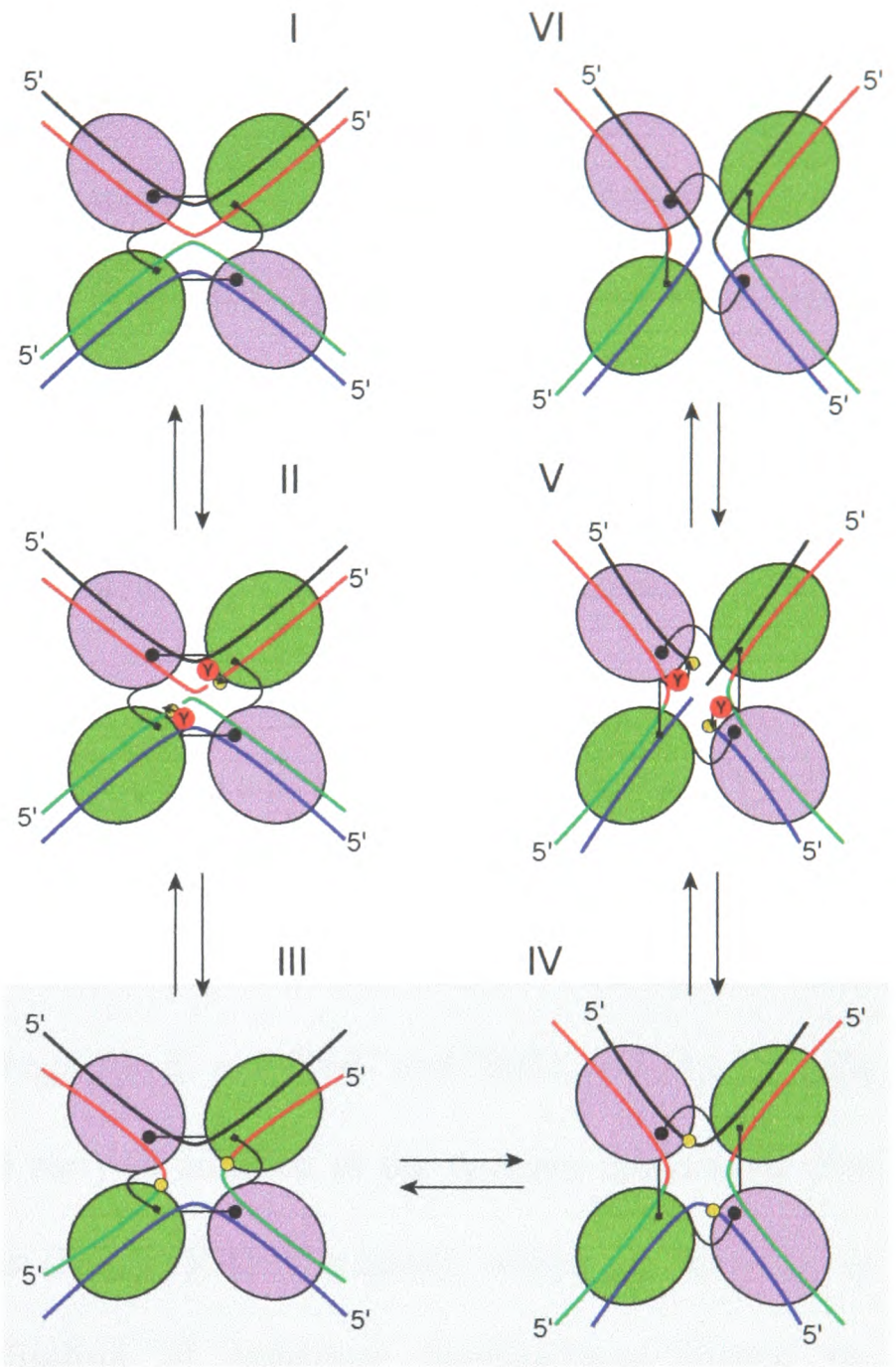

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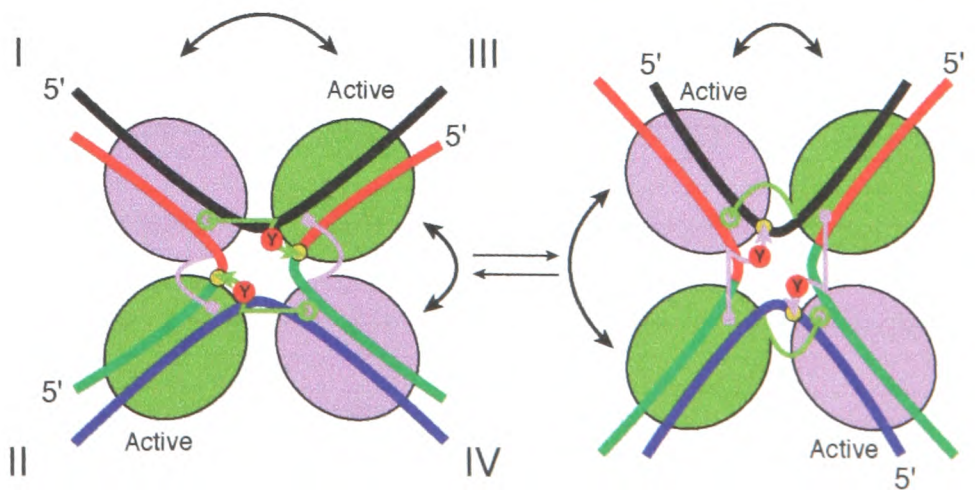


This model implies the disruption of the dimer interface between monomers bound on the same core site. The second "static subunit" model conserves the protein-protein contacts and recombination would occur by localised conformational and topological rearrangement of the DNA molecules within the complex (Rice & Steitz, 1994; Yang & Steitz, 1995). However, the subunit rotation model is supported by the observation that Tn3 resolvase recombining sites in which the central regions are not homologous to each other can carry out multiple rounds of "rotation" without rejoining the first single 180^{o} rotation intermediate (McIlwraith et al., 1997). The heterology formed upon the first strand exchanges seems to prevent rejoining and that seems to lead to the following rounds of rotation/exchange.

1.5. Tyrosine recombinases

Members of the tyrosine recombinase family include the integrase of phage λ (λ-Int), and the recombinases Cre from phage P1, *E. coli* XerC and XerD, and Flp from the yeast 2μ plasmid. These enzymes carry in addition to the tyrosine nucleophile five other conserved catalytic residues (R-K-H-R-H/W pentad), which are involved in phosphate activation and stabilisation of transition intermediates during the recombination reaction (Cao & Hayes, 1999; Gopaul & Duyne, 1999; Grainge & Jayaram, 1999; Krogh & Shuman, 2000; Nunes-Duby et al., 1998; Sherratt & Wigley, 1998).

Recombination sites for tyrosine recombinases consist of two inverted ~12 bp recombinase-binding elements separated by a 6-8 bp central region, at the outer boundaries of which DNA cleavage occurs. As for serine recombinases, additional accessory sequences that are located adjacent to the core recombination site may be required to facilitate synapsis as well as modulate the recombination outcome. Some

tyrosine recombinases have evolved to mediate rearrangements between non-identical core recombination sites and therefore their action leads to new genetic combinations by "conjugative transposition" or "integron cassette mobilization" {(Scott & Churchward, 1995; Stokes & Hall, 1989) and G.D. Recchia & D.J. Sherratt, *in press*}.

The recombination reaction catalysed by the tyrosine family members takes place in a tetrameric protein-DNA complex consisting of two anti-parallel aligned core sites, each bound by two recombinase subunits (Figure 1.2-B). The recombination mechanism differs greatly from that of serine recombinases in which it is carried out by two steps of strand exchanges separated in time and space, and having a HJ-containing molecule as an intermediate (Gopaul et al., 1998; Guo et al., 1997; Hallet et al., 1999; Nunes-Duby et al., 1995). First, just one recombinase becomes active for catalysis per core site and its tyrosine nucleophile attacks a scissile phosphate in one strand of the core, producing a free 5'-OH end and a 3' phosphotyrosyl-linked recombinase-DNA complex. Note that the polarity of this cleavage reaction is reversed when compared to that mediated by the serine recombinases. Following the first cleavages, ~3 nucleotides are swapped between the recombining core sites and the free 5'-OH ends attack the recombinase-DNA phosphotyrosyl bonds of the partner duplexes to generate the HJ intermediate. Sequence homology is required between the central regions of the recombining core sites in order to re-anneal and orient the swapped 5'-OH ends for the rejoining step. To complete recombination, the two other strands are exchanged by repeating the same cleavage-religation mechanism (Figure 1.2-B).

A key point in the recombination mechanism catalysed by tyrosine recombinases is how within a recombinase tetramer-DNA complex, catalysis is coordinated so that the two pairs of strand exchanges, which occur between the

recombining DNA sites in a complete recombination reaction, are separated in time and space. This demands that only two of the four catalytic sites are active in recombination at any one time. Furthermore, once two active catalytic sites have completed the initial strand exchange reaction to produce the HJ intermediate, a reciprocal switching process is required to inactivate the two initially active catalytic sites and activate the two initially inactive catalytic sites. Biochemical experiments and crystal structures have begun to reveal that this sophisticated control of catalysis is attained through subtle changes in conformation of the HJ DNA between two alternative forms, each of which is a substrate for strand exchange by one specific pair of recombinase molecules (Figure 1.2, B (grey box) and C). This change of conformation is accompanied by readjustments of the extreme C-termini of the recombinases, which are believed to align the catalytic tyrosines of the cleaving subunits for the attack of their scissile phosphates, while shifting the tyrosines of the non-cleaving subunits away from their target phosphates (Arciszewska et al., 2000; Chen et al., 2000; Gopaul & Duyne, 1999; Gopaul et al., 1998; Guo et al., 1999; Guo et al., 1997; Hallet et al., 1999). The mechanism that drives the change of conformation of the tetrameric recombinase-HJ complex is still a matter of investigation (see below).

1.5.1. Structure of tyrosine recombinases

Tyrosine recombinases are C-shaped proteins that bind DNA like a clamp, with their N- and C-terminal domains occupying different faces of the DNA-binding site {Figure 1.3 and (Chen et al., 2000; Guo et al., 1997; Subramanya et al., 1997)}. While the N-terminal domains have been implicated mainly with stabilization of binding to DNA, the C-terminal domains are responsible for catalysis, specific binding to DNA

and contain most of the elements required for inter-subunit interactions within the tetrameric complex.

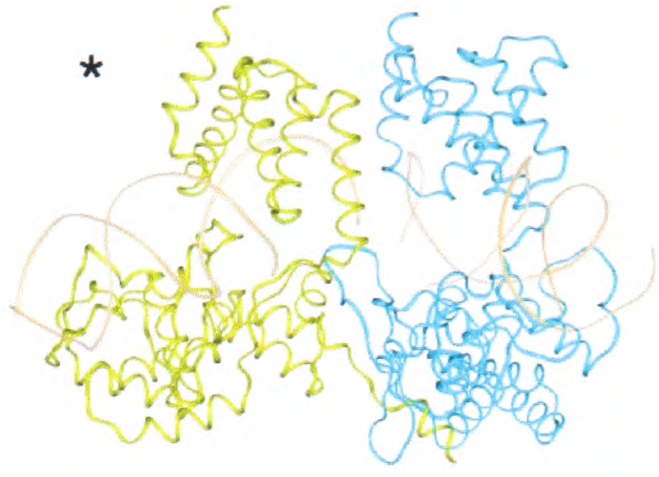


Figure 1.3. Side view of the Cre-*loxP* synaptic complex (Guo et al., 1997). Only one *loxP* core site is shown for clarity. The cleaving subunit is coloured in yellow, and the N-terminal face of the complex is marked with an asterisk.

Crystal structures of five members of this family have been determined: the catalytic domains of λ-Int and HP1 integrases, the inactive form of XerD, and the entire recombinase-DNA tetrameric complexes of Cre and Flp (Chen et al., 2000; Guo et al., 1997; Hickman et al., 1997; Kwon et al., 1997; Subramanya et al., 1997). Despite the amino acid sequence dissimilarity among family members, which might reflect their diversity of functions, these enzymes share a similar folding of their catalytic domains. This structural conservation is responsible for the assembly of the catalytic sites of these enzymes by bringing all the six conserved catalytic residues together (Figure 1.4). The conformation of the active sites of the tyrosine recombinases is also very similar to the active sites of type IB topoisomerases, which reflects a mechanistic relationship (Cheng et al., 1998; Redinbo et al., 1998; Sherratt & Wigley, 1998; Shuman, 1998).

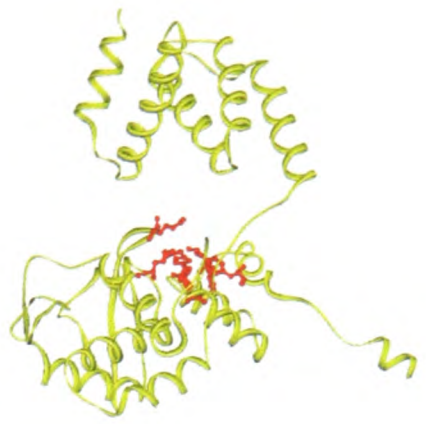


Figure 1.4. Cre recombinase (Guo et al., 1997) showing the catalytic residues in red.

The co-crystal structures of Cre-*loxP* and Flp-*FRT* supplied valuable information about the assembly of tyrosine recombinases tetrameric complexes (Chen et al., 2000; Gopaul et al., 1998; Guo et al., 1997). First, an extensive network of protein-protein interactions was revealed between the N- and C-terminal domains of the recombinases, which is believed to contribute to cooperative binding to DNA, synapsis formation, coordination of the catalytic activity and it might also help to hold the tetrameric complex together during the recombination reaction (Figure 1.5-A). In the Cre-*loxP* synaptic complex, recombinases are bound to two anti-parallel aligned core recombination sites that are bent at the start of the reaction to provide an architecture that resembles a nearly planar four-way junction (Figure 1.5-A). In both Cre-*loxP* and Flp-*FRT* complexes, the extreme C-terminus of each recombinase, which carries the catalytic tyrosine residue, can be seen extended away from the enzyme and contacting another partner recombinase in a cyclic arrangement (Figure 1.5, A and B). Note that in Flp-*FRT*, the C-terminal region that contacts the partner recombinase carries with it the tyrosine nucleophile (Figure 1.5-B) that will participate in the assembly of the catalytic site of the partner (Chen et al., 2000).

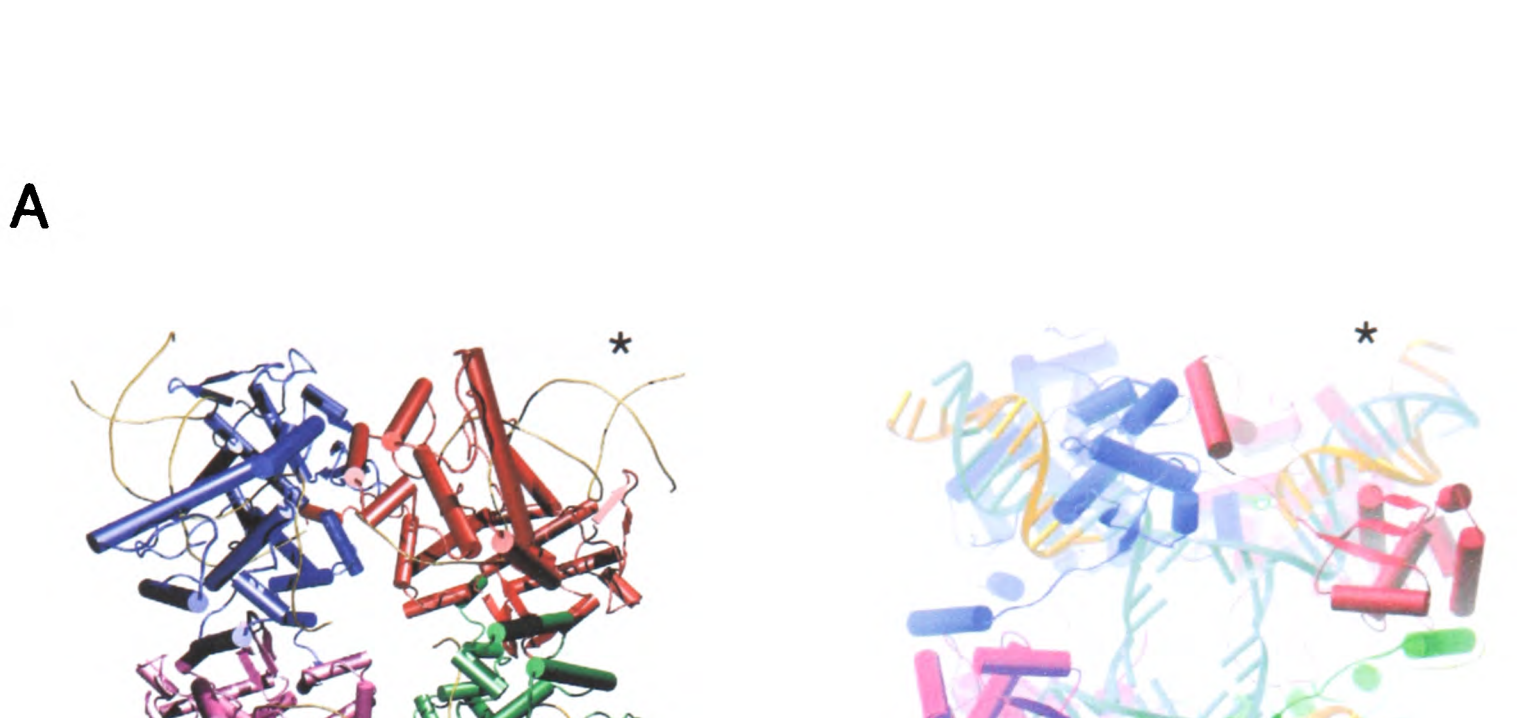


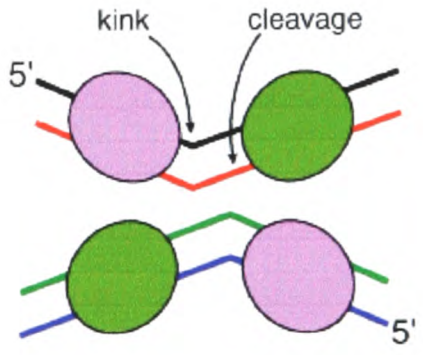
Figure 1.5. Structures of Cre-*loxP* and Flp-*FRT* complexes. **A)** Cre-*loxP* synaptic complex and Flp-*FRT* HJ; view from the N-terminal face of the complexes. Cleaving subunits are marked with asterisks. **B)** Side view of Flp-*FRT* and Cre-*loxP* HJ complexes; the N-terminal domains are at the top. The catalytic site residues in both complexes are coloured in green, and the scissile phosphates (yellow balls) that will be cleaved are indicated with small arrows.

Therefore, the active site within Flp is composed of amino acid residues from two different monomers and cleavage occurs *in trans* (Chen et al., 1992; Lee et al., 1999). On other hand, all the catalytic residues in Cre come from the same recombinase subunit and the phosphodiester cleaved is adjacent to the half-site bound by the enzyme (cleavage *in cis*) {Figure 1.5-B and (Gopaul & Duyne, 1999; Guo et al., 1997). Similar to Cre, Xer recombinases and HP1 integrase were also proposed to cleave their target phosphodiesters *in cis* (Arciszewska & Sherratt, 1995; Hickman et al., 1997). However, λ-Int can apparently cleave both *in cis* and *in trans* (Han et al., 1993; Kwon et al., 1997; Nunes-Duby et al., 1994). A possible explanation for this was given by the fact that the catalytic tyrosine in λ-Int is located on an exposed and relatively flexible 17 amino acids loop, which could participate in the assembly of two alternative active sites (Kwon et al., 1997).

In the rather compact quaternary structures of Cre-*loxP* and Flp-*FRT*, the cleavage and strand exchange steps are believed to take place by simply swapping DNA strands between substrates, with only modest changes of structure of either the protein subunits or the DNA arms throughout the entire reaction pathway (Chen et al., 2000; Gopaul & Duyne, 1999; Gopaul et al., 1998; Hallet et al., 1999). An important feature of this mechanism is that branch migration of the HJ intermediate formed after the first step of strand exchanges is not required in order to reorganise the complex to be resolved to recombinant products, and the extensive interface formed between the recombinase subunits in the tetrameric complex would be preserved.

Although the current model of recombination catalysed by the tyrosine recombinases suggests a link between the isomeric state of the recombinase-HJ complex and the activation of a given pair of recombinases for catalysis (Gopaul et al., 1998; Hallet et al., 1999), it does not address how cleavage preferences and

directionality of the recombination reaction take place. The available evidence suggest that the nature of DNA bending induced by recombinase binding can determine which of the two DNA strands will be cleaved and exchanged in the initial stages of the recombination reaction (Grainge & Jayaram, 1999; Guo et al., 1999; Lee et al., 1997). Support for this idea comes from the tertiary structures of Cre-*loxS* synaptic complexes, in which the *loxS* sites are asymmetrically bent containing a kink in the crossover region close to the non-cleaving subunit of Cre {*loxS* is a symmetrised *loxP* site (Guo et al., 1999)}. The scissile phosphate that becomes activated for cleavage is that close to the Cre-binding site opposite to the kink (Figure 1.6). In agreement with this are the biochemical data showing that Cre and Flp recombinases preferentially cleave DNA strands (in linear duplex substrates) containing bulges that can mimic the bends observed in the Cre-*loxS* synaptic complexes (Grainge & Jayaram, 1999; Guo et al., 1999; Lee et al., 1997).



***loxP* site:** 5′-ATAACTTCGTATAG **CATACA** TTATACGAAGTTAT-3′
3′-TATTGAAGCATATC **GTATGT** AATATGCTTCAATA-5′

Figure 1.6. Model of the asymmetrically bent Cre-*loxP* synaptic complex (Guo et al., 1999). Active recombinase subunits are coloured in green. The sequence of the *loxP* site is shown with the central region in bold. Cleavage occurs in the phosphodiester bonds between the underlined bases.

Following the first step of strand exchanges, directionality in the Cre-*loxP* system might be achieved by a biased resolution of the HJ intermediate formed. This idea is

based on the observation that Cre preferentially resolves synthetic HJ substrates on a particular pair of strands (Lee & Sadowski, 2001). This bias was proposed to be dictated by the asymmetric *loxP* sequence (Figure 1.6) since the resolution preference can be abolished or reversed when the bases immediately 5' to the scissile phosphodiester bonds are made identical or interchanged, respectively. On other hand, the Xer system (in which the bases 5' to the scissile phosphodiester bonds are identical in the core sites *dif, psi* and *cer*) utilises two related but functionally distinct recombinases plus accessory sequences and proteins in order to impose directionality to its recombination reaction (details about the Xer system will be given in the next section and in the following Chapters). Finally, the DNA sequence in the central region of the core site, which seems to determine the preferred conformation adopted by HJ intermediates (Arciszewska et al., 2000; Arciszewska et al., 1997; Azaro & Landy, 1997; Gopaul et al., 1998; Hallet et al., 1999), might also contribute in the geometry of linear synapsed core sites influencing the first step of cleavages.

1.6. The Xer system

Chromosome and plasmid dimers can be generated in *E. coli* by homologous recombination. To ensure the correct partitioning of the genetic material to the daughter cells at cell division, Xer site-specific recombination converts these dimers back to their monomeric forms (Sherratt et al., 1995). Whereas in most tyrosine recombinase reactions, four molecules of a single recombinase mediate a complete recombination reaction, in XerCD site-specific recombination, two related recombinases, XerC and XerD, each catalyse the exchange of one pair of strands (Figure 1.2-B). On supercoiled DNA substrates containing two recombination sites, the first pair of strand exchanges, mediated by XerC, leads to the formation of a HJ

intermediate. Progression to a complete recombination reaction by XerD-mediated strand exchange requires a conformational change of the HJ intermediate in order to activate XerD and reciprocally inactivate XerC (Colloms et al., 1996; Hallet et al., 1999).

Xer recombinases act on a variety of naturally occurring sites, in which the recombination cores consist of two related ~11 bp recombinase-binding elements, separated by 6-8 bp central regions (*dif* and *psi* have a 6 bp central region, whereas *cer* has 8 bp). These sites evolved to have very distinct recombination properties that direct their subtly different biological roles. For example, a complete recombination reaction at *psi* {from plasmid pSC101 (Cornet et al., 1994)} occurs by sequential strand exchanges by XerC and XerD. In contrast, at the plasmid site *cer* {from ColE1 (Summers & Sherratt, 1984)}, XerC forms HJs, which are unable to undergo the conformational change that would allow them to be resolved by XerD. Therefore, the HJ intermediate appears to be the Xer recombination reaction endpoint at *cer*-containing plasmids (McCulloch et al., 1994), and other cellular factors might be responsible for the resolution of these intermediates to recombinant products (Colloms et al., 1996). Recombination at both *cer* and *psi* requires additional ~200 bp of accessory sequences adjacent to their XerC-binding sites to which the accessory proteins PepA/ArgR and PepA/ArcA bind to, respectively (Alén et al., 1997; Colloms et al., 1998; Colloms et al., 1997; Colloms et al., 1996; Strater et al., 1999). The function of these accessory sequences and proteins is to assemble productive synaptic complexes of defined geometries, which also contribute to impose directionality to the recombination reaction {M. Bregu, D.J. Sherratt & S.D. Colloms, *in preparation*, and (Colloms et al., 1997)}.

In recombination at *dif*, a 28 bp site located close to the replication terminus of the *E. coli* chromosome (Blakely et al., 1991; Kuempel et al., 1991), XerC was proposed to mediate cycles of HJ formation and resolution unless chromosomal dimers were present at the time of cell division, when FtsK (a septum-located protein) would facilitate the conversion of the HJ intermediate formed by XerC to a conformation suitable for XerD-mediated resolution (Barre et al., 2000; Recchia et al., 1999). Recently, the observation that XerD can mediate the first step of strand exchanges at *dif* in the presence of FtsK (*in vivo* and *in vitro*), and the demonstration that FtsK uses ATP hydrolysis to translocate along duplex DNA as a multimer *in vitro*, which is consistent with the idea that it pumps DNA through the closing division septum *in vivo*, have led to the proposal of a new model of chromosome dimer resolution in *E. coli* (L. Aussel, F.X. Barre, M. Arroyo & D. Sherratt, *in press*). In this model, FtsK would work as a molecular sensor, which scans the DNA at the septum and activates chromosome dimer resolution when it encounters *dif* sites synapsed by XerCD. The start of the recombination reaction by XerD strand exchanges at *dif* in the presence of FtsK would require a HJ isomerisation step that could reorganise the intermediate to a conformation suitable for XerC-mediated resolution. Since *dif*-containing HJ intermediates normally adopt a conformation that is suitable for XerC-mediated resolution (see below), the pathway followed during the XerCD/*dif*/FtsK recombination might just rely on the natural ability of the *dif*-HJ to assume the "XerC conformation" in order to be resolved to recombinant products. In Chapter 3, it will be demonstrated that the XerD-DNA interactions also participate in setting the conformation of the recombinase-HJ complex for XerC-mediated resolution.

The different recombination properties of *psi*, *cer* and *dif* sites are, at least in part, determined by the nucleotide sequence of their central regions close to the HJ branchpoint; purine-richness in a strand predisposes it to be a preferential substrate for recombinase-mediated catalysis (Arciszewska et al., 2000; Arciszewska et al., 1997; Azaro & Landy, 1997; Gopaul et al., 1998; Hallet et al., 1999). The *cer* central region (5'-TTAAGGGA), being purine-rich in the strand exchanged by XerC, is only a substrate for XerC under all conditions tested. In contrast, *psi* has an equal amount of purines and pyrimidines in both strands of the central region, which makes it a potential good substrate for XerD strand exchanges (the *psi* strands cleaved by XerC and XerD have the central regions 5'-GATCCA and 5'-TGGATC, respectively). However, a *psi*-containing HJ is still preferentially resolved by XerC and appears to require the accessory protein PepA in order to adopt a conformation that is suitable for XerD-mediated resolution (M. Robertson. & D.J. Sherratt, *in preparation*). Similar to *psi*, the central region of *dif* (5'-TGTATA) is also 50% purine-rich, and *dif*-containing HJs adopt preferentially a conformation suitable for XerC-mediated strand exchanges. Finally, regardless of the central region sequence, XerC normally initiates catalysis on duplex DNA containing the wild type sites *cer*, *psi* or *dif* (in the absence of FtsK); this is presumably an effect of asymmetric XerC-XerD interactions after binding to duplex core sites (Spiers & Sherratt, 1999).

1.7. Objectives

The objective of this project was to study the mechanism of XerCD site-specific recombination by focusing on the contribution of the Xer recombinases in the coordination of catalysis. To perform these analyses, chimeric Xer recombinases were constructed in which N- and C-terminal regions were exchanged between XerC and

XerD. Their phenotypes were examined in experiments of resolution of synthetic HJ substrates, recombination of plasmids containing directly repeated recombination sites and binding and cleavage of linear duplex DNA substrates *in vitro*. The results obtained suggest that the N-terminal domains of XerC and XerD participate in the coordination of catalysis, and also extend the analyses of Hallet et al. (1999) about the interactions and roles of the C-terminal acceptor and donor regions of Xer recombinases during recombination. Finally, the recombination reaction catalysed by the tyrosine recombinase ResD, which is encoded by the *E. coli* F plasmid and that is believed to function in F plasmid dimer resolution, was reconstituted *in vitro*. ResD lacks a region corresponding to the N-terminal domains of XerC and XerD, and hence the characterisation of its recombination reaction might supply further insights about the roles of the N-terminal domains of tyrosine recombinases.

CHAPTER 2

Materials and methods

2.1. Bacterial strains and growth conditions

DS9040 (DS941, *xerC* and *xerD*; D.J. Sherratt, unpublished) was used for the expression of XerCD recombinases and their variants. The *E. coli* strains DS957 {DS941, *pepA::*Tn5 (Stirling et al., 1989)} and DS956 {DS941, *argR::*Tp^r (Colloms et al., 1990)} were used for over-expressing PepA and ArgR. The *E. coli* strain DS650 was used as a source of F-plasmid DNA. The strain JC8679 {AB1157, *recBC sbcA* (Summers & Sherratt, 1988)} was used for plasmid multimerisation. DS9041 {DS941, *ftsK* (Recchia et al., 1999)}, DS887 and DS888 (*fis*$^-$ and *fis*$^+$ *E. coli* strains, respectively) were used for the ResD resolution assays *in vivo*. The *E. coli* strains DH5α and Epicurian Coli XL1-Blue were used for sub-cloning purposes. All cells were cultured in LB or LB-agar medium with the required antibiotics at 37^{O}C (Sambrook et al., 1989).

Genotypes:

AB1157: *E. coli* K12, *thr-1, leuB6, hisG4, thi-1, ara-14, Δ(gpt-proA)62, argE3, galK2, supE44, xyl-5, mtl-1, tsx-33, lacY1, rpsL31* (Bachmann, 1972).

DS941: AB1157, *recF143, lacZΔM15, lacI*^{q} (Summers & Sherratt, 1988).

DH5α: *deoR, endA1, gyrA96, hsdR17, recA1, relA1, supE44, glnV44, thi-1, Δ(lacIZYA-argF)U169, ϕ-80ΔlacZΔM15* (New England BioLabs).

Epicurian Coli XL1-Blue: *recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac* [F′*proAB lacI*^{q}*ZΔM15* Tn*10*(Tet^r)] (Stratagene).

2.2. Plasmids

The plasmids used are listed in Table 2.2

Table 2.1. Plasmids used and some of their characteristics.

Plasmids	Relevant Characteristics	Source/Reference
pTrc99a	Expression plasmid carrying a strong hybrid *trp/lac* promoter; [1] Ap^r	(Amann et al., 1988)
pBAD24	Expression plasmid containing the arabinose P_{BAD} promoter; Ap^r	(Guzman et al., 1995)
pK184	Cloning vector carrying the p15a replicon; [2] Km^r	(Jobling & Holmes, 1990)
pUC18/pUC19	Cloning vectors carrying ColEI replicons, Ap^r	New England BioLabs
pUC4K	Kanamycin cassette cloned into pUC18	Amersham-Pharmacia
pBH400*	*xerC* in pTrc99a	B. Hallet and D.J. Sherratt, unpublished.
pRM132*	pRM130 derivative; *xerD* in pUC19	(Blakely et al., 1993)
pAS105	*xerD* in pTrc99a	A.J. Spiers and D.J. Sherratt, unpublished.
pSDC104	*xerC* in pUC18	(Colloms et al., 1990)
pSDC134	*psi* reporter plasmid	(Colloms et al., 1996)
pSDC124	*dif* reporter plasmid	(Blakely et al., 1991)
pMIG103	*psi* reporter plasmid; contains directly repeated *psi* recombination sites	M. Bregu, D.J. Sherratt and S.D. Colloms, unpublished.
pMIG104K	pMIG103 variant; *psi* accessory sequences are placed adjacent to the XerD-binding sites	
pMIG121	*cer* reporter plasmid	
pMIG122	Inverted-core *cer* reporter plasmid	
pOS13*	*xerD[CIIe]* in pUC19	A.J. Spiers and D.J. Sherratt, unpublished.
pOS16	*xerC[DIIe]* in pUC18	
pOS14-32	*xerC[De]* in pUC18	O. Schimidt-Kittler, B. Hallet, D.J. Sherratt, unpublished.
pOS15*	*xerD[CII]* in pUC19	
pOS32/30*	*xerD[Ce]* in pUC19	
pOS16e32/30	*xerC[DII]* in pUC18	This work
pB14-32*	*xerC[De]* in pTrc99a	
pB16*	*xerC[DIIe]* in pTrc99a	
pB30S*	*xerC[DII]* in pTrc99a	
M5'	pAS105; *xerD-Sal*I at position 340 (encodes XerDS114V,E115D)	
D43*	*xerD[CKRGK]* in pTrc99a	
CD*	*xerD[CABCD]* in pTrc99a	
DC*	*xerC[DABCD]* in pTrc99a	
1A2B*	*xerD[CAB]* in pTrc99a	
1B2A*	*xerC[DAB]* in pTrc99a	
1A2B-2	*xerD[CAB]-Bgl*II at position 262 (encodes XerD[CAB]E89S)	
1B2A-2	*xerC[DAB]-Bgl*II at position 265	

	(encodes XerC[DAB]S88R/Q89S)	
CCD*	*xerC[DCD]* in pTrc99a	
DCD*	*xerD[CCD]* in pTrc99a	
CA*	*xerC[DA]* in pTrc99a	
CB*	*xerC[DB]* in pTrc99a	
DA*	*xerD[CA]* in pTrc99a	
DB*	*xerD[CB]* in pTrc99a	
CC4*	*xerC[ΔABCD]* in pTrc99a	
DC3*	*xerD[ΔABCD]* in pTrc99a	
XerCN-ter*	pBH400; *xerC*-TGA at position 346	
XerDN-ter*	M5'; *xerD*-TGA at position 340	
pB*resD**	*resD* in pBAD24	
pU13	ResD recombination site cloned in pUC18	
pKDseq	ResD recombination site cloned in pK184	
p*resD*b	pUC18-based ResD reporter plasmid	
pk*resD*b	pK184-based ResD reporter plasmid	

[1] Ampicillin resistance; [2] kanamycin resistance

* Plasmids used for protein purification.

2.3. DNA manipulation *in vitro*

Standard DNA manipulations and molecular biology procedures were carried out as (Sambrook et al., 1989). Restriction and modifying enzymes were used as recommended by the manufacturers (New England BioLabs, BRL-GIBCO, Stratagene, Amersham-Pharmacia).

2.3.1. Construction of XerCD recombinase variants

Site-directed mutagenesis (QuikChange™, Stratagene) was used for the introduction of point mutations in *xerC* and *xerD* genes cloned in pBH400 and pAS105, respectively (relevant primers are indicated in Table 2.2). The PCR(Polymerase Chain Reaction)-based domain exchange technique employed to construct XerC and XerD chimeric recombinases is depicted in Figure 2.1. PCR conditions were as follows: 95°C/1', followed by 30 cycles of 95°C/1', annealing temperature calculated for each combination of primers/1', and 68°C/2'.

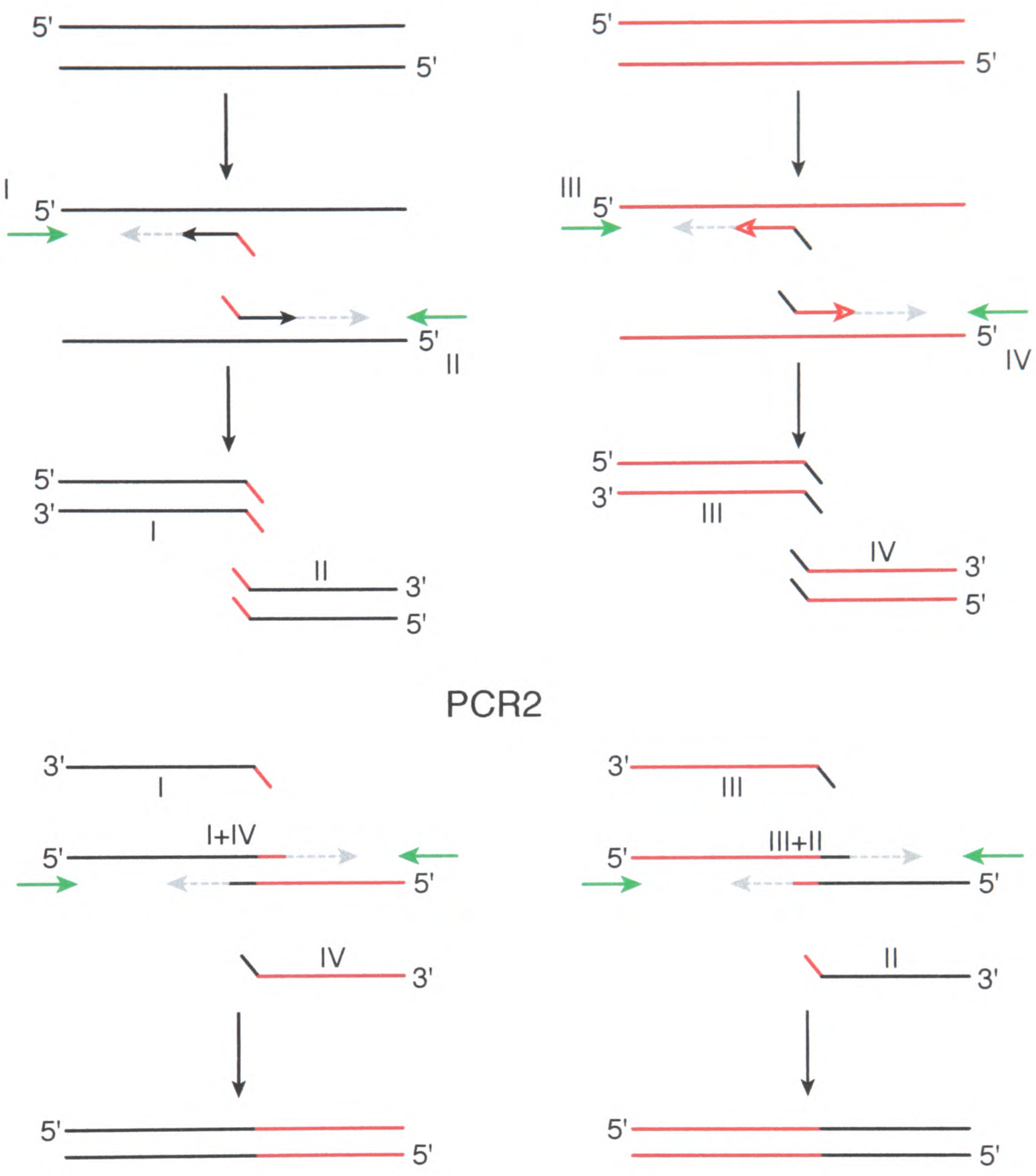


Figure 2.1. In the first step (PCR1), the regions to be exchanged were amplified by PCR using combinations of external (green arrows) and internal primers (red-black arrows). The internal primers are 40-80 bases long, in which each half is complementary to one of the sides immediately to the right or to the left of the boundary that will be created in the chimeric gene. The products from the first four PCRs (I, II, III, and IV) are then mixed as shown above (I+IV and III+II) and the chimeric genes are concluded in a second step (PCR2) using now only the external primers. The PCR products are separated by electrophoresis in 1.4% agarose-TAE gel, purified and cloned into appropriate expression vectors.

The reaction mixture contained: 2.5 units of Pfu DNA polymerase (Stratagene), 100μM of each dNTP, 0.6μM of each primer, buffer as recommended, the appropriate DNA templates and water to 30μl final.

The chimeric genes *xerC[DIIe]*, *xerD[CIIe]*, *xerC[De]*, and *xerD[Ce]* were constructed by the two-steps PCR method described (A. Spiers and D.J. Sherratt, unpublished data; O. Schimidt-Kittler, B. Hallet and D.J. Sherratt, unpublished data). *xerC[DII]* was constructed by replacing a *Mlu*I-*Bam*HI fragment of ~120bp from plasmid pOS16 {carrying *xerC[DIIe]*} with the *Mlu*I-*Bam*HI fragment from plasmid pOS32 {*xerD[Ce]*}. *xerD[CII]* was constructed by a similar procedure, replacing a *Nde*I-*Hind*III fragment of ~150bp from pOS13 {*xerD[CIIe]*} with the *Nde*I-*Hind*III fragment from pOS14-32 {*xerC[De]*}. In order to increase the expression levels of the XerC C-terminal chimeras their DNA coding sequences were transferred from plasmids pOS14, pOS16, and pOS16e32/30 to pTrc99a, which carries a strong hybrid *trp/lac* promoter (Amann et al., 1988). This transference was carried out by replacing a *Sal*I-*Hind*III fragment of ~600 bp from pBH400 with the *Sal*I-*Hind*III fragments from plasmids pOS14, pOS16 and pOS16e32/30, giving rise to pB14-32, pB16 and pB30S, respectively (Table 2.1). The chimeric gene *xerD[CKRGK]* was constructed by site-directed mutagenesis (QuikChange™, Stratagene), in which the DNA sequence from *xerC* encoding for the last four amino acid residues KRGK was inserted at the 3'-end of the *xerD* gene (DNA template was pAS105, and primers were XerD4aac-for and XerD4aac-rev, Table 2.2).

The chimeric genes encoding for the N-terminal variants XerC[DABCD] and XerD[CABCD] were constructed by swapping the *Sal*I-*Hind*III fragments of ~600bp from plasmids pBH400 and M5' (*Sal*I was inserted in *xerD* by site-directed mutagenesis using primers XerD-SalI-for and XerD-SalI-rev, Table 2.2, and pAS105

as DNA template); *xerC[ΔABCD]* and *xerD[ΔABCD]* were obtained by sub-cloning the *Sal*I-*Hind*III fragments from pBH400 and M5', which encode for the C-terminal domains of XerC and XerD recombinases, respectively, into the pTrc99a expression vector. In order to over-express the N-terminal domains of XerC and XerD (XerCN-ter and XerDN-ter, respectively), the stop codon TGA was inserted by site-directed mutagenesis in *xerC* and *xerD* genes at the 3'-end of the DNA sequences encoding for α-helices D of these recombinases (DNA templates were pBH400 and pAS105; primers were XerC-TGA-for, XerC-TGA-rev, XerD-TGA-for and XerD-TGA-rev, Table2.2). *xerC[DAB]* and *xerD[CAB]* were constructed by PCR using as DNA templates plasmids pBH400 and M5', and primers newCD-for, newCD-rev, newDC-for, newDC-rev, pTrc-for and pTrc-rev (Table 2.2). *xerC[DCD]* and *xerD[CCD]* were constructed by swapping the *Bgl*II-*Hind*III fragments of ~680bp from plasmids 1A2B-2 and 1B2A-2 {*Bgl*II restriction sites were inserted in *xerC[DAB]* and *xerD[CAB]* by site-directed mutagenesis using plasmids 1B2A and 1A2B, respectively, as DNA templates, and primers CBglII-for, CBglII-rev, DBglII-for and DBglII-rev, Table 2.2}. Finally, *xerC[DA]*, *xerC[DB]*, *xerD[CA]* and *xerD[CB]* were constructed by PCR using as DNA templates plasmids pBH400, pAS105, 1A2B and 1B2A, and primers CaA-for, CaA-rev, DaA-for, DaA-rev, pTrc-for and pTrc-rev (Table 2.2).

All *xerC* and *xerD* variants used in the present work were verified by DNA sequencing (the sequencing primers used are listed in Table 2.2).

Table 2.2. PCR primers used

Primer	Sequence/comments
#S1201S	5'AACAGCTATACCATG
#S1211S	5'GTAAAACGACGGCCAGT

	(Sequencing primers for pUC18 and pUC19 - New England BioLabs)
pTrc-for	5'GACAATTAATCATCCGGCTCG
pTrc-rev	5'CTGTTTTATCAGACCGCTTC
	(Sequencing primers for the expression vector pTrc99a)
XerD-*Sal*I-for	5'GCCCCAGCGTTTGCCAAAAGATTTAGTCGACGCGCAGGTCGAACG
XerD-*Sal*I-rev	5'GTAATAAACGTTCGACCTGCGCGTCGACTAAATCTTTTGGCAAACG CTGGGGC (*Sal*I is underlined)
XerD4aac-for	5'CGCGGGCGAAACGGGGGAAATGAGGATCCTCTAGAGTCGACC
XerD4aac-rev	5'GACTCTAGAGGATCCTCATTTCCCACGTTTCGCCCGCGGATGAT GCTGTTGATGAAGTTG (Nucleotide insertions are underlined)
XerD-TGA-for	5'CCAAAAGATTTATGAGAAGCGCAGG
XerD-TGA-rev	5'CCTGCGCTTCTCATAAATCTTTTGG
XerC-TGA-for	5'CGAAAAACATCGACTGAGACGATATGAATCG
XerC-TGA-rev	5'CGATTCATATCGTCTCAGTCGATGTTTTTCG
NewDC-for	5'CGGGTTGACGCTGGCGCAGCAATGTGATGTGACGATGG
NewDC-rev	5'CCATCGTCACATCACATTGCTGCGCCAGCGTCAACCCG
NewCD-for	5'GAAAACGGCCTGCAAAGCTGGACGGCGCAAAGTGACG
NewCD-rev	5'CAAATCGTCACTTTGCGCCGTCCAGCTTTGCAGGCCGTTTTCG
	(Highlighted and underlined DNA sequences correspond to *xerC* and *xerD*, respectively)
C*Bgl*II-for	5'GCTTTTTTGACTGGCTGGTCAGATCTAACGAACTCAAAGC
C*Bgl*II-rev	5'GTTAGCTTTGAGTTCGTTAGATCTGACCAGCCAG
D*Bgl*II-for	5'GATTGTTCCAGTATCTTTATAGATCTAAGTTTCGTGAAG
D*Bgl*II-rev	5'CGTCTTCACGAAACTTAGATCTATAAAGATAC (*Bgl*II is underlined)
DaA-for	5'GTTATCTGAGCGTGGAGAAAAATCTGGCTGAAAATACG
DaA-rev	5'GTATTTTCAGCCAGATTTTTCTCCACGCTCAGATAACG
CaA-rev	5'GCTAAGCTGGCGTTCCAGCCACAGAGC
CaA-for	5'GGCTGGAACGCCAGCTTAGCCCG
	(Highlighted and underlined DNA sequences correspond to *xerC* and *xerD*, respectively)
XerD[K19A, N20A]-for	5'GATGCTCTGTGGCTGGAAGCGGCGCTGGCTGAAAATACG
XerD[K19A,	5'CGTATTTTCAGCCAGCGCCGCTTCCAGCCACAGAGC

N20A]-rev	
XerDK19A-for	5'GATGCTCTGTGGCTGGAAGCGAATCTGGCTGAAAATACG
XerDK19A-rev	5'CGTATTTTCAGCCAGATTCGCTTCCAGCCACAGAGC
XerDK19F-for	5'GATGCTCTGTGGCTGGAATTCAATCTGGCTGAAAATACG
XerDK19F-rev	5'CGTATTTTCAGCCAGATTGAATTCCAGCCACAGAGCATC
XerDK19N-for	5'GATGCTCTGTGGCTGGAAAACAATCTGGCTGAAAATACG
XerDK19N-rev	5'CGTATTTTCAGCCAGATTGTTTTCCAGCCACAGAGC
XerDK19R-for	5'GTTTCTTGATGCTCTGTGGCTGGAACGCAATCTGGCTGAAA ATACGTTGAACGCTTACCG
XerDK19R-rev	5'CGGTAAGCGTTCAACGTATTTTCAGCCAGATTGCGTTCCAG CCACAGAGCATCAAGAAACTG
XerDN20A-for	5'GATGCTCTGTGGCTGGAAAAGGCGCTGGCTGAAAATACG
XerDN20A-rev	5'CGTATTTTCAGCCAGCGCCTTTTCCAGCCACAGAGC
XerDN20F-for	5'GATGCTCTGTGGCTGGAAAAGTTCCTGGCTGAAAATACG
XerDN20F-rev	5'CGTATTTTCAGCCAGGAACTTTTCCAGCCACAGAGC
XerDN20R-for	5'GATGCTCTGTGGCTGGAAAAGCGTCTGGCTGAAAATACG
XerDN20R-rev	5'CGTATTTTCAGCCAGACGCTTTTCCAGCCACAGAGC
XerDN20Q-for	5'GTTTCTTGATGCTCTGTGGCTGGAAAAGCAACTGGCTGAAA ATACGTTGAACGCTTACC
XerDN20Q-rev	5'GGTAAGCGTTCAACGTATTTTCAGCCAGTTGCTTTTCCAGCC ACAGAGCATCAAGAAACTG
XerDE23P-for	5'GTTTCTTGATGCTCTGTGGCTGGAAAAGAATCTGGTTCCAAA TACGTTGAACGCTTACCGTCG
XerDE23P-rev	5'CGACGGTAAGCGTTCAACGTATTTGGAACCAGATTCTTTTCC AGCCACAGAGCATCAAGAAAC
XerDA22S-for	5'GCAGTTTCTTGATGCTCTGTGGCTGGAAAAAAATCTGAGCGAA AATACGTTGAACGCTTACCGTCGC
XerDA22S-rev	5'GCGACGGTAAGCGTTCAACGTATTTTCGCTCAGATTTTTTTCCA GCCACAGAGCATCAAGAAACTGC
XerDN24I-for	5'GCTGGAAAAAAATCTGGCTGAAATAACGTTGAACG
XerDN24I-rev	5'CGTTCAACGTTATTTCAGCCAGATTTTTTTCC
XerDN27L-for	5'CTGGCTGAAAATACGTTGCTCGCTTACCGTCGC
XerDN27L-rev	5'GCGACGGTAAGCGAGCAACGTATTTTCAGCCAG
XerDA28N-for	5'GCAGTTTCTTGATGCTCTGTGGCTGGAAAAAAATCTGGCTGAA AATACGTTGAACAACTACCGTCGCGATCTGTCAATGATGG
XerDA28N-rev	5'CCATCATTGACAGATCGCGACGGTAGTTGTTCAACGTATTTTCA GCCAGATTTTTTTCCAGCCACAGAGCATCAAGAAACTGC
XerDR30Q-for	5'CTGGCTGAAAATACCTTGAATGCTTACCAACGCGATCTGTCAAT GATGGTGGAGTGGTTGCATCACC
XerDR30Q-rev	5'GGTGATGCAACCACTCCACCATCATTGACAGATCGCGTTGGTAA

	GCATTCAAGGTATTTTCAGCCAG
XerDD32Q-for	5'GTTGAACGCTTACCGTCGCCAGCTGTCAATGATGGTGG
XerDD32Q-rev	5'CCACCATCATTGACAGCTGGCGACGGTAAGCGTTCAACG
XerDS34E-for	5'GCTTACCGTCGCGATCTGGAGATGATGGTGGAGTGG
XerDS34E-rev	5'CCACTCCACCATCATCTCCAGATCGCGACGGTAAGC
XerD[A22S,N24I,	5'GGAAAAAAATCTGAGCGAAATAACGTTGAACAACTACCGTCGC
A28N,S34E]-for	GATCTGGAGATGATGGTGG
XerD[A22S,N24I,	5'CCACCATCATCTCCAGATCGCGACGGTAGTTGTTCAACGTTATT
A28N,S34E]-rev	TCGCTCAGATTTTTTTCC
	(Alterations are underlined)
ResD recombination site-for	5'ACTCGTCGACGCTATTAATCAGTTTTTTAG
ResD recombination site-rev	5'GCTCTAGAGTTAACCAATAAAAATTAAAATCTGAC
resD-for	5'GGAGGAATTCACCATGTCAGGCTCCGTTATACACAGCCAGTCTGC
resD-rev	5'GCTGTAGACCCGGGTCAGGATAATTGTTTCAGCATCGCAACCGCAT CAGACTCC
pKDseq Deletions:	
Forward primer	5'GTCAGATTTTAATTTTTATTGGTTAACTCTAGAGC
rfsF-reverse	5'GAGGGAGAGGAAGATCCGTAACTTGG
R1-reverse	5'CGACTTTCCGGAAGTGTACAACGCTGAG
R2-reverse	5'CGTTTCGTATGGATCCGACTTTATTTGAATAAACG

2.3.2. Cloning *resD* and the ResD recombination site

The *resD* gene and the ResD recombination site were isolated by PCR. PCR conditions were: 95^{O}C/ 1 min; followed by a touch down cycle of 95^{O}C/ 30 sec, annealing temperatures ranging from 68^{O}C to 66^{O}C/ 1 min, and extension of 68^{O}C/ 2.5 min; a final 20 cycles PCR of 95^{O}C/ 30 sec, 64^{O}C/ 1 min, and 68^{O}C/ 2.5 min was carried out to amplify the products obtained on the touch down step. The PCR reactions contained: 1-5 ng of total genomic DNA from the *E. coli* strain DS650, which carries F-plasmid, 100 μM of each dNTP, 0.6 μM of each primer (Table 2.2),

2.5 U of Pfu DNA-polymerase, Pfu buffer, and water to 25 µl. The ResD recombination sequence and the *resD* gene were cloned into the vectors pK184 and pBAD24, respectively, producing pKDseq and pB*resD* (Figure 2.2).

2.3.3. Construction of the ResD reporter plasmid substrates

pKDseq plasmids (Figure 2.2) were multimerised upon their transformation into the *E. coli* strain JC8679. The internal deletions of the ResD recombination site were prepared by PCR using the pKDseq plasmid as DNA template and primers rfsF-reverse, R1-reverse or R2-reverse in combination with the Forward primer (Table 2.2 and Figure 5.3). PCR conditions were: 95^{O}C/1', followed by 20 cycles of 95^{O}C/1', annealing temperature calculated for each combination of primers/1', and 68^{O}C/11'. The reaction mixture contained: 2.5 units of Pfu DNA polymerase (Stratagene), 100µM of each dNTP, 0.6µM of each primer, buffer as recommended, 1-5 ng of DNA template, and water to 30µl final.

The construction of the deletion reporter plasmid pK*resD*b is illustrated in Figure 2.3. The restriction map of pK*resD*b using the enzymes *Eco*RI, *Hind*III and *Nco*I, which cut once, twice and three times, respectively, and in relevant positions within the reporter plasmid, is illustrated in Figure 2.4.

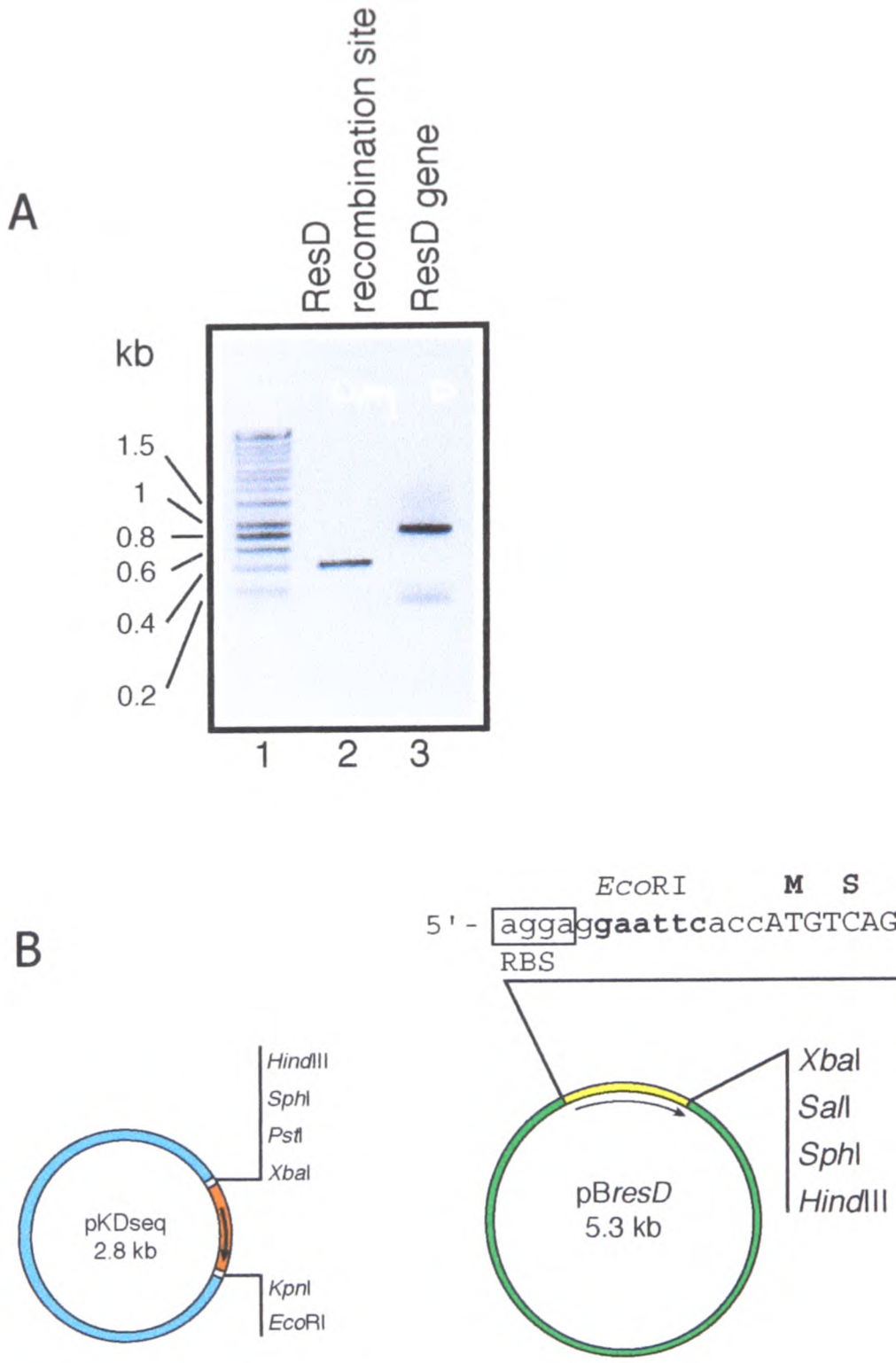


Figure 2.2. A) Ethidium bromide-stained 1% agarose gel electrophoresis showing the PCR amplification products corresponding to ResD recombination site (lane 2) and *resD* gene (lane 3). **B)** The ResD recombination sequence and the *resD* gene were cloned into the vectors pK184 and pBAD24, respectively, producing pKDseq and pB*resD*. The 5'-end of *resD* is shown above pB*resD*; RBS, ribosome-binding site. **C)** The nucleotide sequences of the ResD recombination site (top) and the *resD* gene (bottom); the amino acid sequence of ResD recombinase is displayed at the top of the *resD* gene. The sequences of the primers used in the PCR reactions are highlighted in both nucleotide sequences. The nucleotide coordinates of both sequences in F-plasmid are shown at the top of their sequences.

C

ResD recombination sequence (at *oriV1* region)

(F-plasmid coordinates: 45817-46209)

```
  1 actcgtcgac GCTATTAATC AGTTTTTTAG TTTTTGAAAA TTGTGATGCT TCTAAAATTA
 61 CTAAAATTGA GGATTTTAAT GCTACAACAA TGCCTGCCTC TTCTTATTTC TCCGGAGATC
121 CGAAAACCCC AAGTTACGGA TCTTCCTCTC CCTCCGCACA GCGTTACATC CCGTCAGCAC
181 AGCATGTAGT GCCTCATACA GTTGCCCATG GCACTATATG TTGTGTTGTA TCTCTGGACT
241 GTGATGCGCC GCGCAGGGGC GGAAAACAGC GATATGATGA TTTTCTCAGC GTTGTACACT
301 TCCGGAAAGT CGTTTATTCA AATAAAGTCG GATCCATACG AAACGGGAAT GCGGTAATTA
361 CGCTTTGTTT TTATAAGTCA GATTTTAATT TTTATTGGTT AACtctagag c...3'
                ... CAGT CTAAAATTAA AAATAACCAA TTGagatctc g...5' XbaI
```

***resD* gene (F-plasmid coordinates: 46866-47672)**

```
       EcoRI      M  S  G  S  V  I  H  S  Q  S  A  V  M  V  P  A
5'-1 ggaggaattc accATGTCAG GCTCCGTTAT ACACAGCCAG TCTGCAGTCA TGGTACCGGC

       V  Y  S  A  G  Q  P  A  S  L  P  V  A  I  D  Y  P  A  A  L
 61 AGTGTATTCT GCCGGGCAGC CTGCATCGCT GCCTGTTGCC ATTGATTATC CGGCAGCTCT

       A  L  R  Q  M  S  M  V  H  D  E  L  P  K  Y  L  L  A  P  E
121 GGCACTTCGC CAGATGTCGA TGGTCCATGA TGAACTGCCA AAATATCTGC TGGCTCCGGA

       V  S  A  L  L  H  Y  V  P  D  L  H  R  K  M  L  L  A  T  L
181 AGTGAGTGCC CTGCTCCATT ACGTCCCGGA TCTGCACCGC AAGATGCTGC TGGCCACACT

       W  N  T  G  A  R  I  N  E  A  L  A  L  T  R  G  D  F  S  L
241 GTGGAACACC GGAGCACGCA TTAATGAAGC ACTGGCGCTG ACGCGGGGGG ATTTTTCGCT

       A  P  P  Y  P  F  V  Q  L  A  T  L  K  Q  R  T  E  K  A  A
301 TGCGCCTCCG TATCCGTTTG TGCAGCTTGC GACCCTGAAA CAACGGACCG AAAAAGCTGC

       R  T  A  G  R  M  P  A  G  Q  Q  T  H  R  L  V  P  L  S  D
361 CAGGACGGCG GGGAGAATGC CCGCCGGTCA GCAGACTCAC CGGCTGGTTC CGCTCTCTGA

       S  W  Y  V  S  Q  L  Q  T  M  V  A  T  L  K  I  P  M  E  R
421 CTCCTGGTAC GTCAGCCAGC TGCAGACGAT GGTGGCAACA CTGAAAATAC CCATGGAGCG

       R  N  R  R  T  G  R  T  E  K  A  R  I  W  E  V  T  D  R  T
481 GCGTAACCGT CGCACAGGAA GGACAGAGAA AGCGCGGATC TGGGAAGTGA CGGACAGAAC

       V  R  T  W  I  G  E  A  V  A  A  A  A  D  G  V  T  F  S
541 GGTCAGGACC TGGATTGGGG AGGCGGTTGC CGCCGCTGCT GCTGACGGTG TGACGTTCTC

       V  P  V  T  P  H  T  F  R  H  S  Y  A  M  H  M  L  Y  A  G
601 TGTTCCGGTC ACACCACATA CGTTCCGCCA TTCCTATGCG ATGCACATGC TGTATGCCGG

       I  P  L  K  V  L  Q  S  L  M  G  H  K  S  I  S  S  T  E  V
661 TATACCGCTG AAAGTTCTGC AAAGCCTGAT GGGACATAAG TCCATCAGTT CAACGGAAGT

       Y  T  K  V  F  A  L  D  V  A  A  R  H  R  V  Q  F  A  M  P
721 CTACACGAAG GTTTTTGCGC TGGATGTGGC TGCCCGGCAC CGGGTGCAGT TTGCGATGCC

       E  S  D  A  V  A  M  L  K  Q  L  S  *                  XbaI
781 GGAGTCTGAT GCGGTTGCGA TGCTGAAACA ATTATCCTGA cccgggtcta gagc...3'
... CCTCAGACTA CGCCAACGCT ACGACTTTGT TAATAGGACT gggcccagat ctcg...5'
```

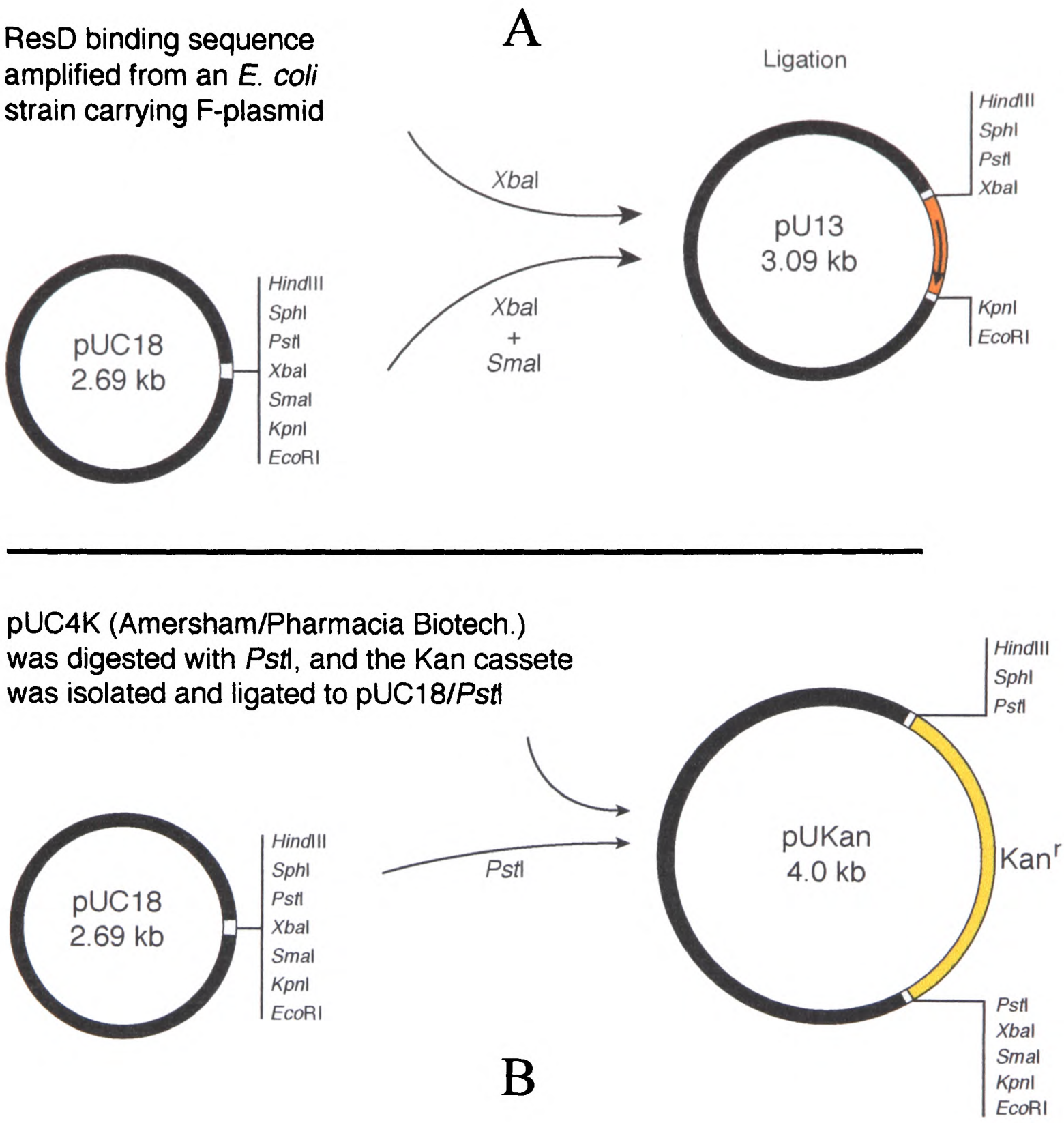
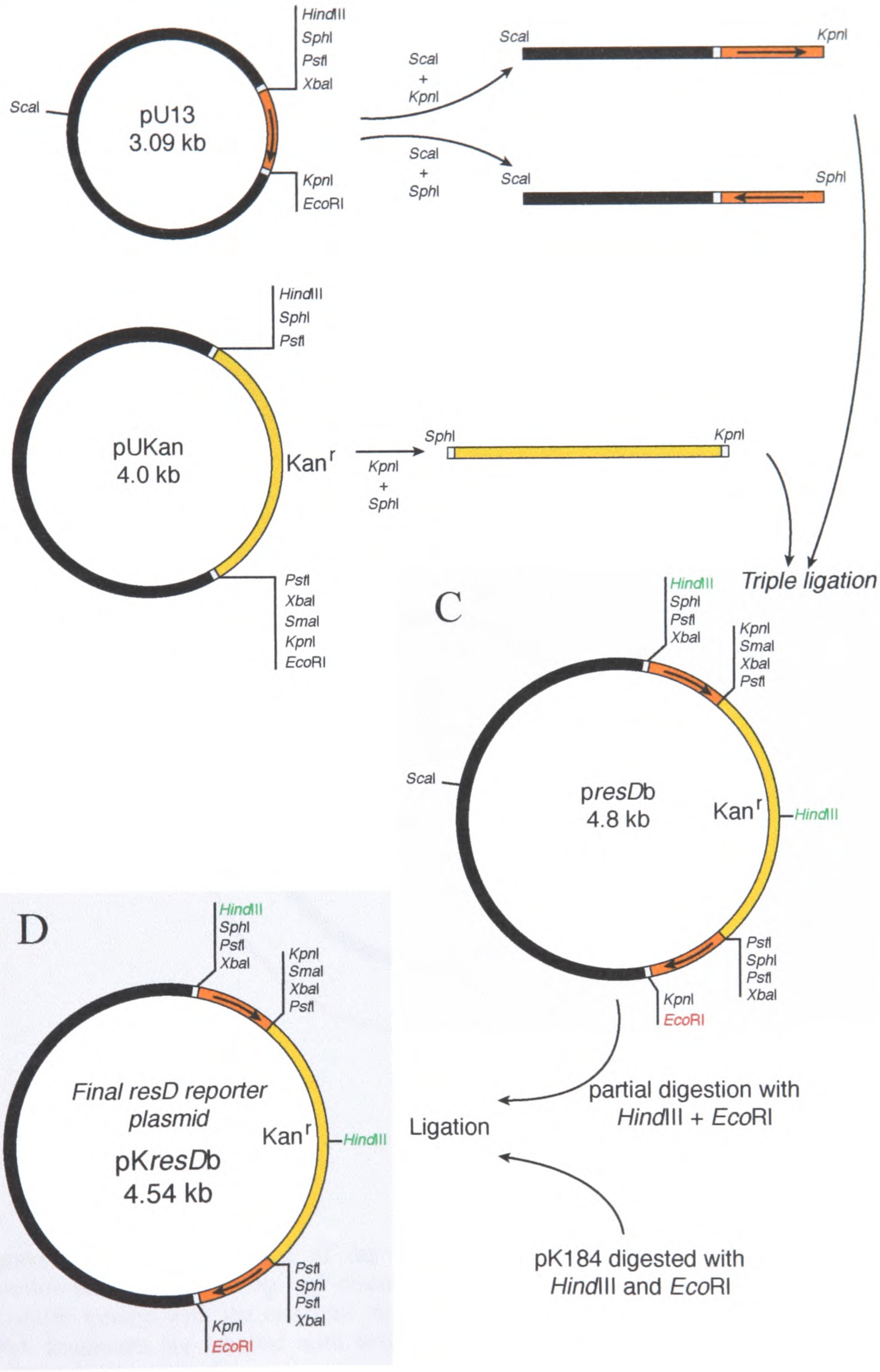


Figure 2.3. The strategy used to construct the ResD reporter plasmid pK*resD*b. **A)** The ResD binding sequence was isolated by PCR and cloned into pUC18 vector, giving rise to pU13. **B)** The gene conferring resistance to kanamycin from pUC4K was transferred to pUC18 producing pUKan. **C)** In a triple ligation, the DNA fragments *Sca*I-*Kpn*I, *Sca*I-*Sph*I (from pU13) and *Sph*I-*Kpn*I (from pUKan) were joined together to produce the pUC18-based reporter plasmid p*resD*b. **D)** The fragment *Hind*III-*Eco*RI from p*resD*b, containing the two directly repeated ResD binding sites and the Kan cassette, was transferred to the vector pK184, which carries the p15a origin of replication. The last step was carried out to ensure that pK*resD*b could be transformed into recipient cells harbouring recombinase-expressing vectors carrying ColEI-based replicons.


HindIII
SphI
PstI
XbaI
ScaI
pU13
3.09 kb
KpnI
EcoRI
ScaI
+
KpnI
ScaI
KpnI
ScaI
+
SphI
ScaI
SphI
HindIII
SphI
PstI
pUKan
4.0 kb
Kanr
PstI
XbaI
SmaI
KpnI
EcoRI
SphI
KpnI
KpnI
+
SphI
Triple ligation
C
HindIII
SphI
PstI
XbaI
KpnI
SmaI
XbaI
PstI
ScaI
presDb
4.8 kb
Kanr
HindIII
PstI
SphI
PstI
XbaI
KpnI
EcoRI
partial digestion with
HindIII + EcoRI
Ligation
pK184 digested with
HindIII and EcoRI
D
HindIII
SphI
PstI
XbaI
KpnI
SmaI
XbaI
PstI
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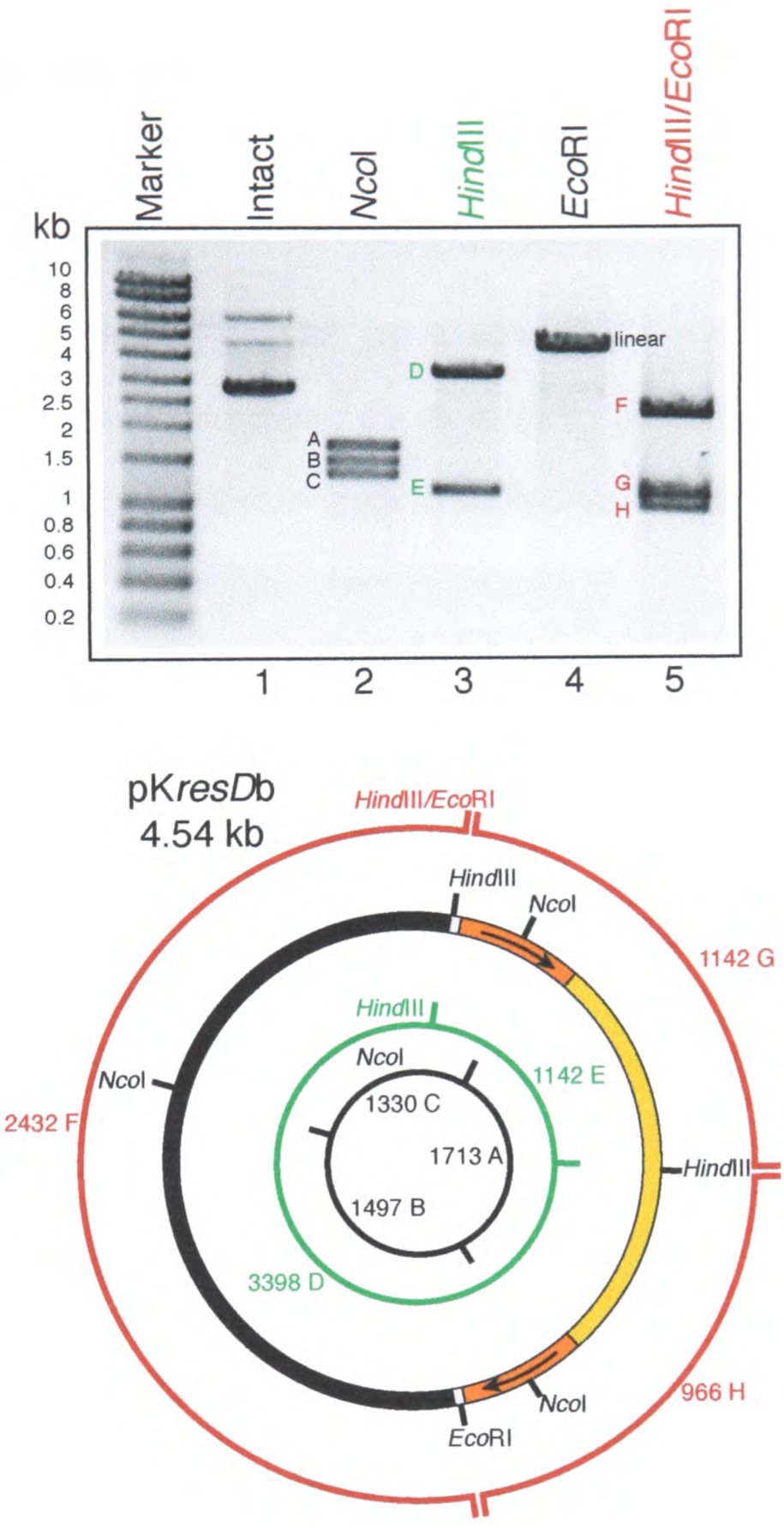


Figure 2.4. Restriction map of the reporter plasmid pK*resD*b. Top, ethidium bromide-stained 1% agarose gel electrophoresis of DNA restriction fragments of pK*resD*b treated with the enzymes *Nco*I, *Hind*III, *Eco*RI and *Hind*III/*Eco*RI. The DNA fragments are labelled with letters A to H corresponding to the fragments depicted in the map, bottom, and that would be expected from the digestions of pK*resD*b with *Nco*I, central black circle, *Hind*III, green circle, *Eco*RI, not shown (linearises plasmid), and *Hind*III/*Eco*RI, external red circle.

2.3.4. Radiolabelling of DNA

DNA was labelled at the 5'-end using T4 polynucleotide kinase (New England BioLabs - NEB) and [γ^{32}P] ATP (Amersham-Pharmacia). 10 pmol of DNA was incubated with 40μCi [γ^{32}P] ATP and 5 units of T4 polynucleotide kinase, as recommended by the manufacturer, for 1h at 37^{O}C. Labelled DNA was separated from unincorporated radiolabel by 1 min centrifugation at 700 xg using gel filtration columns MicroSpin^{TM} G-25 (Amersham-Pharmacia).

2.4. Electrophoresis of DNA (Sambrook et al., 1989)

Electrophoresis in 0.7, 1 or 1.4% agarose-1X-TAE gel was used for separating DNA samples after recombination reactions and general manipulations *in vitro*. DNA migration was at 2-5v/cm in 1X-TAE.

DNA samples from HJ resolution experiments and binding reactions were separated in 6% polyacrylamide-1X-TBE gels (30:1 acrylamide: bisacrylamide). Electrophoresis was at 250V for 3h (room temperature) in 1X-TBE or 1X-TBE/0.1% SDS for binding and HJ resolution experiments, respectively.

Denaturing gels for oligonucleotide purification were 10-20% polyacrylamide (30:1 acrylamide: bisacrylamide) containing 7M-urea. Electrophoresis was at 45W in 1X-TBE.

2.5. Chemicals

All chemicals were obtained from BDH, Boehringer Mannheim or Sigma. Radio-chemicals were obtained from Amersham-Pharmacia Biotech. Oligonucleotides were from Oswel (Oswel Research Products Ltd).

2.6. Preparation of Holliday junction (HJ) and linear *dif* DNA substrates

HJ substrates were prepared by annealing together four synthetic oligonucleotides (Arciszewska et al., 1995; Arciszewska et al., 1997; Arciszewska & Sherratt, 1995). Each oligonucleotide (I, II, II and IV; Table 2.3) was gel purified on a denaturing 10% polyacrylamide gel (section 2.4), visualised using Stains-all (Sigma), excised, eluted and ethanol precipitated before use (Sambrook et al., 1989). Before the annealing reactions, strands III were labelled as described in section 2.3.4. Labelled strands were mixed with a 3-fold excess of the other strands (I, II and IV), heated for 2h at 70^{O}C, and allowed to cool down slowly to room temperature. The final HJ substrates (four-way junctions) were purified from other undesired products (such as three-way junctions and free oligonucleotides) by electrophoresis in 6% polyacrylamide gels, followed by excision and elution in STE buffer (Sambrook et al., 1989). To ensure that similar amounts of different HJ substrates were used in recombination reactions, the amounts of radioactivity in the purified substrates were measured after recovery and the volume of the resuspending solution was adjusted accordingly.

The HJ substrates were named according to the central regions that they carry (Arciszewska et al., 2000; Arciszewska & Sherratt, 1995). *dif*TGTATA is a HJ containing the wild type *dif* chromosomal site; *dif*TGTCTA is a *dif*TGTATA carrying the nucleotide substitution indicated, and *dif*GGGATG is a variant carrying the central region *cer6* ("GGGATG"). BSX is a *dif*TGTATA in which two arms of the HJ substrate (IV and I) are linked by a 9-thymine tether and constrained in a conformation that is suitable for XerD-mediated strand exchange {(Arciszewska et al., 1997) and Table 2.3}.

The preparation of linear *dif* duplex DNA and nicked suicide substrates for binding and cleavage analyses was performed as reported previously (Blakely et al.,

1993; Blakely et al., 1997; Spiers & Sherratt, 1997; Spiers & Sherratt, 1999). The linear *dif* duplex was made by annealing two 60-mer oligonucleotides in the same conditions as used for the annealing of HJ substrates. The suicide substrates consisted of three annealed oligonucleotides (one of 60 and two of 30 bases), producing a nicked linear substrate identical in sequence to the linear *dif* duplex, but bearing a nick on either the top or bottom strands in the middle of the central region. The sequences of the oligonucleotides used are shown in Table 2.3.

Table 2.3. Oligonucleotides used for the assembly of HJ and linear *dif* substrates

Name	Sequence
*dif*TGTATA:	
I (84 nucleotides)	5'GATCCGTGATCACGCTGAACGCGTTTTAGCGGTGCGACTAA**TGTATA**T TATGTTAAATGGCCTAACGCCTAAAGCGGCCGCCTA
II (76)	5'ATCGTAGGCGGCCGCTTTAGGCGTTAGGCCATTTAACATAA**TATACA**T TATGCGCACCCGACCGTTGCCGGATCCG
III (76)	5'TCGATCGAGATCTCAGGATGTCATTTAACATAA**TATACA**TTATGCGCA CCGCTAAAACGCGTTCAGCGTGATCACG
IV (68)	5'CGATCGGATCCGGCAACGGTCGGGTGCGCATAA**TGTATA**TTATGTTAA ATGACATCCTGAGATCTCGA
IV* in BSX	5'CGATCGGATCCGGCAACGGTCGGGTGCGCATAA**TGTATA**TTATGTTAA ATGACATCCTGAGATCTCGATTTTTTTTTTGATCCGTGATCACGCTGAACG CGTTTTAGCGGTGCGACTAA**TGTATA**TTATGTTAAATGGCCTAACGCCT AAAGCGGCCGCCTA
Linear *dif* duplex:	
Top strand	5'GGGGATCCTCTAGATTGGTGCGCATAA**TGTATA**TTATGTTAAATCAGT CGACCTGCAGGC
Bottom strand	5'GCCTGCAGGTCGACTGATTTAACATAA**TATACA**TTATGCGCACCAATC TAGAGGATCCCC
	(The suicide linear *dif* duplexes have a nick either at the top or bottom strands in the middle of the central region - between the underlined nucleotides)

The central regions are shown in bold.

* In BSX, strand IV is linked to strand I, but separated by a 9-thymine tether (underlined). The BSX-HJ is assembled by the annealing of strands IV*, II and III.

2.7. Protein purification

Wild type XerCD recombinases were purified using established methods (Colloms et al., 1996; Subramanya et al., 1997). Cells were grown at 37^{O}C in LB medium supplemented with carbenicillin (200μg/ml). At O.D.$_{600nm}$ of 0.5-0.6, IPTG was added

(1mM) and cells were induced for 4h (pUC-based constructions) or 1h (pTrc99a-based constructions) at 30°C {the N-terminal domains of XerC and XerD (XerCN-ter and XerDN-ter, respectively) were induced for 4h at 30°C}. After induction, cells were harvested by centrifugation at 3000 xg. Cell pellets were resuspended in buffer A (300mM NaCl for XerD or 1M for XerC variants, 50mM Tris-HCl pH 8.0), lysozyme was added to 400μg/ml, and lysis was carried out with the help of a sonicator. After spinning down cell debris, the supernatant was incubated with the IMAC-Talon resin (cobalt-IMAC Talon, Clontech) in a batch system (~4ml resin for each litre of cell culture). The resin was washed as recommended by the manufacturer using buffer A, and proteins were eluted in the same buffer containing 100mM imidazole.

IMAC-Talon eluates containing XerC C-terminal variants were further purified using a Native DNA-cellulose resin (Pharmacia) (loaded and washed in 50mM Tris-HCl pH8.0 buffer containing 100mM NaCl, and eluted in 1M NaCl), and again through IMAC-Talon to remove possible DNA contaminants (Figure 2.5). Talon eluates containing XerD C-terminal variants were further purified using HiTrap heparin-resin affinity columns (Pharmacia) (loaded in 50mM Tris-HCl pH8.0 buffer containing 300mM NaCl; washed in 400mM NaCl, and eluted using a linear gradient from 400mM to 1M NaCl - Figure 2.5).

The Xer N-terminal variants were partially purified using just a single IMAC-Talon resin chromatography step (Figure 2.5). The only exceptions were the chimeras XerC[DABCD] and XerD[CABCD], which were further purified using native DNA-cellulose + a second IMAC-Talon (as described for the XerC C-terminal variants above). The truncated N-terminal domains of XerC and XerD (XerCN-ter and

XerDN-ter, respectively) were tested in crude extracts of the *E. coli* strain DS9040, since they do not bind to the IMAC-Talon resin.

ResD recombinase was purified as follows. One litre culture of DH5α harbouring pB*resD* was grown, under agitation, until $O.D._{600\ nm}$ 0.5. After the addition of arabinose to 0.2% (final concentration), cells were induced for three hours at 37°C. Following induction, cells were harvested by centrifugation, suspended in lysis buffer (50 mM Tris-HCl 8, 300 mM NaCl, 10% glycerol) and sheared by sonication. The cell lysate was clarified by centrifugation and applied to the first chromatography column using the IMAC-Talon resin (~4ml resin for each litre of cell culture). The eluate from Talon was subsequently loaded onto a second affinity chromatography column containing heparin resin. Elution from heparin was carried out by applying a linear gradient of NaCl ranging from 0.4 M to 1.5 M. ResD (29 kDa) co-eluted with a protein species of ~26 kDa, and in a NaCl concentration of around 1 M (Figure 2.5).

ArgR and PepA were purified from the *E. coli* strains DS957 (carrying the ArgR expression plasmid pX3) and DS956 (carrying the PepA expression plasmid pCS126) as described by (Burke et al., 1994; McCulloch et al., 1994).

Protein concentration was estimated by UV measurements at λ280 nm {molar extinction coefficients were determined as described by (Pace et al., 1995)}, and by SDS-PAGE. Glycerol was added to the purified proteins to 50%, which were stored at –70°C.

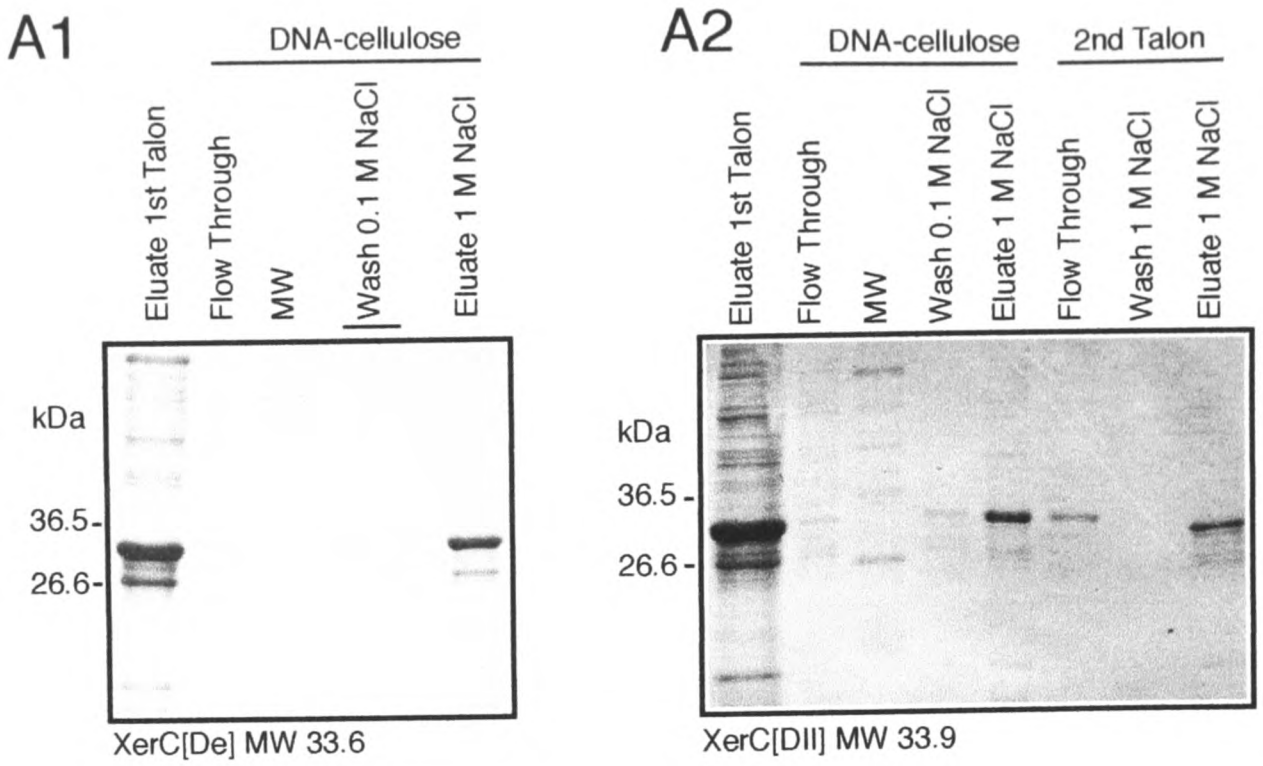
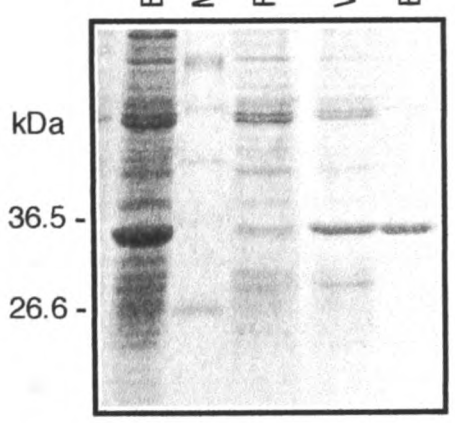


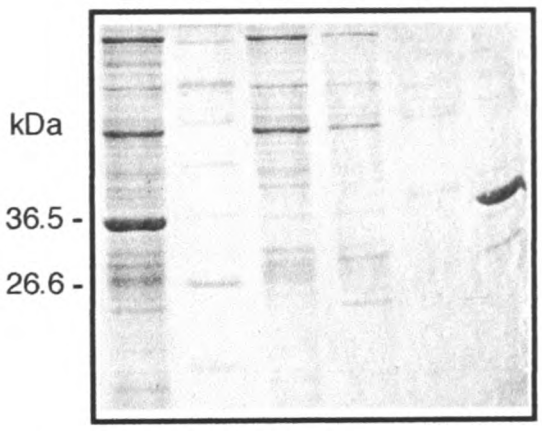
Figure 2.5. Coomassie blue-stained 12% SDS-PAGE of samples from recombinase purification. (A1-7) XerCD C-terminal variants; the purification steps are indicated at the top of the gels. Samples from the second Talon of XerC[De] are not shown; XerD[CKRGK] was partially purified in a single chromatography step using Talon. (B1-3) XerCD N-terminal variants; the molecular weights are indicated after the names of the recombinase variants. (C1-2) ResD purification. The bands corresponding to the recombinase are indicated with arrows.

A4



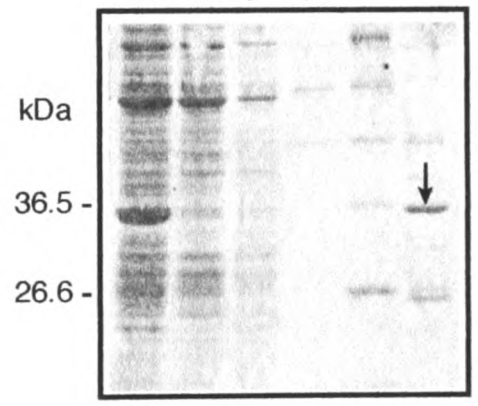
XerD[Ce] MW 34.5

A5



XerD[CII] MW 34.2

A6

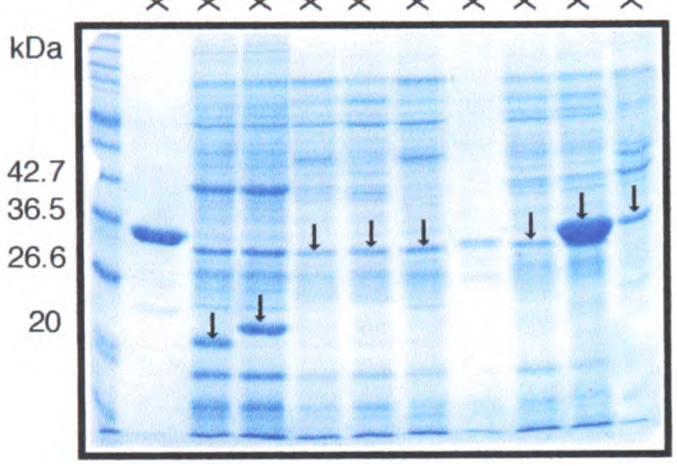


XerD[CIIe] MW 34.5

A7

XerD[CKRGK]
MW 34.7

B1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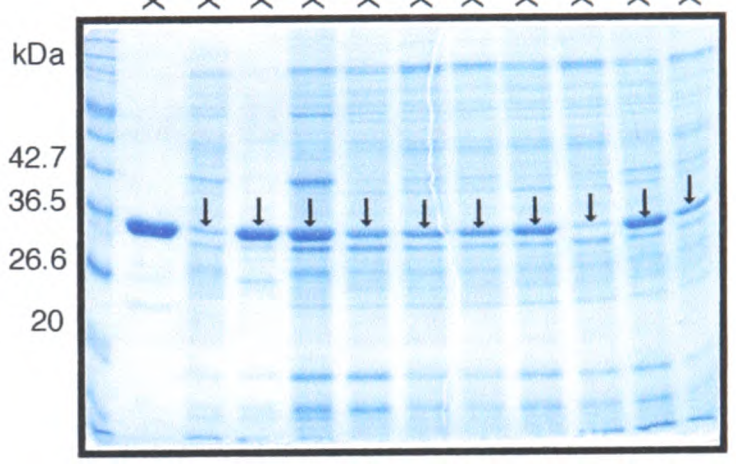
XerD[ΔABCD], 21.4
XerC[DA], 33.9
XerC[DB], 33.9
XerC[DAB], 33.8
XerC[DABCD], 33.9
XerC[DCD], 33.7
XerD[CA], 34.3
XerD[CB], 34.2
kDa
42.7
36.5
26.6
20

B2


XerD
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XerD[CABCD], 34.2
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XerDK19N, 34.2
XerDK19R, 34.2
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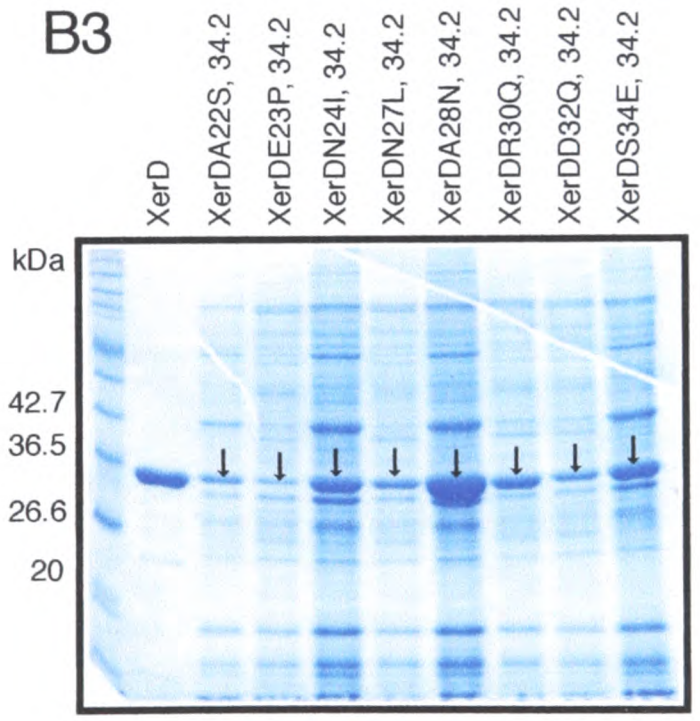
XerDN20Q, 34.2
XerDN20R, 34.2
kDa
42.7
36.5
26.6
20

B3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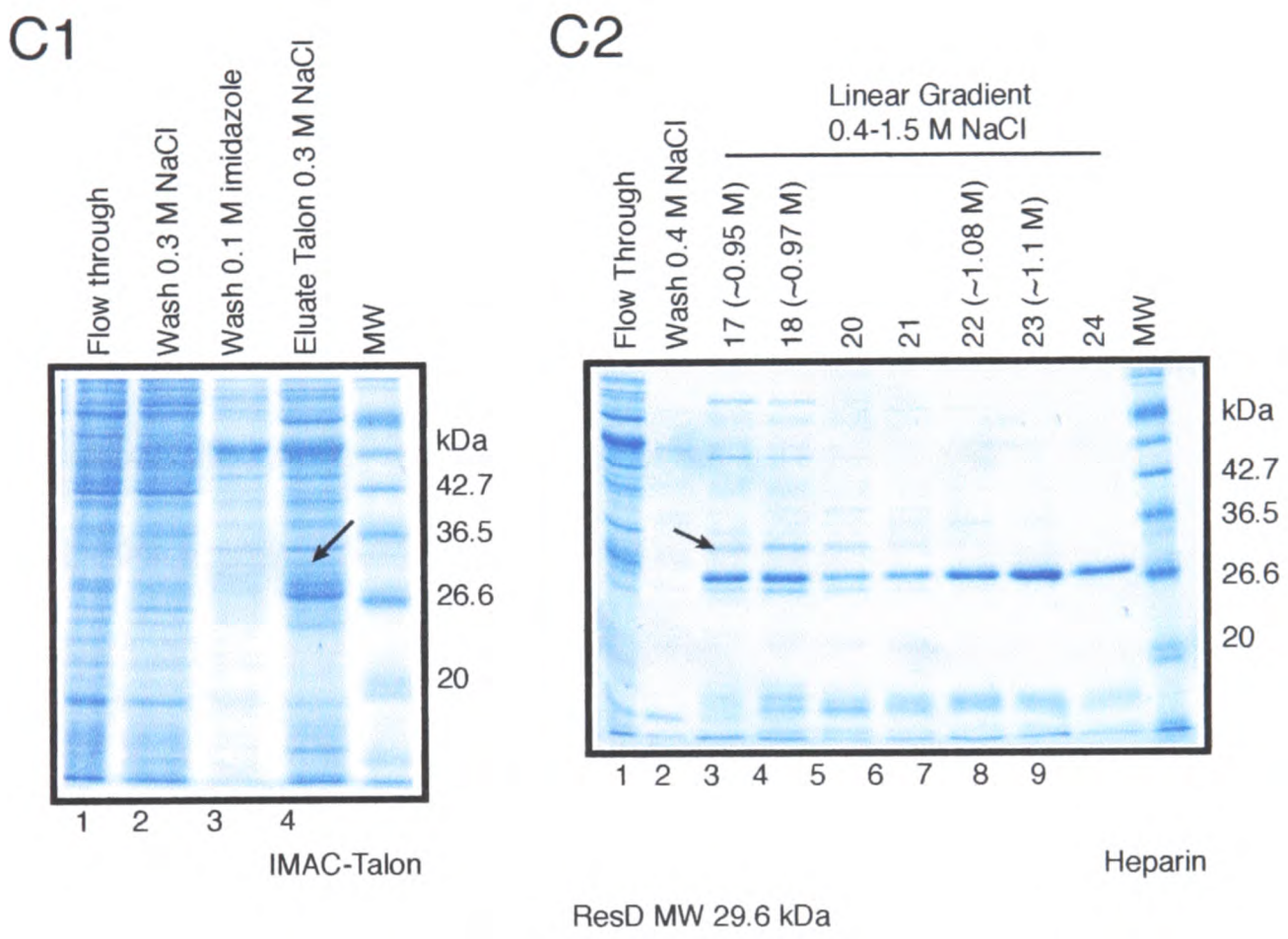
kDa
42.7
36.5
26.6
20



ResD MW 29.6 kDa

2.8. *In vitro* assays of cooperative binding and recombinase-mediated cleavage and strand exchange

Binding reactions were carried out in a volume of 10µl containing: 20mM Tris-HCl pH7.5, 50mM NaCl, 1mM EDTA, 5-10% glycerol, 0.5 mg/ml Poly(dI-dC)/Poly(dI-dC), 100µg/ml BSA, and ~0.1pmol of labelled substrate. Following 5min of incubation on ice, products were analysed by electrophoresis in 6% polyacrylamide-1X-TBE gels and bands were quantified using a PhosphorImager (Molecular Dynamics). Determination of equilibrium dissociation constants and cooperativity were carried out according to (Blakely et al., 1993).

HJ resolution experiments were carried out under standard conditions at 37^{O}C for 30 min {10µl reactions containing: 20mM Tris-HCl pH7.5, 50mM NaCl, 1mM EDTA, 10% glycerol, 0.5 mg/ml Poly(dI-dC)/Poly(dI-dC), 100µg/ml BSA, and ~2 ng of labelled substrate (Arciszewska & Sherratt, 1995)}. Reactions were stopped by the addition of 2µl of 50mM Tris-HCl pH7.5, 1% SDS. Products were then separated in 6% polyacrylamide-1X-TBE gels containing 0.1% SDS, and radioactive bands were quantified on a Molecular Dynamics PhosphorImager.

Recombination reactions of supercoiled reporter plasmids were conducted as described by (Colloms et al., 1996). After 1h incubation, DNA samples were purified by phenol extraction, treated with the appropriate restriction enzymes, separated by electrophoresis in 1% agarose-1X-TAE gels, stained with SYBR (Molecular Probes) and visualised using a Molecular Dynamics FluorImager. The *dif* recombination buffer was: 50mM Tris-HCl pH 8.0, 50mM NaCl, 1.25 mM EDTA, 5 mM spermidine, and 25 µg/ml BSA (reactions were supplemented with glycerol to 40% final); *cer*-buffer was: 50mM Tris-HCl pH 8.0, 50mM NaCl, 1.25 mM EDTA, 5 mM spermidine, 1 mM L-arginine, 10% glycerol and 25 µg/ml BSA; *psi*-buffer: similar to

cer-buffer, but L-arginine was omitted and the NaCl was substituted by 25 mM KCl; ResD recombination buffer: 50 mM Tris-HCl pH 8.0, 50 mM KCl, 1 mM EDTA, 5 mM spermidine, and 25 μg/ml BSA.

2.9. Bioinformatics

Analysis of polypeptide and DNA sequences, and the design of oligonucleotides for polymerase chain reactions (PCR) were carried out using Generunner (Hastings Software Inc.). Amino acid sequence alignments were done using ClustalX (Thompson et al., 1997). Protein secondary structures were predicted using PHDsec {http://www.embl-heidelberg.de/predictprotein/predictprotein.html (Rost & Sander, 1993)}. 3D structure analyses and figures were done using InsightII (Accelrys-Molecular Simulations Inc.).

CHAPTER 3

Switching catalytic activity in XerCD site-specific recombination

Part of this Chapter was published as: Henrique Ferreira, David Sherratt and Lidia Arciszewska, 2001. *Journal of Molecular Biology* **312**: 45-57

3.1. Introduction

The crystal structures of XerD and the related recombinase Cre, complexed with *loxP* sites, revealed that they are C-shaped proteins, which clamp the DNA by the positioning of their N-terminal and C-terminal domains on opposite faces of the DNA {(Guo et al., 1997; Subramanya et al., 1997) and Figure 3.1-A}. The N-terminal and C-terminal domains are connected by a linker, which in the XerD structure is disordered. Despite the lack of homology between these two recombinases at the primary amino acid sequence level, which is also observed among other members of the tyrosine recombinase family (Esposito & Scocca, 1997; Nunes-Duby et al., 1998), XerD and Cre exhibit striking similarities in their N-terminal domain tertiary structures (Figure 3.1-B). These similarities allowed the modelling of XerD onto *loxP*, as illustrated in Figure 3.1-A.

An important feature revealed with the crystal structures of Cre, XerD, and some other members of the tyrosine recombinase family (HP1, λ-int and Flp) is that they all exhibit a similar arrangement of the five conserved catalytic residues (R K H R H/W pentad), which are involved in phosphate activation and stabilisation of transition intermediates during the recombination reaction catalysed by them {(Chen et al., 2000; Guo et al., 1997; Hickman et al., 1997; Kwon et al., 1997; Sherratt & Wigley, 1998; Subramanya et al., 1997) and Figure 3.1-B}. The general folding of their C-terminal or catalytic domains is also similar and the active sites formed by these residues resemble the active sites of Type IB topoisomerases, to which tyrosine recombinases are mechanistically related (Cheng et al., 1998; Redinbo et al., 1998; Sherratt & Wigley, 1998; Shuman, 1998).

A

non-cleaving Cre
bound to *loxP*

inactive form of XerD
modelled onto *loxP*

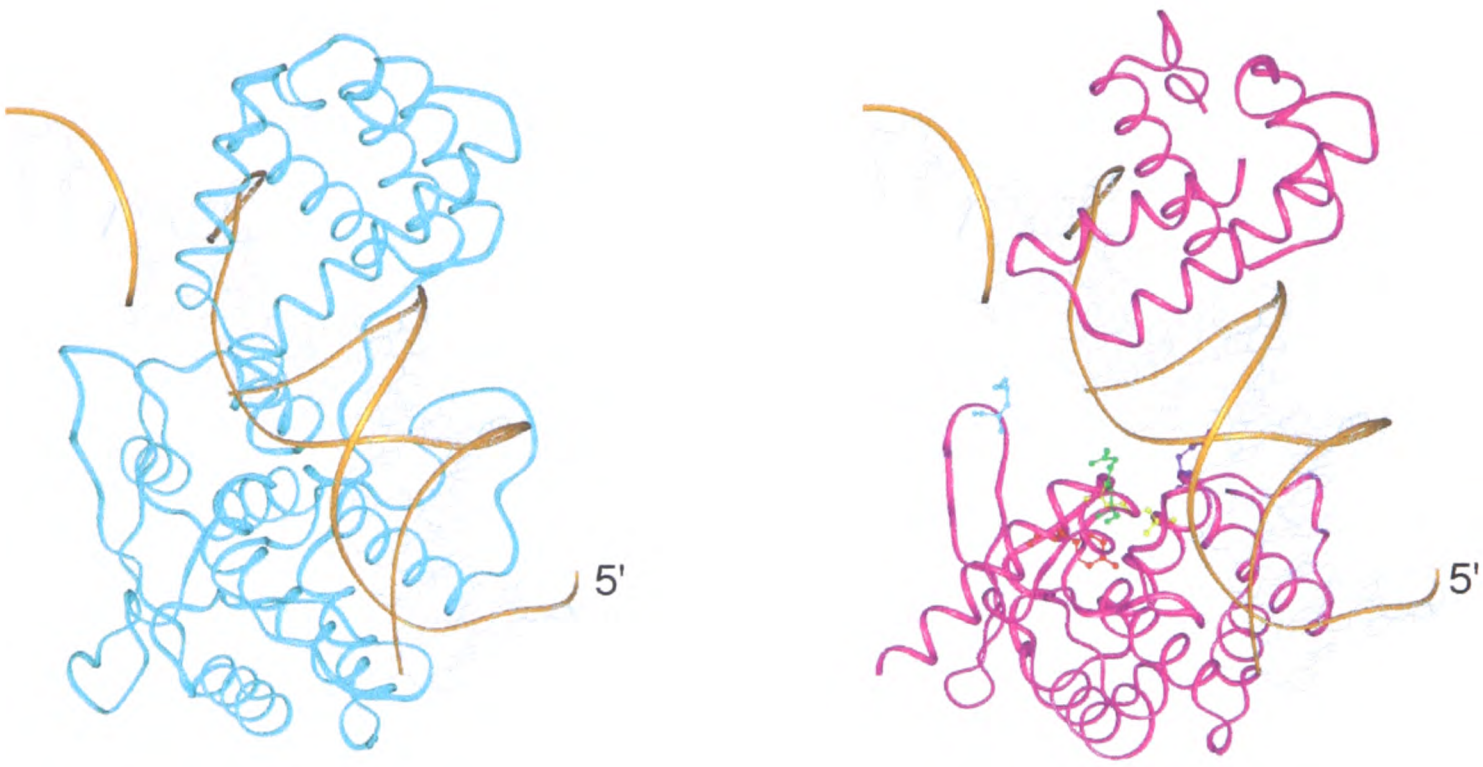
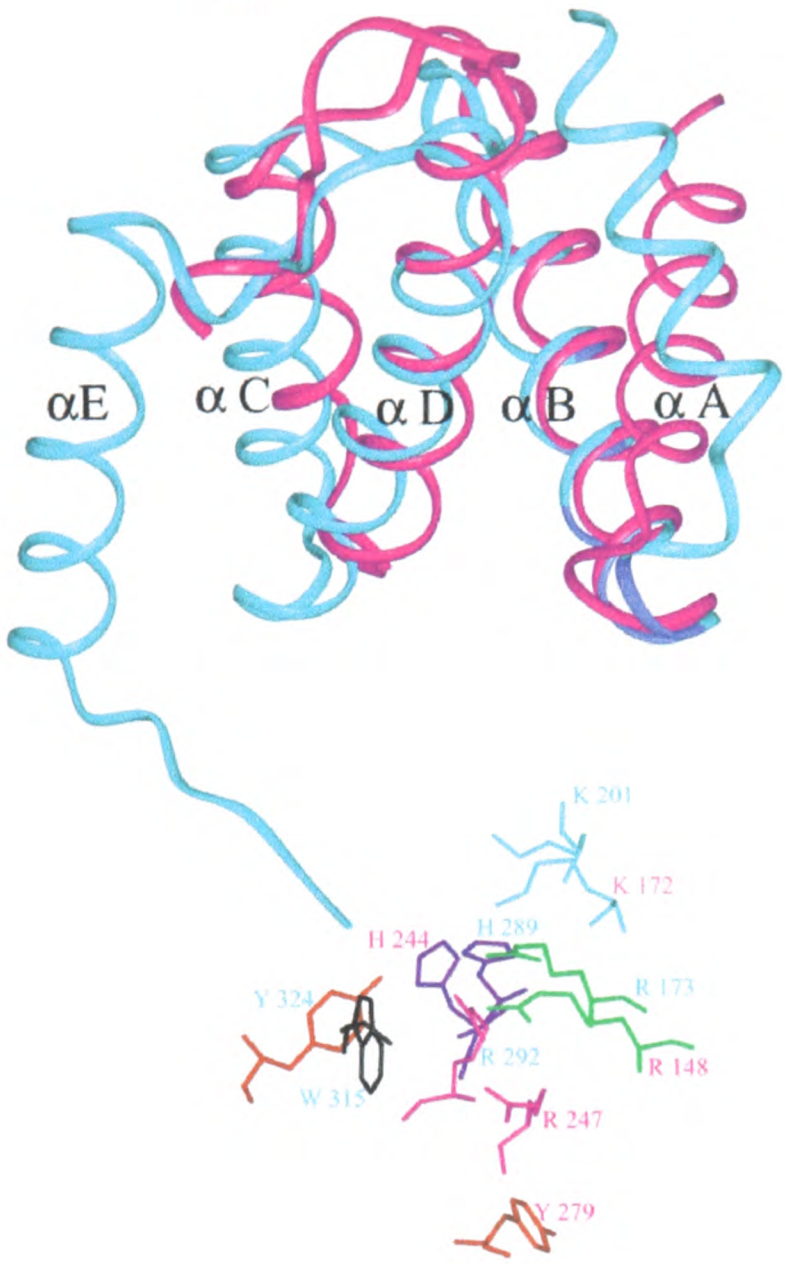


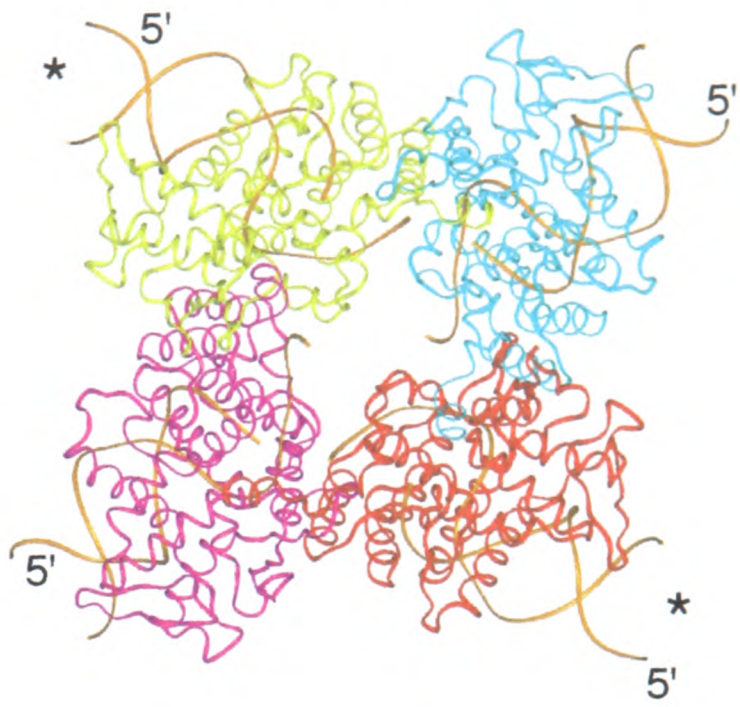
Figure 3.1. The crystal structures of Cre-*loxP* and XerD. **A) Left**, a non-cleaving subunit of Cre bound to a *loxP* half site; **right**, XerD modelled onto *loxP* half site. **B)** Alignment of the N-terminal domains of XerD and Cre recombinases, pink and cyan, respectively; the smaller view illustrates the placement of the upside down "V" formed by α-helices B and D of Cre and XerD into the major groove of the *loxP* DNA binding-site; the catalytic residues are colour-coded to demonstrate their correspondence (pink and cyan labels refer to XerD and Cre, respectively); XerD H270 was missing in the crystal structure. **C)** Three views of the tetrameric complex formed by Cre-*loxP*; cleaving subunits are marked with asterisks.

B

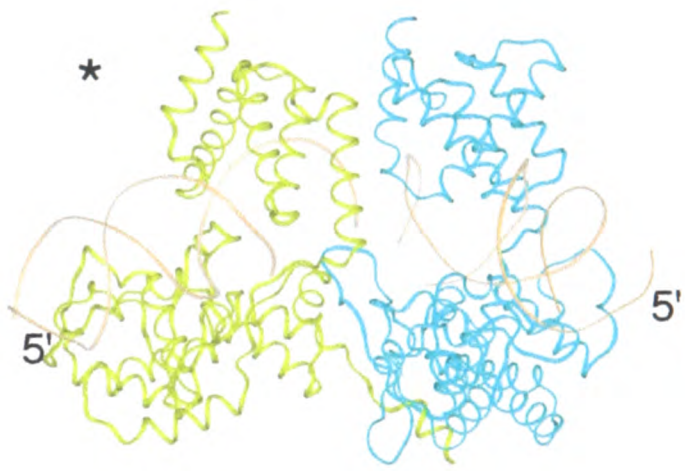


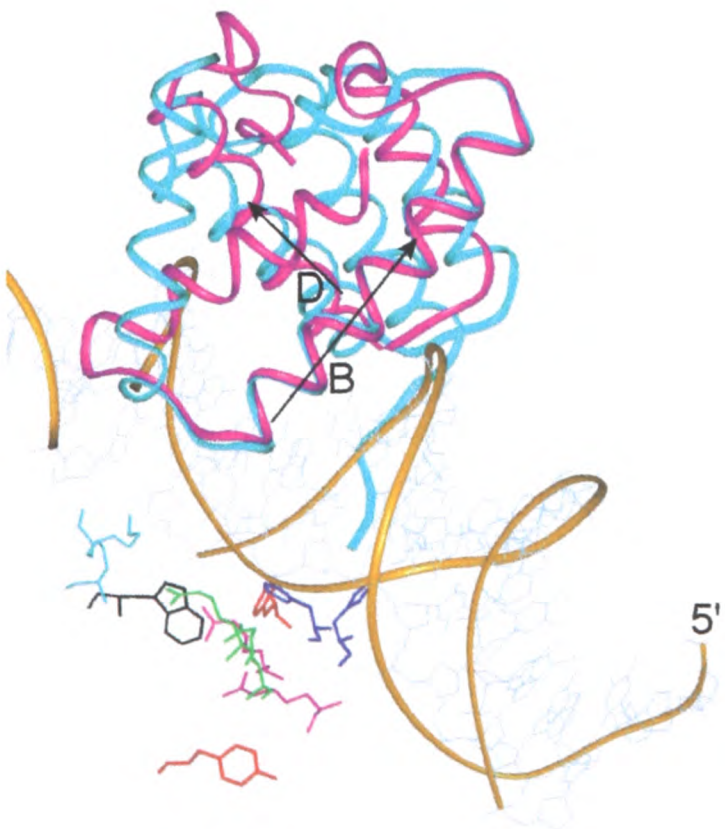
C

Cre-*loxP* C-terminal view

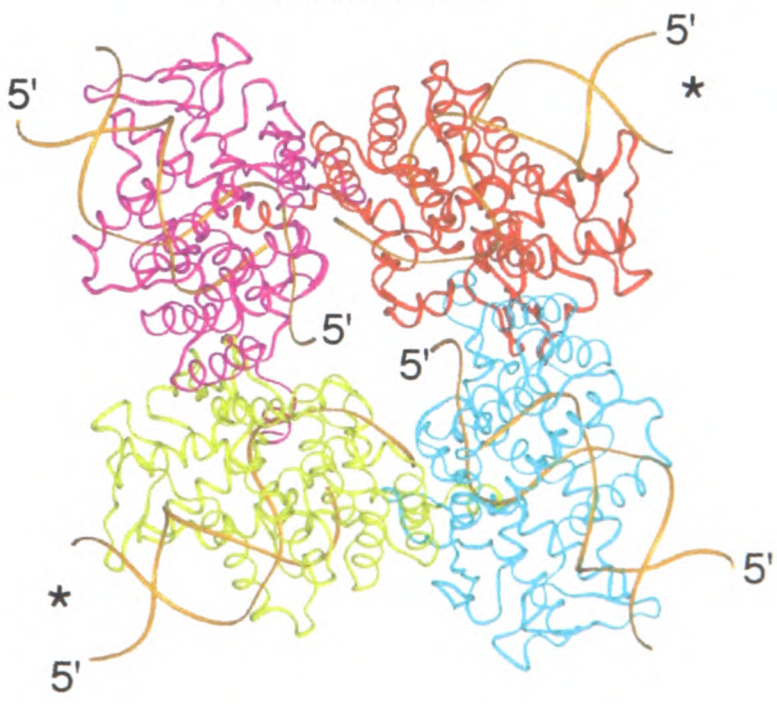


side view





N-terminal view



Besides its catalytic activity, the C-terminal domain also provides sequence-specific DNA recognition to the outer portions of the recombinase-binding sites and contributes to interactions involved in cooperative binding to DNA (Guo et al., 1997; Hallet et al., 1999; Spiers & Sherratt, 1997; Spiers & Sherratt, 1999; Subramanya et al., 1997). Cyclic interactions between the four recombinase monomers, which involve their extreme C-terminal domains, are crucial to the coordination of catalysis {(Chen et al., 2000; Guo et al., 1997; Hallet et al., 1999) and Figure 3.1-C}.

The N-terminal domains of the tyrosine recombinases are the most divergent regions in primary amino acid sequence (Esposito & Scocca, 1997; Nunes-Duby et al., 1998), and they have been implicated mainly in stabilisation of binding to DNA (Hoess et al., 1990; Pan & Sadowski, 1993; Panigrahi & Sadowski, 1994; Tirumalai et al., 1997). However, the availability of crystal structures of Cre and Flp recombinases bound to their DNA recognition sites have brought to light a series of protein-protein interactions, which suggest that the N-terminal domains could participate effectively in cooperative binding to DNA, as well as in the formation of the synaptic complex between the two core sites required for the recombination reaction (Chen et al., 2000; Gopaul et al., 1998; Guo et al., 1997). With respect to protein-DNA interactions, the N-terminal domains engaged in a heterotetrameric recombination complex contact the inner palindromic portions of the recombinase binding-sites (Figure 3.1-A).

In this Chapter, the roles of the N-terminal domains of XerC and XerD during the Xer recombination reaction are investigated. It was found that XerCD variants exchanged or deleted for their N-terminal domains are active in recombination, and can switch the direction of HJ resolution in experiments *in vitro*. This property was exploited in order to demonstrate a complete *in vitro* XerCD recombination reaction

on a plasmid containing two directly repeated *dif* sites, thereby providing further support for a reaction scheme in which a change in conformation of the HJ intermediate formed by XerC generates a substrate for XerD, which can then complete the recombination reaction. A model is proposed in which the N-terminal domains of XerCD may change locally the conformation of the DNA upon binding, by introducing asymmetry to the core recombination site. This asymmetry would be responsible for the activation of catalysis of just one pair of enzymes at any given time in a synaptic or HJ complex. Asymmetry has also been demonstrated for the Cre-*loxP* system (Guo et al., 1999), where a kink produced at one end of the central region that separates the two binding-sites for the enzymes dictates which recombinase will undergo cleavage first; the recombinases distal to the kink are active for catalysis.

3.2. Results

3.2.1. XerC and XerD recombinases lacking their N-terminal domains are active in recombination on Holliday junction substrates

XerD, like the other characterised tyrosine recombinases, is a C-shaped protein that is able to clamp the DNA substrate between its N- and C-terminal domains. The predicted secondary structure of XerC suggests that it too will interact with DNA in the same way (Figure 3.2). In order to define the roles of the N-terminal domains of XerC and XerD in site-specific recombination, recombinase variants lacking their entire N-terminal domains were constructed (Figure 3.2), and their activities were tested *in vitro* on a synthetic HJ substrate containing the recombinase-binding sites of *dif* separated by the central region sequence TGTCTA (5'-GGTGCGCATAA TGTCTA TTATGTTAAAT-3' or *dif*TGTCTA).

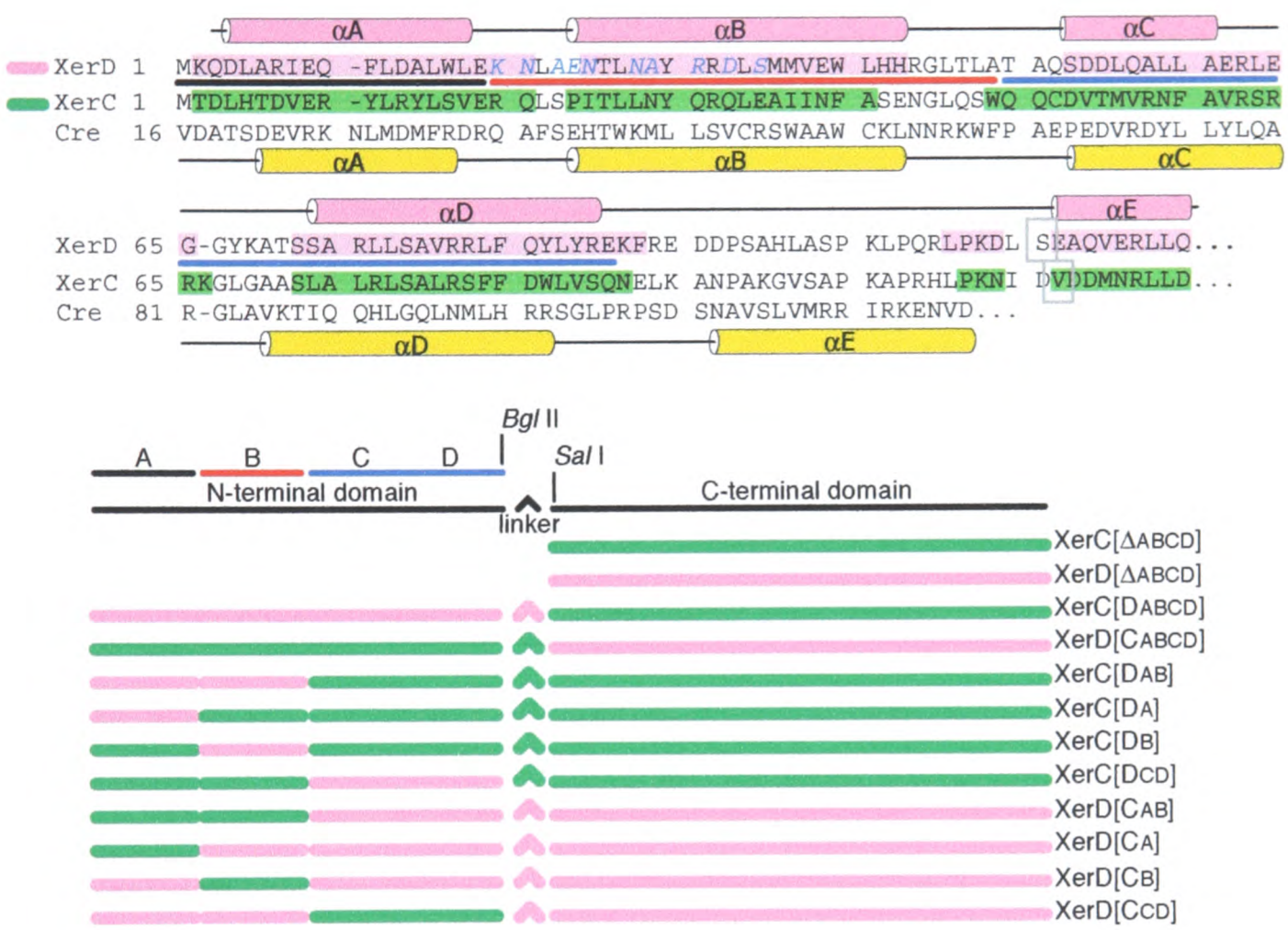


Figure 3.2. Top: amino acid sequence alignment of the N-terminal domains of XerC, XerD and Cre. Pink and yellow cylinders correspond to α-helices of XerD and Cre, respectively. The amino acid residues of XerD and XerC highlighted in pink and green, respectively, show α-helices defined by secondary structure prediction (PHDsec, Rost and Sander, 1993). The regions exchanged between XerC and XerD in the N-terminal chimeras are indicated by black, red, and blue lines which correspond to regions A, B, and CD, respectively. Amino acid residues in blue were substituted in the point mutants. Boxed residues indicate the positions where stop codons "TGA" were inserted into *xerC* or *xerD* genes for the expression of their truncated N-terminal domains. **Bottom:** schematic representation of the XerC-XerD chimeras. The segments derived from XerC and XerD are indicated in green and pink, respectively.

This substrate was chosen because it undergoes a high level of XerD-mediated strand exchange when compared to HJ substrates with different central regions. For example, the ratio of strand exchange by XerC to that by XerD on this substrate is ~10, whereas it is greater than 50 for wild type *dif* (Arciszewska et al., 2000).

Combination of wild type XerC with a XerD variant lacking its N-terminal domain, XerD[ΔABCD], led to a huge stimulation of XerD-mediated strand exchange and a complete impairment of strand exchange by XerC in 30 min reactions (Figure 3.3, lane 4; the ratio of strand exchange is switched to ≤ 0.02 with the variant-wild type recombinase combination, compared to that of ~10 obtained with the wild type recombinases). Similarly, combination of XerD with a XerC variant lacking its N-terminal domain, XerC[ΔABCD], led to increased levels of XerC recombination and impairment of strand exchange by XerD (Figure 3.3, lane 3). Similar results were also evident at the earliest times of the reactions (1-5 min; not shown).

These results demonstrate that a Xer heterotetramer lacking two of the four N-terminal domains can still yield high levels of strand exchange on a HJ substrate. However, the outcome of recombination is profoundly altered, with strand exchange by the recombinase lacking the N-terminal domain being stimulated while that of the wild type partner recombinase is impaired. Similar phenotypes have been observed with Xer recombinase variants carrying changes within regions of the C-terminal domain implicated in recombinase-recombinase interactions (Arciszewska et al., 2000; Hallet et al., 1999).

The combination of XerC[ΔABCD] and XerD[ΔABCD] did not produce detectable strand exchange products (Figure 3.3, lane 5), probably because of an inability of the two truncated recombinases to form a productive heterotetramer-DNA complex (see below).

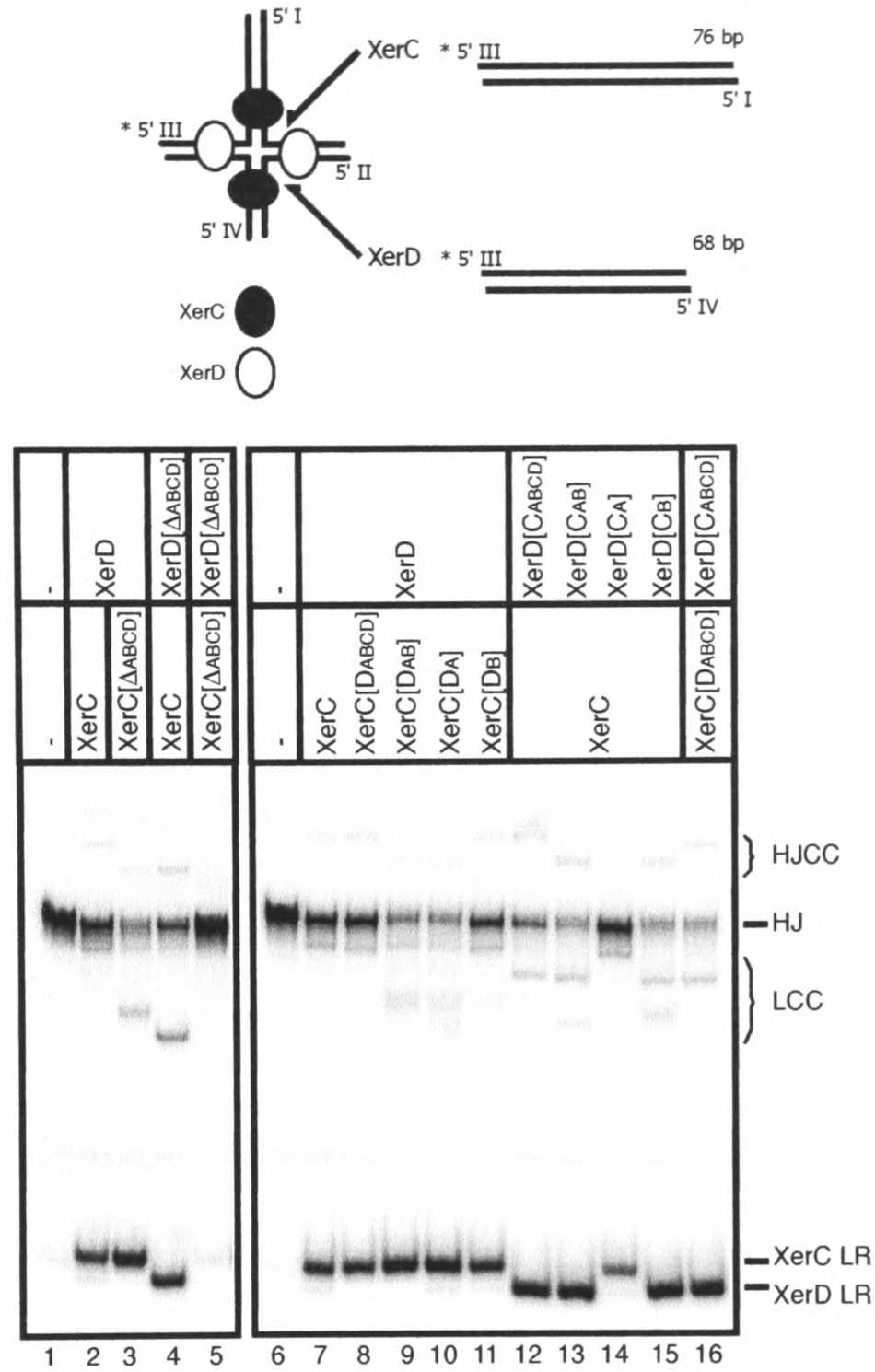


Figure 3.3. XerC and XerD recombinases deleted or exchanged for their N-terminal domains alter the resolution of a *dif*TGTCTA HJ substrate. The HJ substrate, radiolabelled at the 5'-end of strand III, was reacted with the indicated recombinases for 30 minutes at 37^{O}C. Linear rejoined duplex products are indicated XerC LR and XerD LR. HJCC and LCC bands indicate recombinase-HJ DNA and recombinase-linear duplex DNA covalent complexes, respectively. DNA bands running ahead of HJCC and LCC in lanes 9, 10, 13 and 15 are most likely covalent complexes lacking DNA strands 3' of the cleavage point. The loss of DNA strands presumably resulted from denaturation caused by the presence of urea (final concentration 0.08 M) introduced with the recombinase variants XerC[DAB], XerC[DA], XerD[CAB] and XerD[CB]. Electrophoresis was on a 6% polyacrylamide gel containing 0.1% SDS.

3.2.2. The N-terminal domain determinants that control Xer recombinase catalytic activity are located within the region containing α-helix B

In order to determine which regions of the XerCD N-terminal domains are responsible for the reciprocal switch in HJ resolution outcome, chimeras of both recombinases, in which segments within their N-terminal domains were exchanged, were constructed. The basis of this approach was to utilise the primary sequence differences between the N-terminal domains of XerC and XerD, along with the structural similarities of XerD and Cre and the predicted secondary structure for XerC (Figure 3.2). Exchanges of XerD helical regions with predicted helical regions of XerC were therefore expected to cause a minimal perturbation of the overall tertiary structure within a given recombinase chimera. A chimera is defined as XerC or XerD by virtue of its DNA binding specificity, which resides in its C-terminal domain together with the determinants for DNA cleavage and strand exchange (Subramanya et al., 1997). The exchanged segments encompassed the whole N-terminal domain, or the regions containing α-helices AB, α CD, α A, or α B. The origin of the linker region, which connects the N- and C-terminal domains and is disordered in XerD crystal structure, appears not to influence the properties of the chimeras in the assays reported here.

Analysis of the strand exchange activity of the chimeras in which the whole N-terminal domain has been exchanged revealed phenotypes similar to those observed with recombinase variants lacking their complete N-terminal domains. A switch in the direction of HJ resolution, to preferred strand exchange by XerD, was observed in the presence of XerD[CABCD] and XerC as compared to wild type XerCD (Figure 3.3, compare lane 12 with lanes 2 and 4). Impairment of XerD strand exchange was observed in the presence of XerC[DABCD] and XerD, although a reciprocal stimulation of strand exchange by the variant XerC was not evident with this substrate

(Figure 3.3, compare lane 8 with lanes 2 and 3). Nevertheless, the reciprocal nature of the stimulation-impairment phenotype of XerC[DABCD] is revealed with a HJ substrate whose conformation is constrained to give resolution predominantly by XerD with wild type recombinases (see below). From these experiments it was concluded that a XerCD heterotetramer containing four XerC or four XerD N-terminal domains can support high levels of strand exchange on a HJ substrate. However, the reciprocal switch in resolution outcome demonstrates that the N-terminal domain of XerC cannot substitute for the N-terminal domain of XerD and *vice versa*, thus these domains are not functionally identical, despite interacting with the same DNA sequences in the inner portion of the recombinase-binding sites as proposed above by the modelling of XerD on a *loxP* site.

Combination of XerC[DABCD] and XerD[CABCD] leads to HJ resolution exclusively by XerD-mediated strand exchange (Figure 3.3, lane 16), showing that the effect of XerD[CABCD] is dominant. Note that in this combination, two XerC and two XerD N-terminal domains are present in their correct positions with respect to each other, but they are inappropriately connected to their cognate C-terminal domains and therefore to their recombinase binding sites.

XerD chimeras carrying the regions of XerC that contain predicted α-helices AB, XerD[CAB], or α B, XerD[CB], had similar recombination properties to those observed with XerD[CABCD] and XerD[ΔABCD] (Figure 3.3, lanes 13 and 15). In contrast, the XerD variant carrying the region of XerC encompassing predicted α-helices CD {XerD[CCD]}, or α A {XerD[CA]}, had similar activities to wild type XerD in the presence of XerC (data not shown, Figure 3.3 lane 14, respectively). These results suggest that the loss or replacement of the region encompassing α-helix B in XerD leads to a loss of the normal preference for strand exchange by XerC on a

HJ substrate. It can be concluded that this XerD region is involved in interactions required to maintain the HJ DNA-heterotetramer conformation suitable for catalysis by XerC.

The complementary experiments with XerC[DAB] and XerD gave an equivalent result, with the phenotype being similar to that of XerC[ΔABCD] and XerD (Figure 3.3, lane 9). However, XerC[DA] and XerC[DB] showed intermediate phenotypes (lanes 10 and 11). An increase in strand exchange by XerC, but no impairment of XerD recombination was observed with XerC[DA], while some impairment of XerD recombination was observed with XerC[DB].

In an attempt to determine more precisely what interactions within the B region of XerD are important, a series of XerD amino acid substitutions that lie in this region were constructed. The following substitutions were tested: XerDK19A, K19N, K19R, N20F, N20Q, N20R, K19A-N20A, A22S, E23P, N24I, N27L, A28N, R30Q, D32Q, S34E, A22S-N24I-A28N-S34E (Figure 3.2). Extensive analysis of the properties of these XerD variants in recombination on either HJ *dif*TGTCTA or *dif*TGTATA or in cleavage of linear suicide substrates, and analysis of their ability to bind linear duplex DNA in the presence or absence of the partner revealed no detectable differences between these recombinase variants and the wild type XerD (data not shown), indicating that the phenotype arising from the exchange of region B is a consequence of changes in overall conformation of that region.

3.2.3. XerCD recombinase variants lacking or exchanged for their N-terminal domains exhibit altered binding to linear *dif* DNA and an altered pattern of recombinase-mediated cleavage

N-terminal deletions and chimeras of XerC or XerD were able to bind to a linear DNA fragment carrying a *dif* core recombination site in the presence of their wild type partner recombinases (Figure 3.4; band T; compare lane 4 to lanes 6, 8, 10, 12, 14 and 16). They bound poorly or in an undetectable manner to the same fragment when tested alone at concentrations equal or greater than those required to give substantial wild type XerC-DNA or XerD-DNA binary complexes (Figure 3.4; band B; compare lanes 5, 7, and 9 to lane 2 and lanes 11, 13 and 15 to lane 3). Furthermore, the variant recombinase concentrations were sufficient to convert a proportion of the binary DNA complex with wild type recombinase partner to a ternary complex containing one molecule each of XerC and XerD bound to DNA, thereby indicating that they retain some cooperativity in binding with their wild type partner. Indeed, the cooperativity of binding of XerC[DABCD], XerC[DB] and XerC[ΔABCD] with XerD appears to be relatively normal (Figure 3.4; compare lanes 6, 8, 10 with lane 4). In contrast, the binding of XerD[ΔABCD], XerD[CABCD], and XerD[CB] to DNA in the presence of XerC was poor (compare lanes 12, 14 and 16 with 4). Combination of a deleted or chimeric recombinase with a partner containing an equivalent alteration led to no detectable ternary complex formation (data not shown).

All of the chimeric ternary complexes showed a reduced electrophoretic mobility as compared to the wild type XerCD ternary complexes. Those containing XerD chimeras had the slowest mobility. This is indicative of an altered DNA conformation in the complexes. The deleted variants show equivalent mobility differences (compare lanes 10 and 16).

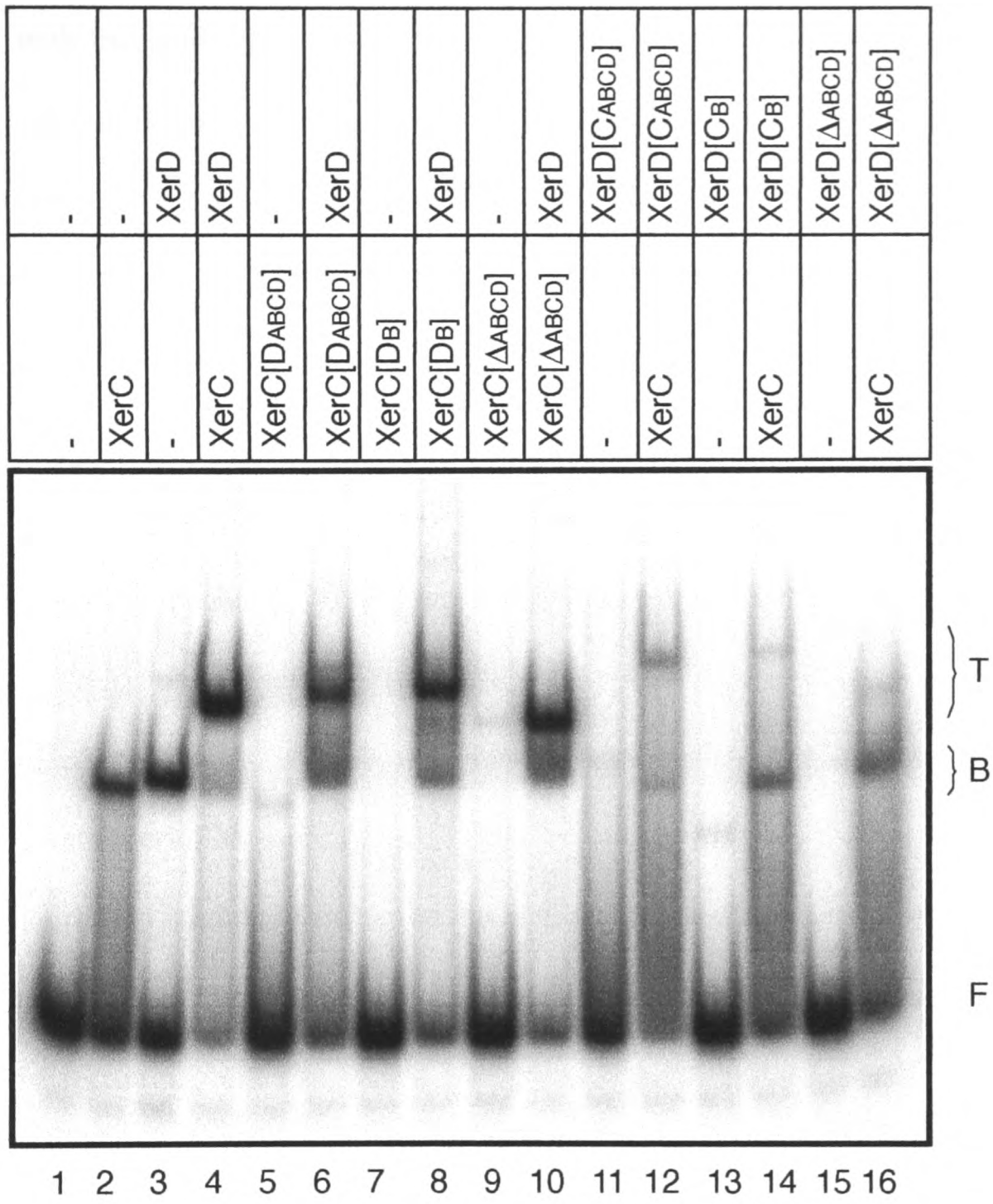


Figure 3.4. XerC and XerD recombinase N-terminal chimeras and deletion variants exhibit altered binding to linear *dif* duplex DNA. The indicated recombinase combinations were incubated on ice with linear *dif*TGTATA DNA. The concentration of recombinases was estimated to be at saturating levels as judged by activity of recombinase samples in HJ resolution assays and by the intensity of recombinase bands in Coomassie blue-stained SDS-PAGE. Recombinase-DNA binary (B) and ternary (T) complexes and free DNA (F) were separated by 6% PAGE.

Taken together, these results show that although the N-terminal chimeras and deletion variants have reduced binding affinity alone they retain some cooperativity in binding with the wild type partner. Previous studies have shown that the XerCD C-terminal domains provide the DNA sequence-specific contacts and contribute substantially to the cooperativity of recombinase binding (Spiers & Sherratt, 1997; Spiers & Sherratt, 1999; Subramanya et al., 1997). The results presented here demonstrate that the N-terminal domains also play an important role in binding and stability of the recombinase DNA complexes, presumably by contributing intermolecular protein-protein contacts and protein-DNA interactions that help anchor the C-clamp shape protein onto the DNA. This would be consistent with the Cre-DNA structures, which show that the N-terminal domains of Cre contact *loxP* DNA and make extensive contacts with the N-terminal domains of adjacent Cre monomers within the tetrameric-DNA complex.

In order to evaluate further the interactions of the chimeric recombinases with DNA, their cleavage activities were tested on linear suicide DNA substrates carrying a *dif* core recombination site. These substrates contain nicks in the middle of the recombination site central region either in the top or bottom strand, and thereby trap the products of recombinase-mediated cleavage by virtue of the diffusion away of the three nucleotides released upon cleavage (Blakely & Sherratt, 1996; Nunes-Duby et al., 1987).

In the presence of the wild type recombinases, a bias of XerC over XerD cleavage is observed with TSN (top strand nicked) and BSN (bottom strand nicked) substrates. The ratio of cleavage by XerC to that by XerD ranges between ~5 and ~3 fold, respectively for TSN and BSN (Figure 3.5, lanes 2 and 6).

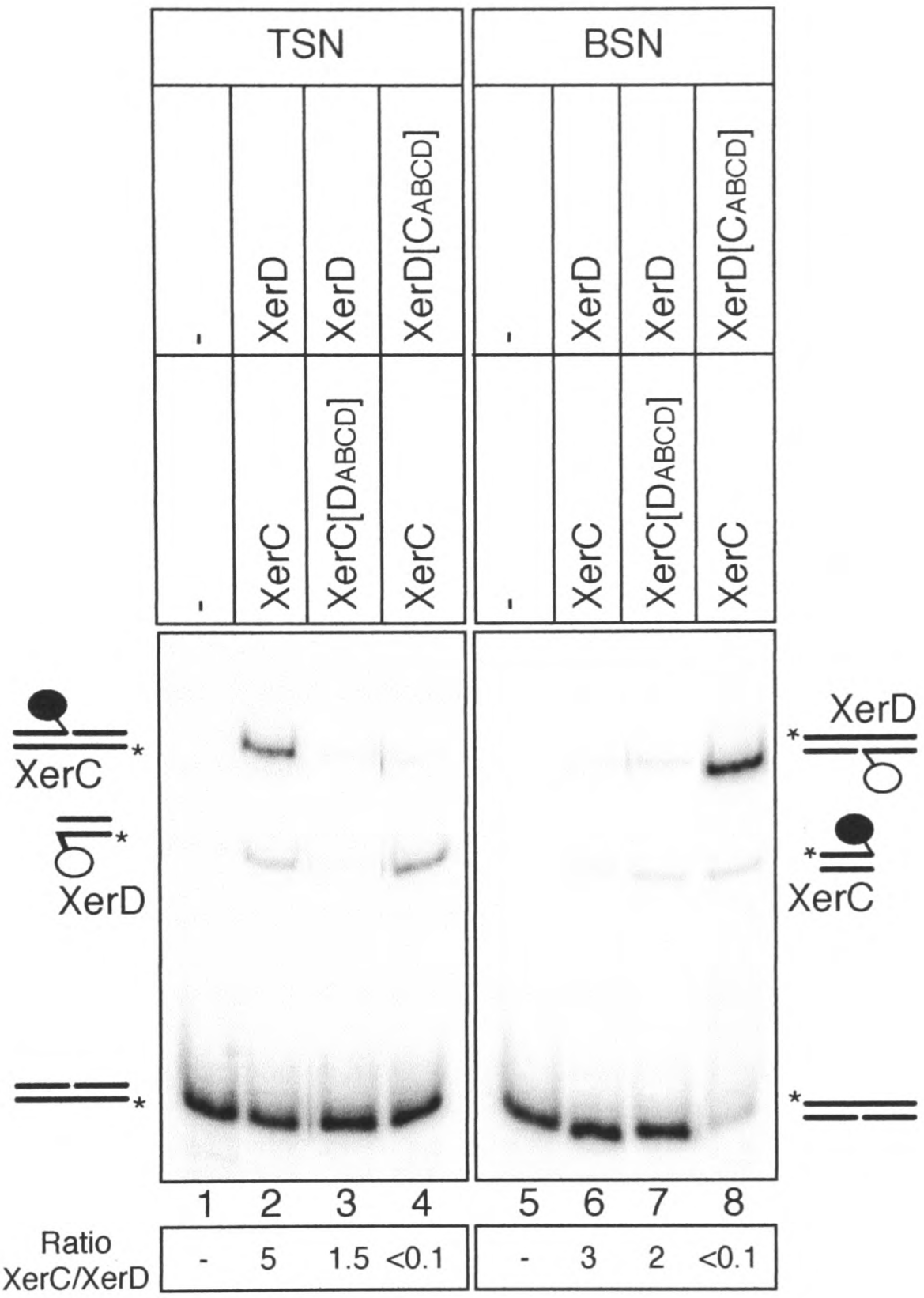


Figure 3.5. XerC and XerD recombinase variants lacking or exchanged for their N-terminal domains display altered patterns of recombinase-mediated cleavage. The indicated recombinase combinations were incubated with *dif*TGTATA TSN (top strand nick) or BSN (bottom strand nick) substrates at 25^{O}C for 1h. The recombinase-DNA covalent complexes generated from cleavage by XerC and XerD and non-cleaved DNA were separated on 6% SDS-PAGE. The substrates were radiolabelled at the 5'-end of the continuous strand, as indicated.

This bias mirrors that observed in resolution of HJ substrates, and is consistent with the observation that XerC normally initiates Xer recombination. An increase in variant XerD-mediated cleavage and a simultaneous decrease in cleavage by XerC was observed in the presence of XerC and XerD[CABCD] on TSN; the ratio of XerC to XerD cleavage changed to <0.1 fold (Figure 3.5, lane 4). A striking increase in XerD cleavage was also observed on BSN, which is normally poorly reactive (lane 8; XerC to XerD cleavage ratio of <0.1 fold). This increase in cleavage by the XerD chimera and the impairment of XerC-mediated strand cleavage in the presence of XerD[CABCD] is consistent with the phenotype of this recombinase variant observed on the HJ substrate. Similar switches in cleavage patterns have been observed with XerCD derivatives mutated in their donor-acceptor regions of their C-terminal domains (Arciszewska et al., 2000; Hallet et al., 1999).

In reactions with XerC[DABCD] and XerD on TSN, a large decrease in cleavage by the variant XerC and modest decrease in XerD cleavage was observed, thereby reducing the ratio of XerC to XerD cleavage to 1.5 fold (Figure 3.5, lane 3). Similarly on BSN, a reduction in the ratio of cleavage by XerC to that by XerD was observed, although in this case it was generated by stimulating cleavage by XerD, while leaving the level of cleavage by the XerC variant about the same as that obtained with wild type XerC (lane 7). These results contrast with those obtained with the HJ substrate and may reflect a difference in activity of XerC[DABCD] within the dimeric XerCD-linear DNA complex as compared to the heterotetrameric-HJ complex.

3.2.4. The nature of the phenotype of the N-terminal domain exchange and deletion recombinase variants depends on the central region sequence of the HJ substrate

It has been shown previously that the central region sequence of a *dif* core recombination site influences the relative levels of strand exchanges by XerC and XerD, apparently by determining the relative concentrations of two alternative HJ intermediate conformations, one of which gives XerC-mediated resolution products, while the other gives products formed by XerD (Arciszewska et al., 2000; Hallet et al., 1999).

In order to find out how the N-terminal domains of XerC and XerD control the catalytic activity within the heterotetramer, the resolution of different synthetic HJ substrates by N-terminal domain chimeras and deletion variants was analysed. With wild type XerCD, the wild type *dif* site, *dif*TGTATA, undergoes relatively less strand exchange by XerD than *dif*TGTCTA, whereas *dif*GGGATG undergoes strand exchange by XerC exclusively (Arciszewska et al., 2000). These differences appear to arise as a consequence of the nucleotide sequence at the HJ branchpoint. The purine-rich strand has a higher propensity to adopt a conformation suitable for cleavage and strand exchange by the recombinase that acts on that strand (Azaro & Landy, 1997; Gopaul et al., 1998). Previous studies have shown that recombinase variants that switch the resolution of the HJ intermediate from resolution by XerC to XerD have greatest effects with *dif*TGTCTA, least with *dif*GGGATG, and intermediate effects with wild type *dif* (*dif*TGTATA). N-terminal domain chimeras and deletion variants exhibited the same general behaviour (Figure 3.6).

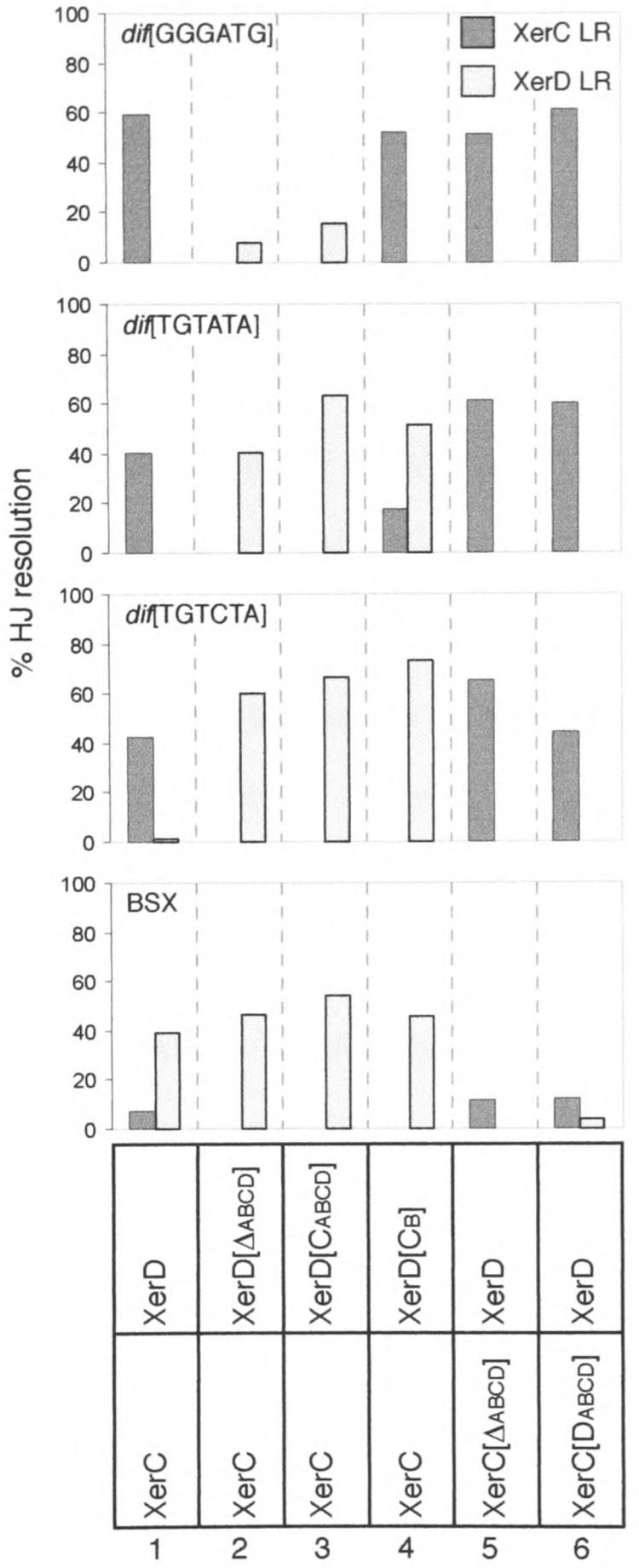


Figure 3.6. The nature of the phenotype of XerCD N-terminal domain variants depends on the HJ central region sequence. The bars indicate the percentage of the input HJ DNA converted to strand exchange product by XerC or XerD (dark and light grey, respectively) in 30 min reactions. The results derive from a single representative experiment.

Strand exchange by XerC is completely impaired in all three substrates in the presence of XerD[ΔABCD] and XerD[CABCD] (compare lane 1 with 2 and 3). This impairment is accompanied by stimulation of XerD catalysis; the highest levels of stimulation being observed with *dif*TGTCTA, the lowest with *dif*GGGATG and intermediate levels with *dif*TGTATA. The phenotype of XerD[CB] with the two new substrates was weaker, with less impairment in strand exchange by XerC and weaker stimulation of XerD strand exchange in *dif*TGTATA and no significant change in resolution of *dif*GGGATG (lane 4). The latter result indicates that α-helix B does not contain all of the determinants for the altered phenotype.

Recombination was also analysed on BSX, a *dif*TGTATA derivative in which two arms are tethered to adopt the conformation appropriate for XerD-mediated resolution. Consequently, this substrate undergoes more strand exchange by wild type XerD than by XerC (Figure 3.6, lane 1). In reactions containing XerD and either XerC[ΔABCD] or XerC[DABCD], strand exchange by XerD was impaired while XerC activity was stimulated (lanes 5 and 6). Note, that the extent of stimulation of strand exchange by XerC is likely to be limited by the presence of the molecular tether. This result demonstrates that XerC and XerD N-terminal domain chimeras and deletion variants have reciprocal phenotypes on HJ substrates. Consistent with all of the above data XerC strand exchange was impaired in BSX samples containing XerC and XerD[ΔABCD], XerD[CABCD], or XerD[CB] (lanes 2, 3, and 4).

3.2.5. The XerD N-terminal domain variants facilitate a HJ conformational change during recombination at *dif* on a supercoiled substrate

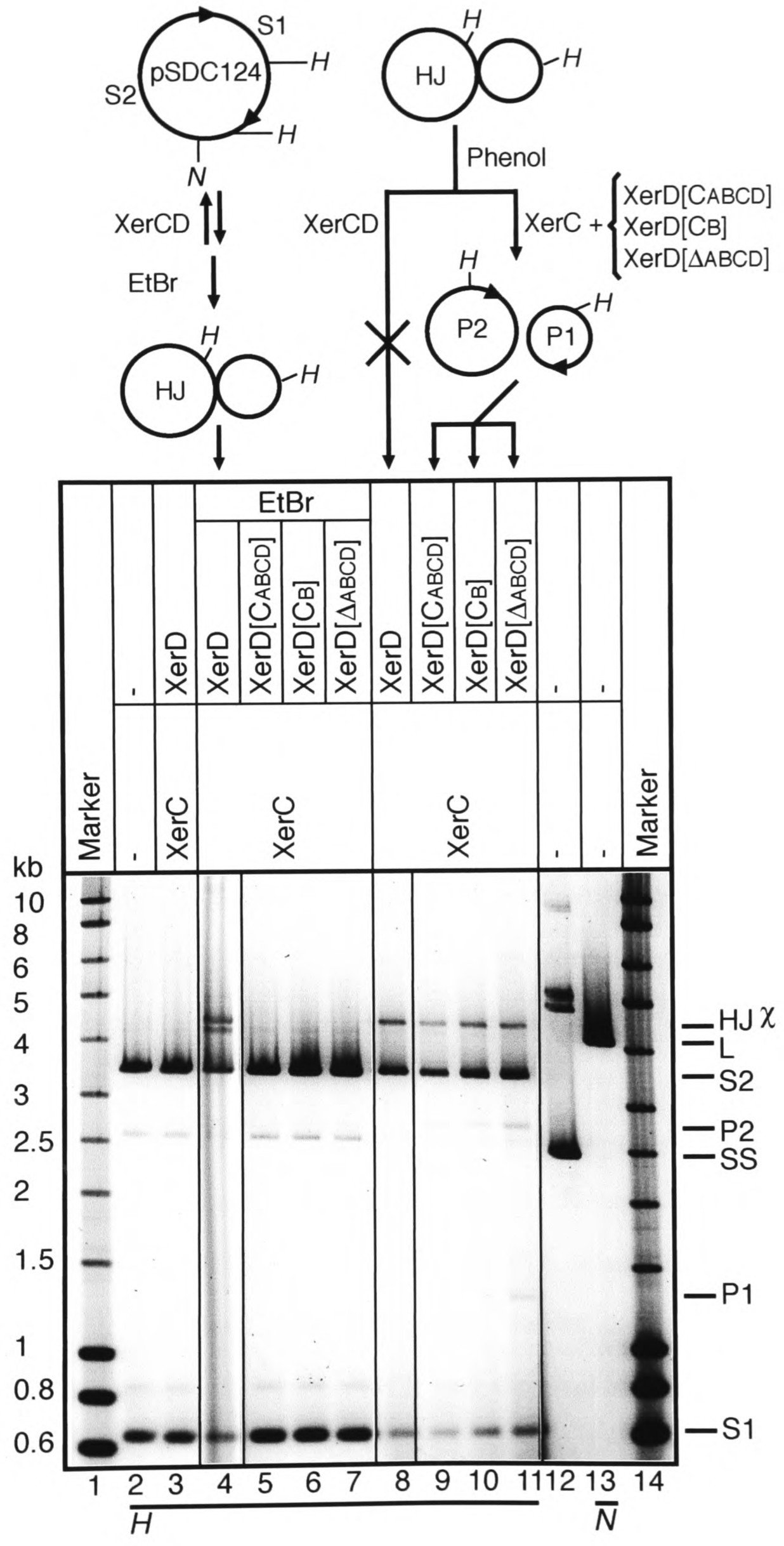
If the N-terminal domain variants that stimulate their own catalyses and impair their partners' catalyses do this as a consequence of altering the conformation of the HJ

intermediate, then this should be reflected in their ability to modulate the outcome of complete recombination reactions on supercoiled substrates containing two recombination sites.

Incubation of a plasmid containing two directly repeated *dif* sites with wild type *E. coli* XerC and XerD leads to cycles of HJ formation and resolution by XerC, reactions that are revealed by the presence of ethidium bromide, which allows HJ formation but prevents HJ processing (Barre et al., 2000). Strand exchange by XerD is not observed because there is a barrier to the conformational change needed to provide a HJ substrate for XerD. *In vivo*, the C-terminal domain of FtsK appears to facilitate this conformational change. On the basis of the experiments presented above, XerD[ΔABCD], XerD[CABCD], or XerD[CB] might well be able to convert the HJs formed by XerC on supercoiled *dif* sites to complete recombinant products, since these variants switch the direction of resolution on synthetic HJ substrates.

A supercoiled plasmid containing two directly repeated *dif* sites was incubated with XerC and XerD, XerD[CABCD], XerD[CB] or XerD[ΔABCD]. After 1 h incubation, reactions were treated with ethidium bromide in order to further prevent HJ processing. Restriction digestion of DNA recovered from these reactions shows that the HJ intermediate was produced only in the presence of wild type XerC and XerD (Figure 3.7, lanes 4-7). This result was anticipated since the XerD N-terminal variants impair strand exchange by XerC. Purified DNA containing a high proportion of supercoiled HJ molecules was recovered and incubated with XerC and XerD, XerD[CABCD], XerD[CB] or XerD[ΔABCD] (lanes 8-11, respectively). Only HJ intermediate and supercoiled plasmid DNA was detected in samples incubated with XerC and XerD (lane 8).

Figure 3.7. N-terminal domain variants of XerD facilitate a conformational change of the *dif* HJ in supercoiled DNA. pSDC124, containing two directly repeated *dif* sites, was reacted with the indicated recombinase combinations (lanes 2-7) for 1h. Ethidium bromide (0.25mg/ml final concentration) was then added to the reactions represented in lanes 4-7, to inhibit reaction on any HJ intermediate. DNA recovered after phenol extraction from the reaction shown in lane 4 was further reacted with XerC and either wild type XerD or XerD N-terminal variants (lanes 8-11). DNA was separated by 1% agarose gel electrophoresis. Supercoiled and linear pSDC124 DNA are shown in lanes 12 and 13, respectively. SS indicates the position of supercoiled pSDC124 DNA, while S1 and S2 indicate the positions of the two DNA fragments obtained from pSDC124 digestion with *Hind*III (H); L indicates the position of the linearised substrate (digested with *Nde*I [N]). HJ indicates position of the χ structure obtained after digestion of the HJ-containing pSDC124 molecule with *Hind*III. P1 and P2 indicate the positions of the linearised products of a complete recombination reaction.


S1
pSDC124
S2
H
H
N
XerCD
EtBr
H
HJ
H
H
Phenol
XerCD
XerC +
XerD[CABCD]
XerD[CB]
XerD[ΔABCD]
H
P2
P1
H
Marker
-
XerD
EtBr
XerD
XerD[CABCD]
XerD[CB]
XerD[ΔABCD]
XerD
XerD[CABCD]
XerD[CB]
XerD[ΔABCD]
-
-
Marker
XerC
XerC
XerC
kb
10
8
6
5
4
3
2.5
2
1.5
1
0.8
0.6
HJ χ
L
S2
P2
SS
P1
S1
1 2 3 4 5 6 7 8 9 10 11 12 13 14
H
N

However, resolution products generated by XerD-mediated strand exchange were detected in samples incubated with all three XerD variants (lanes 9-11). These data demonstrate that the XerD N-terminal variants facilitate the conformational change of the HJ intermediate in a plasmid substrate, and thereby allow strand exchange by XerD. This is the first demonstration of a complete *E. coli* XerCD-mediated recombination reaction on a supercoiled *dif* reporter plasmid *in vitro*, albeit with different recombinases present for the two separate stages of the reaction. The result provides strong support for a reaction scheme that requires a change in conformation of the *dif*-HJ, formed by XerC, to generate a substrate for XerD, which can then complete the recombination reaction.

3.2.6. A functional tetrameric complex can be assembled on a HJ substrate from the separate/truncated N- and C-terminal domains of XerCD

It was proposed above, based on the comparison between the crystal structures of XerD and Cre-*loxP*, that the N- and C-terminal domains of XerCD would occupy different faces of the DNA upon binding. It is also expected that the heterotetrameric complex formed by XerCD-*dif* will be very similar to the one of Cre-*loxP* (Guo et al., 1997). From the Cre-*loxP* structures, the recombinases form ring-like complexes on opposite faces of the DNA due to extensive protein-protein contacts involving their N- and C-terminal domains (Figure 3.1-C; (Gopaul & Duyne, 1999; Gopaul et al., 1998; Guo et al., 1997)). In order to investigate the organisation of the N- and C-terminal rings on a XerCD-*dif* heterotetrameric complex, and their inter-relations, the truncated N-terminal domains of XerCD were expressed and tested in combinations with their catalytic domains (XerC[ΔABCD] and XerD[ΔABCD], respectively) on HJ

resolution experiments. Note that in these experiments there was no covalent linkage between the N- and C-terminal domains of XerC and XerD.

The XerCD N-terminal domains were prepared by the insertion of stop codons at the end of the linker regions that separate the N- and C-terminal domains in the *xerCD* genes (Figure 3.2). The linkers were retained because it has been shown that the equivalent regions in Cre are needed for the proper functioning of its recombination system, together with α-helix A and the β-hairpin turn containing the catalytic lysine in the C-terminal domain of the enzyme (Guo et al., 1997; Shaikh & Sadowski, 2000).

The first step was to verify if it is possible to reconstruct the N-terminal ring on a heterotetrameric complex formed by XerC, XerD[ΔABCD] and *dif*TGTCTA. In this combination, only the N-terminal domains of XerC are present, and as already shown above, just XerD strand exchange products are detected (Figure 3.8, lane 4). However, when the N-terminal domain of XerD was added to this reaction (XerDN-ter), a partial reversion of the resolution bias to the wild type phenotype was observed (compare lanes 4 and 6). The yield of XerC strand exchange products in lane 6 is low when compared to the wild type combination (lane 2) probably because the N-terminal domain of XerD is not covalently attached to its catalytic domain, which might suppress, in part, the effect the N-terminal domain of XerD has over its binding-site. This effect would be that of setting the conformation of the HJ complex for XerC strand exchange when wild type enzymes are used.

When the combination of XerC[ΔABCD], XerD and *dif*TGTCTA was tested in the presence or absence of the N-terminal domain of XerC (XerCN-ter), no significant differences were observed in the pattern of resolution products (compare lanes 3 and 5). This was expected since the substrate used already has a propensity to adopt a

conformation that is suitable for XerC strand exchange. However, a slight decrease in the amount of recombinase-linear duplex covalent complexes (LCC) was observed in lane 5, which might reflect a better interaction of XerC[ΔABCD] with its binding-site, and a better rejoining activity by the presence of its N-terminal domain *in trans*.

So far, it has been demonstrated that the catalytic domains of both XerC and XerD form productive tetrameric complexes with their wild type partners on a HJ substrate. When given *in trans*, the N-terminal domain of XerD is apparently able to penetrate into the heterotetrameric complex, and to reset it to the wild type conformation, judged by the appearance of XerC strand exchange products in the reaction shown in lane 6 of Figure 3.8. But would it be possible for the N- and C-terminal domains of XerCD, when not covalently attached, to associate in solution, or even on a HJ substrate, and assemble a functional recombination machinery? Since both XerC[ΔABCD] and XerD[ΔABCD] do not exhibit detectable binding to *dif* linear DNA on their own, as binding requires their N-terminal domains or the presence of a wild type partner recombinase to anchor them to the DNA, it would not be expected to detect any resolution products in a reaction combining two XerCD N-terminal deleted variants (Figure 3.8, lane 7). However, when the XerCD N-terminal domains are added *in trans* to this reaction, resolution products can be detected (lane 8). In this reaction, a N-terminal ring might be reconstituted and positioned in its correct place respective to the C-terminal ring, since this reaction seems also to be reverting to the wild type phenotype (compare lanes 4, 6 and 8). It can be concluded from these experiments that: a) it is possible to assemble a functional Xer recombination machine from separate modules corresponding to the N- and C-terminal domains of XerC and XerD; b) by re-assembling the N-terminal ring on a heterotetrameric complex formed by XerC, XerD[ΔABCD] and *dif*TGTCTA with the addition of the N-terminal domain

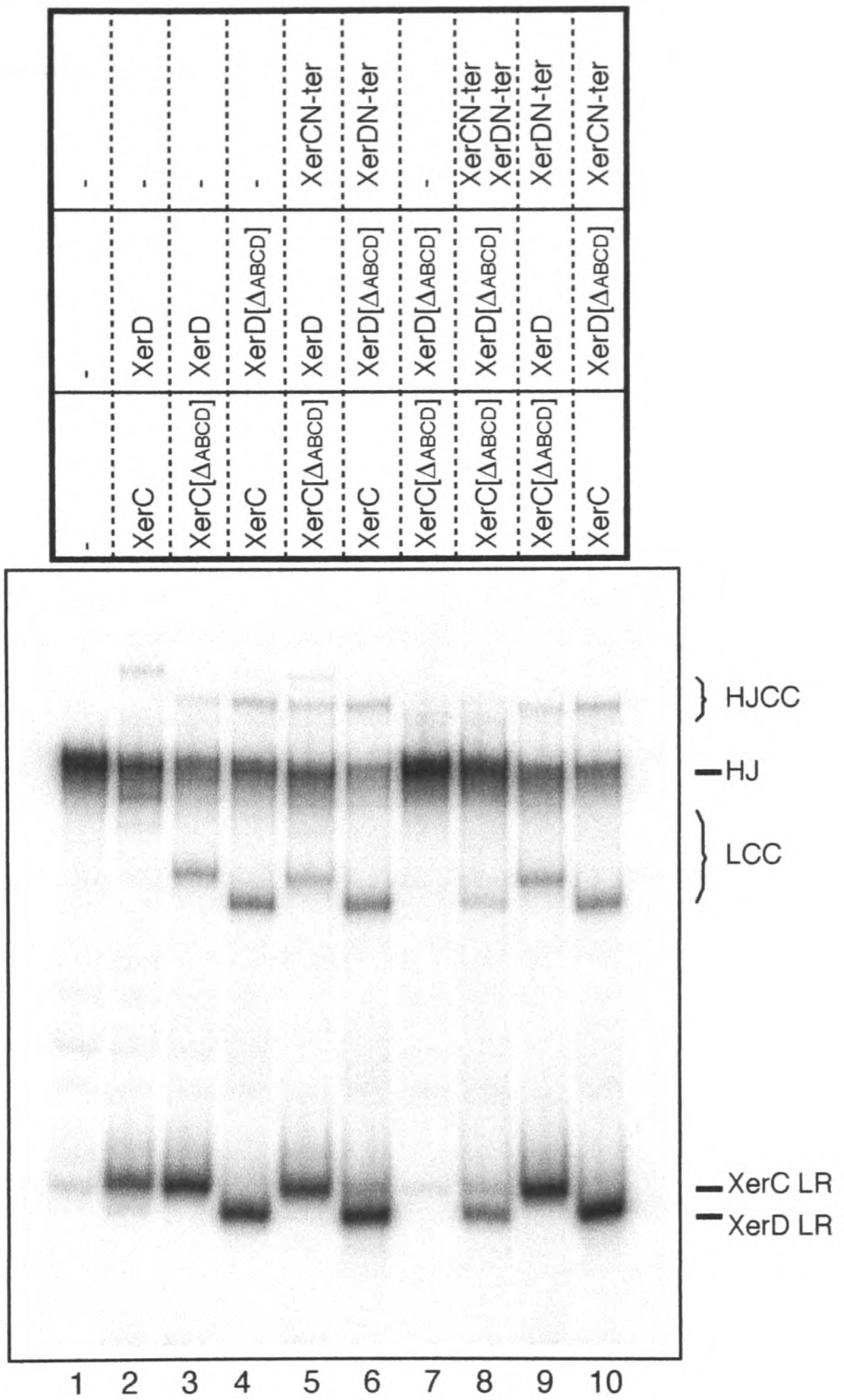


Figure 3.8. A functional tetrameric complex can be assembled on a HJ substrate using XerC and XerD truncated N- and C-terminal domains. The HJ substrate, the same *dif*TGTCTA-HJ illustrated in Figure 3.4, was radiolabelled at the 5' end of strand III, and reacted with the indicated recombinases for 30 minutes at 37^{O}C. Linear rejoined duplex products are indicated XerC LR and XerD LR. HJCC and LCC bands indicate recombinase-HJ DNA and recombinase-linear duplex DNA covalent complexes, respectively. Electrophoresis was on a 6% polyacrylamide gel containing 0.1% SDS.

of XerD, it is possible to reset the wild type bias of HJ resolution, and c) the interactions between XerDN-ter and DNA are responsible for setting the HJ complex to a conformation suitable for XerC strand exchange.

The addition of XerDN-ter to a reaction containing XerC[ΔABCD], XerD and *dif*TGTCTA, or XerCN-ter to the reaction containing XerC, XerD[ΔABCD] and *dif*TGTCTA, had no effect in the outcome of HJ resolution (Figure 3.8, compare lanes 9 and 3, 10 and 4). These results show that the N-terminal domains of XerCD cannot substitute for one another, although productive tetrameric complexes can be formed between wild type recombinases and N-terminal variants in which there is a covalent linkage attaching their N- and C-terminal domains. This is the case when XerC[DABCD] or XerD[CABCD] were used in combination with their wild type partners in experiments described in the previous sections. In such cases, it is expected that the whole complex will be set to accommodate the small variations introduced by these recombinase variants.

3.3. Discussion

In this Chapter it was demonstrated that the N-terminal domains of XerC and XerD, although dispensable for catalytic activity, play a key role in controlling strand exchange by the XerCD recombinases. Previous biochemical studies of recombination by XerCD, and related recombinases, and analysis of crystal structures of Cre-DNA complexes have revealed that catalysis in the recombining tetramer is tightly coordinated, with only two of the four recombinase catalytic sites being active at any one time, as expected for a mechanism in which the two pairs of strand exchanges are separated in time and space. Central to the coordination of catalysis are reciprocal switches between recombinase activity and inactivity at the HJ intermediate stage of

the recombination reaction. These switches correlate with a subtle conformational change between two alternative forms of the Holliday junction intermediate (Gopaul et al., 1998). In Xer recombination, one form is a substrate for strand exchange by XerC and the other for strand exchange by XerD. Cyclic interactions between donor and acceptor regions in the C-terminal domains of Cre and XerCD play a crucial role in mediating this coordination of catalysis (Guo et al., 1997; Hallet et al., 1999). We extend these earlier observations by showing that the N-terminal domains of XerC and XerD also contribute to the reciprocal activation-inactivation of XerC and XerD, by directing the formation of the preferred HJ DNA conformation within a recombining heterotetramer. Furthermore, we have presented new evidence to support a mechanism in which XerC continually forms and resolves HJs at synapsed duplex *dif* sites, because the conformational change in the HJ required to form a substrate for strand exchange by XerD is not favourable. We have also demonstrated that a recombinase combination that switches catalysis on a *dif* HJ from that by XerC to that by XerD, mediates conversion of these HJs to complete recombinant product in a circular plasmid substrate, thereby accomplishing a reaction step that is mediated by the C-terminal domain of FtsK *in vivo* (Barre et al., 2000).

DNA binding, synapsis and recombination by XerC and XerD recombinases deleted or exchanged for their N-terminal domains

XerC and XerD derivatives lacking their N-terminal domains exhibit reduced affinity in binding to linear *dif* DNA when tested alone, or in combination with each other. Nevertheless, they were able to bind DNA in the presence of the wild type partner recombinase, indicating that they retain some cooperative DNA binding. Similar results have been observed for other tyrosine recombinases. For example, the C-

terminal domains of Cre, λ and Flp recombinases have a reduced DNA binding affinity (Hoess et al., 1990; Pan & Sadowski, 1993; Tirumalai et al., 1997).

The N-terminal domains of tyrosine recombinases contact the inner 5 nucleotides of the recombinase binding site and the adjacent 1-2 nucleotides of the central region (Chen et al., 2000; Guo et al., 1997). In Cre, these contacts are made into the DNA major groove by the crossed α-helices B and D. These same helices in the XerD structure superimpose well onto the Cre helices and therefore it is likely that XerD adopts a similar configuration when bound to DNA. Since the N-terminal and C-terminal domains interact with the opposite faces of DNA, the DNA contacts made by each N-terminal domain should be important for the ability of a recombinase to clamp its DNA substrate. Indeed, the XerCD chimeras in which the N-terminal domains have been exchanged, although impaired in DNA binding, bind better than their deleted derivatives.

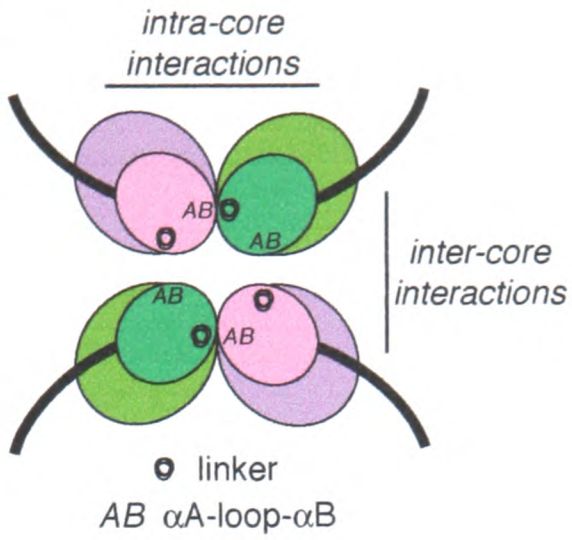
In the Cre-*loxP* structures, the four N-terminal domains form an almost 4-fold symmetric structure that changes little during HJ conformational change. Each domain contacts its two neighbours through cyclical interactions between α-helix A (including the loop connecting it to helix B) and α-helix E. Helix E forms the N-terminal part of the linker, which provides the covalent connection with the C-terminal domain. Equivalent interactions that link the N-terminal domains of Flp are revealed in the crystal structure of the Flp-*frt* complex, despite these domains having little sequence similarity to those of Cre-XerCD (Chen et al., 2000). In Cre, an additional contact between the N- and C-terminal domains is made between α-helix E and the β-hairpin turn of an adjacent C-terminal domain. Therefore, just as cyclical interactions connect the C-terminal domains of tyrosine recombinases, an equivalent set of interactions interconnects the four N-terminal domains. These, along with the

DNA contacts, will contribute to recombinase binding to duplex DNA, and to the overall stability of the recombinase tetramer-DNA complex. It can be assumed that equivalent interactions will be important for the assembly of a functional XerCD recombination complex, and that the linker-β-hairpin turn interactions are most probably the ones responsible for the re-assembly of the XerCD N- and C-terminal rings on the HJ substrate when the N- and C-terminal domains are not covalently attached to each other. Nevertheless, it is likely that the N-terminal domains of XerCD are interconnected by interactions between the linker region (L) and the α-helices A-B region (Figure 3.9, top). The intra-core interactions that link the two N-terminal domains bound to a given duplex segment occur between a region of α-helices A-B of XerD and the linker region of XerC, while the inter-core interactions that link two duplexes involve the linker region of XerD and the region encompassing α-helices A-B of XerC. It can therefore be suggested that synapsis may be dependent on the N-terminal domain inter-core interactions, whereas cooperative binding of XerCD to duplex DNA may require the intra-core interactions.

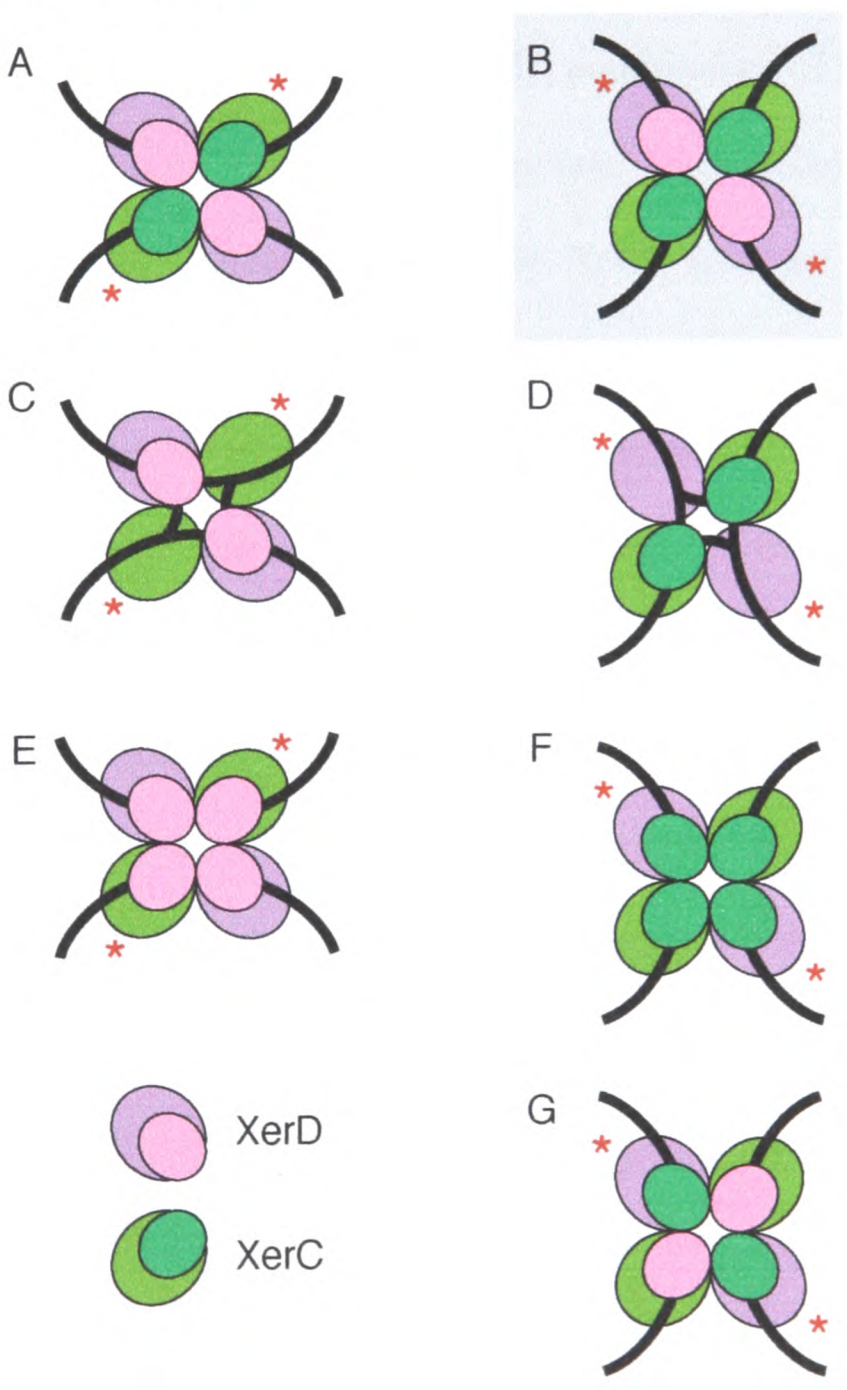
The deletion of one pair of N-terminal domains, which removes two sets of DNA binding interactions and all four sets of N-terminal domain protein-protein contacts, would be expected to compromise the assembly of a productive recombination complex on duplex DNA. Consistent with this, no synapsis of duplex sites was detected for the N-terminal deletions in combination with their wild type partner, as measured by the failure to form accessory factor-dependent HJs on normal and variant *psi* substrates, upon which recombination is initiated by XerC- or XerD-mediated strand exchange, respectively (HF, LKA, M. Bregu and DJS, unpublished observations). Furthermore, the same assays indicate that the XerC[DABCD]-XerD combination is defective in synapsis. This suggests that the two sets of inter-core N-

Figure 3.9. Top Synapsis. Schematic of the cyclic N-terminal domain interactions involved in synapsis of two XerCD duplex DNA complexes. In a ternary complex composed of one molecule of XerC and XerD bound to duplex DNA, the N-terminal domain intra-core interactions are formed between distal part of α-helix A, the loop connecting helices A and B and perhaps the proximal part of helix B of XerD (AB), and the linker region of XerC. The N-terminal domain inter-core interactions necessary to support formation of a synaptic complex involve XerC AB and the linker of XerD. The view is from the N-terminal domain side of the complex. The DNA conformation in the complex is set for catalysis by XerC. Larger ovals represent C-terminal domains while smaller ovals represent N-terminal domains. The black curved lines represent DNA. **Bottom** HJ resolution. Schematic of the conformation adopted by the HJ DNA upon binding by different combinations of XerCD recombinases. The view is as above. A HJ heterotetramer containing wild type XerC and XerD recombinases is set for catalysis by XerC (A). The heterotetramer does not adopt the conformation for catalysis by XerD (B), unless the conformational change is facilitated by interactions with other factors or by the presence of a molecular tether. Combination of wild type XerD and XerC[ΔABCD] (C), or XerC[DABCD] (E), leads to a HJ-heterotetramer in a conformation for strand exchange by XerC. Combination of wild type XerC and XerD[ΔABCD] (D), or XerD[CABCD] (F), leads to a HJ-heterotetramer in a conformation for strand exchange by XerD, as does the combination of XerC[DABCD] and XerD[CABCD] (G).

Synapsis



HJ resolution



terminal domain protein-protein interactions are disrupted when all four N-terminal domains derive from XerD. In contrast, XerC and XerD[CABCD] are able to synapse and form HJs with supercoiled variant *psi* sites in which recombination is initiated by strand exchange mediated by XerD. This indicates that the inter-core contacts are preserved when all four N-terminal domains are derived from XerC. However, this combination may be impaired in intra-core contacts since only a low level of ternary complex was visualised in DNA binding experiments, while the XerC[DABCD]-XerD combination produced higher levels of ternary complexes, consistent with better intra-core contacts (Figure 3.4, Figure 3.9, top). Finally, combination of XerC[DABCD] and XerD[CABCD] appears to be defective in inter-core interactions for the synaptic configuration appropriate for strand exchange by XerD, since no HJ formation was detected on variant supercoiled *psi* substrates that initiate catalysis with XerD.

A role for cyclical N-terminal domain interactions in synapsis has also been suggested by experiments with Cre. Variants lacking the N-terminal domain, or mutated at A36, a residue in the loop between α-helices A and B that is predicted by the crystal structure to interact with R118 in α-helix E of its N-terminal domain neighbour, are inactive in recombination and unable to form higher order recombinase-DNA complexes, both of which are indicative of impaired synapsis (Hoess et al., 1990; Shaikh & Sadowski, 2000).

Just as for XerCD, λ-Int, Cre and Flp fail to mediate a complete recombination reaction when the four N-terminal domains of a putative recombination complex are deleted. Nevertheless, the C-terminal domains of λ-Int and Flp mediate cleavage and/or topoisomerase reactions (Tirumalai *et al.*, 1997; Pan and Sadowski, 1993). It is therefore intriguing that some tyrosine recombinases, for example, FimBE and ResD, which naturally lack a region equivalent to an intact Cre N-terminal domain, can

support a complete recombination reaction (Disque-Kochem & Eichenlaub, 1993; Klemm, 1986). Presumably in these recombinases, the absence of a complete N-terminal domain has been compensated by changes in their C-terminal domains. In Chapter 5, ResD is purified and an initial characterisation of its recombination properties is made.

A switch in the direction of HJ resolution

The switch in the direction of HJ resolution, observed with the XerCD variants deleted or exchanged for their N-terminal domains, demonstrates that these domains make an important contribution to the normal preferred heterotetramer-HJ structure. Examination of the recombination properties of the N-terminal variants provides a simple set of rules and a possible mechanism of action for these domains (Figure 3.9, bottom). In normal Xer recombination reactions, the system appears to be "set up" to do strand exchange by XerC, with XerC initiating recombination on duplex substrates, and with the HJ-intermediate being a preferred substrate for XerC, unless other proteins or conditions facilitate the formation of a substrate for XerD (Barre et al., 2000; Colloms et al., 1996; Recchia et al., 1999). The observations presented here show that the normal operation of this system for preferred XerC activity on HJs requires that the XerD N-terminal domain be present and correctly positioned (Figure 3.9; bottom, A, C, E); the normal reaction is insensitive to the presence of the XerC N-terminal domain. In contrast, on a HJ conformation suitable for strand exchange by XerD, the presence of the XerC N-terminal domain is important; its absence leads to a switch to XerC catalytic activity as a consequence of a change in HJ conformation (Figure 3.9, bottom, switch from B to C). The only exception to this is when the XerC recombinase has a XerD N-terminal domain, and the XerC N-terminal domain is

present on XerD; this leaves the complex set for strand exchange by XerD (Figure 3.9; bottom, G).

How do the XerCD N-terminal domains contribute to the structure of the HJ intermediate and its preferred conformation, and hence determine which recombinase is active? This cannot require the cyclic interactions between the N-terminal domains, since recombination complexes need only two of the normal four N-terminal domains present in order for HJ resolution, a situation where the cyclic interactions are not possible (Figure 3.9, C and D). The other possibilities are therefore: (i) the interactions that the XerD N-terminal domains make with their target DNA, and in this case, the covalent linkage between the N- and C-terminal domains of XerD is of great importance; (ii) the non-covalent contacts of a N-terminal domain with a C-terminal domain (for example, the contact seen in Cre where the N-terminal domain proximal linker region contacts the β-hairpin loop of an adjacent inactive C-terminal domain catalytic site), and (iii) a combination of all of these mechanisms.

Option (ii) can be eliminated because it fails to explain the properties of complexes in which two N-terminal domains have been deleted; the β-hairpin of an active molecule contacts the scissile phosphate and is distant from the linker in the available Cre-*loxP* crystal structures. In contrast, option (i) has a number of attractive features. A role for a DNA interaction that alters the DNA structure locally in a way that prevents the recombinase molecule's own activity while activating the partner for catalysis, is consistent with the observation that a kink in the region of the DNA contacted by a given N-terminal domain activates cleavage by the partner recombinase in a synapse of Cre-duplex DNA (Guo et al., 1999). During recombination this kink is translated into the HJ branchpoint in the middle of the central region. The altered electrophoretic mobilities of the XerCD ternary complexes,

containing either chimeric or deleted recombinases are consistent with this hypothesis, as is the demonstration that re-positioning the N-terminal domain of XerD ("*trans* complementation") on a tetrameric complex formed by XerC-XerD[ΔABCD]-DNA reverses, to some extent, the outcome of the reaction to the wild type phenotype. Further support for this hypothesis is the requirement of the α-helix B region for the determination of the normal pattern of catalytic activity. However, the efforts made to identify the contacts between XerD N-terminal region and DNA, by substitutions at residues within the region of α-helix B including the loop region between A and B helices, have failed. Also consistent with hypothesis (i) are the properties of all of the variants cartooned in Figure 3.9 (bottom). In panels A, C, and E, the XerD N-terminal domains activate the intra-core partner, XerC, while in panels D and F, the XerC N-terminal domains activate the intra-core partner, XerD. In panel G, the XerD N-terminal domain activates its intra-core partner, which is a XerD catalytic domain. The failure of the XerC N-terminal domain to activate XerD (B) is then possibly a consequence of inhibition mediated through contacts between the N-terminal domains of XerC and XerD.

Consistent with the above interpretation of the switches in HJ resolution, a switch in cleavage activity in a dimeric linear suicide substrate complex observed when the XerD N-terminal domain is replaced with that of XerC, mirrors that observed with the HJ substrate. Similarly, replacement of the XerC N-terminal domain with the XerD N-terminal domain in a dimeric complex resulted in impairment of cleavage by XerD, as expected, but surprisingly no substantial stimulation of cleavage by the variant XerC was observed. Indeed on TSN, low levels of cleavage by both XerC and XerD were obtained, possibly because interactions of the two XerD N-terminal domains on the dimeric complex make it difficult for it to

adopt a conformation suitable for catalysis. In contrast, on a HJ XerCD complex that contains 4 N-terminal domains of XerD, other constraints on the overall HJ structure may allow it to adopt a configuration that makes it a substrate for efficient recombination.

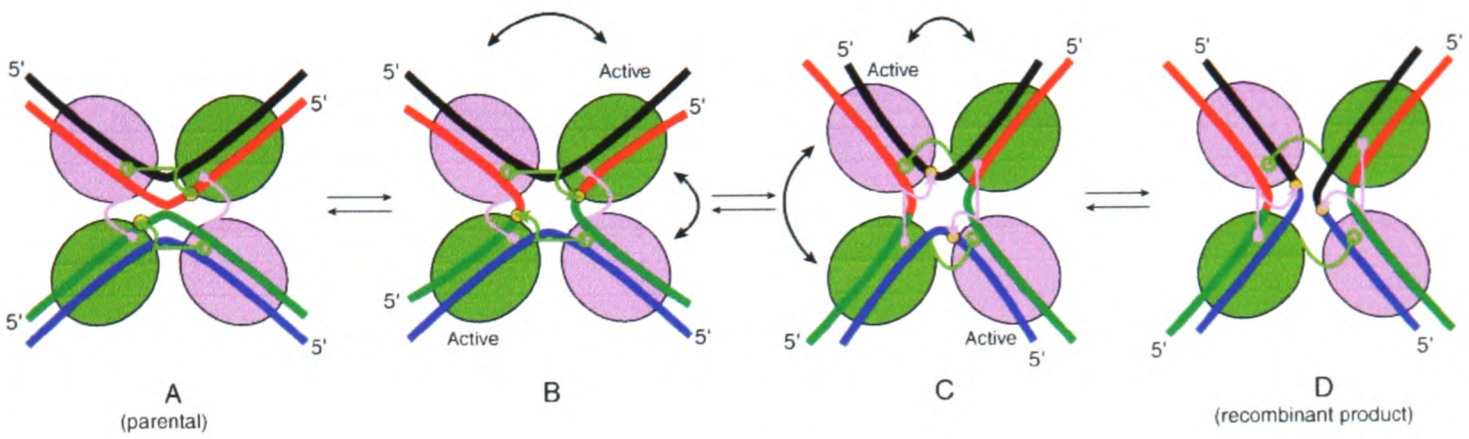
The results presented here, along with previous analyses, demonstrate that the structure and preferred conformation of the HJ intermediate is maintained by a spring-like tension within the recombination complex. This maintains the HJ intermediate in one of two alternative conformations that differ in free energy. All other possible transition states will have higher free energy and be unstable. DNA and recombinase structure and the multitude of interactions present in the recombination complex contribute to the free energy differences that dictate that the XerC HJ substrate is normally the preferred configuration for catalysis. The biological reasons for this are beginning to emerge since it is now evident that Xer recombination on both plasmid and chromosomal recombination sites often involves just a single pair of strand exchanges that lead to cycles of HJ formation and resolution by the same recombinase, there being a barrier to the HJ conformational change required to produce a substrate for the second pair of strand exchanges. It is only under special conditions, which involve the participation of additional proteins, that the change in HJ conformation is facilitated {e.g. see (Barre et al., 2000)}. Such efficient control of a recombination reaction could only be achieved by using two different recombinases, since the two alternative HJ conformations in a recombination complex containing four identical recombinase molecules will be close to isoenergetic.

CHAPTER 4

XerCD C-terminal donor-acceptor interactions

4.1. Introduction

The recombination reaction catalysed by the tyrosine recombinases is mediated by a tetramer of enzymes bound to two synapsed core sites (diagram below, A).



The recombination reaction catalysed by the tyrosine recombinases showing the two different conformations adopted by the HJ intermediate (B and C). View from the N-terminal domain side of the complex.

Recombination proceeds through two sequential steps of strand exchanges separated in time and space, and having a HJ-containing molecule as an intermediate (Gopaul & Duyne, 1999; Nunes-Duby et al., 1995). In the first step, only one pair of recombinases becomes activated for catalysis (green pair, A), and strand exchange generates the HJ intermediate (B). The resolution of this intermediate to recombinant products requires the inactivation of the green pair and activation of the purple pair of recombinases, which is accomplished by a change of conformation of the HJ complex (the scissoring process illustrated from B to C, curved arrows). Note that this proposed HJ conformational change is accompanied by rearrangements of the extreme C-termini of these enzymes (purple and green lines), which participate in cyclic interactions among recombinases and contain the catalytic site residues tyrosine. Cleaving subunits have their extreme C-termini in an extended conformation (active), in which the tyrosines (green and purple arrows in A-B and C-D, respectively) are

positioned for the attack of their target scissile phosphates. In other hand, non-cleaving subunits have their C-termini in a more contracted form (inactive) with their tyrosines located away from their target phosphates. Therefore, this reciprocal activation and inactivation of different pairs of recombinases for catalysis is coupled to the change of conformation of the HJ intermediate (Gopaul & Duyne, 1999; Gopaul et al., 1998; Guo et al., 1997; Hallet et al., 1999). In the present Chapter, the region of a given recombinase that is contacted by the C-terminus of another recombinase will be referred to as acceptor region, while the extreme C-termini of these enzymes will be called donor regions, as proposed by Hallet et al. (1999).

In the Xer recombination, which has two recombinases working co-ordinately during recombination, each enzyme catalyses one of the two steps of strand exchanges: the first pair of strands is exchanged by the action of XerC generating the HJ intermediate, which then undergoes a change of conformation in order to activate the XerD-mediated resolution of the HJ to products. Coordination of catalysis during Xer recombination has been extensively studied by the isolation and characterisation of several XerC and XerD acceptor and donor mutants, which display altered coordination of catalysis or even a complete disruption of the recombination reaction (Arciszewska et al., 2000; Hallet et al., 1999; Spiers & Sherratt, 1999). One of the most striking phenotypes was described for the acceptor mutants XerCNHG and XerDESS (Hallet et al., 1999). These mutants carry triple amino acid substitutions ("swaps" between XerC and XerD) within the conserved Motif II, which also contains the catalytic site residues H-R-H-Y (Figure 4.1).

The tripeptides NHG and ESS, identified by sequence comparison, were proposed to be part of the acceptor regions of XerD and XerC, respectively. When combined with their wild type partners in HJ resolution experiments or in recombination of *psi*-containing reporter plasmids, XerCNHG and XerDESS have the ability to stimulate their own activities, while impairing the catalysis by the partner recombinase (Hallet et al., 1999). The impairment observed was proposed to be a result of perturbations of the interactions between the mutant-acceptor and partner-donor regions.

The donor mutants comprise a second class of Xer recombinase variants that were identified by alterations at the C-termini of these enzymes (Hallet et al., 1999; Spiers & Sherratt, 1999). These mutants were obtained by C-terminal deletions of different amino acid segments of XerC and XerD (Spiers & Sherratt, 1997; Spiers & Sherratt, 1999). XerC C-terminal variants in which five or ten amino acids were deleted exhibited a considerable decrease in cooperative binding to linear *dif* DNA, in the presence of the wild type partner XerD, while similar deletions in XerD had no measurable effects on cooperative interactions with XerC (Spiers & Sherratt, 1997; Spiers & Sherratt, 1999). This asymmetry of binding implies that XerC would contact the partner XerD on a linear *dif* DNA via donor-acceptor interactions, while the donor of XerD would be free to probably contact the acceptor of another XerC bound to a different core-recombination site, and thereby facilitate synapsis (Hallet et al., 1999; Spiers & Sherratt, 1999). When tested in HJ resolution assays, XerC and XerD C-terminal deletion variants exhibited a phenotype which is opposite to that of the acceptor mutants XerCNHG and XerDESS; the C-terminal variants stimulated catalysis by the partner recombinase, while displaying an impaired self-catalysis (Hallet et al., 1999).

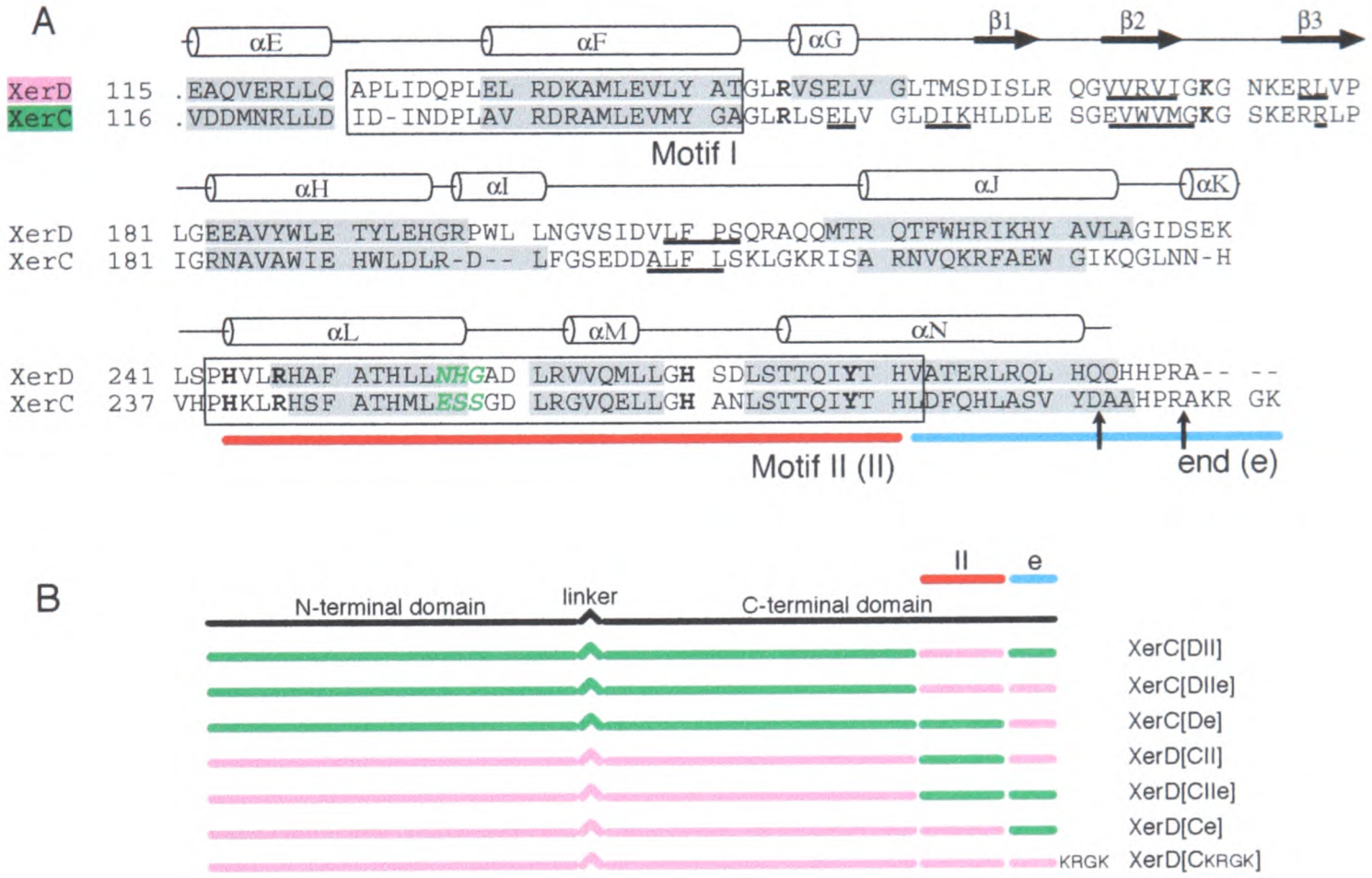


Figure 4.1. A) Amino acid sequence alignment of the C-terminal domains of XerC and XerD. The cylinders and arrows at the top of XerD sequence correspond to α-helices and β-sheets, respectively. The amino acid sequences highlighted in grey and underlined show α-helices and β-sheets, respectively, defined by secondary structure prediction (PHDsec; Rost and Sander, 1993). The regions exchanged between XerC and XerD in the construction of the C-terminal chimeras are indicated by red and blue lines (corresponding to the conserved Motifs II (**II**) and the donor regions of XerC and XerD (**e**), respectively). The amino acid triplets NHG and ESS, swapped in the construction of XerCNHG and XerDESS are indicated in green. The residues located to the right of the vertical arrows at the end of XerC were deleted in XerCΔ5 and XerCΔ10. **B)** Schematic representation of the XerC-XerD C-terminal chimeras. The segments derived from XerC and XerD are indicated in green and pink, respectively.

In the present Chapter, the roles of the acceptor and donor regions of XerC and XerD on the coordination of catalysis were further investigated. Chimeric Xer recombinases were constructed in which domains believed to compose the acceptor and donor regions were exchanged between XerC and XerD. Analysis of these variants in HJ resolution experiments and in recombination of reporter plasmids *in vitro* showed that both acceptor and donor regions of XerC and XerD are functionally distinct and not interchangeable.

4.2. Results

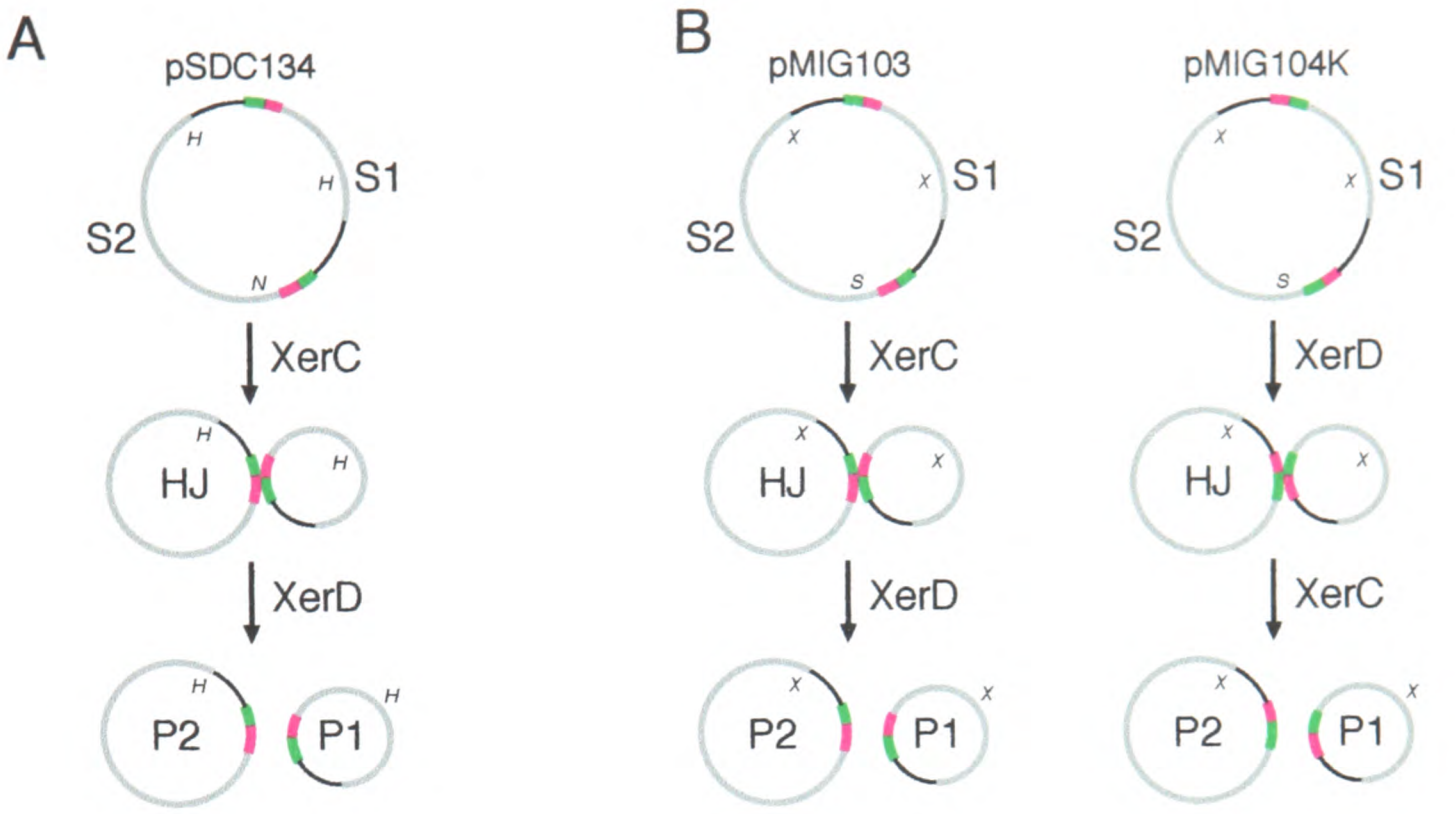
4.2.1. The C-termini of XerCD are functionally distinct

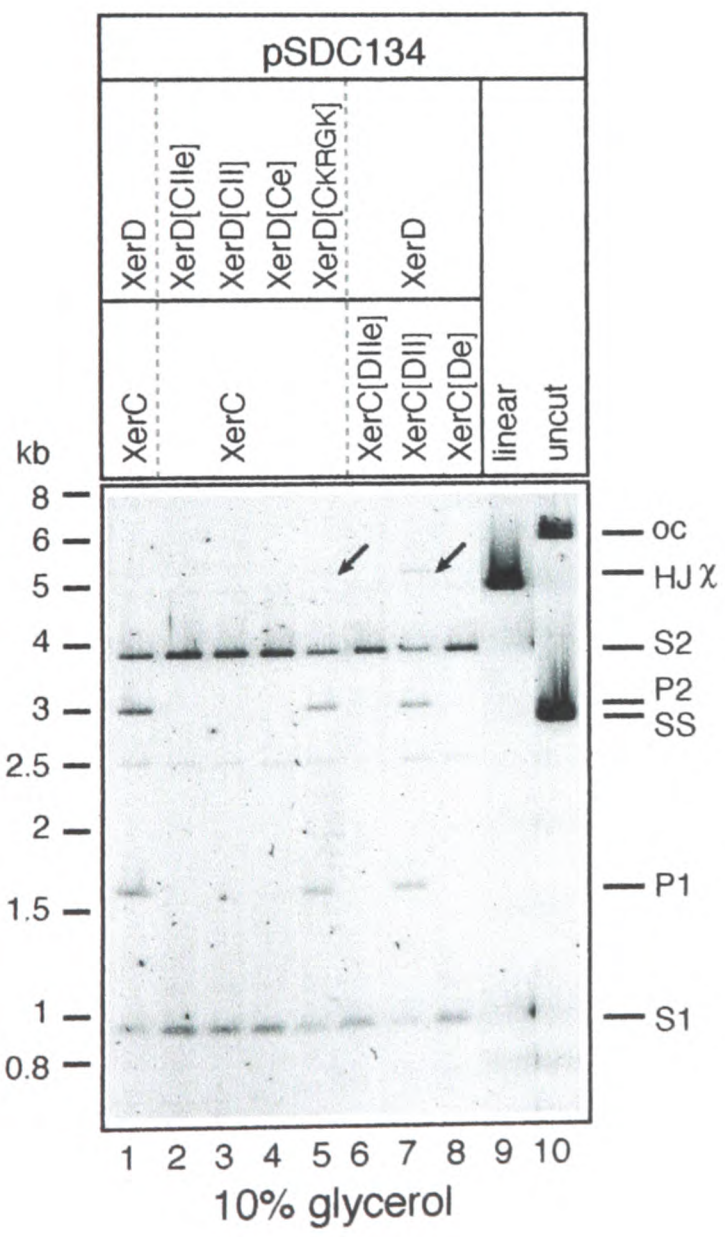
In order to determine functional specificities within XerC and XerD, chimeras of these recombinases were constructed in which regions at their C-terminal domains were exchanged. Two regions were chosen for this analysis: Motif II (**II**) and the region that extends from the 3'-end of Motif II until the C-terminus of the recombinase (end or **e**) (Figure 4.1). Motif II is highly conserved among the tyrosine recombinases and contains the active site residues H-R-H-Y, besides the tripeptides ESS and NHG identified previously to be located within or close to the acceptor interface of XerC and XerD recombinases, respectively (Esposito & Scocca, 1997; Hallet et al., 1999; Nunes-Duby et al., 1998). Region **e** has been recognised as essential for the activation of the catalytic activity of these recombinases, and it is also important for cooperative binding of XerC and XerD to linear DNA (Hallet et al., 1999; Spiers & Sherratt, 1997; Spiers & Sherratt, 1999). Based on primary amino acid sequence alignments, the region **e** of XerC is four residues longer than the equivalent region of XerD, and sequence identity between these two enzymes in this region is

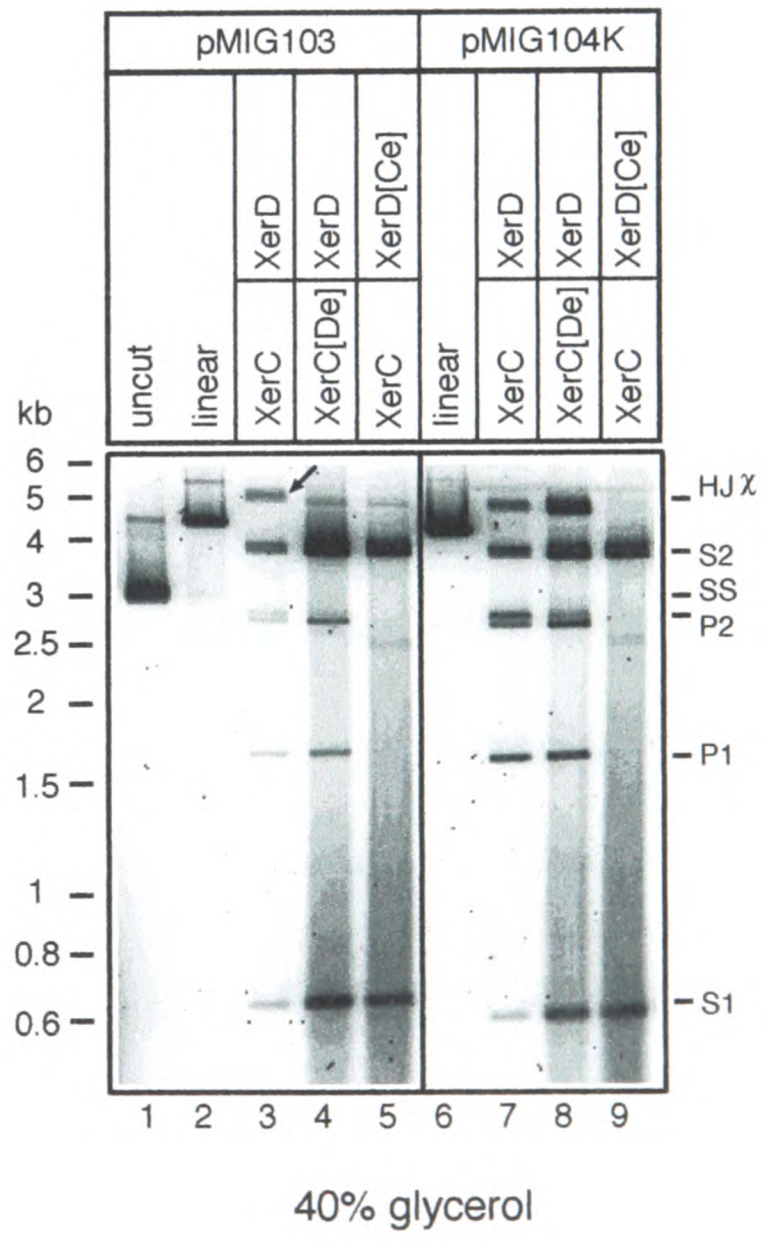
limited to the four sub-terminal residues HPRA (Figure 4.1-A). The XerC and XerD chimeric recombinases had either region **II** or region **e** or both exchanged between them (Figure 4.1-B). In addition, a XerD recombinase variant containing the four extra amino acid residues KRGK, which are present at the C-terminus of XerC, was constructed.

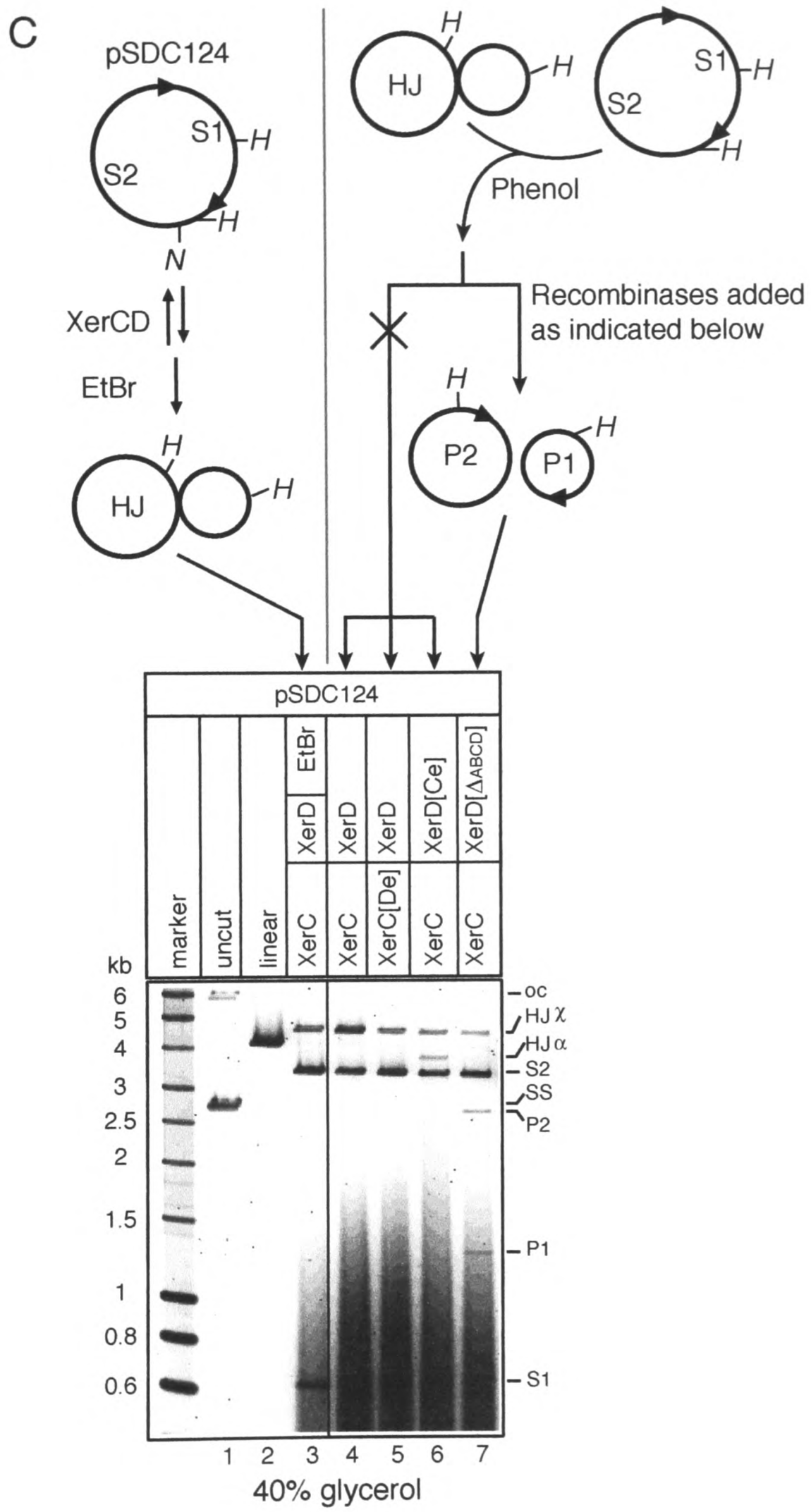
The XerCD C-terminal chimeras were tested for their activities in recombination *in vitro* using reporter plasmid substrates containing two *psi* {pSDC134 (Colloms et al., 1996), pMIG103 and pMIG104K (M. Bregu, D.J. Sherratt and S.D. Colloms, *in preparation*)} or two *dif* {pSDC124 (Blakely et al., 1991)} recombination sites oriented in direct repeat (diagrams of these plasmids illustrating the order of XerCD-mediated strand exchange are shown in Figure 4.2-ABC). First, pSDC134 was incubated with the accessory protein PepA and different combinations of chimeric recombinases and their wild type partners at 10% glycerol, and for 1h at 37^{O}C. Reactions were stopped by phenol extraction, and the DNA samples were treated with the appropriate restriction enzymes prior to separation in agarose gel. In these experiments, only chimeras XerC[DII] and XerD[CKRGK] were active in supporting a complete recombination reaction (Figure 4.2-A, lanes 5 and 7). Note, however, that HJ intermediates (arrows) were also accumulated in these reactions, which might suggest an impairment of the second step of strand exchange by XerD and XerD[CKRGK] (lanes 5 and 7, respectively). No recombination products were observed when the *psi* reporter plasmid was incubated with XerC[DIIe] or XerC[De] in combination with XerD, nor when XerD[CII], XerD[CIIe] or XerD[Ce] were combined with XerC (Figure 4.2-A). These results indicate that both regions **II** and **e** of XerC and XerD are not interchangeable.

Figure 4.2. Assessing the ability of the XerCD C-terminal chimeras to recombine reporter plasmid substrates containing two directly repeated *psi* or *dif* recombination sites. Diagrams of the reporter plasmids used, illustrating the order of XerCD strand exchanges, are shown above each gel. **A)** XerCD C-terminal chimeras were incubated with the indicated wild type recombinases (lanes 2–8) and a supercoiled *psi*-containing reporter plasmid (pSDC134) for 1h at 37^{O}C, in the presence of 10% glycerol. Reactions were stopped by phenol extraction, and digested with the appropriated restriction enzymes prior to separation in 1% agarose gel electrophoresis. **B)** The recombinase combinations were incubated with the reporter plasmid pMIG103 (a *psi*-containing reporter plasmid similar to pSDC134) and pMIG104K (identical to pMIG103, but in which the accessory sequences are adjacent to the XerD-binding site; see text) in the same conditions as above, but in the presence of 40% glycerol. **C)** Supercoiled pSDC124, containing two directly repeated *dif* sites, was reacted with XerCD wild type recombinases for 1h at 37^{O}C. Ethidium bromide (0.25mg/ml final concentration) was then added to this reaction to inhibit further recombination of the HJ intermediates produced. DNA recovered after phenol extraction from the reaction shown in lane 3 was subsequently reacted with the recombinase combinations indicated (lanes 4–7). DNA samples were separated by 1% agarose gel electrophoresis. SS corresponds to the supercoiled plasmids in A, B and C. oc, open circle; S1 and S2 indicate the positions of the two DNA fragments obtained from the digestion of the reporter plasmid substrates with *Hind*III (H) or *Xho*I (X); plasmids were linearised with *Nde*I (N) or *Sca*I (S). HJχ indicates the position of the χ structure. The HJα in lane 6-gel C is probably a resultant of a partial digestion of the pSDC124-HJ substrate by *Hind*III. P1 and P2 indicate the positions of the linearised recombinant products.


A
pSDC134
H
H
S1
S2
N
XerC
HJ
H
H
XerD
P2
P1
H
H
B
pMIG103
pMIG104K
X
X
S1
S2
S
XerC
XerD
HJ
X
X
XerD
XerC
P2
P1
X
X


pSDC134
XerD
XerD[CIIe]
XerD[CII]
XerD[Ce]
XerD[CKRGK]
XerD
XerC
XerC
XerC[DIIe]
XerC[DII]
XerC[De]
linear
uncut
kb
8
6
5
4
3
2.5
2
1.5
1
0.8
oc
HJ χ
S2
P2
SS
P1
S1
1 2 3 4 5 6 7 8 9 10
10% glycerol


pMIG103
pMIG104K
XerD
XerD
XerD[Ce]
XerD
XerD
XerD[Ce]
uncut
linear
XerC
XerC[De]
XerC
linear
XerC
XerC[De]
XerC
kb
6
5
4
3
2.5
2
1.5
1
0.8
0.6
HJ χ
S2
SS
P2
P1
S1
1 2 3 4 5 6 7 8 9
40% glycerol


C
pSDC124
S1
S2
H
N
XerCD
EtBr
HJ
Phenol
Recombinases added
as indicated below
P2
P1
pSDC124
marker
uncut
linear
XerC
XerC
XerC[De]
XerC
XerC
EtBr
XerD
XerD
XerD
XerD[Ce]
XerD[ΔABCD]
kb
6
5
4
3
2.5
2
1.5
1
0.8
0.6
oc
HJ χ
HJ α
S2
SS
P2
P1
S1
1 2 3 4 5 6 7
40% glycerol

Recombination of reporter plasmids was also performed at increased levels of glycerol (40%) and in the presence of *E. coli* cell lysates containing the C-terminal chimeras XerC[De] and XerD[Ce]. At 40% glycerol, HJ intermediates generated by the first pair of strand exchanges may accumulate as compared to reactions at 10% glycerol (Barre et al., 2000; Hallet et al., 1999). Two *psi*-containing reporter plasmids were used in these analyses: pMIG103, which is a standard *psi* reporter plasmid similar to pSDC134, and pMIG104K, which is a derivative of pMIG103 in which the XerCD core sites have been inverted with respect to the accessory sequences {Figure 4.2-B (M. Bregu, D.J. Sherratt and S.D. Colloms, *in preparation*)}. In pMIG104K, the XerD-binding site is adjacent to the accessory sequences, and recombination starts by XerD-mediated strand exchange followed by XerC resolution of the HJ intermediate. Recombination of both plasmids was observed in the presence of XerC[De] and XerD (Figure 4.2-B). However, the pattern of recombination was different from that obtained with the use of the wild type recombinases XerCD (compare lanes 3-4 and 7-8). While the incubation of pMIG103 with XerCD led to an accumulation of HJ intermediates (lane 3, arrow), only complete recombinant products were observed for the combination XerC[De] + XerD (lane 4). This might indicate that the HJ intermediates generated by XerC[De]-mediated strand exchange are rapidly resolved to recombinant products by XerD. On other hand, the incubation of XerC[De] and XerD with a substrate in which recombination starts by XerD-mediated strand exchange (pMIG104K), led to a significant accumulation of HJ intermediates, which is consistent with a stimulation of XerD catalysis and a less efficient XerC[De]-mediated resolution of these intermediates to recombinant products (lane 8). No recombination was observed in the presence of XerD[Ce] and XerC on either of the reporter plasmids tested.

The activities of these two C-terminal chimeras were further examined using the *dif* reporter plasmid pSDC124 (Figure 4.2-C). This plasmid does not undergo a complete recombination reaction *in vitro* to produce recombinant products in the presence of wild type XerC and XerD, unless the FtsK protein is present in the reaction (L. Aussel, F.X. Barre, M. Aroyo and D.J. Sherratt, *in press*). However, low amounts of HJ intermediates, generated by XerC-mediated strand exchange, can be detected in the absence of FtsK and at 40% glycerol (Barre et al., 2000). These can be further increased if ethidium bromide is added to the reaction {Chapter 3 and (Barre et al., 2000)}. In this analysis, the *dif* reporter plasmids used had been previously treated with XerCD recombinases and ethidium bromide. Therefore, the DNA substrate samples contained both the unreacted reporter plasmid and HJ-containing molecules, so that the activities of the C-terminal recombinase variants could be tested in both kinds of substrates at the same time (see Chapter 3 and Figure 4.2-C, lane 3). Neither XerC[De] nor XerD[Ce] were able to support recombination on these DNA substrates when combined with their wild type partners (Figure 4.2-C, lanes 5-6). Note that, complete recombinant products can be obtained when this DNA substrate mix is incubated with XerC and XerD[ΔABCD], a XerD variant with increased XerD catalytic activity (lane 7) (Chapter 3).

In summary, the inability of most of the Xer C-terminal chimeras to recombine *psi* reporter plasmids at 10% glycerol shows that the regions **II** and **e** of XerC and XerD are functionally distinct. This is supported by the fact that at 40% glycerol, the pattern of recombination exhibited by XerC[De] differs from that of XerC, which indicates an alteration of the intermolecular interactions within the XerC(donor)-XerD(acceptor) interface. This alteration does not seem to interfere with the assembly of a productive synaptic complex when accessory sequences and

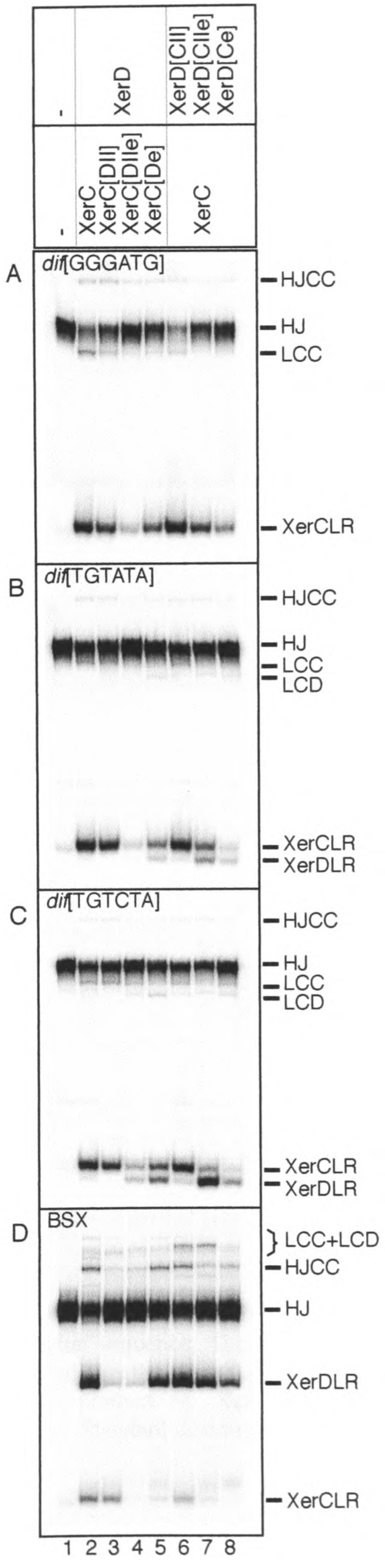
proteins are involved, as is the case when *psi*-containing reporter plasmids are used. However, in the absence of accessory sequences and proteins, which was the case when the *dif* reporter plasmid was used, this C-terminal variant cannot support recombination. In other hand, XerD[Ce] was unable to support recombination on any of the reporter plasmids tested, which might suggest that this chimera is deficient in synapsis formation.

4.2.2. Resolution of synthetic Holliday junction substrates by the XerCD C-terminal chimeras

The catalytic activities of the C-terminal chimeras were further analysed by resolution of synthetic HJ substrates *in vitro*. The HJ substrates contained *dif* core sites carrying three different central regions (*dif*GGGATG, *dif*TGTATA and *dif*TGTCTA). In reactions combining wild type XerCD and these HJ substrates, resolution is predominantly mediated by XerC strand exchange. XerD-mediated resolution is very poor, or cannot be detected, and depends on the central region present: *dif*GGGATG, *dif*TGTATA and *dif*TGTCTA undergo undetectable, ~1% and <5% of XerD-mediated resolution, respectively {Chapter 3 and (Arciszewska et al., 2000)}. BSX, a *dif*TGTATA-HJ in which two arms are tethered and the HJ molecule is constrained in a conformation suitable for XerD-mediated resolution, was also used in this analysis (Arciszewska et al., 1997).

All the C-terminal chimeras were able to support resolution of the synthetic HJ substrates. However, the patterns of resolution differed greatly from those when wild type XerC and XerD recombinases are used. The XerC chimera carrying the Motif II of XerD, XerC[DII], did not support XerD strand exchange (Figure 4.3-BCD, lane 3, and summary Figure 4.4), while XerD[CII] caused a slight impairment of XerC-

Figure 4.3. XerC and XerD C-terminal chimeras alter the resolution of synthetic HJ substrates. The HJ substrates were reacted with the indicated recombinases for 30 minutes at 37^{O}C. Linear rejoined duplex products are indicated XerC LR and XerD LR. HJCC and LCC bands indicate recombinase-HJ DNA and recombinase-linear duplex DNA covalent complexes, respectively. Electrophoresis was on a 6% polyacrylamide gel containing 0.1% SDS.


-
XerD
XerD[CII]
XerD[CIIe]
XerD[Ce]
-
XerC
XerC[DII]
XerC[DIIe]
XerC[De]
XerC
A
dif[GGGATG]
HJCC
HJ
LCC
XerCLR
B
dif[TGTATA]
HJCC
HJ
LCC
LCD
XerCLR
XerDLR
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HJ
LCC
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HJ
XerDLR
XerCLR
1 2 3 4 5 6 7 8

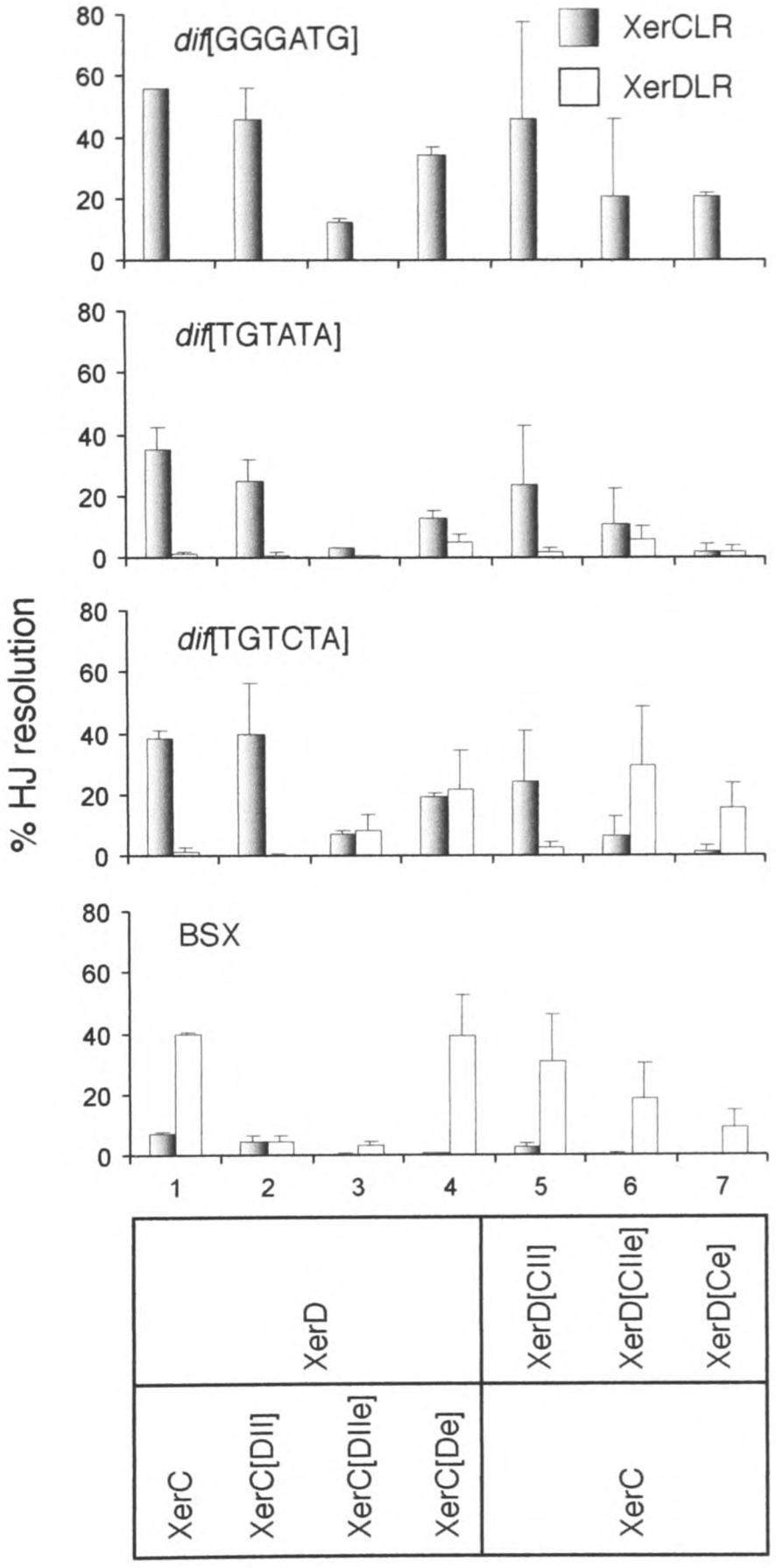


Figure 4.4. The nature of the phenotype of XerCD C-terminal domain variants depends on the HJ central region sequence. The results shown derive from two distinct experiments. The bars indicate the average percentage of the input HJ DNA converted to strand exchange product by XerC or XerD (dark and white, respectively) in 30 min reactions. Standard deviation lines are displayed above each bar.

mediated strand exchange (Figure 4.3, lane 6, and Figure 4.4). These results show that the Motif II regions of XerC and XerD are not functionally similar, and are consistent with previous observations indicating that these regions contribute to the acceptor interfaces of Xer recombinases (Hallet et al., 1999).

Chimeras XerC[De] and XerD[Ce], in which regions **e** were exchanged, stimulated XerD (or XerD-like) strand exchange on substrates that normally allow little XerD resolution, accompanied by a decrease in XerC-mediated catalysis (Figure 4.3, lanes 5 and 8 and Figure 4.4). Note that the higher degree of stimulation with *dif*TGTCTA than with *dif*TGTATA and the absence of stimulation with *dif*GGGATG can be correlated with the central region carried by these HJ substrates (see Chapter 3 and above). The phenotype exhibited by XerC[De] in HJ resolution experiments is comparable to those of XerC C-terminal deletions (Hallet et al., 1999), and is consistent with an alteration of the donor region in this chimera. However, the increased XerD-mediated catalysis shown by XerD[Ce] was not expected for a XerD variant that supposedly carry alterations in its donor region (**"e"**). The phenotype exhibited by XerD[Ce] was equivalent, although less pronounced, to that of the acceptor mutant XerDESS (Hallet et al., 1999). Finally, the phenotypes of XerC[De] and XerD[Ce] are consistent with these chimeras producing a switch of the HJ substrates to a conformation that is suitable for XerD catalysis. These data also suggest that the activation of both recombinases, XerC and XerD, has different requirements and show that the interactions within the XerC(donor) and XerD(acceptor), and XerD(donor) and XerC(acceptor) are distinct.

It was mentioned above that the most striking difference between the C-termini of XerC and XerD is the presence of four extra amino acid residues at the C-terminus of XerC (Figure 4.1-A). In order to evaluate if the phenotype exhibited by the chimera XerD[Ce] could be due to the presence of these four residues from XerC, the variant XerD[CKRGK] was tested in HJ resolution assays. However, XerD[CKRGK] exhibited a phenotype similar to that of the wild type XerD recombinase (data not shown). Although, a moderate decrease in XerD-mediated strand exchange was observed in these HJ resolution assays, which would be consistent with an alteration of the donor region in this chimera.

The chimera XerC[DIIe], in which both regions **II** and **e** were exchanged, exhibited a phenotype which is relatively similar to that of XerC[De] (Figure 4.3, lane 4-5, and Figure 4.4). This chimera is impaired in self-catalysis, but unlike XerC[De], it is not able to stimulate the partner XerD for catalysis, which would be expected of a XerC variant carrying an alteration in the "acceptor" region for XerD. Therefore, XerC[DIIe] appears to combine the phenotypes of XerC[DII] and XerC[De]. In other hand, the phenotypes of XerD[CIIe] and XerD[Ce] were apparently identical (Figure 4.3, lanes 7-8, and Figure 4.4).

4.2.3. Cooperative binding of the XerCD C-terminal chimeras to DNA

The ability of these recombinases to bind a linear *dif* recombination site was analysed by gel retardation experiments. Each chimeric recombinase was incubated on its own or in the presence of the wild type partner recombinase with a ^{32}P-labelled duplex substrate, containing the core *dif* site. Cooperative binding was measured by determining the ratio between the amount of single protein-DNA complexes (*dif* + Xer recombinase/chimera) over the amount of double complexes (*dif* + chimera + the

wild type partner) (Blakely et al., 1993; Spiers & Sherratt, 1997; Spiers & Sherratt, 1999).

Previous studies have demonstrated that the interactions between the C-terminus of XerC and the acceptor region of XerD are vital for cooperative binding (Spiers & Sherratt, 1999). Consistently, the XerC variants XerC[DIIe] and XerC[De] exhibited a drastic reduction in their ability to bind cooperatively in the presence of XerD (Figure 4.5, 3 and 5). Note also that these phenotypes are very similar to the phenotypes displayed by the XerC variants XerCΔ5 and XerCΔ10 {Figure 4.5, 4, and (Spiers & Sherratt, 1999)}, in which the last 5 or 10 C-terminal amino acid residues have been deleted, respectively (Figure 4.1-A, residues to the 3'-end of the arrows). The chimeras XerD[CII] and XerD[CIIe] also exhibited decreased cooperative binding in the presence of XerC, which would be expected for recombinases carrying acceptor mutations (Figure 4.5, 7-8). But surprisingly, chimeras XerC[DII] and XerD[Ce] exhibited noticeable decreases in cooperative binding to *dif* with XerD and XerC, respectively (Figure 4.5, 2 and 9). These results were not expected since the extrapolation from the cyclic interactions within the Cre nucleoprotein complex to the XerCD heterotetrameric complex did not envision that the XerD C-terminus would be necessary for cooperative binding to linear DNA with XerC (in the case of XerD[Ce]), nor that the acceptor of XerC would be required for cooperative binding with XerD (in the case of XerC[DII]; see below).

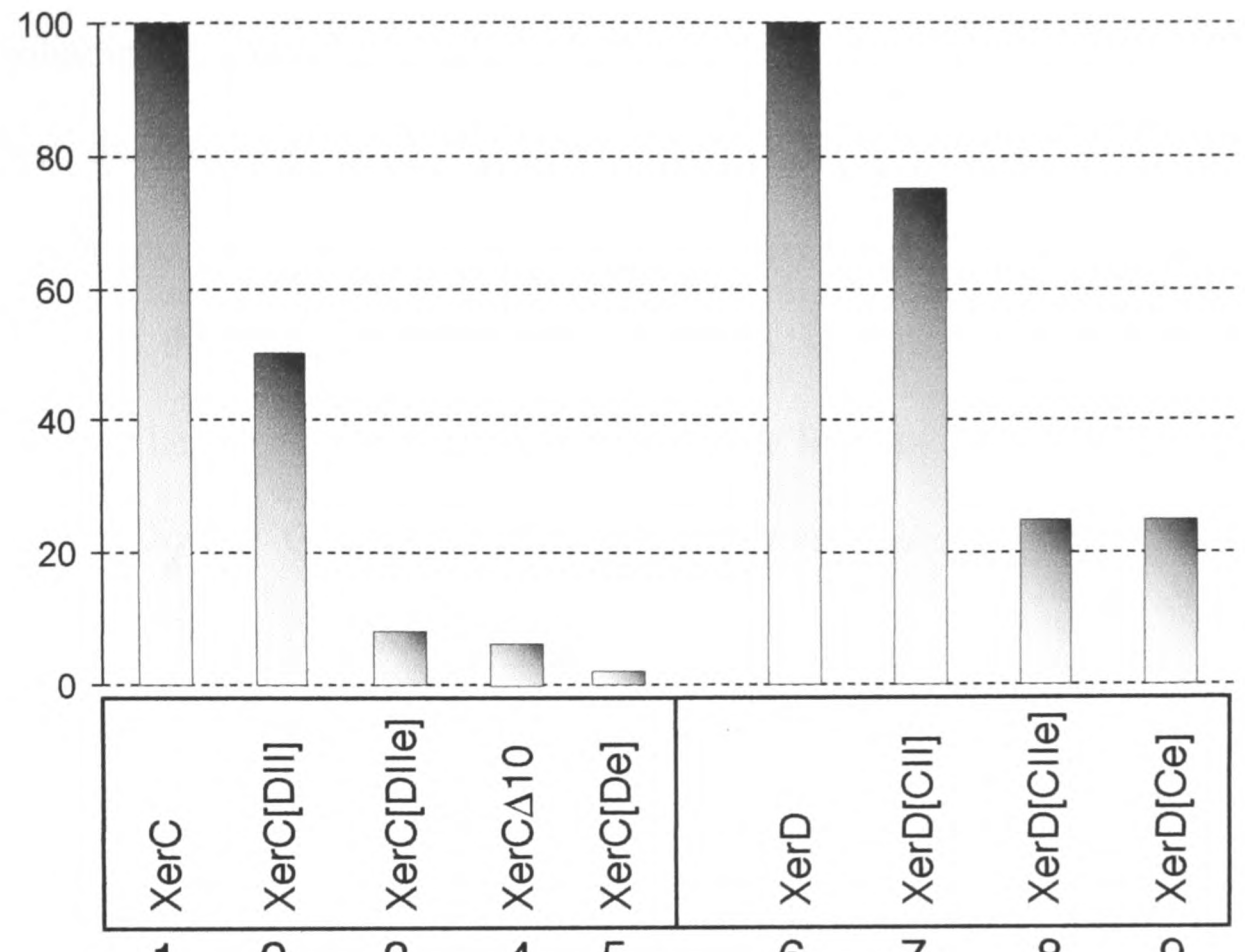
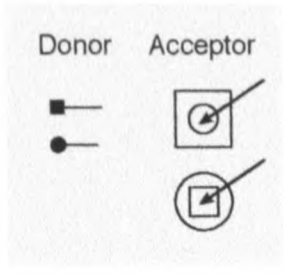


Figure 4.5. XerC and XerD C-terminal chimeras, and a XerC deletion variant, exhibit altered cooperative binding to linear *dif* DNA. The indicated recombinases were incubated with their wild type partners (XerC or XerD) on ice with linear *dif*TGTATA DNA. The concentration of the recombinases was determined by UV absorption at $O.D._{280nm}$ and estimated to be at saturating levels as judged by the activity of recombinase samples in HJ resolution assays. The bars indicate the average cooperative values estimated from three independent experiments using the ratios between the amount of recombinase-DNA binary complexes (*dif*TGTATA + Xer chimera or Xer wild type recombinase) over the amount of recombinase-DNA ternary complexes (*dif*TGTATA + Xer chimera + the Xer wild type partner).

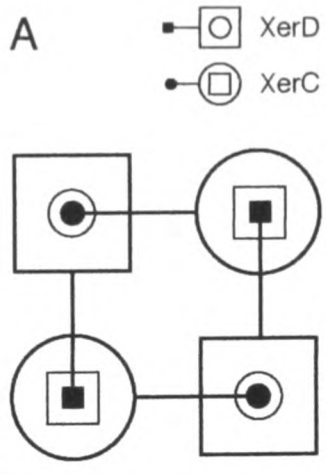
4.3. Discussion

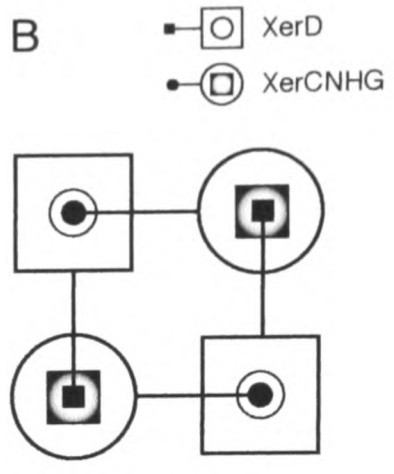
This Chapter describes experiments of recombination of reporter plasmids and resolution of synthetic HJ substrates *in vitro* using Xer chimeric recombinases in which the proposed acceptor and donor regions of XerC and XerD were exchanged. In both sets of experiments the phenotypes exhibited by these C-terminal variants suggested that the XerC and XerD acceptor and donor regions are functionally distinct. These propositions were based on the fact that the recombination reactions catalysed by the C-terminal chimeras, in conjunction with their wild type partner recombinases, are uncoordinated.

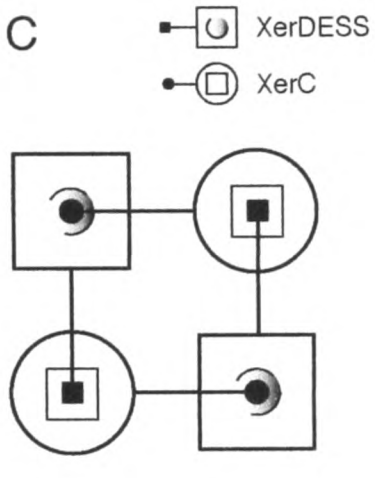
The currently favoured model for Xer recombination relies on the coordinated activation and inactivation of different pairs of recombinases during the reaction, which was proposed to be triggered by small changes in the conformation of the HJ-recombinase tetrameric complex {see diagram in section 4.1 and (Hallet et al., 1999)}. This coordination is believed to be dependent on the integrity of the cyclic "acceptor-donor" interactions among the four recombinases bound to a synaptic or HJ complex {Figure 4.6-A and (Gopaul & Duyne, 1999; Gopaul et al., 1998; Hallet et al., 1999)}. In support of this model is the phenotype exhibited by two classes of Xer mutants carrying alterations in their proposed acceptor or donor regions (Hallet et al., 1999; Spiers & Sherratt, 1999). The acceptor mutants, carrying amino acid substitutions within the conserved Motif II (Figure 4.6, XerCNHG and XerDESS, B and C, respectively), exhibit a stimulated self-catalysis, while impairing the catalysis by their partner recombinases. Opposite to that, the donor mutants, produced by C-terminal deletions of the Xer recombinases (Figure 4.6, XerCΔ5 or 10 and XerDΔ5 or 10, D and E, respectively), display an impaired self-catalysis, while stimulating the catalysis by the partner. When the acceptor-donor interactions are perturbed, the

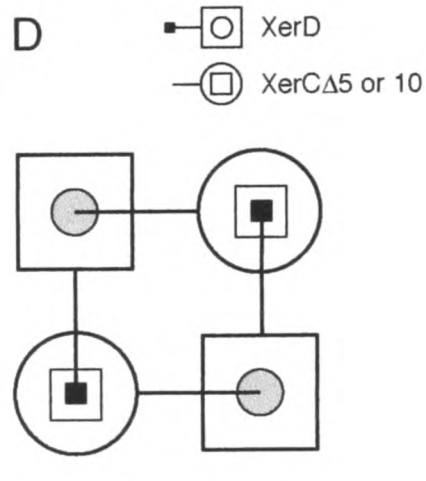


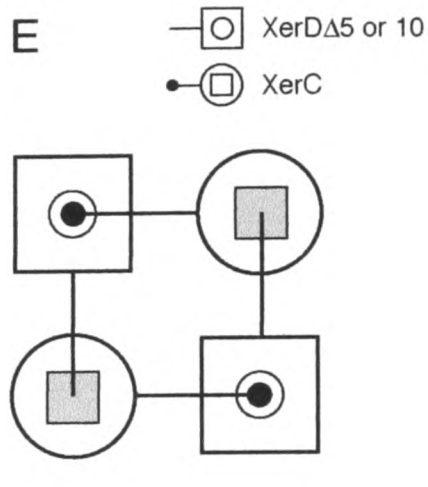
Wild type donor-acceptor interactions. View from the N-terminal domain side of the complex

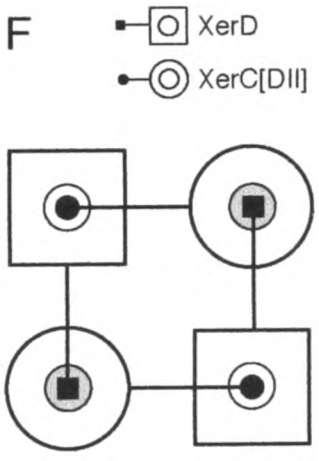


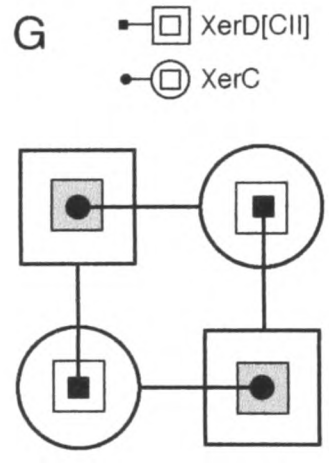


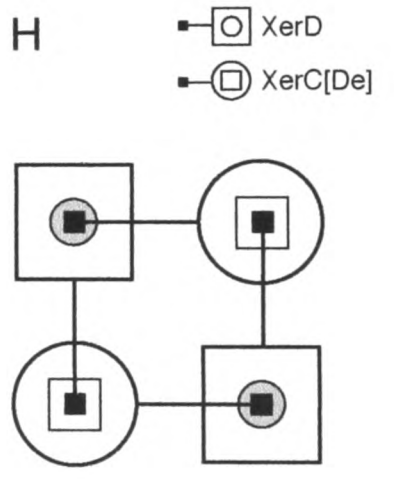


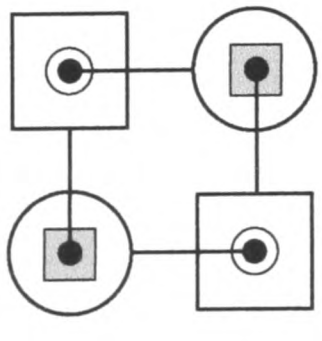


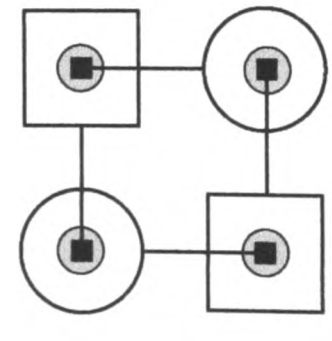












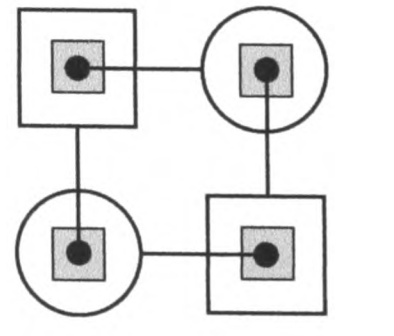


Figure 4.6. A model for the cyclic acceptor-donor interactions at the C-termini of XerCD recombinases. The schematic representation of the wild type XerC and XerD recombinases, and their acceptor and donor regions, is shown in A. The wild type cyclic acceptor-donor interactions are illustrated in (A), in which the donor region of each recombinase (black lines with either balls or squares, for XerC and XerD, respectively) contacts the acceptor region of the adjacent partner recombinase (white inner squares or circles, for XerC and XerD, respectively). Recombinases are viewed from their N-terminal domains. Altered acceptor-donor interactions are indicated by grey or dark acceptor regions, while normal interactions are demonstrated by clear acceptor regions (B-K). Phenotypes are described in the text.

coordination of catalysis might be altered or the recombination reaction is abolished (Hallet et al., 1999).

Based on the acceptor-donor model, the predicted phenotypes of the Xer C-terminal chimeras would be as follows: XerC[DII] and XerD[CII] (Figure 4.6, F and G, respectively) should exhibit stimulated catalysis, while impairing the activity of their partners, since they carry replaced Motif II regions, which are alterations expected to be equivalent to the tripeptide substitutions in the acceptor mutants XerCNHG and XerDESS (Hallet et al., 1999). XerC[De] and XerD[Ce] (Figure 4.6, H and I, respectively), with replaced donor regions ("**e**"), would exhibit the phenotypes of the C-terminal deletions of XerC and XerD, in which 5 or 10 C-terminal residues have been removed (Hallet et al., 1999; Spiers & Sherratt, 1999). The chimera XerD[CKRGK] could be allocated in the same category as XerC[De] and XerD[Ce], since it carries an altered donor region (Figure 4.6, I). Finally, chimeras XerC[DIIe] and XerD[CIIe] would combine both phenotypes of acceptor and donor mutants, since they carry a double replacement of Motif II and donor regions (Figure 4.6, J and K, respectively). These enzymes would be expected to exhibit an impaired self-catalysis and should not support strand exchange by their partner recombinases. However, the observed phenotypes for the Xer C-terminal chimeras differed from the predicted ones in some aspects (see below).

First of all, chimeras XerC[DII] and XerD[CII] did not display increased catalytic activities as was observed for the acceptor mutants XerCNHG and XerDESS (Hallet et al., 1999). One possible explanation for this could be that the exchange of the entire Motif II region in these chimeras (38 residues composing two and a half α-helices; Figure 4.1-A) might have caused perturbations on their donor regions. These perturbations could consequently alter their ability to stimulate their own catalytic

activities, in addition to the expected capacity to inhibit the partner. Consistent with this is the decreased cooperative binding of XerC[DII] and XerD to linear *dif* DNA, even though this chimera carries a XerC donor region. Nevertheless, XerC[DII] and XerD[CII] still exhibited a strong inhibition of catalysis by their wild type partners in HJ resolution experiments (Figure 4.4). Besides, the phenotypes of XerC[DII] and XerD[CII] on recombination of *psi* reporter plasmids were consistent with the alterations of the acceptor regions that they carry (Figure 4.2-A). XerC[DII] was able to initiate the recombination reaction on reporter plasmids, and the impairment of the second step of strand exchange by XerD led to a slight accumulation of HJ intermediates. In other hand, the lack of recombination observed when XerC was combined with XerD[CII] is consistent with this chimera inhibiting the initiation of the reaction by XerC.

Both XerC[DIIe] and XerC[De], which carry the donor regions of XerD, were impaired in self-catalysis and stimulated the activity of the wild type partner XerD in resolution of HJ substrates that are normally processed by XerC (Figure 4.4). In addition to this, the ability to bind co-operatively to linear *dif* DNA in the presence of XerD was abolished. As predicted, these phenotypes are very similar to the ones exhibited by the proposed donor mutants XerCΔ5 and XerCΔ10 (Hallet et al., 1999; Spiers & Sherratt, 1999). Note also that XerC[DIIe], which accumulates both acceptor and donor mutations, showed a very poor overall recombination. Despite the donor mutation, the chimera XerC[De] was able to support recombination of *psi* reporter plasmids (Figure 4.2-B). However, this recombination was only possible in the presence of 40% glycerol and with the participation of accessory sequences and proteins. These conditions are believed to help synapsis formation and might stimulate the accumulation of HJ intermediates (Barre et al., 2000; Colloms et al.,

1996). In a substrate in which recombination initiates by XerC-mediated strand exchange (pMIG103), HJs are rapidly converted to products by the stimulation of XerD strand exchange in the presence of XerC[De]; while on a substrate where recombination starts by XerD strand exchange (pMIG104K), HJs are accumulated by the increased XerD catalytic activity and the resolution to products by XerC[De] is impaired. Without the help of accessory sequences and proteins in the recombination of *dif*-containing reporter plasmids, the chimera XerC[De] was unable to support the formation of HJs. Finally, XerD[CKRGK] could also be allocated in this class of donor mutants, since it seems to be slightly impaired in XerD-like resolution of HJ intermediates. This is supported by the moderate accumulation of HJ intermediates in the recombination reaction using the *psi* reporter plasmid pSDC134 (Figure 4.2-A).

Surprisingly, the XerD C-terminal chimeras carrying the donor regions of XerC, XerD[CIIe] and XerD[Ce], did not exhibited phenotypes consistent with the donor mutations that they are supposed to harbour. The total lack of recombination of the *psi* reporter plasmids pSDC134 and pMIG103 at 10 or 40% glycerol, respectively, suggests that these chimeras could be inhibiting the first step of strand exchange by the wild type partner XerC, a phenotype that would be expected from an acceptor mutant such as XerDESS. And consistent with this is the apparent stimulation of XerD-mediated strand exchange by these chimeras on HJ resolution experiments, accompanied by an inhibition of catalysis by the partner XerC (Figure 4.4). However, when the chimera XerD[Ce] was tested with the partner XerC on pMIG104K, the *psi* reporter in which recombination is initiated by XerD-mediated strand exchange, no recombination was observed. Based on these results, it appears that the replacement of the donor region of XerD for the equivalent region from XerC caused a perturbation of the acceptor region of XerD[Ce]. This would explain, at least in part, why

XerD[Ce] does not support recombination of normal *psi* reporter plasmids and its stimulated catalysis on synthetic HJ resolution experiments. Also supporting this explanation is that XerD[Ce] exhibited a significant decrease in cooperative binding to linear *dif* DNA in the presence of XerC (which would require an intact acceptor in XerD[Ce]), and the similarities found in the phenotypes of XerD[Ce] and the "acceptor/donor" mutant XerD[CIIe] (Figures 4.4 and 4.5). The incapacity of XerD[Ce] to recombine pMIG104K might be due to the fact that this chimera still carries an altered donor region, which probably compromises its ability to assemble a productive synaptic complex with XerC {the XerD(donor) and XerC(acceptor) interactions are believed to be vital for synapsis formation; see Chapter 3 and (Spiers & Sherratt, 1999)}.

Although XerC[De] and XerD[Ce] were able to resolve synthetic HJ substrates *in vitro*, none of them could support resolution of HJs contained in the reporter plasmid pSDC124. One possible explanation for this would be that these C-terminal chimeras may not be able to operate any change of conformation of the HJ-containing plasmids to allow the XerD-mediated resolution (note that both XerC[De] and XerD[Ce] would stimulate XerD-mediated strand exchange). The HJ-containing reporter plasmids were produced by XerC-mediated strand exchange in the presence of 40% glycerol and ethidium bromide, and so far, these HJs could only be resolved to recombinant products by their re-incubation with XerC and XerD[ΔABCD] (Chapter 3). This XerD N-terminal deleted variant is believed to produce an overall change of conformation of the HJ-recombinase complex in which only XerD-mediated strand exchange can occur. Despite the strong ability exhibited by XerD[ΔABCD] to resolve synthetic HJ substrates (>80% XerD strand exchange), the resolution of HJ-containing plasmids is believed to be possible only if these substrates are not actually

supercoiled. This would imply that only relaxed HJ-containing molecules would be re-set to a conformation suitable for XerD-mediated strand exchange, whereas supercoiled intermediates of recombination would keep a conformation suitable for XerC-mediated catalysis (a barrier that might not be overcome by XerD[ΔABCD]). Since the resolution levels of synthetic HJs displayed by XerC[De] and XerD[Ce] are far less pronounced than those shown by XerD[ΔABCD], it is likely that the stimulation of XerD catalysis that can be produced by these C-terminal chimeras would not be sufficient to support the resolution of HJ-containing plasmids (the total recombination observed for these chimeras on synthetic *dif*TGTATA-HJ, which is the same HJ present in the HJ-containing plasmids pSDC124, was: <20% for XerC[De] + XerD, <4% for XerD[Ce] + XerC, and >80% for XerD[ΔABCD] + XerC; Figure 4.4 and Chapter 3).

The Xer C-terminal chimeras characterised in the present Chapter constitute valuable tools for the study of the mechanism of site-specific recombination mediated by XerC and XerD. These variants could be grouped into two previously defined classes of Xer mutants, the acceptor and donor mutants, and their phenotypes supplied further evidence to support the model in which recombination is achieved by a synchronised two-steps strand exchange process. Therefore, coordination of catalysis depends on the cyclic donor-acceptor interactions among the four recombinases bound to a synaptic or HJ complex. It was shown above that the acceptor and donor regions of the Xer recombinases are functionally distinct and that their replacements can lead to a complete loss of the recombination control.

CHAPTER 5

The ResD site-specific recombination reaction

5.1. Introduction

ResD is a tyrosine recombinase encoded by the F-plasmid of *E. coli* (Lane et al., 1986). The *resD* gene is the third component of the *ccd* (for coupled cell division) operon of F, which also carries genes *H* and *G*, whose products are involved in a killing process of plasmid-free cells (de Feyter et al., 1989; Lane et al., 1986; Ogura & Hiraga, 1983). ResD has been implicated in the conversion of F-plasmid dimers to monomers, and therefore, it could be involved in the stable maintenance of F-plasmid by ensuring its proper segregation into the daughter cells upon cell division (Disque-Kochem & Eichenlaub, 1993; Lane et al., 1986).

The DNA target or recombination site for ResD is located near the origin of replication *oriV1* of F-plasmid (also known as *ori-1* or *oriV*), which is the primary replication initiation site (O'Connor et al., 1986; O'Connor & Malamy, 1984). The identification of the ResD recombination site was based on extensive analyses of the ability of F-plasmid, and fusion derivatives between F and other replicons carrying the *oriV1* region, to participate in cointegrate/dimer formation and resolution. The recombination events leading to the monomerisation of F-plasmid dimers depend on the presence of an inverted repeat named *rfsF* (for replicon fusion site) within *oriV1*, since the deletion of part or the whole *rfsF* site can cause the complete abolishment of the ResD-mediated site-specific recombination observed (O'Connor et al., 1986). Besides, the analysis of several nucleotide sequences derived from junctions produced upon illegitimate recombination within *oriV1* of F-plasmid and other replicons led the authors to propose that *rfsF* could be the crossover site for the RecA-independent site-specific recombination mediated by ResD (O'Connor et al., 1986). O'Connor et al. (1986) have also shown that the deletion of sequences to the 3'-end of *rfsF* resulted in a significant decrease in recombination.

The ResD recombinase contains all the six catalytic residues that are considered a fingerprint of the tyrosine recombinase family {red labelled R-K-H-R-H-Y in Figure 5.1 (Sherratt & Wigley, 1998)}. ResD is also expected to share similarities in tertiary structure with other family members, since the similar folding of catalytic domains, necessary for the assembly of the catalytic site of these enzymes, has been proposed to be a general feature of this group (Chen et al., 2000; Guo et al., 1997; Hickman et al., 1997; Kwon et al., 1997; Sherratt & Wigley, 1998; Subramanya et al., 1997). This suggestion is supported by the fact that the predicted secondary structure of ResD matches almost perfectly the determined secondary structures of XerD and Cre recombinases (Figure 5.1). Note, however, that ResD lacks most of the region corresponding to the N-terminal domains of Cre and XerD (Figure 5.1).

The absence of N-terminal domains is also observed in the tyrosine recombinases FimB and FimE, which are the smallest members of this family (Nunes-Duby et al., 1998). Despite the fact that ResD and FimBE seem to be composed mainly of catalytic domains, and in the light of the emerging data showing that the N-terminal domains of the tyrosine recombinases might be involved in coordination of catalysis (Chapter 3), ResD and FimBE are apparently able to catalyse complete recombination reactions (Disque-Kochem & Eichenlaub, 1993; Klemm, 1986; Lane et al., 1986). Most notably, FimE is known to exhibit a strong directionality in recombination {see below (Gally et al., 1993; McClain et al., 1991)}.

The two related recombinases FimBE control the phase variation of type 1 fimbriae in *E. coli* (Klemm, 1986). This phase variation is dependent on the orientation of an invertible DNA segment (the *fim* switch), which contains the promoter that directs transcription of the *fim* subunit genes in two possible orientations: ON and OFF (Abraham et al., 1985; Klemm, 1984). DNA inversion

```
XerC   1   MTDLHTDVER -YLRYLSVER QLSPITLLNY QRQLEAIINF ASENGLQSWQ QCDVTMVRNF AVRSRRKGLG
XerD   1   MKQDLARIEQ -FLDALWLEK NLAENTLNAY RRDLSMMVEW LHHRGLTLAT AQSDDLQALL AERLEG-GYK
Cre   16   VDATSDEVRK NLMDMFRDRQ AFSEHTWKML LSVCRSWAAW CKLNNRKWFP AEPEDVRDYL LYLQAR-GLA
FimB       ---------- ---------- ---------- ---------- ---------- ---------- ----------
FimE       ---------- ---------- ---------- ---------- ---------- ---------- ----------
ResD       ---------- ---------- ---------- ---------- ---------- ---------- ---- 1 MSG

XerC   70  AASLALRLSA LRSFFDWLVS QNELKANPAK GVSAPKAPRH LPKN------ --IDVDDMNR LLDI--DIND
XerD   69  ATSSARLLSA VRRLFQYLYR EKFREDDPSA HLASPKLPQR LPKD------ --LSEAQVER LLQAP-LIDQ
Cre    85  VKTIQQHLGQ LNMLHRRSGL PRPSDSNAVS LVMRRIRKEN VDAGERAKQA LAFERTDFDQ VRSLMENSDR
FimB       ---------- ---------- ---------- 1 MKNKADNK KRNF------ --LTHSEIES LLKA--ANTG
FimE       ---------- ---------- ---------- 1 MS------ KRRY------ --LTGKEVQA MMQA--VCYG
ResD   4   SVIHSQSAVM VPAVYSAGQP ASLPVAIDYP AALALRQMSM VHDE------ --LPKYLLAP EVSALL-HYV

XerC   130 PLAVRDRAML EVMYGAGLRL SELVGLDIKH LDLES--GEV WVM---GKGS ---------- ------KERR
XerD   130 PLELRDKAML EVLYATGLRV SELVGLTMSD ISLRQ--GVV RVI---GKGN ---------- ------KERL
Cre    155 CQDIRNLAFL GIAYNTLLRI AEIARIRVKD ISRTDGGRML IHIGR-TKTL ---------- -VSTAG--VE
FimB   29  PHAARNYCLT LLCFIHGFRA SEICRLRISD IDLKA--KCI YIHR--LKKG ---------- ------FSTT
FimE   23  ATGARDYCLI LLAYRHGMRI SELLDLHYQD LDLNE--GRI NIRR--LKNG ---------- ------FSTV
ResD   65  P-DLHRKMLL ATLWNTGARI NEALALTRGD FSLAP--PYP FVQLATLKQR TEKAARTAGR MPAGQQTHRL

XerC   179 LPIGRN-AVA WIEHWLDL-- -RD---LFGS ED---DALFL SKLGKRISAR NVQKRFAEWG IKQGLNN-HV
XerD   179 VPLGEE-AVY WLETYLEH-- -GRPWLLNGV SI---DVLFP SQRAQQMTRQ TFWHRIKHYA VLAGIDSEKL
Cre    211 KALSLGVTKL VERWISVSGV ADDPNNYLFC RVRKNGVAAP SATSQLSTRA LEGIFEATHR LIYGAKDDSG
FimB   79  HPLLNK-EVQ ALKNWLSI-- -RTS-YPHAE S----EWVFL SRKGNPLSRQ QFYHIISTSG GNAGLSL-EI
FimE   73  HPLRFD-ERE AVERWTQE-- -RAN-WKGAD RT---DAIFI SRRGSRLSRQ QAYRIIRDAG IEAGTVT-QT
ResD   132 VPLSDSWYVS QLQTMVAT-- -LKIPMERRN RR---TGRTE KARIWEVTDR TVRTWIGEAV AAAAADGVTF

XerC   238 H------PHK LRHSFATHML ESSGDLRGVQ ELLGHANLST TQIYTHLDFQ HLASVYDAAH PRAKRGK--- -----
XerD   242 S------PHV LRHAFATHLL NHGADLRVVQ MLLGHSDLST TQIYTHVATE RLRQLHQQHH PRA------- -----
Cre    281 QRYLAWSGHS ARVGAARDMA RAGVSIPEIM QAGGWTNVNI VMNYIRNLDS ETGAMVRLLE DGD------- -----
FimB   139 H------PHM LRHSCGFALA NMGIDTRLIQ DYLGHRNIRH TVWYTASNAG RFYGIWDR-- --ARGRQRHA VL---
FimE   134 H------PHM LRHACGYELA ERGADTRLIQ DYLGHRNIRH TVRYTASNAA RFAGLWERNN LINEKLKREE V----
ResD   196 SVPVT--PHT FRHSYAMHML YAGIPLKVLQ SLMGHKSISS TEVYTKVFAL DVAARHRVQF AMPESDAVAM LKQLS
```

Figure 5.1. Amino acid sequence alignment of XerC, XerD, Cre, FimB, FimE and ResD tyrosine recombinases. The light and dark yellow boxes in the amino acid sequences of XerD and Cre correspond to α-helices and β-sheets, respectively, determined by X-ray crystallography (Subramanya *et al.*, 1997; Guo *et al.*, 1997). The grey boxes and underlined characters in XerC, FimB, FimE and ResD sequences represent predicted α-helices and β-sheets, respectively (PHDsec; Rost and Sander, 1993). The six catalytic site residues, characteristic of the tyrosine recombinase family, are shown in red. This alignment was optimized for the alignment of the secondary structures of these recombinases.

requires either FimB or FimE (Gally et al., 1996; Klemm, 1986; Kulasekara & Blomfield, 1999). Whereas FimB promotes recombination with little orientation bias, FimE promotes recombination in the ON-to-OFF direction exclusively (Gally et al., 1994; McClain et al., 1993; McClain et al., 1991). In addition to FimBE, normal frequencies of inversion of the *fim* switch also require the presence of other cellular factors such as the integration host factor IHF, the leucine-responsive regulatory protein Lrp, and the histone-like protein H-NS (Blomfield et al., 1993; Blomfield et al., 1997; Dorman & Higgins, 1987; Eisenstein et al., 1987; Gally et al., 1994; Kawula & Orndorff, 1991).

Unlike FimBE, very little is known about the function and the mechanism of recombination mediated by ResD. In this Chapter, a preliminary characterization of the ResD site-specific recombination is described. The *resD* gene was isolated from a F^+ *E. coli* strain and cloned in an expression vector. The DNA recombination site for ResD (Disque-Kochem & Eichenlaub, 1993; O'Connor et al., 1986) was also cloned and used for the construction of reporter plasmid substrates for the study of the ResD system *in vivo* and *in vitro*. ResD was shown to convert dimeric plasmid substrates, which contain two copies of the ResD recombination site oriented in direct repeat, to monomers *in vivo*. This recombination is preferentially intramolecular, since only monomerisation of the dimeric substrates was observed. The site-specific recombination reaction mediated by ResD *in vitro* is demonstrated here for the first time. Recombination of reporter plasmid substrates *in vitro* stalls at the HJ intermediate, with the generation of a product of unique topology. Data from both *in vivo* and *in vitro* experiments support the view that topological selectivity may be utilised by ResD. Deletion analyses of the ResD recombination sequence supply further evidence about the minimum DNA target site required for recombination.

5.2. Results

5.2.1. ResD resolves plasmid multimers *in vivo*

To study the molecular mechanisms of the site-specific recombination mediated by ResD, it was first necessary to isolate and clone its DNA coding region, and also the ResD DNA target recombination site. Both the *resD* gene and the ResD recombination site were isolated from a F^+ *E. coli* strain by PCR (Chapter 2). *resD* was cloned under the control of the arabinose inducible promoter in pBAD24 (Guzman et al., 1995), giving rise to pB*resD*, while the ResD recombination site was cloned into pK184 (Jobling & Holmes, 1990), which carries a p15a replicon compatible with ColEI-based expression vectors (such as pBAD24), producing pKDseq.

The activity of ResD was first evaluated by using a plasmid resolution assay *in vivo*. The F^- *recA*$^-$ *E. coli* strain DH5α was transformed with different combinations of the ResD expression vector (pB*resD*) and dimeric and tetrameric reporter plasmids containing two and four directly repeated ResD recombination sites (pKDseq dimers and tetramers, respectively; Chapter 2). After transformation, cells were grown for 12-14 h in the presence of the inhibitor of the arabinose promoter glucose (0.2% final concentration) or the inductor arabinose (0.2%) (Guzman et al., 1995). Plasmid DNA was purified and separated in 1% agarose gel electrophoresis.

Since DH5α has no DNA coding region for ResD, the pKDseq dimers and tetramers should be stably maintained inside this strain after transformation. As predicted, pKDseq monomers were not detected in plasmid purifications from DH5α transformed with the dimeric or the tetrameric plasmids (Figure 5.2, lanes 8 and 9,

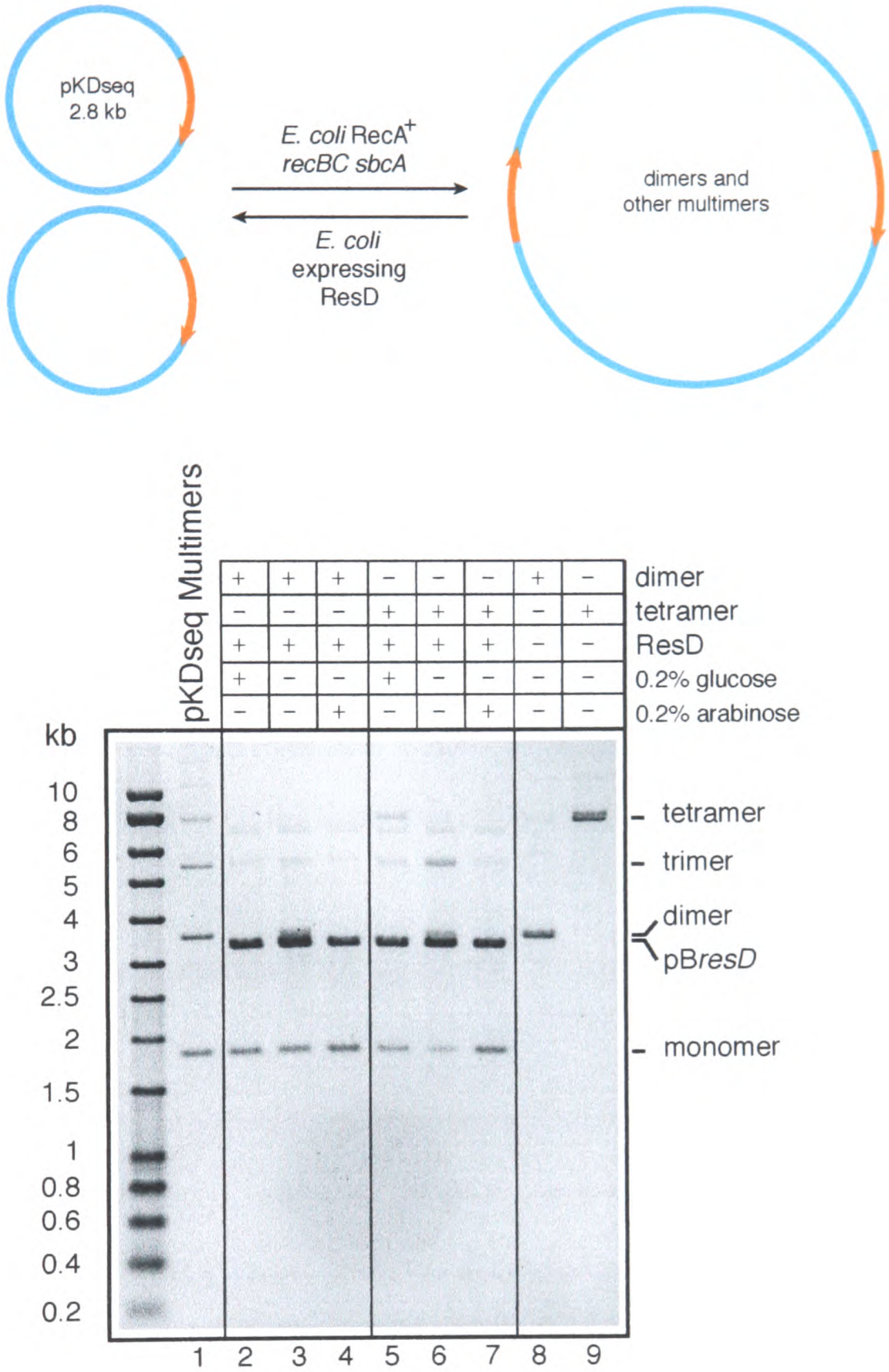


Figure 5.2. ResD resolves plasmid dimers containing two directly repeated ResD recombination sites. The *E. coli* strain DH5α, carrying the ResD expression vector pB*resD*, was transformed with dimeric and tetrameric pKDseq plasmids and grown for 14h in the presence of arabinose or glucose. Following growth, plasmid molecules were extracted and separated by 1% agarose gel electrophoresis. The transformation of dimers and tetramers into DH5α, not harbouring pB*resD*, resulted in the stable maintenance of these molecules inside the cells, since no monomers of pKDseq could be detected (lanes 8 and 9).

respectively). When pKDseq dimers and tetramers were introduced into a DH5α strain carrying pB*resD*, efficient resolution of the multimeric forms to monomers was observed (lanes 2-4 and 5-7). Resolution was detected even in the presence of glucose (lanes 2 and 5), indicating that ResD was being expressed at levels sufficient for the resolution of plasmid multimers. The fact that dimeric DNA species cannot be seen in lane 2 (compare lane 2 and 3) can be related to a lowering of plasmid copy number by the presence of glucose in the culture medium (our unpublished observations). Besides, dimers are expected to be more efficiently converted to monomers than tetramers, which was the case in these experiments (compare lanes 5-7).

These results show that the resolution of pKDseq multimers *in vivo* is dependent on the presence of the ResD expression vector pB*resD*.

5.2.2. Defining the minimum DNA segment of the *oriV1* region required for ResD recombination

The ResD mediated site-specific recombination at *oriV1* depends on the presence of two copies of the proposed crossover site *rfsF* within F-plasmid dimers (O'Connor et al., 1986) {Figure 5.4-A}. Deletion studies have also shown that additional sequences located to the 3'-end of *rfsF* appear to be required for efficient recombination (O'Connor et al., 1986). In these experiments, different segments of DNA were removed between *rfsF* and the region labelled R1 in Figure 5.3-A leading to a significant decrease in the efficiency of dimer resolution. Later, a further ResD-binding site was identified by DNaseI footprint nearby the region R1, which is located at ~150 bp away from the 3'-end of *rfsF* (Disque-Kochem & Eichenlaub, 1993) (Figure 5.3-A; the nucleotides protected from DNaseI cleavage are shown in yellow boxes within the regions R1 and R2 that will be discussed below).

A

rfsF

left arm → ACCCCAAG ← right arm

115 GAGATCCGAAA ACCCCAAG TTACGGATCTT

GAGATACAACA

TTTCGGATCTC
TTGAGGATTTT
TTACGCTTTGT
GTATGGATCCG
TTACTAAAATT
TTCCGGAAAGT
TTCCGGAAGTG
AGTCGGATCCA

```
  1 ACTCGTCGAC GCTATTAATC AGTTTTTTAG TTTTTGAAAA TTGTGATGCT TCTAAAATTA
    TGAGCAGCTG CGATAATTAG TCAAAAAATC AAAAACTTTT AACACTACGA AGATTTTAAT

 61 CTAAAATTGA GGATTTTAAT GCTACAACAA TGCCTGCCTC TTCTTATTTC TCCGGAGATC
    GATTTTAACT CCTAAAATTA CGATGTTGTT ACGGACGGAG AAGAATAAAG AGGCCTCTAG

                rfsF
121 CGAAAACCCC AAGTTACGGA TCTTCCTCTC CCTCCGCACA GCGTTACATC CCGTCAGCAC
    GCTTTTGGGG TTCAATGCCT AGAAGGAGAG GGAGGCGTGT CGCAATGTAG GGCAGTCGTG
                <-- rfsF-reverse primer

181 AGCATGTAGT GCCTCATACA GTTGCCCATG GCACTATATG TTGTGTTGTA TCTCTGGACT
    TCGTACATCA CGGAGTATGT CAACGGGTAC CGTGATATAC AACACAACAT AGAGACCTGA

                                                          R1
241 GTGATGCGCC GCGCAGGGGC GGAAAACAGC GATATGATGA TTTTCTCAGC GTTGTACACT
    CACTACGCGG CGCGTCCCCG CCTTTTGTCG CTATACTACT AAAAGAGTCG CAACATGTGA
                                                <-- R1-reverse primer

                                  R2
301 TCCGGAAAGT CGTTTATTCA AATAAAGTCG GATCCATACG AAACGGGAAT GCGGTAATTA
    AGGCCTTTCA GCAAATAAGT TTATTTCAGC CTAGGTATGC TTTGCCCTTA CGCCATTAAT
                <-- R2-reverse primer

361 CGCTTTGTTT TTATAAGTCA GATTTTAATT TTTATTGGTT AACTCTAGAG C...3'
    GCGAAACAAA AATATTCAGT CTAAAATTAA AAATAACCAA TTGAGATCTC G...5'
                --> Forward primer
```

Number of mismatches in the homologous sequence:

Blue, 2 out of 11 bp; Orange, 3; Violet, 3; Red, 4; Green, 4, and Magenta, 5.

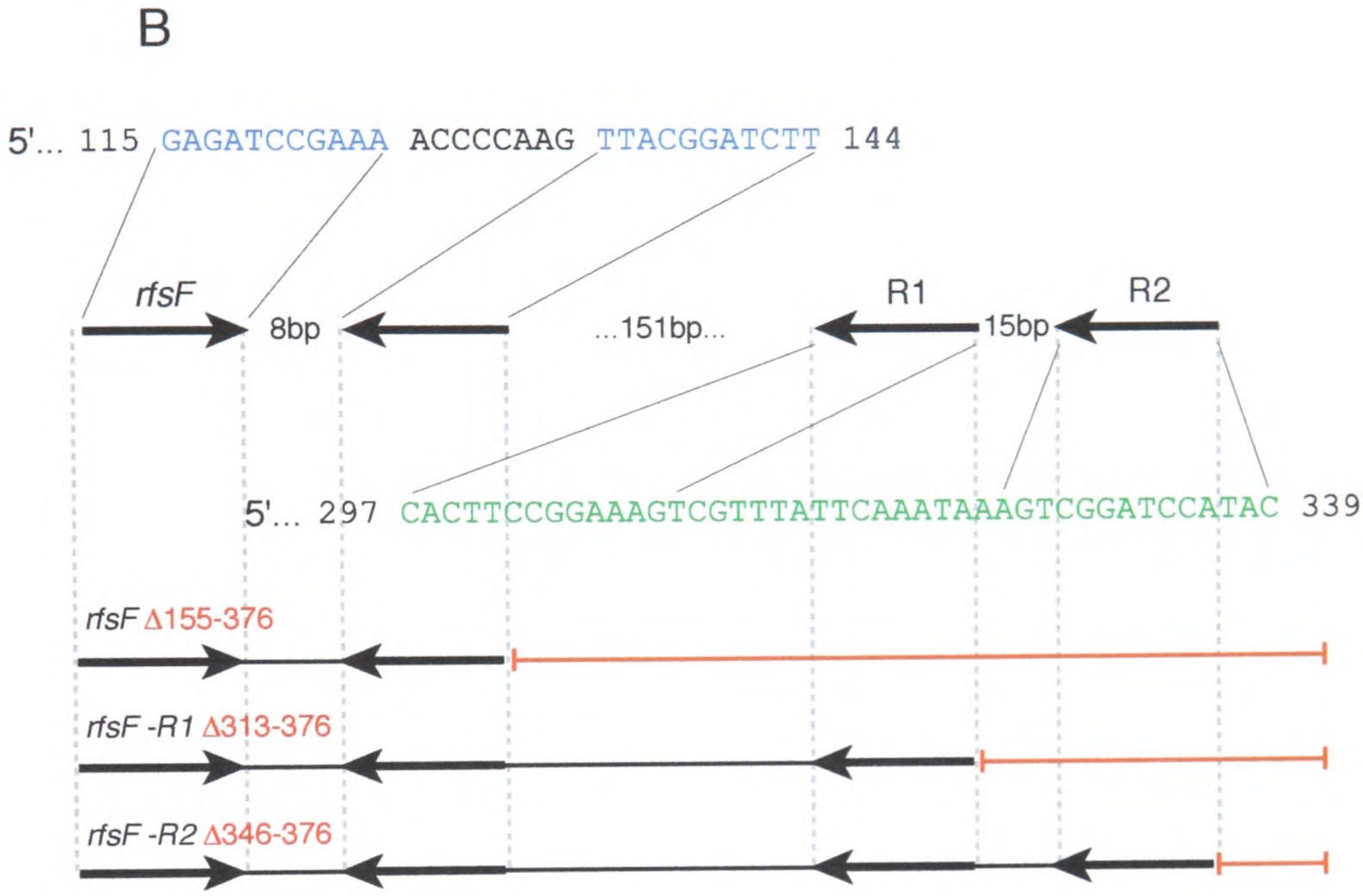


Figure 5.3. Determining the minimum ResD recombination site required for recombination. **A)** The ResD recombination sequence cloned in pKDseq showing the proposed crossover site "*rfsF*" and the regions R1 and R2 (grey boxes). The nucleotide sequences sharing similarities to the right or left arms of *rfsF* are displayed in different colours. Only sequences exhibiting no more than 5 mismatches when compared with either of the *rfsF* inverted repeats were considered in these analyses. The nucleotides protected from cleavage in the DNaseI footprint experiments of Disque-Kochem & Eichenlaub (1993) are shown in yellow boxes within R1 and R2 (see text). Internal deletions of the ResD recombination site were prepared by PCR using as DNA template pKDseq and combinations of the reverse primers "*rfsF*, R1 or R2" plus the "Forward primer" (arrows). **B)** A schematic representation of the internal deletions produced. The black arrows illustrate the inverted repeats of *rfsF*, and the nucleotide sequences within R1 and R2 that are homologous to the right arm of *rfsF*; the relevant DNA sequences and their coordinates are shown.

In an attempt to define precisely the minimum recombination site required by ResD, homology searches looking for nucleotide sequences sharing similarities to the right and left arms of *rfsF* within the *oriV1* region were performed. The rationale for this came from the current knowledge about site-specific recombination systems like γδ and Tn3 resolvase/*res*, in which accessory sequences for binding of further molecules of recombinase are necessary, besides the crossover sites, in order to assemble highly-ordered nucleoprotein synaptic complexes (Hallet & Sherratt, 1997; Sarkis et al., 2001; Stark & Boocock, 1995). At least 7 sequences could be found nearby the *rfsF* site of F-plasmid exhibiting 2 to 5 bp mismatches when compared with the 11 bp of the right arm of *rfsF* (Figure 5.3-A, orange, red, green and magenta nucleotide sequences). Only one sequence homologous to the left arm of *rfsF* was found with the search parameters used (violet). Note that 4 out of the 7 sequences homologous to the right arm of *rfsF* are localised around the ResD-binding site identified by the DNaseI footprint (Disque-Kochem & Eichenlaub, 1993). For simplification, the ResD-binding site and the four homologous sequences that were found flanking it will be divided in two groups: R1 and R2 (Figure 5.3-A, grey boxes).

In order to determine the requirement for the sequences within R1 and the ResD-binding site within R2 in ResD recombination, three pKDseq derivatives were constructed by PCR in which these regions were deleted as follows (Figure 5.3-B, the red lines show the regions that were removed): A) *rfsF*, which contains just the proposed crossover site; B) *rfsF*-R1, which contains the DNA segment from *rfsF* until the 3'-end of R1, and C) *rfsF*-R2, containing the DNA segment from *rfsF* until the 3'-end of R2. The pKDseq derivatives were transformed into the RecA$^+$ *recBC sbcA E.*

coli strain for multimerisation, following the isolation of the dimeric forms of the plasmids for *in vivo* experiments.

After the transformation of the dimeric pKDseq derivatives into the tester strain DH5α/pB*resD*, only the plasmids carrying "*rfsF*-R2" were converted to monomers (Figure 5.4, lanes 8 and 9). Note also that the efficiency of the monomerisation reaction showed here decreased considerably if compared to the first experiments *in vivo* (Figure 5.2). Only 50% of the dimers were converted to monomers in the presence of arabinose, while 100% conversion was observed when the original pKDseq plasmid was used (Figure 5.2, lanes 4 and 7).

In summary, it appears that the proposed crossover site *rfsF*, although strictly required, is not sufficient for ResD recombination. Secondly, the further binding site for ResD (contained within the R2) was demonstrated here to be necessary for recombination. Finally, the minimum site required for ResD recombination correspond to the entire *oriV1* region cloned in pKDseq (Chapter 2), since the absence of the DNA sequence to the 3'-end of R2 in the pKDseq derivative "*rfsF*-R2" led to a significant decrease in the efficiency of dimer resolution.

5.2.3. ResD recombination *in vitro*

In this section, the reconstitution of the ResD-mediated site-specific recombination *in vitro* is described. The reporter plasmid used, pK*resD*b (Figure 5.5-A), contains two copies of the ResD recombination site oriented in direct repeat (red arrows), and separated by a kanamycin cassette (yellow). The complete ResD-mediated recombination of pK*resD*b is expected to generate two smaller circles of different sizes (Figure 5.5-A, P1 and P2 of 2.84 kb and 1.7 kb, respectively), while the HJ intermediate, if it accumulates sufficiently to be visualised, can be differentiated from

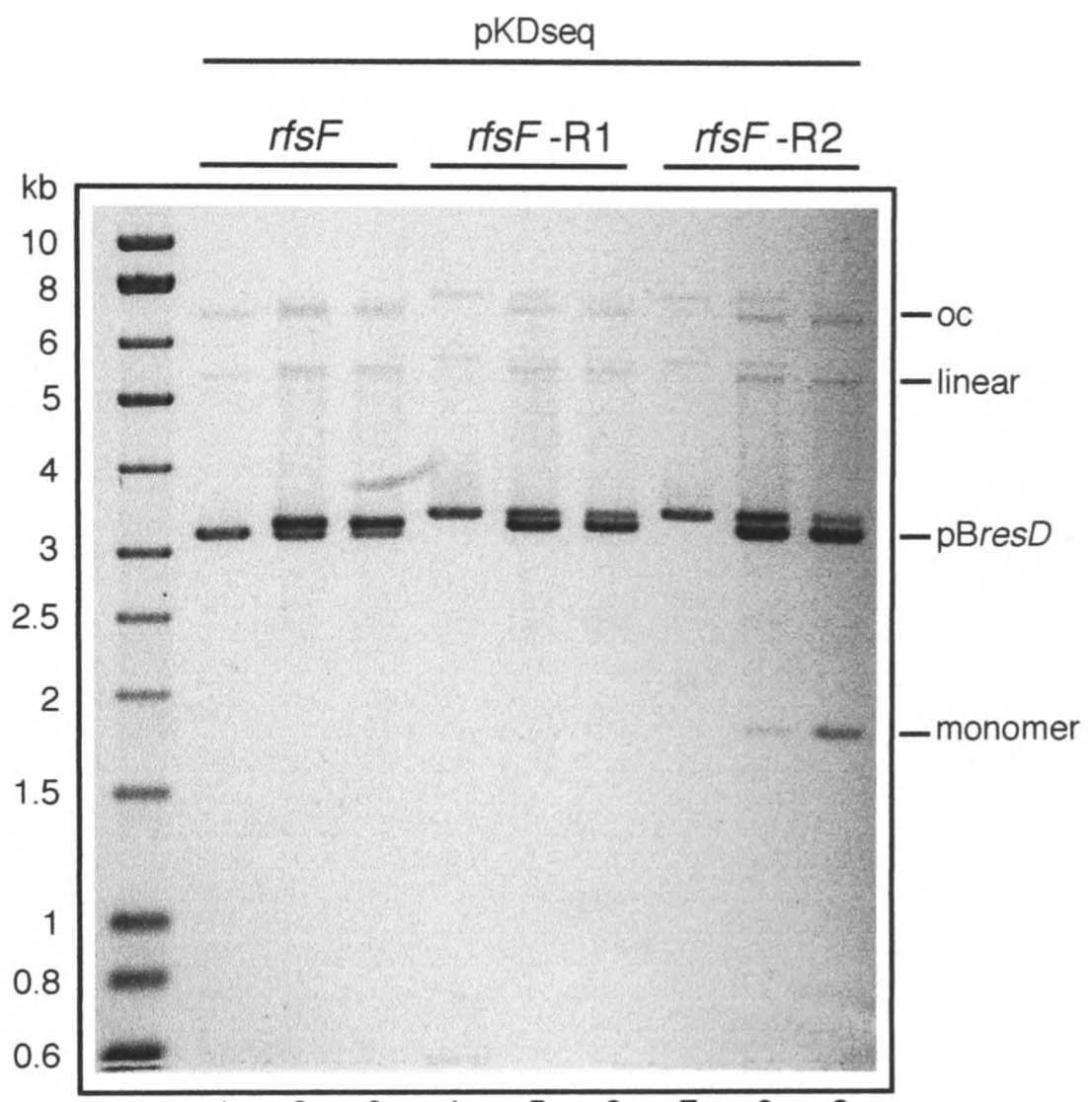
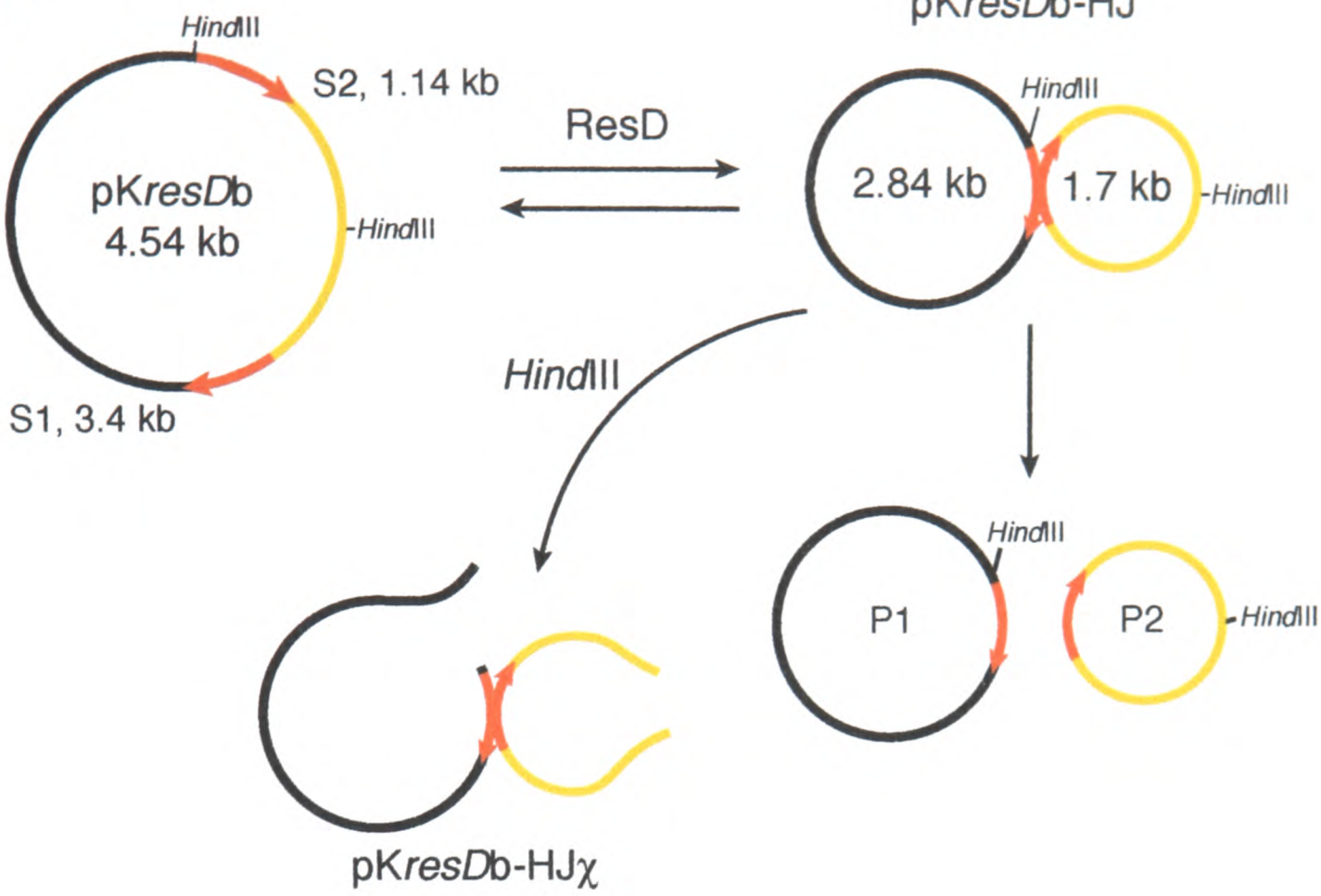


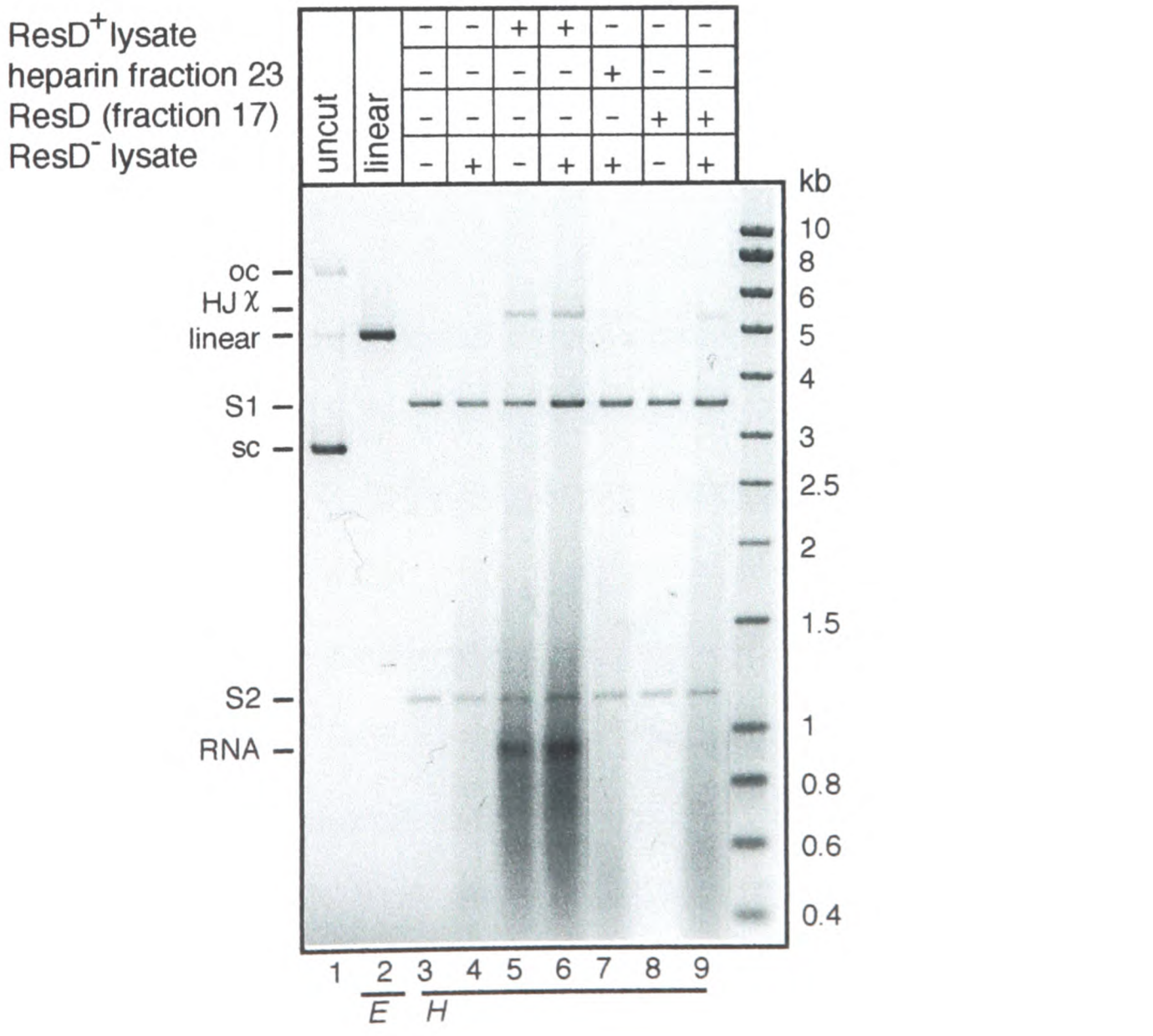
Figure 5.4. The minimum ResD recombination site required for ResD recombination contains the nucleotide sequence from *rfsF* until R2. The ethidium bromide-stained agarose gel above shows the products obtained in the ResD resolution assay *in vivo* using pKDseq dimers containing deletions within the ResD recombination sequence. pKDseq dimers carrying nucleotide deletions between *rfsF* and R2 (Figure 5.3) were transformed into the *E. coli* strain DH5α or DH5α/pB*resD*. Selected transformants were grown for 14h in the presence or absence of the inductor arabinose. After growth, plasmids were purified, separated by 1% agarose gel electrophoresis, and visualised by ethidium bromide staining.

Figure 5.5. ResD recombination *in vitro*. **A)** Schematic representation of the expected ResD-mediated recombination of a deletion reporter plasmid substrate containing two directly repeated ResD recombination sites (red arrows), separated by a kanamycin cassette (yellow segment). The first step of strand exchanges produces a HJ-containing molecule, and a further round of strand exchanges would resolve the HJ intermediate to two smaller circles (P1 and P2). **B)** Ethidium bromide stained 0.7% agarose gel electrophoresis of pK*resD*b samples incubated with different combinations of purified ResD (fraction 17), heparin fraction 23, $ResD^+$ lysate and $ResD^-$ lysate. Reactions were carried out in 50 mM Tris-HCl 7.5, 50 mM KCl, 1 mM EDTA and 5 mM spermidine for 1h at 37^OC. Prior to separation in gel, the reactions were stopped by phenol extraction and digested with the restriction enzyme *Hind*III (*H*). Linear plasmid was obtained by *Eco*RI (*E*) digestion. oc, open circle; HJχ, the *Hind*III-digested pK*resD*b-HJ intermediate of recombination; S1, substrate fragment of 3.4 kb; sc, supercoiled pK*resD*b; S2, substrate fragment of 1.14 kb; RNA, RNA present in the $ResD^+$ lysate.

A


pKresDb-HJ
HindIII
S2, 1.14 kb
ResD
pKresDb
4.54 kb
HindIII
2.84 kb
1.7 kb
HindIII
S1, 3.4 kb
HindIII
HindIII
P1
P2
HindIII
pKresDb-HJχ

B


ResD+ lysate
heparin fraction 23
ResD (fraction 17)
ResD- lysate
uncut
linear
kb
10
8
6
5
4
3
2.5
2
1.5
1
0.8
0.6
0.4
oc
HJ χ
linear
S1
sc
S2
RNA
1 2 3 4 5 6 7 8 9
E
H

fully recombinant products after digestion with the appropriate restriction enzymes (Figure 5.5-A).

The reporter plasmid was incubated with crude extracts from DH5α and DH5α/pB*resD* ($ResD^-$ and $ResD^+$ lysates, respectively), and the partially purified ResD recombinase (heparin fraction 17; see Chapter 2). Since a protein species of 26 kDa was found to be the major component in all of the fractions that were eluted from heparin, fraction 23, which contains mainly this protein, was also included in these analyses. Following the incubation for 1 h at $37^{O}C$, DNA samples were phenol purified, and digested with the restriction enzyme *Hind*III prior to gel electrophoresis. Recombination of the reporter plasmid was detected in the presence of the $ResD^+$ lysate and the purified recombinase by the production of a DNA species that migrates in agarose gel in the position expected for the pK*resD*b-HJ (the pK*resD*b-HJ was converted to a HJχ structure upon *Hind*III digestion; Figure 5.5 A and B, lanes 5 and 9). Approximately 25% and 10% of the input DNA substrate was converted to the pK*resD*b-HJ intermediate in the presence of the $ResD^+$ lysate and the purified recombinase, respectively. In these reactions, the products of a complete recombination reaction (P1 and P2) were not detected. Finally, no recombination products were obtained in the presence of the heparin fraction 23 (lane 7).

Recombination in the presence of the purified enzyme was only detected when the $ResD^-$ lysate was added to the reaction, which might suggest that a cellular cofactor, present in the $ResD^-$ lysate, is required for the ResD-mediated recombination (compare lanes 8 and 9). In an attempt to identify whether this cofactor could be a protein, the reporter plasmid pK*resD*b was incubated with purified ResD and the accessory proteins IHF, PepA and ArgR (Colloms et al., 1996; Hallet & Sherratt, 1997). Cell lysates from *fis*$^+$ and *fis*$^-$ *E. coli* strains (FIS^+ and FIS^- lysates,

respectively) were also included in these analyses, since FIS is a multifunctional factor known to be involved directly or indirectly in many different cellular processes such as replication, transcription, transposition and recombination (Finkel & Johnson, 1992; Hallet & Sherratt, 1997; Travers et al., 2001). None of the purified proteins used (IHF, PepA and ArgR) were able to stimulate recombination by ResD *in vitro* (Figure 5.6, lanes 6-10). However, the FIS^- lysate stimulated the recombination by the purified ResD to a similar level as that obtained in the reaction in which the $ResD^+$ lysate was used (compare lanes 2 and 5). The FIS^+ lysate was also able to stimulate ResD recombination, but to an almost imperceptible level (lane 4).

In order to further investigate any possible effect that FIS might have in the recombination reaction catalysed by ResD, a new set of experiments was carried out in which purified FIS was used instead of cell lysates. The addition of FIS to pK*resD*b recombination reactions containing either $ResD^+$ lysate or purified ResD had no detectable effects (Figure 5.7, lanes 5-8). The band corresponding to the linearised pK*resD*b reporter plasmid in lane 8 is probably a result of an inhibition of the *Hind*III digestion occasioned by interactions between FIS and the DNA substrate. From these experiments it can be concluded that FIS seems to have an indirect effect in the ResD recombination by possibly controlling the cellular levels of another component involved in the ResD-mediated reaction.

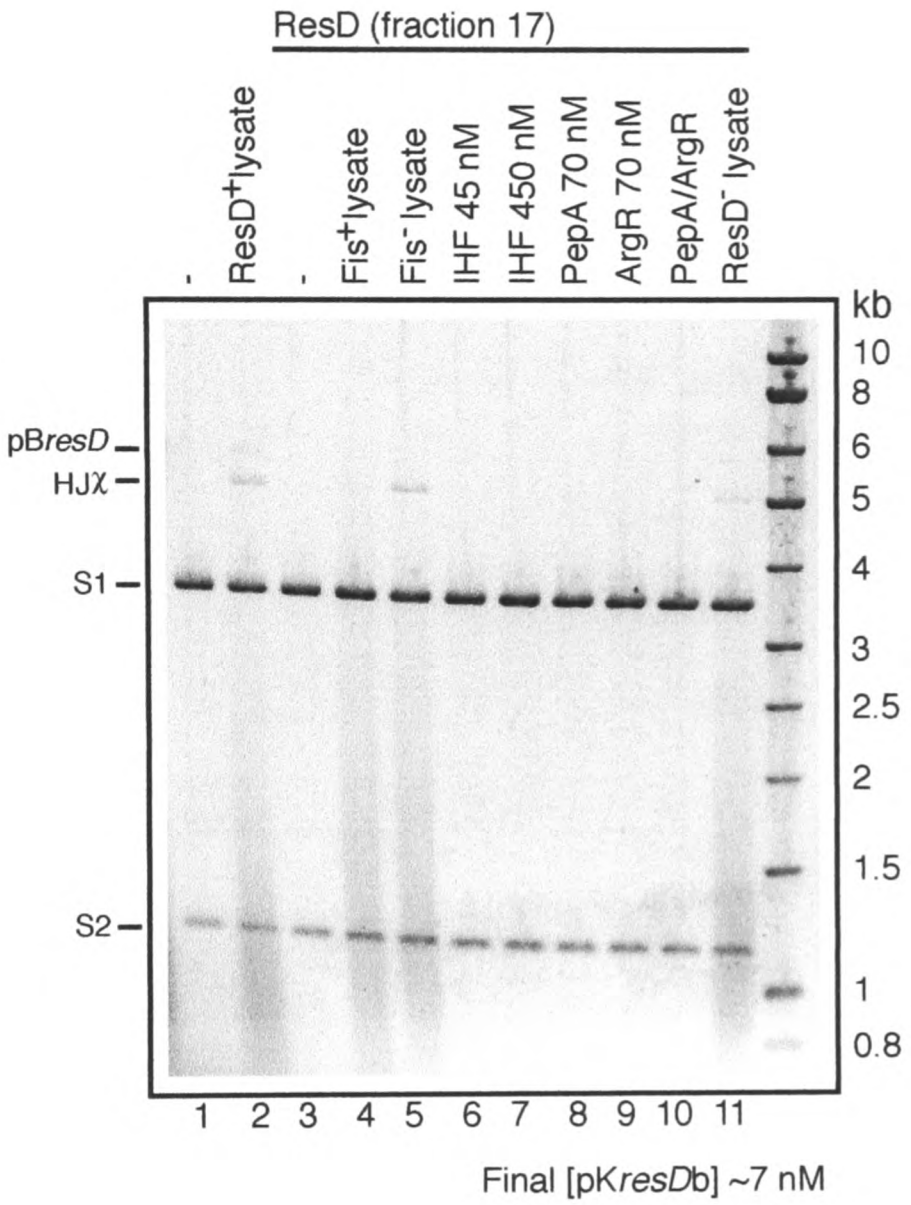


Figure 5.6. A FIS$^-$ cell lysate stimulates ResD recombination *in vitro*. Ethidium bromide stained 1% agarose gel electrophoresis showing the products obtained from the incubation of pK*resD*b with different combinations of purified ResD and IHF, PepA and ArgR, and also cell lysates from *fis*$^-$, *fis*$^+$ and *resD*$^-$ *E. coli* strains. The combinations are indicated at the top of each lane. Reactions were carried out in 50 mM Tris-HCl 7.5, 50 mM KCl, 1 mM EDTA and 5 mM spermidine for 1h at 37^{O}C. After incubation, reactions were stopped by phenol extraction, and DNA samples were digested with the restriction enzyme *Hind*III (*H*). HJχ, the *Hind*III-digested pKresDb-HJ intermediate of recombination; S1, substrate fragment of 3.4 kb; S2, substrate fragment of 1.14 kb.

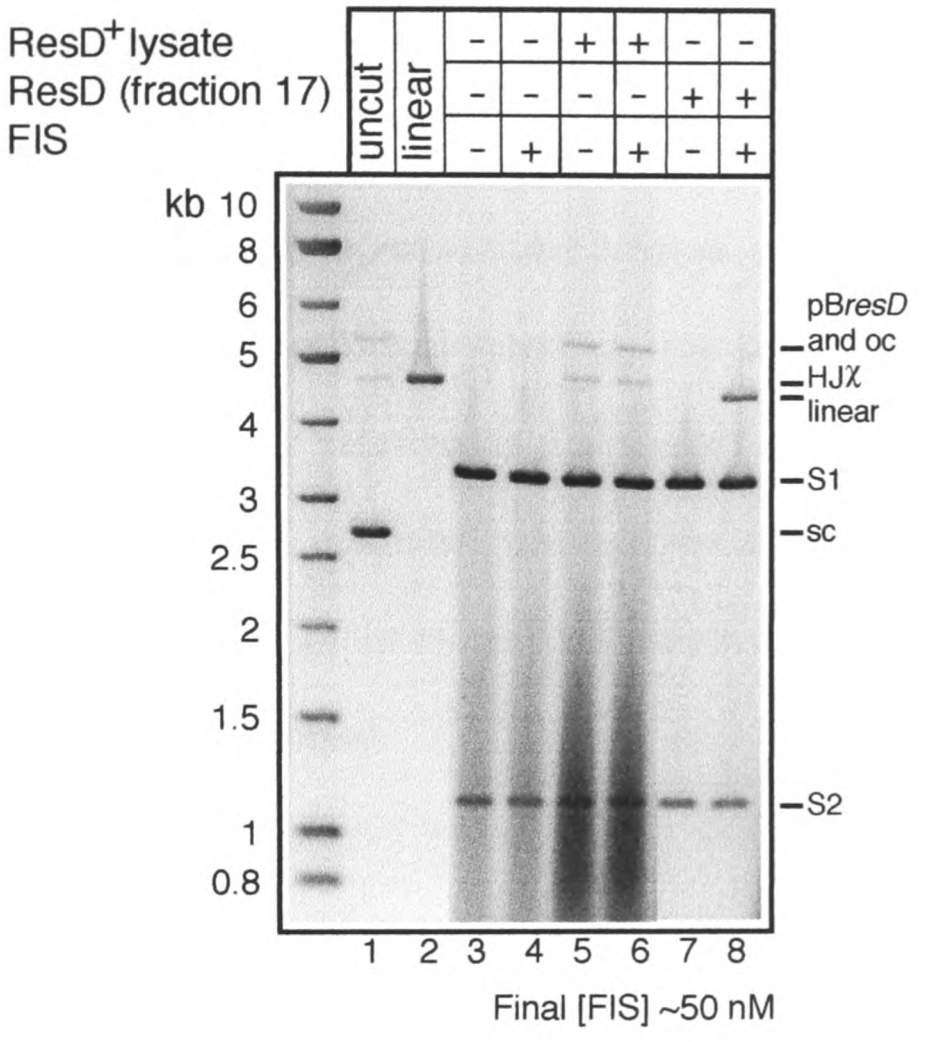


Figure 5.7. Purified FIS has no effect on ResD recombination *in vitro*. The 0.7% agarose gel electrophoresis above shows pK*resD*b samples incubated with either purified ResD or the ResD$^+$ lysate, in the presence or absence of FIS. Reactions were carried out in 50 mM Tris-HCl 7.5, 50 mM KCl, 1 mM EDTA and 5 mM spermidine for 1h at 37^{O}C. Prior to separation in gel, the reactions were stopped by phenol extraction and digested with the restriction enzymes *Hind*III (*H*) and *Eco*RI (*E*) as indicated. oc, open circle; HJχ, the *Hind*III-digested pK*resD*b-HJ intermediate of recombination; S1, substrate fragment of 3.4 kb; sc, supercoiled pK*resD*b; S2, substrate fragment of 1.14 kb.

5.2.4. The HJ intermediate generated by ResD has a unique topology

One important feature of a system that is supposedly involved with dimer resolution is whether it exhibits topological selectivity during recombination. Such a feature would guarantee that multimers formed, for example, by homologous recombination are resolved preferentially by intramolecular deletion, and that intermolecular fusions are avoided. The use of topological selectivity can be inferred by the analysis of the topology of the products generated in a particular recombination reaction (Colloms et al., 1997). In order to determine whether The ResD system exhibits topological selectivity, the topology of the pK*resD*b-HJ intermediate produced by ResD-mediated recombination was examined.

The method employed was previously used for the study of the topology of *cer*-containing HJ intermediates from XerCD-mediated recombination (Colloms et al., 1997). The deletion reporter plasmid pK*resD*b was first incubated with the ResD$^+$ lysate, following the purification of the DNA sample by phenol extraction. One part of this sample was digested with the restriction enzyme *Hind*III, to confirm the formation of the HJ intermediate, and the remaining DNA was nicked with DNaseI to determine the topology of this product. The resulting DNA samples were analysed by 0.7% agarose gel electrophoresis alongside control *cer*-HJ intermediates containing 2, 3, 4, 5, 6 and 8 nodes trapped by the first event of strand exchange. The *cer*-HJ intermediates in which 3 or 5 nodes have been trapped were obtained by XerCD-mediated recombination of an inversion *cer*-containing reporter plasmid in which accessory sequences have been placed adjacent to the XerD-binding site instead of the wild type location by the XerC-binding site (M. Bregu, D.J. Sherratt and S.D. Colloms, unpublished data).

As described in the previous section, the incubation of the reporter plasmid with the ResD$^+$ lysate converted ~25% of the input DNA substrate to the HJ intermediate (Figure 5.8, lane 3, band 6). DNaseI digestion of this sample generated the following bands from top to bottom in lane 5: 11, the nicked open circle pB*resD*; the nicked open circle pK*resD*b (as 8 in lane 4); the linear pK*resD*b (as 9 in lane 4), and bands 12 and 13, which co-migrate relatively with the control *cer*-HJ figure-8 band 15 (lane 6). Note that the appearance of bands 12 and 13 correlated with the disappearance of the pK*resD*b-HJ band 6 (lane 3). The DNA species 12 and 13 are proposed to be the pK*resD*b-HJ figure-8 (the relaxed form of the HJ intermediate produced by DNaseI nicking into the region of homology close to the branch point) and a 2-noded pK*resD*b-HJ, respectively. The rationale for these propositions is as follows: first, the *cer*-reporter plasmid substrates used to produce the various forms of HJs with different numbers of nodes trapped are slightly smaller than pK*resD*b; therefore, the known product *cer*-HJ figure-8 (lane 6, band 15), also generated by DNaseI nicking inside the region of homology of the HJ, would be expected to migrate ahead of the pK*resD*b-HJ figure-8 and behind the 2-noded pK*resD*b-HJ. Second, the 2-noded *cer*-HJ, if visible in this gel, would migrate slightly ahead of the *cer*-HJ figure-8 (band 15) and the 2-noded pK*resD*b-HJ (band 13).

From these analyses it can be concluded that the pK*resD*b-HJ, a product of the ResD-mediated recombination *in vitro*, has a unique topology. Based on these results, it can be suggested that the ResD system, like the Xer/*psi*/*cer* and γδ/Tn3 resolvase/*res* systems {reviewed by (Hallet & Sherratt, 1997)}, may utilise topological selectivity to ensure that its recombination reaction will take just one pathway, which leads to the resolution but not multimerisation of F-plasmid.

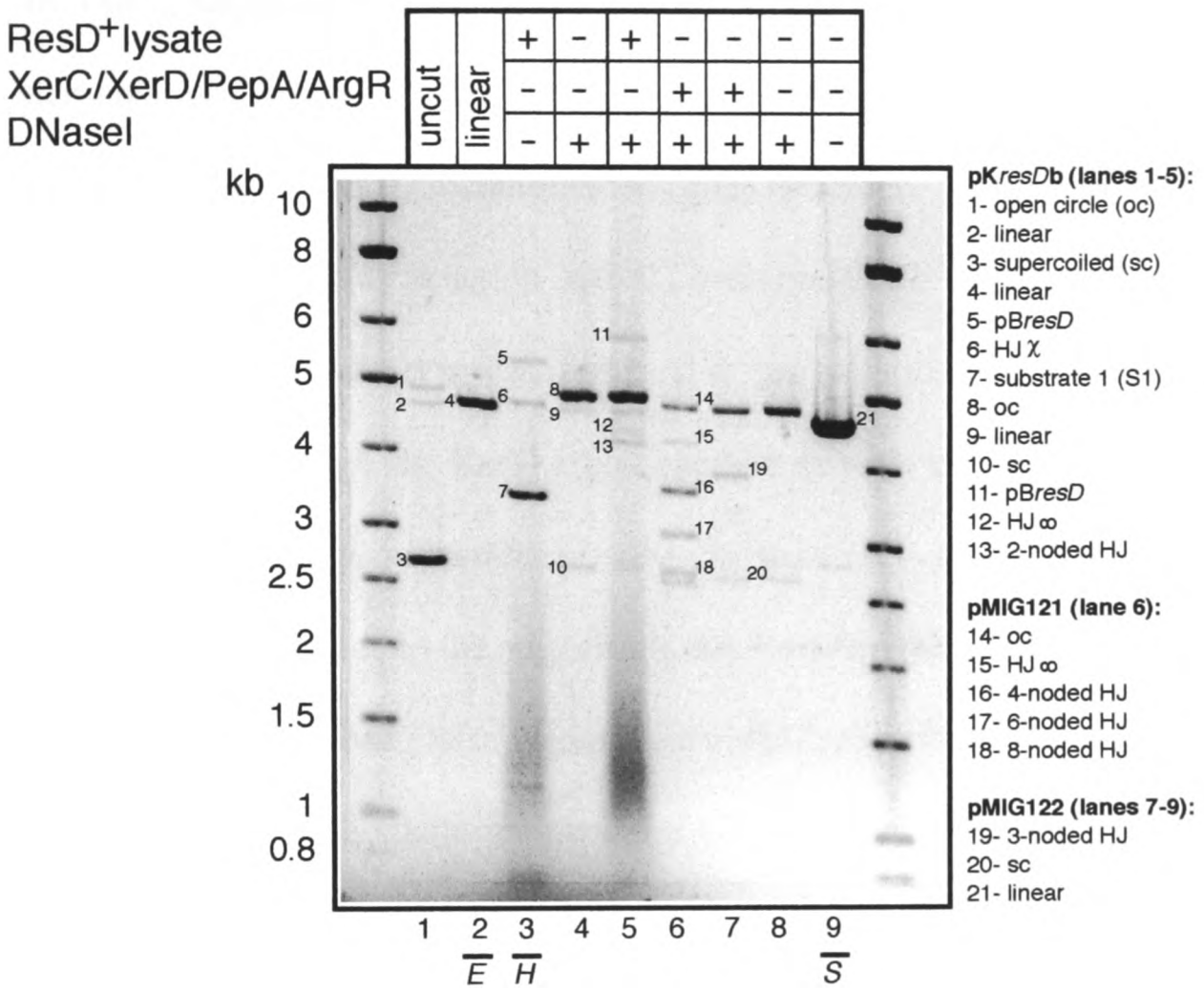


Figure 5.8. The pK*resD*b-HJ intermediate generated by ResD-mediated recombination *in vitro* has a unique topology. The reporter plasmid pK*resD*b was incubated with ResD^{+} cell lysate in 50 mM Tris-HCl 7.5, 50 mM KCl, 1 mM EDTA and 5 mM spermidine for 1h at 37^{O}C. Reactions were stopped by phenol extraction, and recovered DNA was treated with DNaseI as indicated, prior to separation in a 0.7% agarose gel. Relevant DNA species are numbered and described on the right of the figure. The *cer* reporter plasmids pMIG121 and pMIG122 were used as markers for 4-, 6-, 8-, and 3-noded HJ intermediates of recombination. *E, H* and *S* indicate samples treated with the restriction enzymes *Eco*RI, *Hind*III and *Sca*I, respectively.

5.3. Discussion

ResD site-specific recombination

In this Chapter, the site-specific recombination reaction catalysed by ResD was demonstrated *in vitro* for the first time. ResD recombines reporter plasmid substrates containing two ResD recombination sites oriented in direct repeat. The main characteristics of this reaction are: 1) recombination appears to be preferentially intramolecular, since no multimerisation of the reporter plasmid substrates could be detected; 2) although the ResD recombination *in vitro* was reasonably efficient, only HJ intermediates were produced, and 3) these HJ intermediates have a unique topology, which leads to the suggestion that ResD might utilise topological selectivity during recombination. These features are consistent with the proposed function of the ResD system in the stable maintenance of F-plasmid in *E. coli* (Disque-Kochem & Eichenlaub, 1993; Lane et al., 1986). However, the reason why the ResD recombination *in vitro* stalls after the first step of strand exchanges with the formation of the HJ intermediate is less clear.

Similar results have also been reported for the XerCD recombination of *cer*-containing reporter plasmids *in vitro* and *in vivo* (Colloms et al., 1996; McCulloch et al., 1994). The *cer*-HJ intermediates are produced by the first step of strand exchanges mediated by XerC, and they can be resolved to recombinant products *in vivo* by the action of other cellular factors other than XerD (Colloms et al., 1996). It is possible that ResD would use a similar mechanism in which recombination stalls at HJ and other cellular factors are required in order to resolve these intermediates to products. Consistent with this is the fact that only recombinant products were detected in the ResD recombination assays *in vivo*, without the accumulation of HJ intermediates. Another possible explanation could be that the HJ produced is unable to undergo a

required change of conformation, which would allow the second step of strand exchange to take place. The ability of the HJ complex to undergo a change of conformation appears to be related to the nucleotide sequence at the HJ branchpoint, where the purine-rich strand has a higher propensity to adopt a conformation suitable for cleavage and strand exchange (Arciszewska et al., 2000; Azaro & Landy, 1997; Gopaul et al., 1998). These observations were exemplified in the experiments of synthetic HJ resolution described in Chapter 3. By using the wild type XerCD recombinases it was shown that HJ intermediates containing the wild type *dif* site, *dif*TGTATA, are efficiently resolved by XerC, but are very poor substrates for XerD, whereas those HJ intermediates carrying a *cer* central region variant, *dif*GGGATG, undergo strand exchange by XerC exclusively (the DNA sequences TGTATA, 50% purines, and GGGATG, ~85% purines, correspond to the central regions of the strands that are cleaved by XerC). Note that similar to *dif*, the central region of the proposed ResD crossover site *rfsF* also exhibits a 50% purine content (5'-ACCCCAAG-3'; Figure 5.3-A). By analogy to the *dif*-containing HJ substrates, it might be possible that a HJ containing the *rfsF* central region would be less prone to undergo a change of conformation to allow the second step of strand exchange to take place.

The recombination reaction mediated by ResD requires in addition to the crossover site *rfsF* a further ResD binding site located at ~150 bp away from the 3'-end of *rfsF*. The deletion of different DNA segments between *rfsF* and this ResD binding site may lead to a decrease in the efficiency of F-plasmid dimer resolution (O'Connor et al., 1986). Here, it was demonstrated that the deletion of the ResD binding site contained within the region R2 (Figure 5.3-A) completely abolished the recombination by ResD *in vivo*; a result also observed when the crossover site itself

was deleted (O'Connor et al., 1986). Therefore, the ResD binding site at R2 is proposed to be an accessory-binding site for ResD, which is necessary for recombination (Figure 5.3-B).

This particular organisation of recombination sites has been found in other systems such as Xer/*cer*/*psi*, γδ and Tn3 resolvases, DNA invertases and bacteriophage Mu {reviewed by (Hallet & Sherratt, 1997)}. In these systems, recombination is only possible upon the assembly of highly ordered nucleoprotein complexes, which are believed to activate the recombinases for catalysis, and to impose directionality to the reaction (Blake et al., 1997; Colloms et al., 1997; Hallet & Sherratt, 1997; Stark & Boocock, 1995). The assembly of these nucleoprotein complexes requires the participation of DNA accessory sequences adjacent to the crossover sites, which are bound either by the recombinase itself, in the case of γδ and Tn3 resolvases, or by cellular accessory proteins in other system (Hallet & Sherratt, 1997; Stark & Boocock, 1995). It is also likely that ResD, and possibly other accessory proteins, would bind to the accessory sites nearby R2 in order to assemble a highly ordered nucleoprotein complex, which would stimulate the ResD-mediated recombination at *rfsF*. Consistent with this is the fact that the HJ intermediate formed by ResD recombination *in vitro* has a unique topology (see below).

In support of the idea that the ResD recombination site consists of the crossover site *rfsF* and further accessory sequences is the observation that (an)other cellular component(s) appears to be required for ResD recombination. This proposition is based on the fact that the ResD recombination reaction reconstituted *in vitro* was only possible in the presence of *E. coli* cell lysates. Several cellular components have been demonstrated to participate as accessory proteins during site-specific recombination reactions catalysed by members of the tyrosine and serine

recombinase families. Some examples are PepA, ArgR, ArcA-P and FtsK in the Xer system, different combinations of IHF, FIS, Lrp and H-NS in the Fim, λ, Gin- and Hin-invertase and bacteriophage Mu systems, and further molecules of the recombinase in the γδ and Tn3 resolvase systems (Barre et al., 2000; Blomfield et al., 1993; Blomfield et al., 1997; Colloms et al., 1998; Hallet & Sherratt, 1997; Kawula & Orndorff, 1991; Landy, 1989; Stark & Boocock, 1995). A selection of some of these accessory proteins (PepA, ArgR, IHF and FIS) was tested for the ability to activate the purified ResD for catalysis. However, all of them failed to stimulate the ResD recombination *in vitro*. The involvement of FtsK in the activation of ResD was also examined using the ResD/pKDseq dimer resolution assay. FtsK is a septum-located protein that has been demonstrated to stimulate Xer recombination at *dif in vitro* and *in vivo* through a mechanism apparently different from the architectural functions proposed for PepA and ArgR {(Barre et al., 2000; Colloms et al., 1997; Recchia et al., 1999) and L. Aussel, F.X. Barre, M. Aroyo and D.J. Sherratt, *in press*}. When pKDseq dimers were transformed into a *ftsK*⁻ *E. coli* strain expressing ResD, 100% resolution of the reporter plasmid dimers to monomers was observed, showing that this protein is not necessary for ResD recombination either (data not shown).

Surprisingly, a cell lysate prepared from an *E. coli* strain defective for FIS stimulated the purified ResD, which suggests that FIS might have an indirect effect on ResD recombination. FIS is a highly abundant DNA bending protein whose expression levels are regulated depending on the bacterial growth phase (Ball et al., 1992; Ninnemann et al., 1992). FIS is also a multifunctional factor known to be involved directly or indirectly in several cellular processes, such as gene expression, replication, DNA inversion, phage integration and excision, transposition, etc. (Finkel & Johnson, 1992; Richins & Chen, 2001; Travers et al., 2001; Ussery et al., 2001). It

is likely that a cell lysate from a *fis*⁻ *E. coli* strain could stimulate ResD recombination by maybe having an increased amount of a component that is required for this reaction, and that might be negatively controlled in the presence of FIS. However, the cellular component(s) involved cannot be inferred from these preliminary analyses.

A model for the ResD synaptic complex

A direct consequence of the use of topological selectivity, in which recombination can only take place if a defined synaptic complex is assembled, is the unique topology of the recombinant products that will arise from the reaction (Colloms et al., 1997; Stark & Boocock, 1995). The HJ intermediate generated by ResD *in vitro* was proposed to have 2 nodes trapped, whereas the fully recombinant product, if also obtained *in vitro*, would probably be a 2-noded catenane. These results, together with the fact that ResD apparently requires accessory sequences and other cellular accessory factors for recombination, fit perfectly with the idea that ResD may utilise topological selectivity during recombination. The topological filter model was proposed to explain the selectivity and specificity observed in the recombination reactions catalysed by the serine recombinase members γδ and Tn3 resolvases (Stark & Boocock, 1995). In this model, directly repeated recombination sites are brought together with three negative nodes being trapped in the synaptic complex; strand exchange at the core sites introduce a positive node with the generation of a (-) 2-noded catenane product. Lately, a similar model was used to explain the Xer recombination at *cer* and *psi*, in which the products generated are a (-) 4-noded HJ and a (-) 4-noded catenane, respectively {(Colloms et al., 1997) and M. Bregu, D.J. Sherratt ad S.D. Colloms, unpublished data}. The model for Xer is illustrated in Figure 5.9 (A-B-C-D). First, two negative nodes are trapped upon binding of PepA/ArgR (*cer*) or PepA/ArcA-P

(*psi*) to the DNA accessory sequences shown in red (A). After that, the core sites (blue arrows) are aligned in anti-parallel (B) as proposed in the current model of site-specific recombination mediated by the tyrosine recombinases (Gopaul & Duyne, 1999; Guo et al., 1997; Hallet et al., 1999). The first step of strand exchanges mediated by XerC generates a (-) 4-noded HJ (C). Note that in C, two extra negative nodes are introduced after HJ formation (compare C-D). Finally, the second step of strand exchanges mediated by XerD will resolve the HJ intermediates shown in C to the (-) 4-noded catenane product (D). In the Xer recombination at *psi*, the alternative pathway A-E-F-G appears to be used if low levels of PepA are present in the recombination reaction, which will lead to the generation of a (-) 2-noded catenane product (M. Bregu, D.J. Sherratt ad S.D. Colloms, unpublished data). Based on the topological filter models proposed for γδ and Tn3 resolvases and the Xer/*cer*/*psi* systems, two models of synapsis can be suggested to explain the outcome of the ResD recombination reaction (Figure 5.9). In the first model (pathway A-E-F-G), two nodes are trapped in the synaptic complex upon binding of accessory proteins, and possibly ResD itself, to the accessory sequences shown in red (A); the core recombination sites (blue arrows) are aligned in anti-parallel (E), and finally, the first step of strand exchanges would generate the (-) 2-noded HJ intermediate (F). The resolution of this HJ intermediate would generate a (-) 2-noded catenane (G). The second possible model for ResD (H-I-J-K) predicts that one negative node would be held by the accessory sequences and proteins in the synapsis (Figure 5.9-H), and the anti-parallel alignment of the core sites for recombination would require the introduction of a further negative node (an "alignment node"; I). The first step of strand exchanges (J) would generate a (-) 2-noded HJ intermediate indistinguishable from the product shown in F, whereas the final product K, which was not detected in the *in vitro*

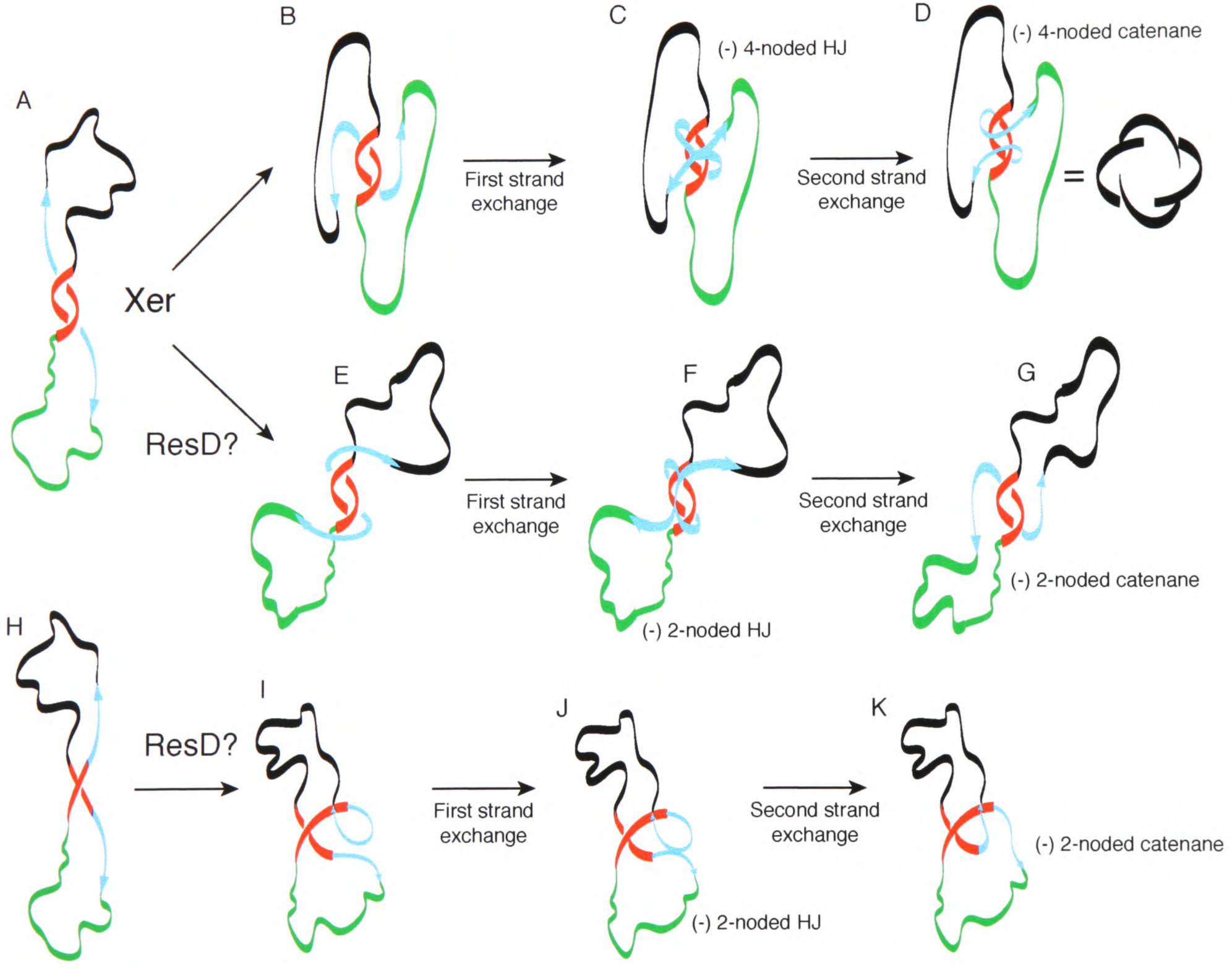

A
B
C
D
(-) 4-noded HJ
(-) 4-noded catenane
Xer
First strand exchange
Second strand exchange
=
ResD?
E
F
G
First strand exchange
Second strand exchange
(-) 2-noded HJ
(-) 2-noded catenane
H
ResD?
I
J
K
First strand exchange
Second strand exchange
(-) 2-noded HJ
(-) 2-noded catenane

Figure 5.9. The ResD synaptic complex. Two models showing how the ResD synaptic complex could be assembled in order to trap two negative nodes after recombination are shown in **A-E-F-G** and **H-I-J-K**. First, **A-E-F-G**, two negative nodes are trapped in the synaptic complex upon binding of accessory proteins to the accessory sequences in red (A); core sites (blue arrows) are aligned in anti-parallel (E), and the first step of strand exchanges generates the (-) 2-noded HJ intermediate (F); a second step of strand exchanges would generate the (-) 2-noded catenane product (G). The model for the Xer-*cer* or Xer-*psi* synapsis is shown for comparison (**A-B-C-D**). Note that the only difference between the synapses shown for Xer (**A-B-C-D**) and ResD (**A-E-F-G**) is the relative orientation of accessory and core recombination sites, which will lead to the formation of completely distinct recombinant products. Second, **H-I-J-K,** only one negative node is trapped in the accessory sequences (H) and a further node is introduced in order to align the core sites in anti-parallel (I; an alignment node); the first step of strand exchanges produces a (-) 2-noded HJ intermediate indistinguishable from F, and the final recombinant product K is also identical to G.

experiments, would be identical to G. Note also that by analogy to Xer/*cer*/*psi* and γδ/Tn3 resolvase/*res* systems the nodes trapped in both models of ResD synapsis are shown as negative nodes (Colloms et al., 1997; Hallet & Sherratt, 1997). So far, the preliminary data supplied in this Chapter are not sufficient to identify which of the two pathways proposed is the one utilised by the ResD system.

CHAPTER 6

Concluding remarks

The main purpose of the work described in this Thesis was to examine the roles of XerC and XerD recombinases in Xer site-specific recombination. The strategy employed was the construction of Xer chimeras by exchanging selected N- or C-terminal domain regions between XerC and XerD, followed by the characterisation of their recombination phenotypes *in vitro*. The analysis of the XerCD variants showed that even the most conserved regions between these recombinases are functionally distinct and not interchangeable. Subsequently, the recombination reaction catalysed by ResD, which is encoded by the *E. coli* F plasmid, was reconstituted *in vitro*. ResD lacks a region correspondent to the N-terminal domains of XerCD, and still, it exhibits directionality during recombination.

Xer recombinase variants in which N-terminal domain regions were exchanged displayed an altered coordination of catalysis in the presence of their wild type partners. This was the first report of the involvement of the N-terminal domains of tyrosine recombinases in the control of the recombination reaction, besides their known function in the stabilisation of recombinase binding to DNA. Analysis of the N-terminal chimeras' phenotypes led to the suggestion that the Xer recombinases impose asymmetry to the core recombination sites upon binding, and this asymmetry in the synaptic complex would favour the activation of just one pair of recombinases for catalysis. Asymmetry was also shown in a Cre/*loxP* synaptic complex, in which each of the *loxP* core sites has a sharp kink at one side of the central region separating the two Cre-binding sites (Guo et al., 1999). The assembly of an asymmetrically bent synaptic complex in the Cre system could be caused or facilitated by the asymmetric nucleotide composition of the *loxP* site (Lee & Sadowski, 2001). The position and direction of these bends were proposed to determine which pair of recombinases will initiate the recombination reaction, which are those occupying the binding sites distal

to the kinks (Guo et al., 1999). In the present work, it was demonstrated that the interactions between the N-terminal domains of XerD and the DNA target site are responsible for the activation of XerC for catalysis. This seems to be the case at least for the XerCD-mediated recombination at the chromosomal site *dif*, which unlike *psi* and *cer* recombination sites does not have accessory sequences and proteins that help to determine the order of strand exchanges (M. Bregu, D.J. Sherratt and S.D. Colloms, *in preparation*). A consequence of this is that the XerCD-*dif* heterotetrameric complex is locked in a conformation only suitable for XerC-mediated strand exchange. In this situation, conformational change and resolution of a HJ intermediate formed by XerC might be achieved by either a modification of the overall structure of the HJ complex mediated by an external factor such as the FtsK protein (Barre et al., 2000; Recchia et al., 1999), or by a direct modification of the XerD recombinase itself. However, another possibility has recently arisen in which recombination at *dif* might start by XerD-mediated strand exchange in the presence of FtsK, followed by XerC-mediated resolution of the HJ intermediate to recombinant products (L. Aussel, F.X. Barre, M. Aroyo and D.J. Sherratt, *in press*). This could imply that in the presence of FtsK, the synaptic complex formed between two *dif* sites in a dimeric chromosome would adopt a conformation suitable for XerD-mediated strand exchanges, and the HJ formed might then undergo the required change of conformation to be resolved by XerC by the action of XerD on the DNA target site (Chapter 3).

Coordination of catalysis in Xer also depends on C-terminal protein-protein interactions between the proposed acceptor and donor regions of XerCD, which are necessary for the concerted activation and inactivation of different pairs of recombinases for catalysis during the recombination reaction (Gopaul & Duyne,

1999; Gopaul et al., 1998; Hallet et al., 1999). This coordination was proposed to be exerted by modest changes in conformation of the HJ intermediate, which reorganise the catalytic sites of the recombinases (Gopaul et al., 1998), and is responsible for the directionality of the reaction (Gopaul & Duyne, 1999; Hallet et al., 1999; Nunes-Duby et al., 1995). Previous analyses of Xer mutants with altered acceptor or donor regions showed that the disruption of the proposed acceptor-donor interactions can lead to a loss of the control of catalysis (Hallet et al., 1999). The acceptor mutants characterised did not support the activity by the partner and exhibited a stimulated self-catalysis, while donor mutants have an exactly opposite phenotype. The phenotypes of the Xer C-terminal chimeras described in the present work, and that were also proposed to carry alterations in their acceptor or donor regions, supplied further evidence in support of the model of coordination of catalysis dependent on the integrity of the acceptor-donor interactions (Hallet et al., 1999). And as proposed for the set of acceptor and donor mutants examined by Hallet et al. (1999), the Xer C-terminal chimeras are believed to express their phenotypes by stabilising alternative conformations of the HJ intermediates other than those normally adopted by HJ heterotetrameric complexes containing wild type XerCD.

Finally, the site-specific recombination reaction catalysed by ResD was preliminarily characterised *in vitro*. ResD is a member of the tyrosine recombinase family, which lacks a region equivalent to the N-terminal domains of XerCD and Cre. Other examples of family members lacking this region are FimBE from *E. coli*, MrpI from *Proteus mirabilis*, and TnpA from *Weeksella zoohelcum* (Bahrani & Mobley, 1994; Brassard et al., 1995; Klemm, 1986; Nunes-Duby et al., 1998; Zhao et al., 1997). FimBE and MrpI are involved with fimbriae phase variation, while TnpA of *Weeksella* is believed to be a transposon resolvase. ResD, in other hand, was proposed

to participate in the monomerisation of F-plasmid dimers, which would ensure its stable segregation in *E. coli* (Disque-Kochem & Eichenlaub, 1993; Lane et al., 1986). The recombination reaction mediated by ResD appears to be preferentially intramolecular with the use topological selectivity. Both features are consistent with the proposed function of ResD, and yet, how the control of catalysis is accomplished in ResD recombination is one of the questions that remain to be answered.

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