NOTES

fpr, a Deficient Xer Recombination Site from a *Salmonella* Plasmid, Fails To Confer Stability by Dimer Resolution: Comparative Studies with the pJHCMW1 *mwr* Site[▽]

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Salmonella **plasmid pFPTB1 includes a Tn3-like transposon and a Xer recombination site, *fpr*, which mediates site-specific recombination at efficiencies lower than those required for stabilizing a plasmid by dimer resolution. Mutagenesis and comparative studies with *mwr*, a site closely related to *fpr*, indicate that there is an interdependence of the sequences in the XerC binding region and the central region in Xer site-specific recombination sites.**

Xer site-specific recombination stabilizes many plasmids by resolving dimers created through recombination events (17, 18). Most plasmids' Xer recombination sites consist of a core recombination site (CRS) that includes two 11-nucleotide binding sites for XerC and XerD, separated by a 6- to 8-nucleotide central region, and a stretch of about 180 bp known as accessory sequences (AS) that bind the architectural proteins PepA and ArgR (Fig. 1A). These elements form a synaptic complex (11, 14) where XerC is activated by interaction with XerD and catalyzes the exchange of the first pair of strands, which results in the formation of a Holliday junction (3, 6) that, in the case of *cer* (ColE1) or *mwr* (pJHCMW1), is resolved by Xer-independent processes (Fig. 1B) (2).

The pJHCMW1 plasmid, originally isolated from *Klebsiella pneumoniae*, includes *mwr* and the Tn3-like transposon Tn1331, which transposes through a replicative pathway (15, 20, 22). The efficiency of Xer-mediated dimer resolution at *mwr* when *Escherichia coli* is cultured in L broth is below the levels needed to stabilize the plasmid; instead, stabilization is mediated by the Tn1331 resolvase acting at the *res* site (13, 21). However, the efficiency of Xer-mediated dimer resolution at *mwr* is substantially increased when the cells are cultured in low-osmolarity broth (4, 13). The low levels of dimer resolution observed when cells are cultured in L broth seem to be due to a weak interaction of the substandard *mwr* ARG box with ArgR, hindering proper formation of the synaptic complex. When the cells are cultured in low-osmolarity growth medium, an increase in the density of negative supercoiling results in an increased stability of the synaptic complex and/or efficiency of catalysis by XerC, leading to a significantly higher efficiency of

dimer resolution (23). These characteristics make pJHCMW1 a very unusual plasmid that includes a Xer recombination site that, under certain conditions, is unable to confer stability by resolution of dimers; instead, that function is performed by the cointegrate resolution system of Tn1331. In this study, we report that another plasmid, *Salmonella* Typhimurium plasmid pFPTB1 (12), includes a Xer recombination site with high homology to *mwr* (Fig. 1C), from here on called *fpr* (p*FP*TB1 Xer *r*ecombination site), and a copy of the replicative transposon Tn3-ΔTn1721 (Fig. 1A and C). Plasmid pFPTB1 is the second case reported in which stabilization by dimer resolution is provided by the insertion of a replicative transposon rather than a resident Xer recombination site, suggesting that rather than being exceptional, pJHCMW1 and pFPTB1 may be part of a group of plasmids with these characteristics.

The *E. coli* strains and plasmids used in this study are described in Table 1. Plasmid pFPRTT1 was generated by inserting a synthetic DNA fragment with the *fpr* site sequence (coordinates 2221 to 2520, accession number AJ634602) from pFPTB1 (12) into the EcoRV site of pUC57 (accession number Y14837). Plasmids pTTT1 through pTTT6 were generated by site-directed mutagenesis using the QuikChange II XL kit (Stratagene). Lennox L broth (containing 2% [wt/vol] agar in the case of solid medium) is called high-osmolarity medium (containing 0.5% NaCl; osmolality, 209 mmol/kg); for low-osmolarity growth medium, NaCl was omitted (osmolality, 87 mmol/kg) (13, 23). *In vivo* resolution assays were carried out as described by Pham et al. (13). Although pFPTB1 has been isolated from *S.* Typhimurium, we decided it was appropriate to carry out the *in vivo* experiments with *E. coli* because it has been shown before that the Xer recombination proteins of *S.* Typhimurium can substitute for and are highly homologous to the corresponding proteins of *E. coli* (7), *K. pneumoniae* Xer recombination proteins share high homology with those of *E. coli* and can complement mutants, we have observed no dif-
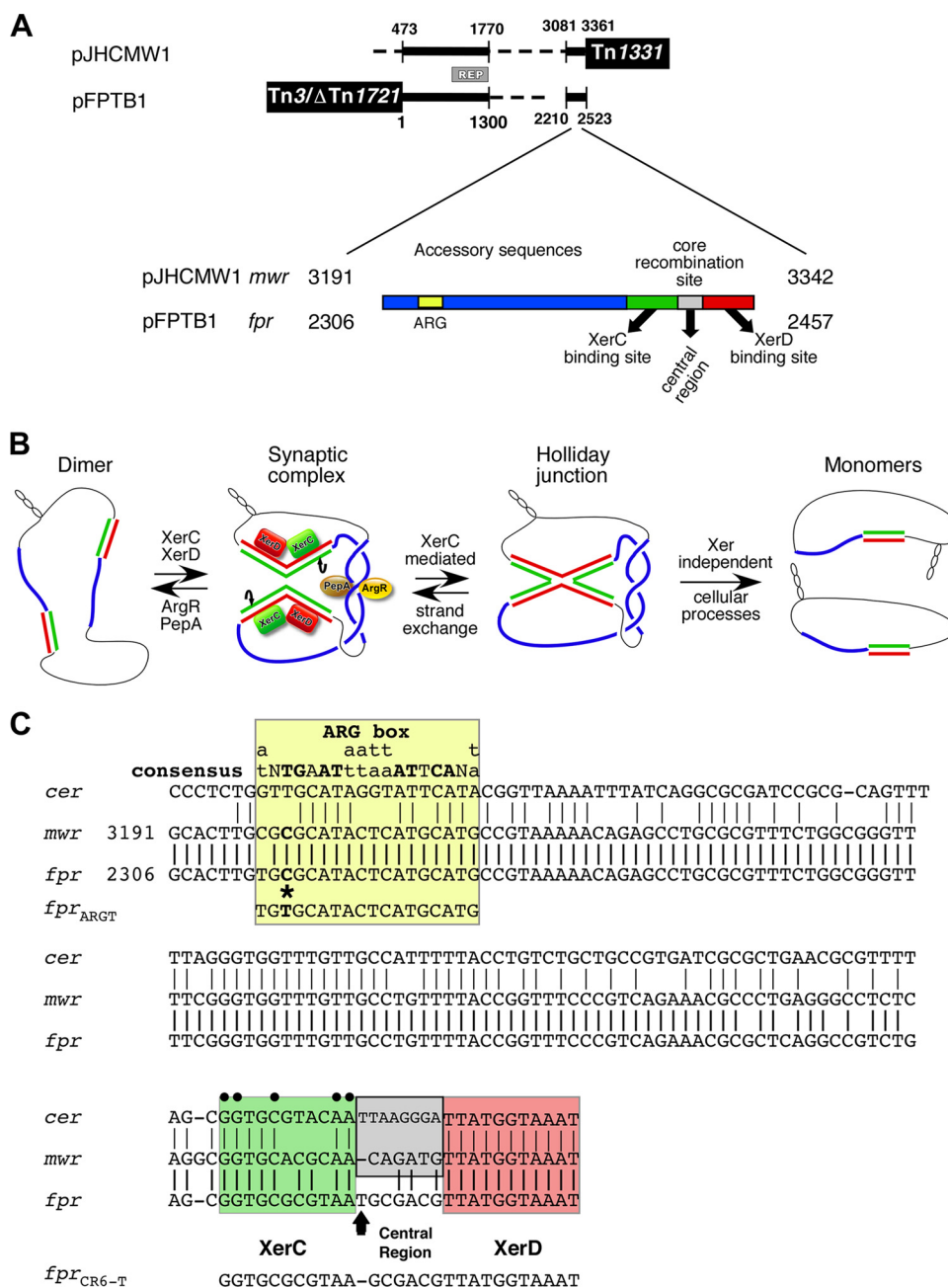
FIG. 1. (A) Comparative diagrams of pFPBT1 and pJHCMW1. The black lines represent regions of homology. The Tn3-like transposons in both plasmids are shown at the correct locations but are not to scale. The gray bar between the plasmid maps identifies the replication (REP) regions, which share 97% homology. The numbers indicate the coordinates in the GenBank database (pJHCMW1, accession number AF479774; pFPBT1, accession number AJ634602). The location and a diagram of the Xer site-specific recombination site are shown below the plasmid diagrams. The different regions of the Xer site-specific recombination site, shown with different colors, are not drawn to scale. ARG, ARG box, ArgR binding site. (B) Schematic representation of dimer resolution mediated by the Xer site-specific recombination reaction. For clarity, the proteins are shown only in the synaptic complex (red rectangle, XerD; green rectangle, XerC; brown oval, PepA; yellow oval, ArgR). Blue lines represent AS, and the CRS is the only region shown with a double line (green and red) representing the two DNA strands. The green line represents the DNA strand exchanged by XerC. Only the Xer-independent pathway of resolution of the Holliday junction (demonstrated for cer and mwr) is shown. (C) Comparison of the nucleotide sequences of mwr, fpr, and cer. The ARG box and different regions of the CRS are individually boxed. The ARG box consensus sequence is shown at the top. Nucleotides mutated in different derivatives are indicated by an arrow (central region) or a star (ARG box). Black dots identify the most conserved nucleotides among several sites (8).

ference in levels of resolution with some sites such as dif and cer and minimal differences with mwr (4), and pJHCMW1-like replicons such as pGY1 (Salmonella) (9), pVI678 (E. coli; accession number NC_008597), and pTKH11 (Klebsiella) (25)

are being found in nature across the members of the family Enterobacteriaceae rather than in one specific genus.

The backbones of pJHCMW1 and pFPTB1 share homology in regions essential for their stable inheritance and include loci

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic or genotype	Relevant characteristics of Xer recombination sites	Reference or origin
E. coli			
DS941	AB1157 *recF143 lacI*^{q} *lacZ*ΔM15; used as a host in dimer resolution assays		19
DS981	DS941 *xerC2::aph*; used to purify dimers		5
JC8679	DS945 *recBC sbcA* (hyperrecombinogenic); used to generate dimers		17
Plasmids			
pES	Recombinant plasmid containing the pJHCMW1 *mwr* site	AS$_{mwr}$, CRS$_{XerCmwr,crmwr}$	13
pKS492	Recombinant plasmid containing the ColE1 *cer* fragment	AS$_{cer}$, CRS$_{cer}$	16
pFPRTT1	Recombinant plasmid containing the pFPBT1 *fpr* site (nucleotides 2221-2520, accession no. AJ634602)	AS$_{fpr}$, CRS$_{XerCfpr,crfpr}$	This work
pTTT1	Mutant derivative of pFPRTT1 containing *fpr*$_{ARGT}$	AS$_{fprARGT}$, CRS$_{XerCfpr,crfpr}$	This work
pTTT2	Mutant derivative of pFPRTT1 containing *fpr*$_{CR6-T}$	AS$_{fpr}$ CRS$_{XerCfpr,crCR6-T}$	This work
pTTT3	Mutant derivative of pFPRTT1 containing *fpr*$_{ARGT/CR6-T}$	AS$_{fprARGT}$, CRS$_{XerCfpr,crCR6-T}$	This work
pTTT4	Mutant derivative of pFPRTT1 containing *fpr*$_{ARGT/CRmwr}$	AS$_{fprARGT}$, CRS$_{XerCfpr,crmwr}$	This work
pTTT5	Mutant derivative of pFPRTT1 containing *fpr*$_{ARGT/CR6-T/XerCmwr}$	AS$_{fprARGT}$, CRS$_{XerCmwr,crCR6-T}$	This work
pTTT6	Mutant derivative of pFPRTT1 containing *fpr*$_{ARGT/XerCmwr/CRmwr}$	AS$_{fprARGT}$, CRS$_{XerCmwr,crmwr}$	This work
pUC18	Plasmid vector; lacks a Xer recombination site; used as a control in stability experiments		Accession no. L09136
pUC57	Plasmid vector that differs from pUC18 in the multiple cloning site; used as a vector to clone *fpr*		24; accession no. Y14837

with the characteristics of Xer recombination sites (*mwr* and *fpr*, respectively) (Fig. 1A). Both *mwr* and *fpr* include an ARG box that is substandard due to the presence of a C instead of a T nucleotide in one of the highly conserved positions (Fig. 1C). We have shown before that replacing the C in *mwr* with a T increased the efficiency of Xer recombination, probably due to better binding of ArgR to the ARG box, facilitating formation of the synaptic complex (Fig. 1B) (13, 23). The AS and the XerC binding site differ by only seven and two nucleotides, respectively (Fig. 1C). The central regions not only have different nucleotide sequences but also differ in length. The *mwr* central region consists of six nucleotides, but the *fpr* site central region possesses seven nucleotides, a rare feature (2, 8).

Figure 2A shows the plasmid content of *E. coli* cells transformed with dimers of plasmid pKS492, pES, or pFPRTT1, which includes *cer*, *mwr*, or *fpr*, respectively, after culturing in low- or high-osmolarity growth medium. While the resolution of pFPRTT1 dimers was marginal, ca. 50% and 100% of the pES dimers were resolved at high and low osmolarities, respectively, and 100% of the pKS492 dimers were resolved under both conditions. Stabilization assays (Fig. 2B) showed that pES and pFPRTT1 were lost at approximately the same rate as plasmid pUC18, which lacks a Xer recombination site, indicating that the levels of recombination at *mwr* or *fpr* were not high enough to stabilize the plasmids. Conversely, pKS492 was stably maintained. As expected, the natural pJHCMW1 plasmid was also stably maintained through the action of the Tn*1331* resolvase.

To identify nucleotides responsible for the difference in recombination efficiency observed between *fpr* and *mwr*, three *fpr* mutant derivatives were generated. (i) *fpr*$_{ARGT}$ (plasmid pTTT1) contains a replacement of the C nucleotide in the *fpr* ARG box with a T (asterisk in Fig. 1C), resulting in a derivative with AS including an ARG box with the appropriate consensus sequence (AS$_{fprARGT}$) and the wild-type CRS (CRS$_{XerCfpr,crfpr}$). (ii) *fpr*$_{CR6-T}$ (plasmid pTTT2) contains a deletion of a T (iden-

tified by an upward arrowhead in Fig. 1C) in the CRS (CRS$_{XerCfpr, crCR6-T}$), generating a six-nucleotide central region from here on referred to as the *fpr* modified central region. (iii) *fpr*$_{ARGT/CR6-T}$ (AS$_{fprARGT}$CRS$_{XerCfpr, crCR6-T}$, plasmid pTTT3) is a derivative including mutations i and ii described above. Although derivatives *fpr*$_{ARGT}$ and *fpr*$_{CR6-T}$ showed a small increase in efficiency of recombination with respect to *fpr*, the cumulative effect of both substitutions was necessary to reach levels comparable to those observed for *mwr* (note that dimers harboring *mwr* or *fpr*$_{ARGT/CR6-T}$ were 100% resolved at low osmolarity [Fig. 2A and C]). These results indicate that a more efficient ARG box leads to an increase in the efficiency of Xer site-specific recombination in *fpr*, as was known to occur with *mwr*. However, this increase was smaller than that found for the same modification in *mwr*, in which case the increase was to the levels observed for *cer* (100% resolution at high and low osmolarities) (13). Although the change in efficiency after modification of a nucleotide in the ARG box could also be due to the fact that the ARG box in the actual host is different from that in *E. coli*, we think that this is not the case because the ARG box has been found to be highly conserved between genomes (10). The results also show that the *fpr* seven-nucleotide central region is detrimental for the recombination reaction because reducing the number of nucleotides to six resulted in an increase in the efficiency of dimer resolution (compare Fig. 2A *fpr* to Fig. 2C *fpr*$_{CR6-T}$). However, this enhancing effect on the recombination levels, although measurable, is not as pronounced as in the case of *mwr* (compare Fig. 2A and C) and is not enough to stabilize the plasmid. These experiments also showed that the recombination efficiency of the *fpr* derivatives is dependent on the osmolarity of the milieu (Fig. 2C).

A series of hybrid sites combining XerC binding sites and central regions was generated to further characterize *fpr* and *mwr*. These hybrid sites consisted of AS$_{fprARGT}$ and combinations of the *fpr* or *mwr* XerC binding site ($_{XerCfpr}$ or $_{XerCmwr}$) with the *mwr* or *fpr* modified central region ($_{crmwr}$ or $_{crCR6-T}$). A
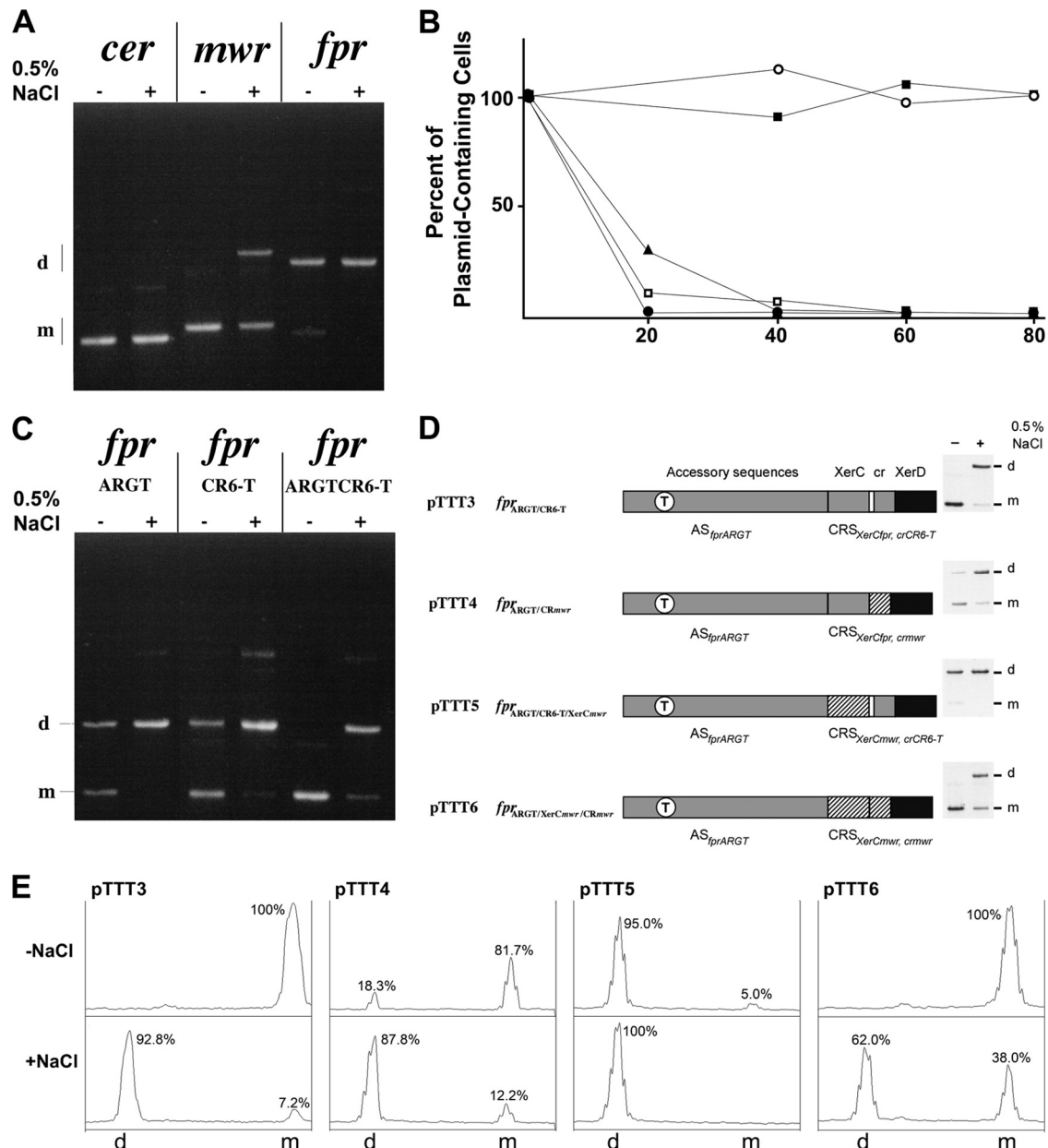
FIG. 2. Dimer resolution and plasmid stability. (A) Dimers of plasmids pKS492 (*cer*), pES (*mwr*), and pFPRTT1 (*fpr*) were introduced by transformation into *E. coli* DS941. The cells were cultured in low-osmolarity medium (− 0.5% NaCl) or high-osmolarity medium (+ 0.5% NaCl) in the presence of 100 μg of ampicillin per ml for 20 generations. Plasmid DNA was isolated and subjected to agarose gel electrophoresis. The locations of dimers (d) and monomers (m) are indicated at the left. (B) Stability of plasmids harboring different Xer recombination sites. Plasmids were introduced by transformation into hyperrecombinogenic *E. coli* JC8679, which was cultured under nonselective conditions for the indicated number of generations, and the plasmid content was analyzed. The plasmids tested were pJHCMW1 (empty circles), pKS492 (filled squares), pFPRTT1 (filled triangles), pUC18 (empty squares), and pES (filled circles). (C) Dimers of plasmids pTTT1 (*fpr*ARGT), pTTT2 (*fpr*CR6-T), and pTTT3 (*fpr*ARGT/CR6-T) were introduced by transformation into *E. coli* DS941. The cells were cultured in low- or high-osmolarity medium in the presence of 100 μg of ampicillin per ml for 20 generations. Plasmid DNA was isolated and subjected to agarose gel electrophoresis. The locations of dimers and monomers are indicated at the left. (D) Resolution of dimers containing hybrid sites. Hybrid sites contain regions from *mwr* (cross-hatched) or *fpr* (gray). The XerD binding sites are identical in both sites (black). The AS are those from *fpr* but with the T substitution that enhances the ARG box. The gray central region with a white gap represents the *fpr* modified central region. The results of dimer resolution assays are shown to the right. (E) Quantification of the gels shown in panel C. The bands were quantified using the ImageJ software (http://rsb.info.nih.gov/ij) (1).

diagram of the different derivatives and the levels of dimer resolution are shown in Fig. 2D and E, respectively. Replacement of the modified *fpr* central region with that of *mwr* generated *fpr*ARGT/CRmwr (AS*fprARGT*CRS*XerCfpr, crmwr*, plas-mid pTTT4). Resolution levels for pTTT4 dimers were similar to those for pTTT3 dimers at 0.5% NaCl but were significantly lower when the cells were cultured in low-osmolarity medium. This result could be interpreted to

mean that the *fpr* modified central region confers slightly higher recombination efficiency on otherwise identical Xer recombination sites. However, comparison of the efficiencies of resolution of dimers of pTTT5 ($fpr_{\text{ARGT/CR6-T/XerC}mwr}$, $\text{AS}_{fpr\text{ARGT}}$, $\text{CRS}_{\text{XerC}mwr,\ cr\text{CR6-T}}$) and pTTT6 ($fpr_{\text{ARGT/CR6-T/XerC}mwr/\text{CR}mwr}$, $\text{AS}_{fpr\text{ARGT}}$, $\text{CRS}_{\text{XerC}mwr,\ cr mwr}$) showed that in the presence of the *mwr* XerC binding region, the site including the *mwr* central region is substantially more efficient as a recombination target than that including the *fpr* modified central region (Fig. 2D and E). These results indicate that there does not seem to be a best central region; instead, there are more efficient combinations of XerC binding regions and central region sequences.

Conclusions. Plasmid pFPTB1 includes *fpr*, a Xer recombination site that shows a recombination efficiency lower than that required for stabilizing the plasmid by dimer resolution. Instead, stabilization must occur through the activity of the resolvase encoded by a resident replicative transposon. To the best of our knowledge, these characteristics were described only in one natural plasmid, pJHCMW1. The findings described here may indicate that pJHCMW1 is not exceptional but that there may be a group of plasmids with these properties. Furthermore, the dimer resolution levels mediated by *fpr* derivatives at high and low osmolarities also suggest that osmoregulation of *mwr* is not a unique phenomenon. The results obtained with the hybrid sites indicate that there is an interdependence of the sequences in the XerC binding region and the central region in Xer site-specific recombination sites; i.e., there is not a best central region, but instead, there are more efficient combinations of the XerC binding regions and central region sequences.

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