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mwr Xer site-specific recombination is hypersensitive to DNA supercoiling

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ABSTRACT

The multiresistance plasmid pJHCMW1, first identified in a *Klebsiella pneumoniae* strain isolated from a neonate with meningitis, includes a Xer recombination site, *mwr*, with unique characteristics. Efficiency of resolution of *mwr*-containing plasmid dimers is strongly dependent on the osmotic pressure of the growth medium. An increase in supercoiling density of plasmid DNA was observed as the osmotic pressure of the growth culture decreased. Reporter plasmids containing directly repeated *mwr*, or the related *cer* sites were used to test if DNA topological changes were correlated with significant changes in efficiency of Xer recombination. Quantification of Holliday junctions showed that while recombination at *cer* was efficient at all levels of negative supercoiling, recombination at *mwr* became markedly less efficient as the level of supercoiling was reduced. These results support a model in which modifications at the level of supercoiling density caused by changes in the osmotic pressure of the culture medium affects resolution of *mwr*-containing plasmid dimers, a property that separates *mwr* from other Xer recombination target sites.

INTRODUCTION

Plasmid dimerization by homologous recombination or other processes reduces the number of plasmid molecules in the cell and leads to segregational instability (1,2). In several plasmids, stabilization occurs through Xer site-specific recombination (3). This process also resolves bacterial chromosome dimers allowing proper segregation at cell division and integrates bacteriophage genomes into the cell's chromosome (4,5). Xer recombination is catalyzed by the heterotetrameric XerCD recombinase complex acting at specific target sites and proceeds by sequential exchange of two pairs of strands via a Holliday junction reaction intermediate (6). Plasmid target sites consist of the core recombination region that includes XerC- and XerD-binding sites separated by a 6–8 bp central region and an adjacent DNA stretch of about 180 bp, known as the accessory sequences (Figure 1A). This region interacts with accessory proteins to form a synaptic complex required for recombination and ensuring that the reaction is exclusively intramolecular (Figure 1A and B) (7–10). Plasmids ColE1 and pJHCMW1 carry highly homologous sites that require the accessory proteins ArgR and PepA (Figure 1A). Xer recombination at plasmid sites is initiated by XerC-catalyzed strand exchange of one pair of strands to form a Holliday junction. In the case of recombination at the sites *cer* (from plasmid ColE1) and *mwr* (from plasmid pJHCMW1), the Xer catalyzed process stops at Holliday junction and these intermediates are resolved by Xer-independent cellular processes (Figure 1B) (11–13).

The plasmid pJHCMW1 was first identified in a *Klebsiella pneumoniae* strain isolated from a neonate with meningitis during a nosocomial infection in the Children's Hospital of Buenos Aires, Argentina (14,15). This plasmid includes *mwr*, a Xer recombination target site that has some unique characteristics (16,17). Resolution of dimers harboring this site is inefficient when the *Escherichia coli* host cells are cultured in standard L broth (containing 0.5% NaCl, osmolality 209 mmol/kg) (17). The low levels of *mwr*-mediated resolution proved insufficient to stabilize the plasmid (13). However, the levels of resolution are substantially

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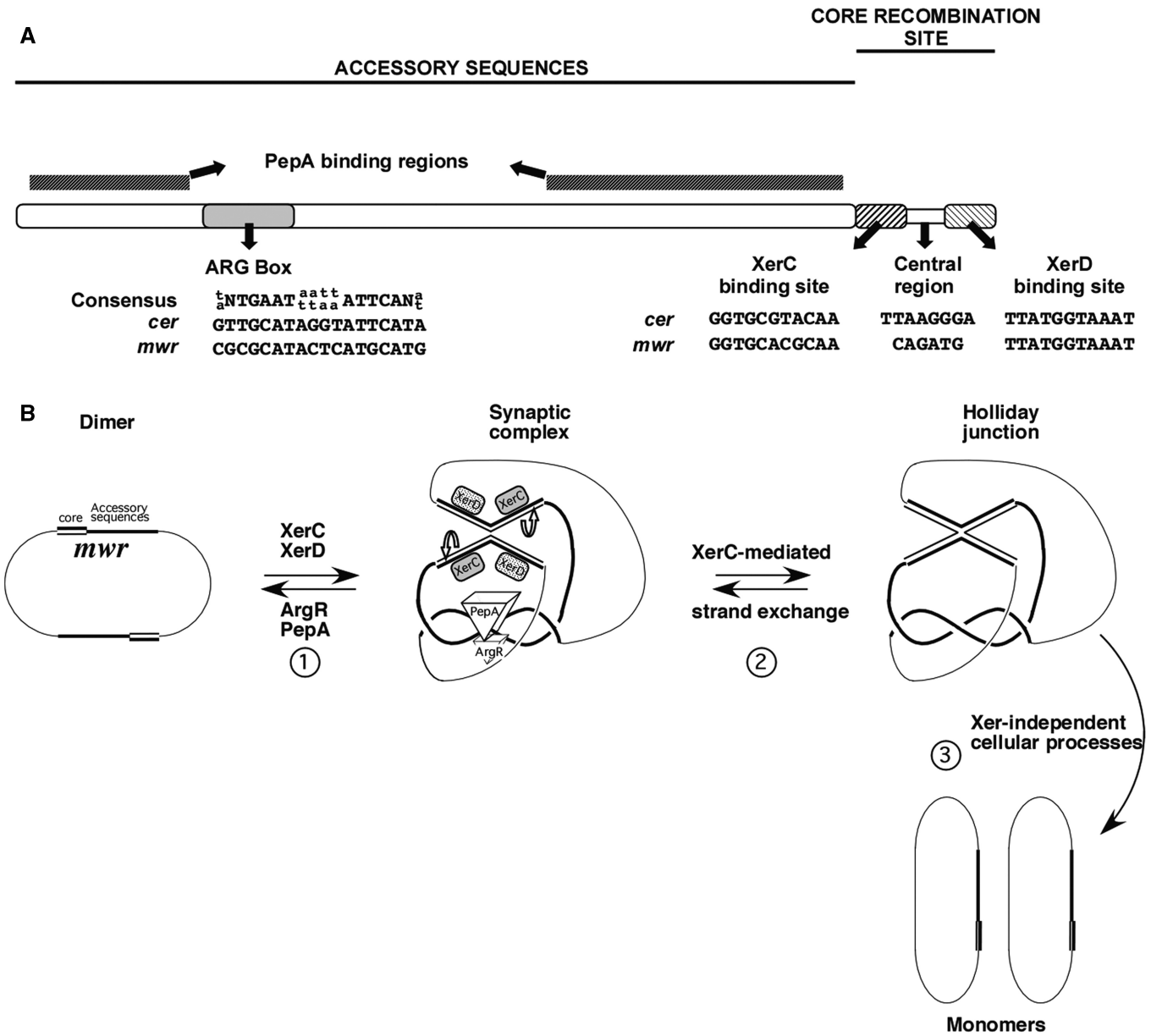

Figure 1. Xer site-specific recombination for *cer* and *mwr* sites. (**A**) Schematic structure of *cer* and *mwr* Xer site-specific recombination sites. The sites contain a core recombination region that includes the XerC and XerD-binding sites (11 bp each) separated by a central region (6–8 bp), and a region known as accessory sequences (180 bp) that provide binding sites for ArgR (ARG box) and PepA. (**B**) Diagram showing the steps of Xer site-specific recombination process for *cer* and *mwr sites*. Proteins and features involved are indicated. For clarity, the proteins are shown only in the synaptic complex. Double lines represent core sequences and bold lines represent accessory sequences. It is still unknown if the functional subunit of PepA and ArgR are single or double hexamers. White arrows indicate the points of action of XerC. For clarity, the supercoiling of the molecules is not shown.

increased in cells cultured in low-osmolarity media (L broth without NaCl added, osmolality 87 mmol/kg) (17). Dimer resolution assays using hybrid sites showed that the *mwr* core site, probably the central region, and the ArgR-binding site in the accessory sequences are directly involved in the dependence of recombination efficiency on osmolarity changes in the growth medium (17).

DNA supercoiling, a feature of almost all DNA molecules inside the cell, has a direct influence on DNA-associated processes *in vivo* (18). DNA isolated from *E. coli* cultured in L broth (0.5% NaCl) has a supercoiling density (σ) of approximately -0.06, indicating that the DNA is underwound (or negatively supercoiled) by ~6 turns for every 100 turns of the DNA helix (19,20). However, the degree of DNA supercoiling is sensitive to environmental stimuli. Local and temporary changes in supercoiling density of plasmids were observed with changes in the cell's growth environment, such as transitions from aerobic to anaerobic growth (21), modification of the osmotic pressure of growth medium, or growth temperature shift (22–24).

In this article, we show that modifications in the plasmid DNA supercoiling density that occur when the cells are cultured in media with different osmolarities correlates with the efficiency of Xer recombination at *mwr*. At high osmolarity, both the level of negative supercoiling and the recombination efficiency at *mwr* were low whereas at low osmolarity there was an increase in recombination efficiency together with an increase in negative supercoiling density. In an *in vitro* experiment, the amount of Holliday Junctions produced by Xer recombination at *mwr* decreased as the plasmid DNA supercoiling density was reduced. Thus, it seems likely that osmolarity affects the efficiency of recombination at *mwr* through its effect on DNA supercoiling. Conversely, efficiency of recombination at the highly related *cer* site was not significantly modified by changes in osmolarity of the growth medium or changes in supercoiling density of the dimer molecule.

MATERIALS AND METHODS

The *E. coli* strains and plasmids

The *E. coli* DS941 (AB1157 *recF143 lac*I^q *lacZ*M15) (3) was used in dimer resolution assays. The *E. coli* DS981 (DS941 *xerC2*::*aph*) (25) was used to culture cells containing reporter plasmids and isolate dimers of pKS492 and pES. The *E. coli* JC8679 (a *recBC sbcA* hyperrecombinogenic strain) (2) was used to generate dimers. Plasmid pBR322 (26) was utilized to determine the effect of osmolarity of the growth medium on plasmid DNA supercoiling. Plasmid pKS492 is pUC18 containing a 280-bp *Hpa*I-*Taq*I including the *cer* site (27) and plasmid pES is pUC18 containing an *Eco*RI-*Sac*I fragment including the pJHCMW1 *mwr* site (17). For supercoiling density analysis and *in vitro* recombination assays, the reporter plasmids pSDC115 and pSDC203, which include directly repeated copies of *cer* and *mwr*, respectively, were used (12,13).

Bacterial growth media and general DNA procedures

Bacteria were cultured in Lennox L broth [1% tryptone, 0.5% yeast extract, 0.5% NaCl (called 'high osmolarity' throughout the text)] or medium containing the same concentrations of tryptone and yeast extract with no NaCl added (called 'low osmolarity' throughout the text). In the case of solid medium, 2% agar was added. Transformations were carried out as described by Cohen *et al*. (28). Nucleotide sequencing was performed at the DNA sequencing facility, Department of Biochemistry, University of Oxford. Restriction endonuclease treatments were carried out as recommended by the suppliers. Plasmid DNA preparations and DNA gel extractions were performed with the QIAspin miniprep kit and QIAquick gel extraction kit, respectively (Qiagen).

Agarose gel electrophoresis

One-dimensional agarose gel electrophoresis was performed in 0.7% agarose gels in Tris–Borate–EDTA buffer for 7 h in 10 cm × 12 cm gels or 16–20 h in 20 cm × 20 cm gels at 2 V/cm. To determine supercoiling density, samples were run in the same conditions but in the presence of the intercalator chloroquine at concentration between 0.5–1 μg/ml in different gels. Two dimensional agarose gel electrophoresis was carried out in 0.6% agarose gel in 0.5 × Tris–Borate–EDTA buffer with the addition of 0.6 μg/ml (first dimension) and 3 μg/ml (second dimension) chloroquine. The first dimension (top to bottom) was run for 16 h at 3 V/cm, and the second dimension (left to right) was run for 6 h at 3.3 V/cm. Gels were stained with Ethidium Bromide or Syber Green and visualized and analyzed using Molecular Imager, Pharos FXtm plus and Quantity One software (BioRad).

In vivo resolution assay

To prepare dimers, *E. coli* JC8679 was transformed with plasmid DNA, cultured and used to extract plasmid DNA, which was present as a collection of multimers. Plasmid DNA was electrophoresed in a 0.7% agarose gel and DNA of the correct size to be plasmid dimer was purified from the gels. The extracted DNA samples were used to transform *E. coli* DS981 and colonies harboring dimer were identified for purification of this form of the plasmid. Resolution of dimers was assayed by transformation of *E. coli* DS941, plating and plasmid DNA isolation and 0.7% agarose gel electrophoresis as described before (17).

Generation of DNA substrates for *in vitro* recombination with different supercoiling density

Negative supercoiled DNA obtained from cells grown at low osmolarity were incubated for 30 min at 37°C (50 mM Tris–HCl, 10 mM $MgCl_2$, 100 mM NaCl 1 mM DTT at pH 7.5) in the presence of vaccinia virus type IB topoisomerase (vaccinia TopIB) (0.1 units/μl). Samples were taken at different times during the incubation, stopped by adding 2 mM EDTA, 0.1% SDS and deproteinized using Protenase K. The DNA samples were further purified by extraction with phenol followed by ethanol precipitation. The DNA pellets were resuspended in H_2O and analyzed by agarose gel electrophoresis.

In vitro recombination assays

XerC, XerD, PepA and ArgR, as well as *in vitro* recombination assays were purified as described previously (12). Briefly, the reactions contained pSDC203 or pSDC115 DNA, XerC, XerD, PepA and ArgR in a buffer containing 50 mM Tris–HCl, pH 8, 50 mM NaCl, 1.25 mM EDTA, 5 mM spermidine, 1 mM L-arginine, 10% (v/v) glycerol and 25 μg/ml bovine serum albumin. The final volume of reaction was 20 μl. The reactions were carried out for 1 h at 37°C and stopped by extraction with phenol followed by ethanol precipitation. The products were resuspended in H_2O, treated with the indicated restriction endonuclease and analyzed by agarose gel electrophoresis.

RESULTS

Modifications in *in vivo* efficiency of dimer resolution and in plasmid DNA supercoiling with the osmolarity of the culture medium

Xer-mediated resolution of pES (carrying *mwr*) and pKS492 (carrying *cer*) plasmid dimers clearly responded differently to changes in osmolarity. The pKS492 dimers were efficiently resolved to monomers in cells grown in both 'low' and 'high' osmolarity medium. In contrast, pES dimers were resolved efficiently at low osmolarity but poorly at high osmolarity (Figure 2A). The level of resolution of pES at high osmolarity was not enough to confer stability by multimer resolution (13). We showed before that this is a general effect of the concentration of the osmolites in the medium and not a specific inhibition by sodium or chloride ions (17). Analysis of the level of resolution of pES dimers at different NaCl concentrations showed that the most drastic reduction in efficiency of resolution occurred as the NaCl concentration was raised from 0% to 0.5% (Figure 2B). Addition of higher concentrations of NaCl to the medium did not significantly increase the inhibitory effect (Figure 2B).

To determine how osmolarity affects plasmid supercoiling, pBR322-harboring cells were cultured in L broth containing different NaCl concentrations and the topological state of plasmid DNA was analyzed by chloroquine agarose gel electrophoresis. The level of negative supercoiling decreased as the NaCl concentration was raised from 0% to 1% but increased again as the NaCl concentration was raised further (Figure 3A). The increase in negative supercoiling at 1.5% and 2% NaCl relative to the supercoiling at 1% NaCl was confirmed by two-dimensional agarose gel electrophoresis (Figure 3B) (see also Supplementary Figure 1A and B). To confirm that the same effects are observed on plasmids containing Xer recombination sites, the levels of negative supercoiling of plasmids pSDC115 and pSDC203, containing directly repeated *cer* and *mwr* sites, respectively, extracted from exponential phase *xerC* mutant *E. coli* cultured in low- and high-osmolarity medium were determined. As expected, there was a significant reduction in the level of negative supercoiling at 0.5% NaCl compared with 0% NaCl (Figure 3C). Although an increase in supercoiling was observed at the higher NaCl concentrations, in these conditions the doubling time of the cells increased significantly suggesting that other biological processes are impaired (Supplementary Figure 1C). These processes could have an effect in the recombination reaction. Therefore, the *in vivo* dimer resolution experiments were carried out varying the NaCl concentration between 0% and 1% NaCl, conditions in which there were significant changes in plasmid DNA negative supercoiling density while the variations in bacterial cell duplication time were relatively small.

Generation of plasmid molecules with different degrees of supercoiling density

Since a reduction in the levels of negative supercoiling and Xer recombination at *mwr* are correlated with changes in the NaCl concentration of the growth medium, an attractive possibility is that the reduction in efficiency of recombination at *mwr* in high-osmolarity medium is caused by the reduction in the level of DNA supercoiling in these conditions. To test this hypothesis, we generated a collection of plasmid DNA molecules *in vitro* that mimic the effect we observed when cells are cultured at different concentration of NaCl between 0% and 0.5%. For this, highly negatively supercoiled pSDC203 and pSDC115 plasmid DNA extracted from cells cultured in low-osmolarity medium were incubated in the presence of vaccinia TopIB. Incubation of supercoiled molecules in the presence of vaccinia TopIB results in a reduction of negative supercoiling over time (29). Relaxation reactions were stopped at various time points (0, 15, 30, 60, 300 and 600 s) and DNA samples were analyzed by one-dimensional agarose gel electrophoresis (Figure 4A). In the absence of chloroquine during the electrophoresis molecules that have lost different levels of negative supercoiling density and fully relaxed DNA run at the

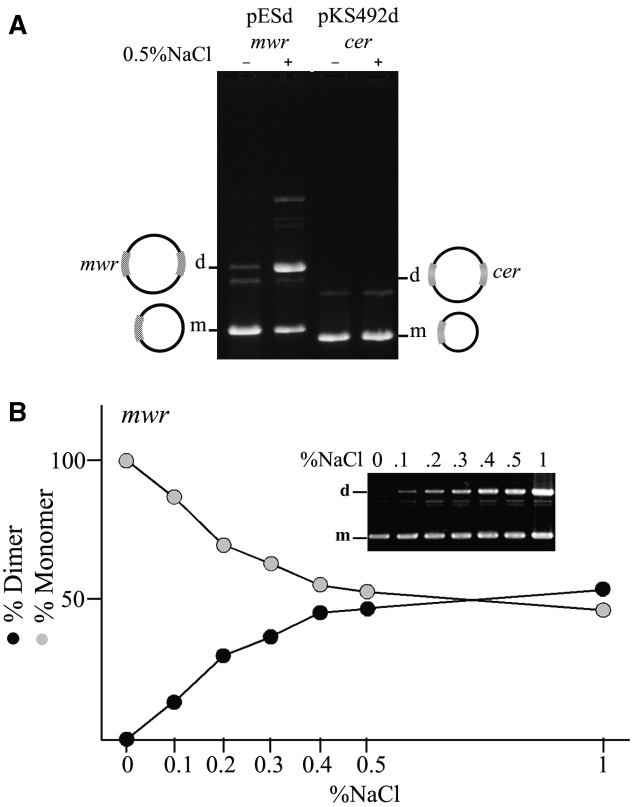

Figure 2. *In vivo* Resolution of pES and pKS492 dimers. (**A**) The pES and pKS492 dimers (pESd and pKS492d) were introduced by transformation into *E. coli* DS941, the cells were cultured in L broth containing 0.5% or no NaCl and plasmid DNA was isolated and subjected to 0.7% agarose gel electrophoresis. The positions of the pES and pKS492 dimers (d) and monomers (m) are shown to the left and to the right, respectively. (**B**) The pESd was introduced by transformation into *E. coli* DS941, the cells were cultured in L broth containing increasing NaCl concentrations, and the plasmid content was analyzed by 0.7% agarose gel electrophoresis. Monomeric and dimeric forms of the plasmids were quantified as described in Materials and Methods section.

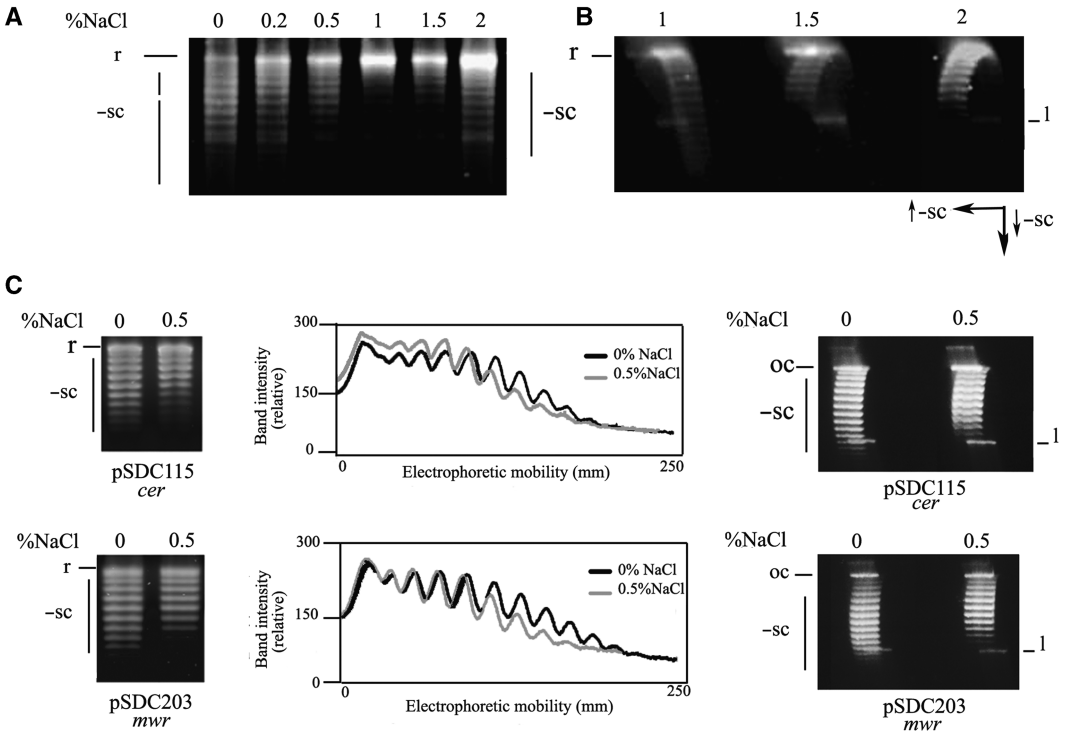

Figure 3. Plasmid DNA supercoiling density. (**A**) Plasmid pBR322 isolated from exponential phase cells cultured at increasing NaCl concentrations. oc, nicked open circle; l, linear DNA; −sc, negative supercoiled DNA. Plasmid DNA was isolated and analyzed on 1% agarose gel electrophoresis in 0.5 × Tris–Borate–EDTA buffer containing 0.5 μg/ml chloroquine. The gel was stained with Syber Green. (**B**) The 2D agarose gel electrophoresis of samples obtained at 1, 1.5 and 2% NaCl. DNA was analyzed on 1% agarose gel electrophoresis in 0.5 × Tris–Borate–EDTA buffer containing 1 μg/ml chloroquine first dimension and 3 μg/ml chloroquine during the second dimension. The gel was stained with Syber Green. (**C**) The effect of the NaCl concentration on supercoiling density of the reporter plasmids was determined using *E. coli* DS981 harboring pSDC115 or pSDC203, cultured in low (0% NaCl) or high (0.5%) osmolarity medium. Plasmid DNA was extracted at exponential phase and analyzed as in 'A' (left) and on two-dimensional gel electrophoresis (right). Quantification of the bands in the gels shown to the left (center). The large arrows adjacent to gels show the direction of increasing negative supercoiling.

same position. To resolve DNA molecules with intermediate conformations, the same samples were subjected to a two-dimensional agarose gel electrophoresis (selected samples are shown in Figure 4B), which can resolve from highly negative supercoiled to fully relaxed DNA molecules. The untreated sample shows bands with the electrophoresis mobility of highly negative supercoiled DNA. Incubation for 30 s results in a decrease of the number of molecules with high-supercoiling density and formation of intermediate conformations. After 300 s, the bands corresponding to highly supercoiled DNA are faint while those corresponding to more relaxed intermediates are brighter. A diagram indicating the nature of the different DNA bands is shown to the right of Figure 4B.

In vitro Xer site-specific recombination of plasmid DNA molecules with different degrees of supercoiling

To test the effect of DNA supercoiling on the efficiency of Xer recombination, samples containing plasmid DNA molecules at different levels of supercoiling were used as substrates for *in vitro* Xer site-specific recombination. Plasmids containing directly repeated *cer* or *mwr* sites yield unresolved Holliday junctions as product (11–13). These Holliday junctions can be observed as X-forms after digestion with a restriction enzyme that cleaves

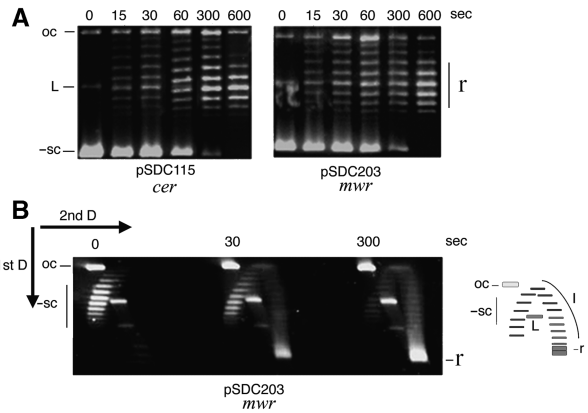

Figure 4. Generation of DNA molecules with different degree of supercoiling. (**A**) One-dimension agarose gel electrophoresis of plasmid DNA with decreasing negative supercoiling density generated by incubation with vaccinia TopIB for 0, 15, 30, 60, 300 and 600 s. −sc, negative supercoiled DNA; r, Boltzmann distribution of relaxed DNA; oc, nicked open circle DNA; l, linear DNA. Electrophoresis was carried out in 0.8% agarose in 0.5 × Tris–Borate–EDTA buffer for 20 h at 2 V/cm at room temperature. (**B**) Two-dimensional agarose gel electrophoresis of plasmid pSDC203 with decresing negative supercoiling density generated by incubation with vaccinia TopIB for 0, 30 and 300 s. The diagram to the right identifies the conformation of the different bands. I, Molecules with intermediate conformations. The '1st D' and '2st D' arrows show the first (0.6% agarose 0.6 μg/ml chloroquine, 16 h at 3 V/cm) and second (3 μg/ml chloroquine, 6 h at 3.3 V/cm) direction of electrophoresis.

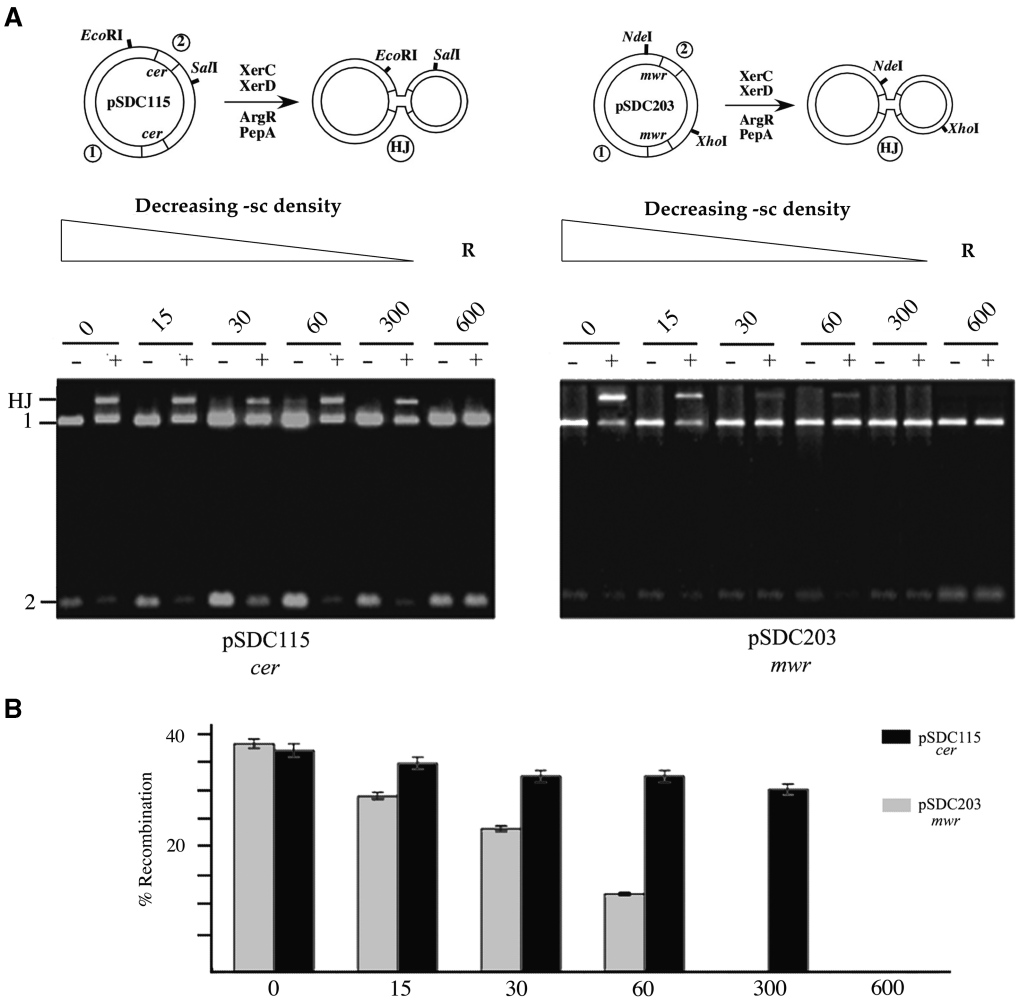

Figure 5. *In vitro* recombination at *cer* and *mwr* sites in plasmids with varying supercoiling densities. (**A**) Plasmid DNA samples with different degrees of supercoiling obtained by treatment with vaccinia TopIB for 0, 15, 30, 60, 300 and 600 s were incubated for 1 h at 37°C with or without addition of XerC, XerD, ArgR and PepA, digested with and *Xho*I and *NdeI* (pSDC203) or *EcoRI* and *SalI* (pSDC115) and subjected to 1% agarose gel electrophoresis. The diagrams on top of the gels describe the substrates and products of recombination and indicate the fragments corresponding to bands labeled 1, 2 and HJ. Bands 1 and 2 correspond to fragments generated after restriction enzyme treatment of unrecombined substrates; the bands labeled HJ correspond to the DNA molecule generated by restriction enzyme treatment of the Holliday junctions produced by recombination. (**B**) Quantification of the Holliday junction bands in the gels shown in (**A**). The quantification was done averaging the values of two gels from two independent experiments.

once on either side of each recombination site (Figure 5A). The level of Holliday junction formation on plasmid pSDC203, which contains two directly repeated *mwr* sites, was strongly dependent on the level of negative supercoiling. Decreasing the level of negative supercoiling reduced the efficiency of the recombination reaction such that there was a large reduction in the level of recombination on pSDC203 that had been relaxed for 60 s, and no detectable recombination on DNA that had been relaxed for 300 or 600 s (Figure 5A and B). In contrast, the level of Holliday junction formation at *cer* was much less sensitive to the degree of negative supercoiling: pSDC115 relaxed for 300 s recombined nearly as efficiently as pSDC115 with native levels of supercoiling (Figure 5A and B). The only condition that resulted in a significant change in levels of recombination in the case of pSDC115 was when the plasmid DNA was fully relaxed (600 s incubation with topoisomerase) (Figure 5A and B). This result is in keeping with the observations by Colloms *et al.* (1996) (12) indicating that fully relaxed dimers are not substrates for Xer recombination.

DISCUSSION

Plasmid DNA supercoiling is very sensitive to the osmotic pressure of the culture medium (22,30,31). While the supercoiling state of the molecules when the cells are cultured in L broth containing the standard concentration of 0.5% NaCl has a σ-value of –0.06 (19,20), an increase or decrease in the osmotic pressure caused by modifying the NaCl concentrations resulted in an increase in negative supercoiling density of plasmid DNA isolated from cells in exponential phase. The mechanisms by which modifications in osmotic pressure induce variations in

supercoiling density on DNA *in vivo* are largely unknown. Possible causes for these modifications are the variations in replication, transcription, and translation levels known to be affected by changes in the osmotic pressure of the environment (32,33).

The differences in efficiency of Xer recombination at *mwr* observed when the cells were cultured in medium with different osmotic pressures could be due to the resulting modifications in plasmid DNA supercoiling density. Since these changes in supercoiling density were identical in plasmids containing directly repeated copies of the highly related *mwr* (pSDC203) and *cer* (pSDC115) sites, comparison of the levels of *in vitro* recombination in reporter plasmids that differ only in the directly repeated sites permitted us to determine if Xer recombination at *mwr* presents a higher degree of dependence on the supercoiling state of the molecule when compared with a well known recombination site like *cer*. We generated pSDC203 and pSDC115 libraries with different average supercoiling densities and used them as substrates in *in vitro* recombination assays. Our results showed that plasmid molecules containing directly repeated *cer* sites recombined *in vitro* at nearly identical efficiency regardless of level of negative supercoiling. Only when all molecules were fully relaxed pSDC115 did not act as substrate for Xer recombination. In contrast, plasmid molecules containing directly repeated *mwr* sites were poorer substrates as the level of supercoiling was reduced. These results showed that in spite of being highly related, *cer* and *mwr* are significantly different in their dependence on supercoiling density. Since all tested plasmid Xer recombination targets showed no or negligible changes in efficiency at different osmolarities (17), the case of *mwr* is unique at the present time.

The accessory sequences of *mwr* include a less than ideal ARG-box and are partly responsible for the low-recombination levels observed when cells are cultured in standard L broth. Weak interaction of the ARG-box with ArgR may interfere with proper formation of the synaptic complex in high-osmolarity conditions (regular L broth). This defect may be compensated by increased levels of negative supercoiling at low osmotic pressure by a number of different mechanisms: (i) an increase in the efficiency of formation of the synaptic complex (by an increase in the rate of the forward reaction or a decrease in the rate of the backward reaction); (ii) an increase in the catalytic XerC-mediated reaction (Holliday junction formation); or (iii) an increase in the efficiency of resolution of the Holliday junction, possibly produced by modifications in the geometry of the Holliday junction brought about by changes in DNA supercoiling density (34) (Figure 1B). The results of the *in vitro* recombination experiment strongly suggest the increase in efficiency of recombination seen *in vivo* occurs at the level of formation of Holliday junction, and not at the level of Holliday junction resolution. On the other hand, these results do not permit us to decide between possibilities (i) and (ii) at this point. The increased recombination at *mwr* at low osmolarity and high-supercoiling density could be brought about by increased stability of the synaptic complex and/or increased efficiency in catalysis by XerC (Figure 1). Formation of the interwrapped synaptic complex necessary for recombination should be favored by increased negative supercoiling, making the former an attractive possibility.

It is of interest that two highly related recombination sites such as *mwr* and *cer* exhibit a very different behavior in response to environmental changes. Besides the contribution to the understanding of Xer site-specific recombination, this or related properties of target sites could be taken advantage of to manipulate and control recombination levels in recently developed technologies that involve site-specific recombination as tool. For example, the Cre/loxP recombination system has been used to genetically modify organisms for analysis of gene function *in vivo* (35). A recent example is the analysis of neuronal network architecture in which site-specific recombination was utilized to create a stochastic choice of expression between three or more fluorescent proteins (36).

SUPPLEMENTARY DATA

Supplementary Data is available at NAR Online.

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