JOURNAL OF BACTERIOLOGY, Mar. 2002, p. 1607–1616 Vol. 184, No. 6
0021-9193/02/$04.00+0 DOI: 10.1128/JB.184.6.1607–1616.2002
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Osmoregulation of Dimer Resolution at the Plasmid pJHCMW1 *mwr* Locus by *Escherichia coli* XerCD Recombination

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Received 21 August 2001/Accepted 21 December 2001

Xer-mediated dimer resolution at the *mwr* site of plasmid pJHCMW1 is osmoregulated in *Escherichia coli*. Whereas under low-salt conditions, the site-specific recombination reaction is efficient, under high-salt conditions, it proceeds inefficiently. Regulation of dimer resolution is independent of H-NS and is mediated by changes in osmolarity rather than ionic effects. The low level of recombination at high salt concentrations can be overcome by high levels of PepA or by mutating the ARG box to a sequence closer to the *E. coli* ARG box consensus. The central region of the *mwr* core recombination site plays a role in regulation of site-specific recombination by the osmotic pressure of the medium.

Plasmids tend to form dimers by homologous recombination, a process that leads to multimer formation, which reduces the number of molecules in the cell and leads to plasmid loss (39, 40). The Xer site-specific recombination system ensures that dimeric plasmids are converted to monomers prior to cell division (4). Xer recombination was first identified through its role in the resolution of ColEl plasmid multimers (40) and has subsequently been demonstrated to mediate resolution in other related plasmids and pSC101 as well as the *Escherichia coli* chromosome (6, 16, 33). The tyrosine family recombinases XerC and XerD act at specific recombination sites present in plasmids and chromosomes to mediate a recombination reaction that proceeds via a Holliday junction intermediate (6, 7, 9, 12, 16, 17, 22, 37, 40). XerCD-mediated recombination at the chromosomal *dif* site requires a 28-bp core site, which includes two 11-bp binding sites for the recombinases XerC and XerD and a 6-bp central region (23, 42). In contrast, recombination at sites present in plasmids, e.g., *psi* (pSC101) and *cer* (ColE1), requires a core site plus about 180-bp additional accessory sequences, which are bound by accessory proteins, PepA and ArgR (*cer*) or PepA and ArcA (*psi*), and ensure that the reaction is exclusively intramolecular (14, 15, 17, 35, 36, 38). The recombination reaction at *psi* occurs by sequential strand exchanges mediated by XerC and then XerD, while for *cer*, only one pair of strand exchanges mediated by XerC to form a Holliday junction has been observed (15). The recombination reaction at *cer* seems to be completed by still unidentified XerD-independent cellular processes (2).

The multiresistance plasmid pJHCMW1, originally isolated from *Klebsiella pneumoniae* (18, 44, 45), contains the recombination site *mwr*. Like *cer* and *psi,* this site contains a core site and accessory sequences, which appear to be related to those of *cer* and which interact with PepA and ArgR. As is the case for *cer*, a reporter plasmid harboring two directly repeated *mwr* sites formed Holliday junctions in vitro in the presence of ArgR and PepA, but did not complete the recombination reaction under the assay conditions (43). On the other hand, resolution of dimers of a recombinant clone consisting of pUC18 and the pJHCMW1 *mwr* site in vivo when *E. coli* cells were growing in L broth (which contains 0.5% NaCl) was inefficient. Stability experiments showed that this low level of resolution efficiency was not enough to prevent loss of the recombinant clone in *E. coli* JC8679 (43). In this work, we show that Xer recombination in *E. coli* between direct repeats of *mwr* is osmoregulated. Resolution of dimers was inefficient in regular L broth, which contains 0.5% NaCl, while it was efficient in cells cultured in L broth lacking NaCl. The low level of recombination observed at high salt concentrations could be overcome by high levels of PepA or by mutating the ARG box to a sequence closer to the *E. coli* ARG box consensus. Mutagenesis experiments showed that the *mwr* central region of the recombination core site plays a role in regulation of site-specific recombination by the osmotic pressure of the medium.

MATERIALS AND METHODS

***E. coli* strains and plasmids.** The *E. coli* strains and plasmids are described in Table 1. Plasmids pMET *cm* and pMET *pm* are pUC18 carrying hybrid sites consisting of the accessory sequences from *cer* or *psi*, respectively, and the *mwr* core recombination site. To generate pMET *cm* and pMET *pm*, the plasmids pLN9 (which contains the *cer* site) and pLN10 (which contains the *psi* site) (15) were treated with *Mlu*I and *Eco*RI fragments to delete the core recombination sites. This treatment resulted in plasmids carrying DNA fragments consisting of the *cer* or *psi* accessory sequences, respectively, ending in an *Mlu*I site. These plasmids were treated with *Mlu*I and *Eco*RI and ligated to a synthetic fragment containing the *mwr* core region flanked by *Mlu*I and *Eco*RI sites. The pMET *mc* plasmid was generated by site-directed mutagenesis of pES to replace the appropriate nucleotides (43) and transform the *mwr* core into the *cer* core region. Plasmid pHP2.3 was generated by site-directed mutagenesis of pMET *mc* to modify the central region of the *cer* core recombination site. Throughout the paper, the sites are described according to the following general nomenclature: A, accessory sequence; C, core recombination site (e.g., A *mwr*-C *cer* describes a hybrid site with the accessory sequences from *mwr* and the core recombination region from *cer*) (Fig. 1). When necessary, further clarification is provided. The

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic or genotype[a]	Source or reference
E. coli strains		
DS941	AB1157 *recF143 lac*I^q *lacZ*ΔM15	41
DS956	DS941 *argR*::*fol* (Tmp^r)	5
DS957	DS941 *pepA*::Tn5 (Kan^r)	35
DS981	DS941 *xerC2*::*aph* (Kan^r)	16
DS9012	DS941 *hns*::Tn *10*	21
JC8679	DS945 *recBC sbcA* (hyperrecombinogenic)	40
Plasmids		
pES	*Eco*RI-*Sac*I fragment containing the pJHCMW1 *mwr* site (A *mwr*-C *mwr*) in pUC18	This work
pKD3	pES with substitution C to T at the ArgR binding site (ARG box)	This work
pMET *pm*	pUC18 derivative carrying A *psi*-C *mwr* (*psi* accessory sequence-*mwr* core site)	This work
pMET *cm*	pUC18 derivative carrying A *cer*-C *mwr*	This work
pMET *mc*	pUC18 derivative carrying A *mwr*-C *cer*	This work
pHP23	pMET *mc* with the *mwr* central region (A *mwr*-C *hp23*) (cr CAGATG)	This work
pMIG103	pUC18 derivative with directly repeated *psi* sites	M. Bregu
pMIG104	pUC18 derivative with directly repeated modified *psi* sites; modification by flipping order of XerC and XerD binding sites with respect to accessory sequence [A *psi*-C *psi*(DC)]	M. Bregu
pMIG107	pUC18 derivative with directly repeated *psi* core sites (C *psi*)	M. Bregu
pSDC124	pUC18 derivative carrying two directly repeated *dif* sites (A *dif*-C *dif*)	S. Colloms
pSDC164	pUC18 derivative with directly repeated *cer* sites with a GATCCA central region [A *cer*-C *cer* (cr, GATCCA)]	S. Colloms
pSDC165	pUC18 derivative with directly repeated A *psi*-C *psi* (cr, GCTCCA)	S. Colloms
pSDC167	pUC18 derivative with directly repeated A *psi*-C *psi* (cr, GACCCA)	S. Colloms
pSDC169	pUC18 derivative with directly repeated A *psi*-C *psi* (cr, GATTCA)	S. Colloms
pLN9	pUC18 derivative with directly repeated A *cer*-C *psi*	15
pLN10	pUC18 derivative with directly repeated A *psi*-C *cer*	15
pCS349	*argR* gene cloned under the control of *plac* (includes its own promoter); λdv replicon; Cm^r	34, 36
pCS119	*pepA* gene cloned under the control of *plac* (includes its own promoter); λdv replicon; Chl^r	34, 36
pCS118	*pepA* gene cloned in opposite orientation with respect to *plac* (includes its own promoter); λdv replicon; Chl^r	34, 36
pKS492	pUC18 containing 280-bp *Hpa*I-*Taq*I *cer* fragment	36

[a] A, accessory sequence; C, core site; cr, central region; Chl, chloramphenicol; Kan, kanamycin; Tmp, trimethoprim.

wild-type sites are mostly identified by the single mention of the name, but when considered helpful for clarity, the A and C names have been detailed (e.g., A *mwr*-C *mwr*).

Bacterial growth media and general DNA procedures. Growth of bacteria was in Lennox L broth (32) (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 either no NaCl added or with other NaCl concentrations indicated in the text. In the case of solid medium, 2% agar was added. Transformations were carried out as described by Cohen et al. (13). Restriction endonuclease and ligase 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). Nucleotide sequencing was performed at the DNA sequencing facility, Department of Biochemistry, University of Oxford. Site-directed mutagenesis was carried out with the Quikchange site-directed mutagenesis kit (Stratagene). Osmolality values were determined in a vapor pressure osmometer (Wescor 5500).

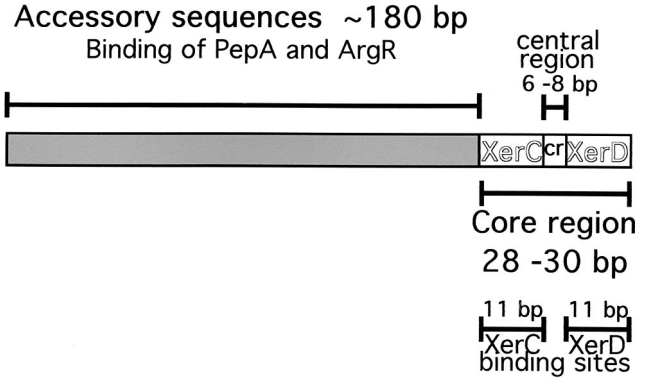

FIG. 1. Schematic structure of plasmid Xer site-specific recombination sites. The sites contain a core recombination region that includes the XerC and XerD binding sites (11 bp each) and a central region (6 to 8 bp), as well as the accessory sequences (~180 bp) with which ArgR and PepA interact (in the case of *psi*, ArcA and PepA). The diagram is not to scale. The hybrid sites utilized in this paper consist of accessory sequences (A) and core recombination regions (C) from different sites.

In vivo resolution assay. To prepare dimers, *E. coli* JC8679 was transformed with plasmid DNA. The transformed strains were cultured in L broth medium in the presence of 100 μg of ampicillin per ml for 20 generations, and plasmid DNA was purified and electrophoresed in a 0.7% agarose gel. DNA of the correct size to be a plasmid dimer was purified from agarose gels with the QIAquick gel extraction kit (Qiagen). Since dimers run close to the position of open circular monomer DNA, the isolated samples were used to transform the XerC-deficient *E. coli* DS981. In this strain, dimers will not be resolved by Xer recombination allowing the isolation of transformants that had obtained a plasmid dimer. Purified plasmid dimers were transformed into *E. coli* DS941 to determine the efficiency of Xer-mediated dimer resolution.

DNA binding assays. Gel mobility shift assays were performed as described by Blakely et al. (7). The oligonucleotides used had the following structure (the putative binding regions are underlined): *cer*, 5′GATCCGCGGTGCGTACAATTAAGGGATTATGGTAAATACG and 5′AATTCGTATTTACCATAATCCCTTAATTGTACGCACCGCG; and *mwr*, 5′GATCCGGCGGTGCACGCAACAGATGTTATGGTAAATACG and 5′AATTCGTATTTACCATAACATCTGTTGCGTGCACCGCCG.

Approximately 10 pmol of oligonucleotide was end labeled with 50 μCi of [γ-^{32}P]ATP and phage T4 polynucleotide kinase (5 U) in kinase buffer (50 mM

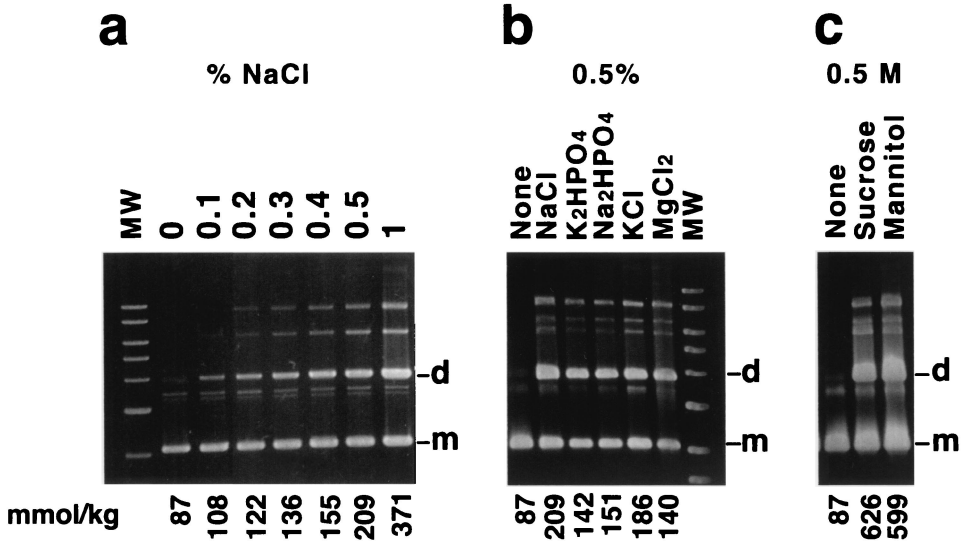

FIG. 2. Resolution of pES dimers. Dimers were introduced by transformation into *E. coli* DS941. The cells were cultured in L medium with the addition of increasing NaCl concentrations (a); 0.5% NaCl, K_2HPO_4, Na_2HPO_4, KCl, and $MgCl_2$ (b); or 0.5 M mannitol or sucrose (c). The osmolality values (millimoles per kilogram) are shown below each lane. The cultures were carried out in the presence of 100 μg of ampicillin per ml for 20 generations. Plasmid DNA was isolated and subjected to agarose gel electrophoresis. The slowly moving bands correspond to multimers (supercoiled and open circular). MW, linear molecular weight standards (10, 8, 6, 5, 4, 3, and 2 kb). d, dimer; m, monomer.

Tris HCl [pH 7.5], 10 mM $MgCl_2$, 5 mM dithiothreitol [DTT], 0.1 mM spermidine) in a final volume of 20 μl. The labeled oligonucleotide was purified with a Nuctrap Probe Purification column (Stratagene) followed by ethanol precipitation. The radiolabeled oligonucleotide was dissolved in 15 μl of H_2O and then made double stranded by annealing with 50 pmol of the complementary oligonucleotide. The mixture was heated to 75°C for a few minutes and then allowed to cool to room temperature overnight. The annealed double-stranded radiolabeled oligonucleotides were purified by electrophoresis on an 8% polyacrylamide gel in Tris borate buffer (100 mM Tris [pH 8], 100 mM boric acid, 2 mM EDTA) as described before (7). The radiolabeled oligonucleotides were mixed with 0.1 mg of poly(dI-dC) per ml and the appropriate protein(s). The binding reaction was carried out for 10 min at 37°C and immediately transferred to ice. The samples were analyzed by electrophoresis in a polyacrylamide gel as described above. The radioactive complexes were detected by exposure to X-ray film. To determine the dissociation constant (K_d), we followed the procedure described by Robinson and Sligar (31). Purified XerD at different concentrations (ranging from 5 μM to 0.25 nM) was incubated with radiolabeled DNA under the conditions described above and the bands corresponding to bound and unbound DNA were quantified. The values were utilized to calculate the fraction of bound DNA (θ), which can be expressed as $\theta \approx 1/(1 + K_d/\mathrm{Pt})$, where Pt is the amount of XerD and K_d is the equilibrium dissociation constant: $K_d = [\mathrm{P}][\mathrm{DNA}]/[\mathrm{P} - \mathrm{DNA}]$. The calculated fraction of bound DNA values was fitted to the equation by nonlinear least-square analysis by using Excel (Microsoft).

In vitro aminopeptidase assays. The enzymatic determinations were carried out basically as described by McCulloch et al. (26). Cells were harvested from overnight cultures; washed with a solution containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 10 mM β-mercaptoethanol; resuspended in a mixture containing 50 mM Tris-HCl (pH 8.2), 1 M NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, and 0.1 mM DTT; and lysed by sonication. The soluble fraction was separated by centrifugation and heated at 75°C for 10 min. This fraction was used to determine aminopeptidase A activity. The reactions were performed in a buffer containing 20 mM Tris-HCl (pH 8.2), 100 mM KCl, 1 mM $MnCl_2$, 0.1 mM EDTA, 1 mM L-leucine-*p*-nitroanilide, and 5 μg of protein at 37°C. Generation of *p*-nitroanilide was determined by measuring the A_{400}.

RESULTS

Dimer resolution at *mwr* is osmoregulated. The efficiency of resolution of pES dimers, which contain directly repeated *mwr* sites, was strongly dependent on the NaCl concentration in the bacterial growth medium. Figure 2a shows the plasmid content of *E. coli* DS941 transformed with pES dimers and cultured at increasing NaCl concentrations (osmolality range, 87 to 371 mmol/kg). While resolution was almost complete in the absence of NaCl, it became more inefficient as the concentration of salt in the medium was increased. Increasing the osmolality of the medium by addition of other salts also resulted in the same inhibition of resolution of dimers carrying *mwr* (Fig. 2b). Therefore, this is a general effect of the concentration of the salts in the medium and not a specific inhibition by sodium or chloride ions. Resolution of dimers of pES in medium supplemented with 0.5 M sucrose or mannitol also led to a decrease in dimer resolution (Fig. 2c), suggesting that Xer site-specific recombination at *mwr* is inhibited by an increase in the external osmolarity and not by an ionic effect.

Resolution of pES dimers in an *hns* mutant is osmoregulated. Since the H-NS protein has been implicated in regulation of osmotically controlled genes (25, 27, 28), we determined the efficiency of resolution of pES dimers in *E. coli* DS9012, an *E. coli* DS941 *hns* mutant derivative (21). Figure 3 shows that resolution was efficient in the mutant in the absence of NaCl while very poor in the presence of 0.5% NaCl. From these results, we concluded that H-NS is not directly involved in the osmoregulation of resolution of pES dimers.

Recombination at sites other than *mwr* is not highly osmoregulated. To find out if recombination at other sites was also osmoregulated, we performed studies on other derivatives. Since resolution of dimers harboring the sites A *psi*-C *psi* (*psi* accessory sequences-*psi* core recombination site) and A *cer*-C *cer* was highly efficient, irrespective of the presence or absence of NaCl, we studied osmoregulation of recombination at derivatives of these Xer recombination sites that were not as efficient. *E. coli* DS941was transformed with several plasmids carrying two directly repeated derivative sites described in Table 1. The transformed strains were cultured in medium with 0

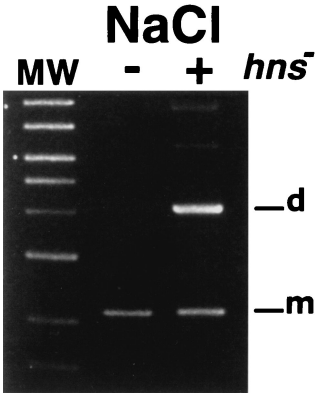

FIG. 3. pES dimer resolution in an H-NS-deficient mutant. *E. coli* DS941 and *E. coli* DS9012 (H-NS deficient) harboring dimers of pES were cultured in L broth containing 0.5% (+) or no (−) NaCl, and plasmid DNA was purified and analyzed by gel agarose electrophoresis. The left lane shows linear DNA molecular weight (MW) standards (10, 8, 6, 5, 4, 3, 2, and 1.5 kb). d, dimer; m, monomer.

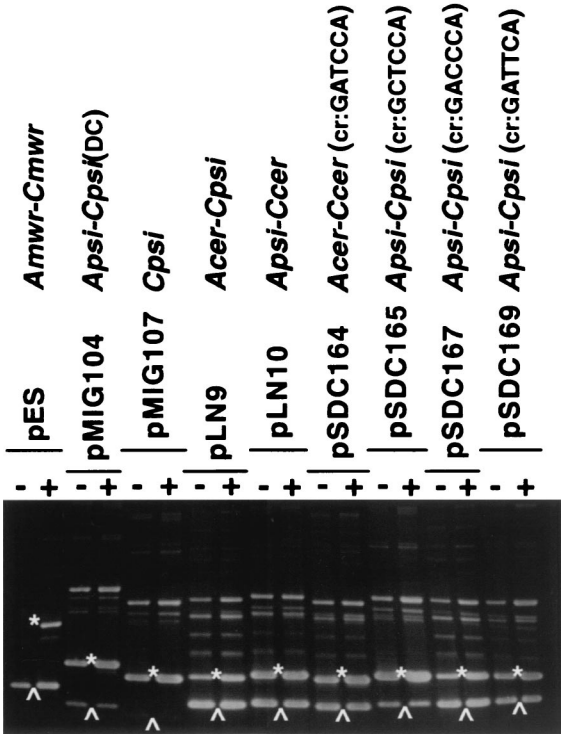

FIG. 4. Xer site-specific recombination at several target sites. Dimers of pES and the different reporter plasmids were introduced by transformation into *E. coli* DS941. The cells were cultured in L medium containing 0.5% NaCl (+) or no NaCl (−). Plasmid DNA was isolated and subjected to agarose gel electrophoresis. The positions of unrecombined and recombined species are shown by asterisks and upward arrowheads, respectively.

or 0.5% NaCl, and plasmid DNA was extracted and analyzed by agarose gel electrophoresis. A plasmid carrying two directly repeated copies of *dif*, pSDC124, showed a small apparent difference in efficiency of resolution between the two conditions. A slightly larger percentage of resolved products was present when no NaCl was added to the growth medium (data not shown). However, the effect was much less than that seen when a dimer with two direct repeats of *mwr* was analyzed. Plasmids pMIG104 and pMIG107 have directly repeated modified *psi* sites and show a lower Xer site-specific recombination efficiency than that of the wild-type *psi* site. Plasmid pMIG104 contains two directly repeated *psi* site derivatives with a modified core site consisting of an inversion of the location of the XerC and XerD binding regions with respect to the accessory sequences [A *psi*-C *psi*(DC)]. Plasmid pMIG107 carries two directly repeated core *psi* sites and no accessory sequences (C *psi*). Figure 4 shows that the levels of resolution for both plasmids are identical in the presence or absence of NaCl in the medium. Plasmid pLN9, which has two directly repeated hybrid regions consisting of the *cer* accessory sequences and the *psi* core region, shows marginally better efficiency of recombination in the absence of NaCl (A *cer*-C *psi*, Fig. 4). Resolution assays using plasmids with directly repeated *cer* sites that have a modified central region (pSDC164), directly repeated *psi* sites with modified central regions (pSDC165, pSDC167, and pSDC169), or a directly repeated hybrid site consisting of *psi* accessory sequences and the *cer* core region (pLN10, A *psi*-C *cer*) showed a marginal difference in efficiency of resolution in the case of pSDC167 and no differences in all other plasmids. These results indicate that, under the assay conditions used, the efficiencies of Xer site-specific recombination at *dif* and derivatives of *cer* and *psi* were not substantially modified by the osmolarity of the external medium. This could reflect the fact that either recombination at wild-type *cer* or *psi* does not respond to a change in osmolarity or recombination at the impaired derivatives tested had lost the ability to be regulated by changes in the osmolarity of the growth medium.

The accessory sequences of *mwr* are responsible for its low dimer resolution efficiency in high-osmolarity medium. To identify the region of *mwr* responsible for the low dimer resolution efficiency under conditions of high osmolarity, we generated hybrid sites containing the accessory sequences of *mwr* and the core region of *cer* (A *mwr*-C *cer*) and the accessory sequences of *cer* and the core region of *mwr* (A *cer*-C *mwr*) (Fig. 5a). Plasmids pMET *mc* and pMET *cm* contain the sites A *mwr*-C *cer* and A *cer*-C *mwr*, respectively. Plasmids pES and pKS492 include an intact copy of *mwr* (A *mwr*-C *mwr*) and *cer* (A *cer*-C *cer*), respectively (Fig. 5a). Dimers of all four plasmids were introduced in *E. coli* DS941 to determine their efficiency of resolution by Xer recombination. Figure 5b shows that efficient recombination was observed in the case of pKS492 (A *cer*-C *cer*) and pMET *cm* (A *cer*-C *mwr*). Conversely, the dimers were poorly resolved in both derivatives carrying the *mwr* accessory sequences (A *mwr*-C *mwr* and A *mwr*-C *cer* [see Fig. 5a]). These results indicate that the accessory sequences of *mwr* are responsible for the low efficiency of Xer recombination at this site in high-osmolarity medium. Furthermore, a comparison of the XerC and XerD binding capabilities of the core sites of *cer* and *mwr* showed that there are no substantial differences in their affinities for *E. coli* XerC and XerD (Fig. 5d). The K_d for XerD binding to *mwr* was 10.6 nM, a value similar to that of the K_d for XerD binding to *cer* (8). Addition of 0.28 μg of partially purified XerC to a reaction mixture containing radiolabeled *mwr* or *cer* DNA and XerD resulted in

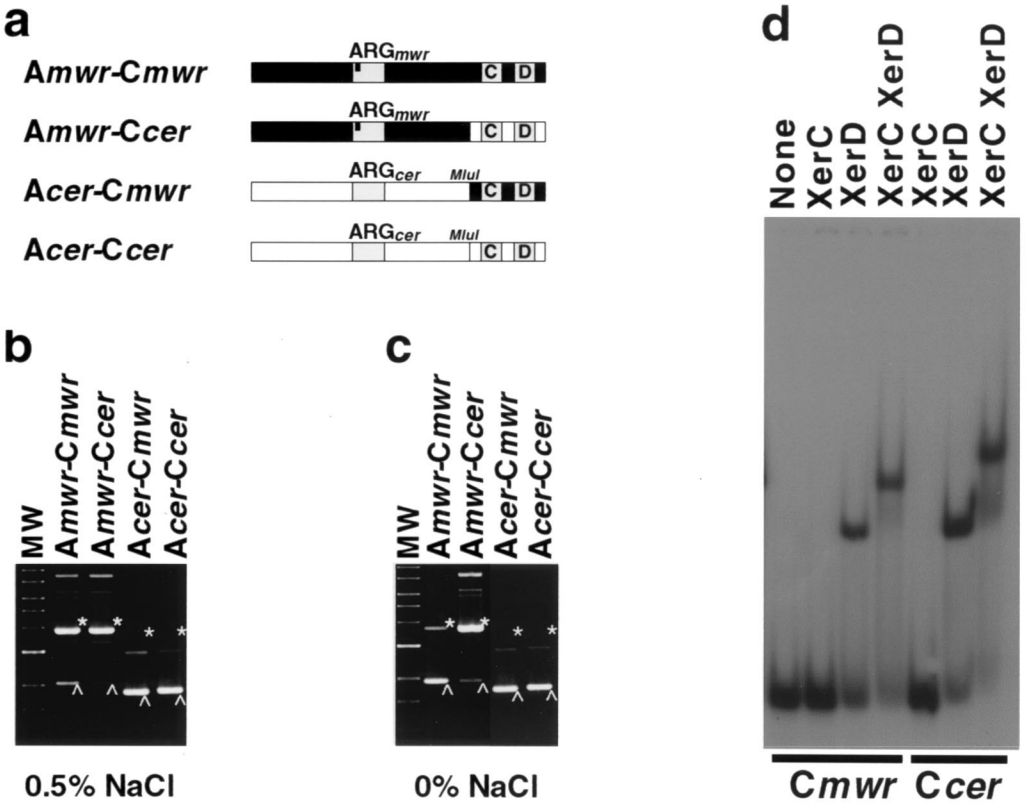

FIG. 5. Resolution of dimers containing hybrid sites. (a) Recombination sites present in plasmids pKS492 (A *cer*-C *cer*), pES (A *mwr*-C *mwr*), pMET *cm* (A *cer*-C *mwr*), and pMET *mc* (A *mwr*-C *cer*). The bar in the ARG box in the *mwr* accessory sequences indicates that 1 nucleotide is different from the conserved consensus ARG box (ArgR binding site) sequence. (b and c) Dimers of pES, pMET *mc*, pMET *cm*, and pKS492 were introduced by transformation into *E. coli* DS941 and cultured in L medium containing 0.5% NaCl (b) or no NaCl (c). Plasmid DNA was isolated and subjected to agarose gel electrophoresis. Linear DNA molecular weight (MW) standards (10, 8, 6, 5, 4, 3, 2, and 1.5 kb) and the positions of dimer (asterisks) and monomer (arrowheads) species are shown. (d) In vitro protein-DNA binding. Oligonucleotides containing the *mwr* and *cer* core regions were end labeled and incubated in the presence of XerC, XerD, or both XerC and XerD. A control with no additions is shown. The products were separated by electrophoresis in 8% polyacrylamide gel.

binding of ~90% of the label in both cases. These results confirmed that the recombinases have similar affinities for the *mwr* and *cer* sites.

A substitution of 1 nucleotide in the *mwr* ARG box results in a target site that recombines at high efficiency in high-osmolarity medium. Unlike operator regions of ArgR-regulated genes, which have two adjacent ARG boxes (20), the *cer* and *mwr* accessory regions contain only one ARG box. In the synaptic complex formed between two recombination sites, one ArgR hexamer is believed to bind to both ARG boxes (1). To test if the apparent impairment of the *mwr* accessory sequences is due to its ARG box, we generated a mutation to create an ArgR binding site more similar to the *E. coli* consensus sequence as defined by Glansdorff (20). Plasmid pKD3 contains a substitution, C to T, at the third nucleotide of the *mwr* ARG box (mwr_T). This is one of 8 nucleotides that are highly conserved in all ARG boxes (boldface in Fig. 6a). Figure 6b shows that dimers of pKD3, which contain two direct repeats of mwr_T, were resolved more efficiently than dimers of pES in *E. coli* DS941. This result indicates that a nonoptimal ARG box may be responsible for the low efficiency of recombination at *mwr* in *E. coli* growing in high-osmolarity medium. It is possible that the *mwr* ARG box has evolved to efficiently bind the ArgR protein from *K. pneumoniae*. However, the *E. coli* ArgR protein shares 94% identity and 98% similarity with the ArgR from *K. pneumoniae* MGH78578, the strain for which the complete genome is being sequenced (http://genome.wustl.edu/gsc/Projects/*K.pneumoniae*/). Cloning of *K. pneumoniae* ArgR and complementation assays will permit us to determine if this is the case.

Increased levels of PepA suppress the poor recombination phenotype at high osmolarity. To determine if higher levels of ArgR or PepA can overcome the low efficiency of recombination at high osmolarity, pES dimers were introduced into $ArgR^-$ and $PepA^-$ mutants of *E. coli* already harboring pCS349 and pSC119. These plasmids overexpress ArgR and PepA from *plac*, respectively. Figure 7a shows that in *E. coli* DS956(pCS349), which has a higher concentration of ArgR in the cell, the dimers were efficiently resolved in the absence of NaCl, while the efficiency was lower in the presence of 0.5% NaCl, as is the case with resolution in *E. coli* DS941. In contrast, in the PepA overproducer *E. coli* DS957(pCS119), the pES dimers were very efficiently resolved when the cells were cultured in medium containing either 0.5% or no NaCl (Fig. 7a). Control assays without the complementing plasmids pCS349 or pCS119 did not show any resolution (Fig. 7a).

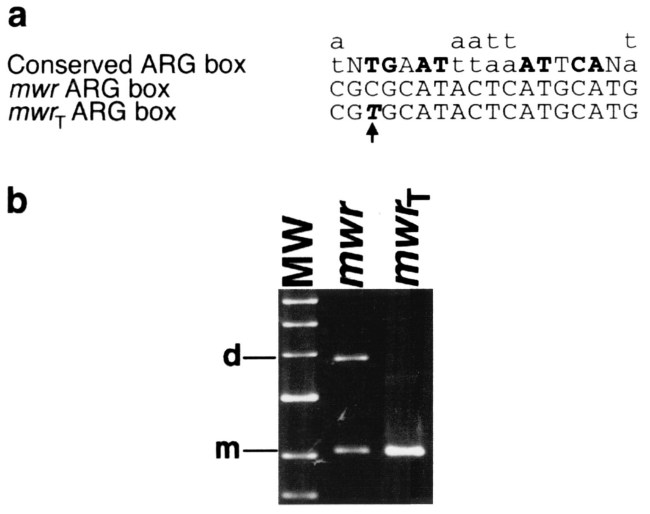

FIG. 6. Mutagenesis of the *mwr* ARG box. (a) Nucleotide sequence of the *mwr* ARG box showing the substitution in mwr_T and the consensus sequence of the ARG box according to Glansdorff (20). Boldface capital letters indicate the most important conserved nucleotides in the ARG box. (b) Dimers of pES (*mwr*) and pKD3 (mwr_T) were introduced by transformation into *E. coli* DS941 and cultured in L broth containing 0.5% NaCl. Plasmid DNA was isolated and subjected to agarose gel electrophoresis. The left lane shows linear DNA molecular weight standards (6, 5, 4, 3, 2, and 1.5 kb). d, dimer; m, monomer.

These experiments show that an excess of PepA can overcome the low efficiency of pES dimer resolution at high osmolarity in the growth medium. It has been shown before that at a high concentration of PepA, the in vitro recombination reaction at *cer* could occur in the absence of ArgR (15). In a control experiment, dimers of pKS492 were efficiently resolved in *E. coli* DS957(pCS119) in the presence or absence of NaCl (Fig. 7a). Figure 7b shows that the concentration of PepA in *E. coli* DS957(pCS119) is substantially higher than that in *E. coli* DS941. Conversely, the PepA concentration in *E. coli* DS957 (pCS118), in which *pepA* was cloned in the orientation so that it could not be expressed from the *lac* promoter, was only slightly higher than that in *E. coli* DS941 (Fig. 7b). (In plasmid pCS118, *pepA* carries its natural promoter.) This result indicates that the high PepA expression from pCS119 is due to transcription from *plac*. Figure 7b also shows that PepA activity in the plasmidless *E. coli* strain DS957 is negligible.

Since a higher concentration of PepA compensates for the lower efficiency of dimer resolution when the cells are cultured in the presence of 0.5% NaCl, we determined if the osmoregulation of pES dimer resolution is mediated by a higher expression of PepA. We measured aminopeptidase A activities in cells growing in L broth containing 0.5% or no NaCl. Figure 7b shows that the aminopeptidase A activity at low osmolarity is slightly lower than at high osmolarity, indicating that a higher concentration of PepA is not responsible for a higher-resolution efficiency at low osmolarity. Therefore, the effect of PepA observed in the experiments described in this section may be due to an ability of high levels of PepA to make the recombination reaction less dependent on ArgR.

The central region of the *mwr* core recombination site is involved in osmoregulation. The results described so far indicate that the *mwr* accessory sequences are responsible for the poor Xer recombination in high osmolarity. A high PepA concentration, a nucleotide substitution in the ARG box that improves ArgR binding, or low osmolarity in the growth medium can overcome this deficiency. To identify features of *mwr* involved in osmoregulation of Xer recombination, the efficiencies of resolution of dimers of plasmids containing intact *cer*, *mwr*, or the hybrid sites A *cer*-C *mwr* and A *mwr*-C *cer* were analyzed in the presence or absence of NaCl in the medium. Figure 5b and c show that while resolution of dimers of pES was substantially enhanced when the NaCl concentration in the medium was reduced, the level of resolution of dimers containing A *mwr*-C *cer* remained constant. Since the only difference between the derivatives A *mwr*-C *mwr* and A *mwr*-C *cer* is their core recombination sites and recombination at the latter is not regulated by the osmolarity of the medium, we generated a substitution in the hybrid A *mwr*-C *cer* derivative, A *mwr*-C *hp23*, in which the central region of the core recombination site has been modified to that of *mwr* (Fig. 8a). The efficiency of resolution of pHP2.3 dimers was increased in the absence of NaCl, although the effect was less than that observed for the wild-type A *mwr*-C *mwr* (Fig. 8b). This result strongly suggests that the core recombination site of *mwr* plays a role in osmoregulation through its central region. To determine if the presence of the *mwr* core recombination site was sufficient to confer the property of being osmoregulated, a site was constructed with the *psi* accessory sequences (A *psi*-C *mwr*, Fig. 8c). A dimer carrying this hybrid site had a low recombination frequency at high osmolarity (Fig. 8d), and only a small improvement in dimer resolution in the absence of NaCl was observed (Fig. 8d), suggesting that the presence of the *mwr* core recombination site is not sufficient for osmoregulation.

DISCUSSION

In this report, we demonstrate that Xer recombination between the directly repeated *mwr* sites in a dimer is osmoregulated. Resolution experiments in vivo at various osmolarities obtained with several osmolites showed an inverse relationship between osmolarity and efficiency of resolution. To the best of our knowledge, osmoregulation of Xer site-specific recombination has not been previously observed. Experiments with hybrid sites showed that the *mwr* accessory sequences are responsible for the low efficiency of recombination in *E. coli* at high osmolarity, apparently because the ARG box in this site interacts poorly with ArgR. A substitution in the *mwr* ARG box that generates a more efficient *E. coli* ArgR binding region resulted in a site that recombines efficiently at high osmotic pressure. The *mwr* ARG box may have evolved to bind the *K. pneumoniae* ArgR more efficiently, thereby increasing the pJHCMW1 stability in this bacterium. However the amino acid sequences of ArgR in both *E. coli* and *K. pneumoniae* MGH78578 (http://genome.wustl.edu/gsc/Projects/K.pneumoniae/) are highly related (94% identity and 98% similarity), and experiments in the presence of the *K. pneumoniae* ArgR will have to be performed to determine if this is the case. The impairment in the pJHCMW1 ARG box could be overcome by increasing the levels of PepA in *E. coli*. Similarly, in vitro recombination assays showed that high levels of PepA can

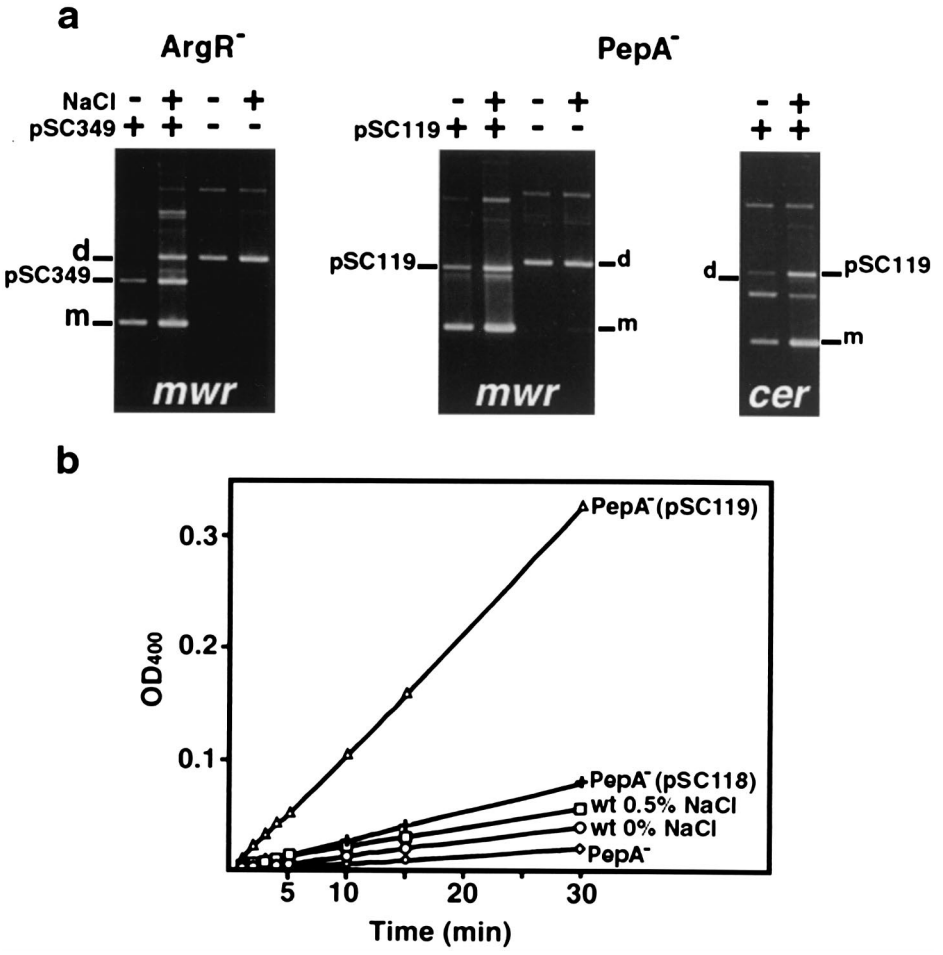

FIG. 7. Complementation of ArgR⁻ and PepA⁻ derivatives. (a) *E. coli* DS956 (*argR*) and DS957 (*pepA*) were transformed with dimers of pES (A *mwr*-C *mwr*) or dimers of pKS492 (A *cer*-C *cer*) and pCS349 (*argR* gene fusion) or pCS119 (*pepA* gene fusion), respectively. These derivatives, as well as control *E. coli* DS956 and DS957 harboring only pES dimers, were cultured in L broth containing 0.5% NaCl (+) or no NaCl (−). Plasmid DNA was isolated and subjected to agarose gel electrophoresis. d, dimer; m, monomer. (b) Aminopeptidase A was partially purified, and its activity was determined as described before (26) for *E. coli* DS957 (◇), *E. coli* DS957(pSC118) (+), *E. coli* DS957(pSC119) (Δ), and *E. coli* DS941 cultured in L broth with no NaCl (○) or 0.5% NaCl (□).

overcome the requirements for ArgR to form Holliday junctions (43). As a consequence of these observations, we considered the possibility that the levels of PepA in a wild-type *E. coli* strain increase at low osmolarity, thereby overcoming the defective interaction of the *mwr* accessory sequences with ArgR. However, our results indicated that this is not the case: the levels of PepA, as determined by aminopeptidase A activity, did not significantly change at high or low osmolarity. On the other hand, these results do not rule out the possibility that at lower osmotic pressure, there is an improvement in the interactions of the accessory proteins with the accessory sequences leading to a more efficient formation of the synaptic complex. Several DNA-binding proteins have been shown to change their interaction properties at different osmotic pressures (30, 31). In other cases, such as some transcriptional regulators, the binding affinity is not highly dependent on the intracellular ion concentration (11).

To cope with osmotic variations, bacteria respond by inducing several adaptation mechanisms. For some of them, such as the osmoprotectant *proU* uptake system, expression is kept at elevated levels as long as the osmotic stimulus persists (24), while for others, like the *kdp* operon, induction is transient (19). Since the higher efficiency of dimer resolution has been observed in several generations of cultures, it is most probable that the elements involved in Xer recombination at *mwr* undergo modifications that last for as long as the osmotic situation persists as is the case for induction of *proU*. Searches for regulators of *proU* failed to identify classical regulators. Instead, DNA-binding proteins such as H-NS, IHF, HU, TopA, or GyrAB were found to be associated with control of transcription of *proU* (10, 21, 29, 46). The involvement of these proteins together with the fact that the degree of DNA supercoiling is lower in cells cultured in low-osmolarity medium suggests that DNA structure may play a role in *proU* expression. Although our results indicate that H-NS seems not to be involved in the higher efficiency of recombination at *mwr* in low-osmolarity-grown cells, it is possible that one or more of these proteins can contribute to osmoregulation. Further analysis of the nature of the *mwr* DNA structure and the role of DNA-binding proteins will help to find out if changes in su-

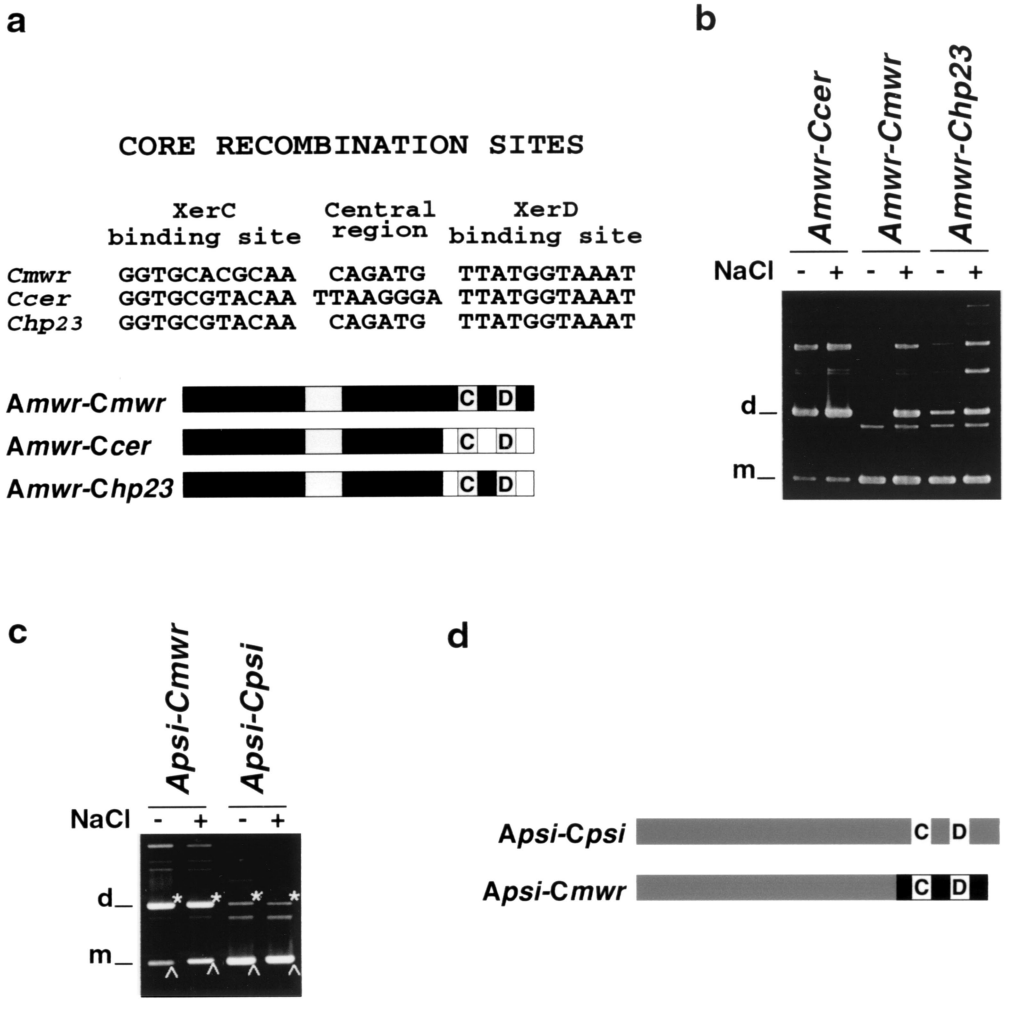

FIG. 8. Resolution of dimers containing a modified core recombination site. (a) Comparison of the nucleotide sequence of core recombination sites of *mwr*, *cer*, and the mutant derivative called *hp23* and diagram of the hybrid recombination site assayed. Gray boxes indicate the location of the XerC (C) and XerD (D) binding sites and the ARG box. (b and c) *E. coli* DS941 transformed with dimers harboring the indicated sites were cultured in L medium containing 0.5% NaCl (+) or no NaCl (−). Plasmid DNA was isolated and subjected to agarose gel electrophoresis. d, dimer; m, monomer.(d) Diagram representing A *psi*-C *psi* and the A *psi*-C *mwr* hybrid sites.

percoiling and interactions with any of these DNA-binding proteins play a role in osmoregulation of recombination at *mwr*. Although we do not know at this time if Xer site-specific recombination at *mwr* is osmoregulated in *K. pneumoniae*, comparisons of the amino acid sequences of the recombinases and accessory proteins with those from *E. coli* showed that they are very closely related. Dimer resolution experiments with plasmids carrying *mwr* in *K. pneumoniae* will permit us to determine if the recombination reaction is osmoregulated and, if it is, what the significance of this property is.

Replacement of the *cer* accessory sequences for those of *mwr* (hybrid site A *mwr*-C *cer*) resulted in a low efficiency of recombination comparable to that of the wild-type *mwr* at high osmolarity. However, unlike A *mwr*-C *mwr*, A *mwr*-C *cer* is not osmoregulated. Therefore, all or a portion of the core recombination site and its interaction with the recombinases may be responsible for this property. Replacement of the central region of the *cer* core recombination site (TTAAGGGA) for that from *mwr* (CAGATG) in the hybrid A *mwr*-C *cer* resulted in a site, A *mwr*-C *hp23*, with an intermediate level of osmoregulation compared to that of A *mwr*-C *mwr* (high osmoregulation) and A *mwr*-C *cer* (no osmoregulation). Therefore, we conclude that the *mwr* core recombination site and in particular the central region are involved in the regulation of recombination by osmotic pressure. The core central region plays an important role in determining the recombination properties of a site (2, 5). While in *psi* the central region favors a complete recombination reaction, in *cer* it favors only a strand exchange by XerC. These two central regions vary in length and purine content at the top strand: 6 nucleotides and 50% purine for *psi* and 8 nucleotides and 75% purine for *cer*. The central region of *mwr* is 6 nucleotides in length and has a 67% purine content. This intermediate purine content could be related to the ability to confer to a core recombination site the property of being osmoregulated. Figure 9 shows the steps for resolution of dimers harboring *psi*, *cer*, or *mwr*. While resolution of the Holliday junction at *cer* proceeds through a Xer-independent pathway, at *psi*, there is a change in conformation mediated by

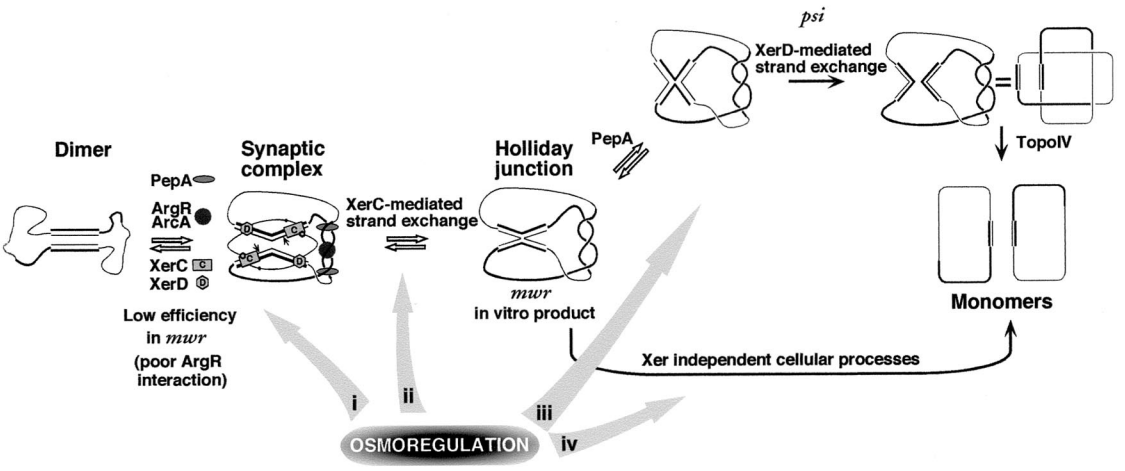

FIG. 9. Osmoregulation of Xer recombination at *mwr*. The diagram shows the steps of the recombination process indicating proteins or features involved. For clarity, the proteins are shown only in the synaptic complex. The accessory proteins are PepA and ArgR (for *cer* and *mwr*) or PepA and ArcA (for *psi*). Bold lines represent accessory sequences. Small hexagons represent the C terminus of XerD activating XerC. The black circles represent the XerC tyrosine residue properly located to exchange the top strands. The Xer-mediated pathway of resolution of the Holliday junction at *psi* is shown at the top. The bottom shows the Xer-independent resolution pathway followed by *cer* (and probably by *psi* and *mwr*). For clarity, the supercoiling of the molecules is not shown. The ability to resolve through the Xer-dependent pathway is determined by the central region of the core recombination site (2). The gray arrows show possible levels of osmoregulation involving the central region.

PepA followed by a XerD-mediated pair of strand exchanges. (It is possible that resolution at *psi* also proceeds through the Xer-independent pathway [2, 3, 15].) Although in vitro recombination at *mwr* yields Holliday junctions (43), we do not know whether they are resolved by Xer recombination (like *psi*), by a Xer-independent pathway (like *cer*), or both. The presence in a site of the accessory sequences of *mwr* results in an inefficient recombination site, probably by poor interaction with ArgR slowing the formation of the synaptic complex (Fig. 9). However, this reduction can be compensated for at low osmotic pressure by 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) or an increase in the efficiency of resolution of the Holliday junction (Fig. 9). We think it is likely that the osmoregulation is mediated through the XerCD-*mwr* core recombination site interaction. However, the stage at which this regulation acts is not known. It could act at the formation of the Xer-mediated synapsis (Fig. 9, arrow i) or at one of the catalytic steps (Fig. 9, arrows ii and iii). Alternatively, the osmoregulation might be mediated by unknown cellular processes, if resolution of the Holliday junction at *mwr* is Xer independent (Fig. 9, arrow iv). Experiments are being carried out to determine the step of the reaction that is regulated by the osmolarity of the growth medium. These possibilities do not rule out an additional contribution to osmoregulation mediated by a better interaction of PepA at a low salt concentration with the *mwr* accessory sequences. Another set of experiments suggests that the sole presence of the *mwr* core recombination site is not enough for osmoregulation of a site. The hybrid site A *psi*-C *mwr*, which recombines at low efficiency, was only barely osmoregulated (Fig. 8). Probably the interwrapping of the *psi* accessory sequences around the accessory proteins (ArcA and PepA) does not form the ideal topology for recombination at the *mwr* core recombination site. This deficiency is only minimally compensated for by osmoregulation mediated by the *mwr* core recombination site.

Bacterial cells may encounter different environments through their life cycle. Upon entering a host, the pathogens' surroundings change dramatically, and they must have systems that enable them to grow or survive. Plasmids harbored by these bacterial cells carry genetic elements to be stably maintained in the various environments, and not all of them may be active under the same conditions. The plasmid pJHCMW1 has two systems for stability by multimer resolution: *tnpR*/*res* and *xer*/*mwr* (43). Although we do not know how by being osmotically regulated *mwr* helps the stability of pJHCMW1 in *K. pneumoniae*, one can envision that the *mwr* locus is not critical for stability under certain environments in which resolution occurs via the resolvase at *res*, but it is essential in others where the osmolarity must be lower. *K. pneumoniae* causes a substantial amount of hospital-acquired urinary tract infections, pneumonia, septicemias, meningitis, and soft tissue infections. One or more of the niches occupied in these diverse infections may present changes in osmolarity that influence the efficiency of Xer recombination at *mwr*.

ACKNOWLEDGMENTS

This work was supported by Public Health grants AI47115-01 (M.E.T.) and LS Basin MIRT T37 TW00048-05 from the National Institutes of Health and a grant from Wellcome Trust (D.J.S.). H.P. and K.D. were supported by MSD grant R25 GM56820-03 and MIRT T37 TW00048-05 from the National Institutes of Health.

We thank Migena Bregu and Sean Colloms for generously providing plasmids and R. Allen for technical help.

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