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Manuscript Draft

Manuscript Number:

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Article Type: Special Issue: ISPB 2012

Keywords: DNA-protein interactions; DNA relaxase; origin of transfer; rolling circle-replicating plasmids

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Abstract: The MobM relaxase (494 amino acids) encoded by the promiscuous streptococcal plasmid pMV158 recognizes the plasmid origin of transfer, oriTpMV158, and converts supercoiled pMV158 DNA into relaxed molecules by cleavage of the phosphodiester bond of a specific dinucleotide within the sequence 5'-GTGTG/TT-3' ("/" being the nick site). After cleavage, the protein remains stably bound to the 5´-end of the nick site. Band-shift assays with single-stranded oligonucleotides and size-exclusion chromatography allowed us to show that MobM was able to generate specific complexes with one of the inverted repeats of the oriTpMV158, presumably extruded as stem-loop structure. A number of tests have been performed to attain a better characterization of the nicking activity of MobM and its linkage with its target DNA. The optimal pH for DNA relaxation was found to be 6.5. Upon nicking, gel retardation assays showed that MobM formed stable complexes with its target DNA. Moreover, MobM bound to relaxed pMV158 molecules were visualized by electron microscopy. The staphylococcal plasmids pUB110 and pE194, and the streptococcal plasmid pDL287 harbour putative oriTs and may encode Mob proteins homologous to MobM. The oriTpUB110, oriTpDL287, and oriTpE194 sequences share 100%, 70%, and 67% (in a 43-nucleotide stretch and allowing a 3-bp gap) identity to oriTpMV158, respectively. Nicking assays using supercoiled DNAs from pUB110, pDL287, and pE194 showed that MobM was able to relax, to a different degree, all plasmid DNAs. Our results suggest that cross-recognition of heterologous oriTs by Mob proteins could play an important role in the plasmid spreading between bacteria.

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Here we present information on the features of the MobM relaxase encoded by the promiscuous plasmid pMV158. The protein binds to oligonucleotides and to supercoiled plasmid DNA containing the origin of transfer. MobM-relaxed pMV158 DNA forms could be visualized by electron microscopy. The MobM protein relaxed supercoiled DNAs from other rolling circle-replicating plasmids which harbor origins of transfer with similarities to the one present in pMV158.

Nicking activity of the pMV158 MobM relaxase on cognate and heterologous origins of transfer

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Key words: DNA-protein interactions; DNA relaxase; origin of transfer; rolling circle-replicating plasmids

Running title: MobM relaxase of pMV158

Category: Plasmid functions

Special issue

Abstract

The MobM relaxase (494 amino acids) encoded by the promiscuous streptococcal plasmid pMV158 recognizes the plasmid origin of transfer, *oriT*$_{pMV158}$, and converts supercoiled pMV158 DNA into relaxed molecules by cleavage of the phosphodiester bond of a specific dinucleotide within the sequence 5'-GTGTG/TT-3' ("/" being the nick site). After cleavage, the protein remains stably bound to the 5´-end of the nick site. Band-shift assays with single-stranded oligonucleotides and size-exclusion chromatography allowed us to show that MobM was able to generate specific complexes with one of the inverted repeats of the *oriT*$_{pMV158}$, presumably extruded as stem-loop structure. A number of tests have been performed to attain a better characterization of the nicking activity of MobM and its linkage with its target DNA. The optimal pH for DNA relaxation was found to be 6.5. Upon nicking, gel retardation assays showed that MobM formed stable complexes with its target DNA. Moreover, MobM bound to relaxed pMV158 molecules were visualized by electron microscopy. The staphylococcal plasmids pUB110 and pE194, and the streptococcal plasmid pDL287 harbour putative *oriT*s and may encode Mob proteins homologous to MobM. The *oriT*$_{pUB110}$, *oriT*$_{pDL287}$, and *oriT*$_{pE194}$ sequences share 100%, 70%, and 67% (in a 43-nucleotide stretch and allowing a 3-bp gap) identity to *oriT*$_{pMV158}$, respectively. Nicking assays using supercoiled DNAs from pUB110, pDL287, and pE194 showed that MobM was able to relax, to a different degree, all plasmid DNAs. Our results suggest that cross-recognition of heterologous *oriT*s by Mob proteins could play an important role in the plasmid spreading between bacteria.

1. Introduction

Bacterial plasmids are genetic entities that replicate in an independent and controlled manner from their hosts (del Solar et al., 1998). Plasmids can be transferred among bacteria mostly by conjugation (self-transmissible plasmids) or mobilization (mobilizable plasmids). The former encode all functions needed for their transfer, whereas the latter make use of functions provided by other (auxiliary) plasmids (Garcillán-Barcia et al., 2011; Grohmann et al., 2003; Smillie et al., 2010). Conjugative and mobilizable plasmids, as well as other self-transmissible DNA elements, like integrative-conjugative elements and phage-like sequences, constitute the mobilome and they play a key role in the spread of genetic information among bacteria by horizontal gene transfer. The mobilome strongly influences the co-evolution of the bacterium-plasmid pair and represents a reservoir of DNA (up to 25% of the total bacterial DNA) that is shared among bacterial species (Thomas, 2000). Transfer requires the assembly of plasmid-encoded proteins on a specific plasmid DNA region, the origin of transfer (*oriT*) to generate a macromolecular complex, the relaxosome (Lanka and Wilkins, 1995). The key player is the plasmid-encoded nicking-closing protein: the DNA relaxase (Francia et al., 2004; Garcillán-Barcia et al., 2009). This protein initiates transfer by cleaving the phosphodiester bond at a specific dinucleotide located within the *oriT* (Datta et al., 2003; Guasch et al., 2003), generating an aminoacyl-DNA adduct that is experimentally recognizable because the 5'-end of the cleaved DNA will be occluded, whereas its 3'-end will be accessible to enzymes (Pansegrau and Lanka, 1996a). The DNA-relaxase complex would be transferred to the recipient cell by means of the coupling protein and the macromolecular protein complex, the transferosome, that constitute a type IV secretion system (Gomis-Rüth and Coll, 2006; Gomis-Ruth et al., 2002; Llosa et al., 2002). Once into the recipient, the relaxase–DNA intermediate would restore the original circular plasmid molecule after termination of transfer through a reversion of the strand transfer reaction, in a mechanism that is similar to that described for termination of rolling circle replication (RCR) (Novick, 1998; Pansegrau and Lanka, 1996b). Finally, conversion of single-stranded DNA molecules into double-stranded plasmid forms in the recipient cells is carried by conjugative replication that initiates from a lagging-strand origin (Lorenzo-Díaz and Espinosa, 2009a; Parker and Meyer, 2005).

Large plasmids may be self-transmissible because they encode the entire machinery for their transfer, even though a large number of plasmids seem to be non-mobilizable (Smillie et al., 2010). However, many small-sized plasmids that replicate by the rolling-circle mechanism (RCR-plasmids) contain a single gene cassette, composed by a gene encoding the *trans*-acting Mob relaxase and a *cis*-acting *oriT*, that allows these plasmids to be transferred by means of helper plasmids (Grohmann et al., 2003). In the case of the streptococcal plasmid pMV158 (Burdett, 1980), the relaxase MobM was purified and several of its interactions with its cognate origin of transfer, $oriT_{pMV158}$, were characterized *in vitro* (de Antonio et al., 2004; Grohmann et al., 1999; Guzmán and Espinosa, 1997). Transfer of pMV158 mediated by plasmids of the Inc18 and IncPα families was shown to occur between different bacterial species in which the plasmid replicates (Farías and Espinosa, 2000; Lorenzo-Díaz and Espinosa, 2009b). The relaxase domain of MobM was further characterized by purification of a truncated protein, MobMN199, which contains the first 199 residues of MobM. Contrary to the dimeric configuration of the native MobM, MobMN199 was shown to be a monomer, so that we could map the dimerization domain as located in the C-terminal moiety of the protein (Lorenzo-Díaz et al., 2011).

In the present work, we have analyzed the interactions of the full length MobM protein with its cognate $oriT_{pMV158}$ and the stability of the linkage between the protein and its target DNA after cleavage the strand to be transferred by gel-shift assays and electron microscopy. Attempts at optimizing the nicking reaction showed that the optimum pH was 6.5, while the temperature (30 ºC), time of reaction (20 min) and divalent cations (8 mM Mn^{2+}) were assessed previously (de Antonio et al., 2004; Guzmán and Espinosa, 1997; Lorenzo-Díaz et al., 2011). Under these optimal conditions, maximum cleavage reached up to ~65% of the total input DNA. Furthermore, we present results showing that MobM protein was able to relax supercoiled DNAs from RCR-plasmids with *oriT*s that share total (plasmid pUB110) or partial (plasmids pDL287 and pE194) homology with the $oriT_{pMV158}$.

2. Materials and Methods

2.1. Bacterial strains, plasmids, and culture conditions

Streptococcus pneumoniae 708 (*end-1, exo-2, trt-1, hex-4, malM594*; (Lacks, 1968) was used as host for plasmids pMV158 and pDL287. The *Bacillus subtilis* 168 MB56 strain (*trpC2*, lab collection (Espinosa et al., 1982) was used as host for plasmids pUB110 and pE194. The RCR-streptococcal plasmids pMV158 (Burdett, 1980) and pDL287 (Le Blanc et al., 1992; LeBlanc et al., 1993; a gift of D. Galli) and the RCR-staphylococcal plasmids pUB110 (McKenzie et al., 1986) and pE194 (Horinouchi and Weisblum, 1982) were used for nicking assays. *Escherichia coli* BL21 (DE3) (r^-_B m^-_B, *gal, ompT, int::*P_{lacUV5}*-T7 gene 1 imm 21 nin5*; a gift of F.W. Studier) was used for purification of the full length MobM protein. This strain harbours a single copy of the T7 RNA polymerase gene integrated into the chromosome, under the control of the inducible *lacUV5* promoter (Studier et al., 1990). For over-expression of the *mobM* gene, plasmid pLGM2 was used; it contains two copies of the *mobM* gene under the control of the ϕ10 promoter of phage T7 (Guzmán and Espinosa, 1997).

E. coli and *B. subtilis* cells were grown in tryptone-yeast extract (TY) medium (Pronadisa), whereas *S. pneumoniae* cells were grown in AGCH medium supplemented with 0.2% yeast extract and 0.3% sucrose (Lacks, 1966; Ruiz-Cruz et al., 2010). When cells harboured plasmids, the medium was supplemented with tetracycline (1 µg/ml, pMV158), erythromycin (10 µg/ml, pE194), ampicillin (100 µg/ml, pLGM2), or kanamycin (10 µg/ml and 30 µg/ml, pUB110 and pDL287, respectively).

2.2. Purification of plasmid DNA

Plasmids pMV158 (Lacks et al., 1986; Priebe and Lacks, 1989) and pE194 were purified by two consecutive CsCl gradients as described (del Solar et al., 1987), whereas pDL287 and pUB110 were isolated from alkaline-lysis preparations using the High Pure Plasmid Isolation Kit (Roche Applied Science). The suspension buffer of this kit was supplemented with 50 mM glucose and 0.1% sodium deoxycholate for *S. pneumoniae* (Ruiz-Cruz et al., 2010) or with 50 mM glucose and 1 mg/ml lysozyme for *B. subtilis* (Espinosa et al., 1982); the NaOH content of the lysis buffer was adjusted to 0.17 N as described (Stassi et al., 1981).

2.3. Overproduction and purification of MobM

MobM was overproduced and purified essentially as described (de Antonio et al., 2004), but with some modifications that allowed us to obtain a higher protein yield (Lorenzo-Díaz et al., 2011). Briefly, cell pellets from 4l-cultures were concentrated (100x) in buffer A (20 mM Tris–HCl pH 7.6, 1 mM EDTA, 1 mM dithiothreitol, 5% (v/v) glycerol) supplemented with 1 M NaCl and a tablet of a protease inhibitor cocktail (Roche Applied Science). Cells were disrupted by passage through a French pressure cell, the whole-cell extract was centrifuged to remove cell debris, and protein MobM was purified essentially as described (Lorenzo-Díaz et al., 2011). Protein concentration was determined with a NanoDrop ND-1000 Spectrophotometer, and the final yield was of 8-10 mg of purified MobM. Protein preparations were stored in buffer A supplemented with 500 mM NaCl and kept at -80 ºC. Analyses of the MobM protein hydrodynamic parameters were done by analytical ultracentrifugation and determination of the molecular mass of the protomer was performed by mass spectrometry (MALDI-TOF-TOF) under the reported conditions (Lorenzo-Díaz et al., 2011; Moreno-Córdoba et al., 2012).

2.4. Nicking activity of MobM

Nicking assays were performed essentially as reported (Guzmán and Espinosa, 1997; Lorenzo-Díaz et al., 2011). Usually the standard reaction mixtures (20 μl) contained 25 mM Tris-HCl pH 7.6, 0.1 mM EDTA, 10% glycerol (v/v), 1 mM dithiothreitol, NaCl 50mM, 8 mM $MnCl_2$ and purified protein (240 nM). Mixtures were incubated in the reaction buffer for 5 min at room temperature before the addition of supercoiled plasmid DNA. The amount of plasmid DNA used was 500 ng, except for the heterologous *oriT* recognition assays, in which 100 ng of each supercoiled DNA was used. Unless otherwise stated, the reaction mixtures were incubated at 30 ºC, 20 min, and reactions were stopped by treatment with SDS (0.5%) and proteinase K (1 mg/ml) followed by incubation at 37 ºC, 30 min. Generation of open circular forms (forms FII) was monitored by agarose (1%) gel electrophoresis. Gels were stained with ethidium bromide (EtBr, 1 μg/ml) and DNA was visualized using a Gel-Doc system (BioRad). The intensity of the bands was quantified using the QuantityOne software. To calculate the percentage of open circular forms, the intensity of the bands corresponding to supercoiled and relaxed forms (FI and FII, respectively) was quantified in the absence and in the presence of MobM.

2.5. Electron microscopy

Reaction mixtures (20 µl) contained 20 mM Tris-HCl pH 7.0, 1 mM dithiothreitol, 1 mM EDTA, 1% glycerol, 50 mM NaCl, 15 mM $MgCl_2$, pMV158 DNA (8 nM) and MobM protein (375 nM). After 30 min at 30 ºC, MobM-DNA complexes were fixed with 0.3% glutaraldehyde, 15 min at the same temperature. Then, reactions were diluted 10-fold in buffer GA (10 mM triethanolamine chloride, pH 7.5, 10 mM $MgCl_2$), adsorbed onto freshly cleaved mica, positively stained with 2% uranyl acetate, rotary shadowed with Pt/Ir, and covered with a carbon film as described previously (Spiess and Lurz, 1988). Micrographs of the carbon film replica were taken using a Philips CM100 (FEI Company, Hillsboro, Oregon) electron microscope at 100 kV on 35-mm film.

2.6. Electrophoretic mobility shift assay (EMSA)

To analyse complex formation between MobM and its target DNA by EMSA, single-stranded or supercoiled DNA harbouring a part of, or the entire *oriT*$_{pMV158}$ sequence, were used as substrates. In the first case, oligonucleotides harbouring either the inverted repeat 2 or the inverted repeat 3 (IR2 and IR3, respectively) of the *oriT*$_{pMV158}$ were employed. Both oligonucleotides were labelled with the fluorophore Cy5 either at their 3' or 5'-ends. Thus, oligonucleotide IR2 was TAAAGTATAGTGTG/TTATACTTTA-Cy5 (coordinates 3582-3605 of pMV158, accession number X15669), and oligonucleotide IR3 was Cy5-GCACACACTTTATGAATATAAAGTATAGTGTG/ (coordinates 3564-3595). Oligonucleotides were heated (80 ºC, 15 min), after which the solution was flash-cooled to 4 ºC. Different amounts of MobM were incubated (20 min, 24 ºC) with 2 nM of each oligonucleotide in buffer containing 20 mM Tris–HCl pH 7.6, 1 mM EDTA, 1 mM dithiothreitol, 5% (v/v) glycerol and 300 mM NaCl. Protein-DNA complexes were separated by electrophoresis on 10% native polyacrylamide gels as described (Lorenzo-Díaz et al., 2011). EMSA assays with supercoiled DNA were performed in the same buffer but supplemented with 50 mM NaCl and 8 mM Mn^{+2}. Mixtures containing 8 nM of pMV158 DNA were incubated with 240 nM of MobM, 20 min, 30 ºC. Then, reactions were treated or not with SDS (0.5%) and proteinase K (1 mg/ml) followed by incubation at 37 ºC, 30 min. Samples were analyzed in agarose (1%) gels and stained with EtBr (1µg/ml). In some cases, as indicated in the Results, reaction mixtures were not treated with proteinase K but were subjected to restriction

with *Eco*RI and *Afl*II enzymes (New England Biolabs). The resulting DNA fragments were separated by electrophoresis on 5% polyacrylamide gels and stained with EtBr (1µg/ml).

2.7. Size exclusion chromatography

In this case, the oligonucleotides used were unlabelled IR2 and IR3. Complex formation of MobM-DNA was performed as follows: after a heat-cooling step (under the above EMSA conditions), a 10% molar excess of the appropriate oligonucleotide was added to 150 nM MobM in buffer GF (300 mM NaCl, 20 mM Tris-HCl pH 7.6, 1 mM dithiothreitol, 1 mM EDTA). The mixture was incubated (24 ºC, 20 min) and passed through a Superdex 200 10/300 column (GE Healthcare) pre-equilibrated with buffer GF. Complex formation was assessed by a shift in elution volume and by changes in the absorbance at 280 nm of the eluting species. The column was previously calibrated by passing only MobM protein or only the oligonucleotides IR2 and IR3 in the same conditions.

2.8. Bioinformatics resources

The following websites were used to perform sequence alignments and calculations of different parameters of the Mob proteins encoded by plasmids pMV158 (MobM), pUB110 (MobU), pDL287 (MobL), and pE194 (MobE). General calculations were done at BIOSHELL (http://www.bioshell.pl/s,servers,58.html). Pfam features were obtained at the Sanger website (http://www.sanger.ac.uk/resources/databases/pfam.html) and protein domain families were identified with the ProDom program (http://prodom.prabi.fr/prodom/current/html/home.php). The presence of coils and coiled coils were done from http://www.ch.embnet.org/software/COILS_form.html, whereas ClustalW2 alignments were obtained from EBI webpage (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

3. Results and Discussion

3.1. Features of the MobM relaxase

The promiscuous plasmid pMV158 is the representative of an entire family of RCR-plasmids (del Solar et al., 1998; del Solar et al., 2002) composed so far by more than 50 replicons isolated from a variety of bacteria (Espinosa, 2013). The

plasmid has been established in nearly twenty bacterial species, and dissection of its functions has shown that it is composed by three main genetic modules (Fig. 1A): i) LIC, which harbours genes and loci involved in leading strand replication and control (del Solar et al., 2002), ii) DET, including a *tetL* gene determinant that provides resistance to tetracycline (Lacks et al., 1986), and iii) MOB, harbouring the relaxase-encoding *mobM* gene and the $oriT_{pMV158}$ (Guzmán and Espinosa, 1997; Lorenzo-Díaz et al., 2011; Priebe and Lacks, 1989). In addition, the plasmid harbours two lagging-strand origins, *ssoU* and *ssoA*, the latter used for replication and transfer between streptococcal species, whereas the former seems to be involved in interspecies transfer, most likely being the responsible for the promiscuity of the replicon (Kramer et al., 1999; Lorenzo-Díaz and Espinosa, 2009a).

The MobM protein (Fig. 1B and Table 1) is a DNA relaxase of 494 amino acid residues, with a theoretical mass of 57,874 Da per protomer, and a determined value of 57,900 Da as determined by MALDI-TOF-TOF. Analytical ultracentrifugation of the purified protein showed a molecular mass of 115,650 ± 1,800 Da, and a frictional ratio of f/f_0 = 1.65, indicative of a dimeric protein with an ellipsoid shape (Table 1). These results agree with our previous determinations (de Antonio et al., 2004) but with a higher accuracy. The same applies for the MALDI-TOF-TOF experiments done with the new protein preparations, which gave a value of 57,900 Da for the mass of the MobM protomer, being 57,707 Da the expected value if we reduce 149 Da for the mass of the first Met residue which was removed (determined by N-terminal sequencing of the protein) and 18 Da for the peptide bond (Table 1). Protein MobM was shown to cleave supercoiled DNAs at the specific sequence 5'-TAGTGTG/TTA-3' ("/" being the phosphodiester bond cleaved by the protein) (de Antonio et al., 2004; Guzmán and Espinosa, 1997; Lorenzo-Díaz et al., 2011). Two main regions (Fig. 1B) have been defined in the full length MobM (Lorenzo-Díaz et al., 2011). The N-terminal moiety (roughly the first 200 residues) contains the catalytic domain with three conserved motifs: i) HxxR (x, any amino acid) of unknown function yet; ii) NYEL, which has been predicted to harbour the catalytic tyrosine, Y44 (Francia et al., 2004; Grohmann et al., 1999), and iii) HxDE…PHUH (U, a hydrophobic residue), which is present in the Rep-initiator proteins of the plasmids of the pMV158 family and which should contain the metal coordination motif (Ilyina and Koonin, 1992; Koonin and Ilyina, 1993). This N-terminal domain is contained within the truncated version of the full length MobM, the so-called MobMN199 protein which has been

previously characterized in terms of cleavage of supercoiled DNA and binding affinity for several single-stranded DNAs (Lorenzo-Díaz et al., 2011). The C-terminal moiety of MobM harbours the functions related to: i) dimerization, since MobMN199 was monomeric in solution (Lorenzo-Díaz et al., 2011); we attribute this fact to the presence of a putative leucine-zipper motif that we have located between residues 317 and 338 (Fig. 1C); ii) association to the cell membrane that was disrupted by a quadruple mutation in one of the predicted coiled coil regions of MobM: the COILS software predicts a high content of coiled coils between residues 420 and 465. Specifically, changes R421P, L423P, L425P, and L427P resulted in a MobM mutant protein that was unable to associate to the cell membrane (de Antonio et al., 2004). A pMV158-derivative harbouring the four mutations was unable to be transferred between pneumococcal strains (not shown). Further mutagenesis studies are needed to refine the distribution of sub-domains within the C-terminal region of MobM.

3.2. Nicking activity of MobM

We have reported that the optimal temperature for MobM-mediated DNA cleavage was 30 ºC, being Mn^{2+} the preferred cation, although it could be substituted by Mg^{2+}, and partially by Ca^{2+} (de Antonio et al., 2004; Guzmán and Espinosa, 1997). We have also discovered that the presence of Mn^{2+} ions greatly augmented the thermal stability of the protein, so that we modified the nicking conditions (Lorenzo-Díaz et al., 2011). Here we wanted to analyze another parameter, not tested before, which was the effect of the pH on the nicking reaction. For this, we used a MobM protein preparation that was purified following a new protocol that increased the yield and purity of the protein (Lorenzo-Díaz et al., 2011). When supercoiled pMV158 DNA (forms FI) was incubated with this protein preparation in the presence of 8 mM Mn^{2+}, 30 ºC, 20 min, and pH 7.2, we found that ~55% of the supercoiled pMV158 molecules were relaxed (not shown). We next analysed the influence of the pH on the nicking activity of MobM in presence of 8 mM Mn^{2+} at 30 ºC, 20 min (Fig. 2). The highest nicking activity (~65% of FII forms) was observed when the pH was 6.5. Such an activity was even higher than that reported previously (~50% of relaxed molecules) not only for MobM (Guzmán and Espinosa, 1997) but also for other relaxases, like those encoded by plasmids RP4 (Pansegrau et al., 1990), R388 (Llosa et al., 1995), and F (Matson et al., 1993).

We next performed a MobM concentration-dependent relaxation assay incubating supercoiled pMV158 DNA with increasing concentrations of MobM (Fig. 3A). The reaction mixtures were incubated under the above optimal conditions (30 ºC, 20 min, pH 6.5, and 8 mM Mn^{2+}). Then, they were treated with proteinase K to remove the MobM protein that remains stably bound to the 5´-end generated by its nicking activity. The FI and FII plasmid forms were separated by electrophoresis on an agarose gel (Fig. 3A). At 175 nM of MobM, 63% of the supercoiled molecules (FI) were relaxed (FII), and such a percentage did not increase at higher MobM concentrations. The appearance of open circular molecules (forms FII) was also monitored by electron microscopy. To this end, pMV158 supercoiled DNA (5540-bp) was incubated with MobM under nicking conditions. Samples were further fixed with glutaraldehyde and prepared for electron microscopy. Under these conditions, supercoiled and relaxed plasmid molecules bound to MobM were visualized. An electron micrograph of this experiment is shown in Figure 3B. To verify the specificity of the MobM-pMV158 complexes, samples were digested with *Stu*I after the fixation with glutaraldehyde and prior to their preparation for electron microscopy. Plasmid pMV158 has a single *Stu*I site (coordinate 4626). Linear DNA molecules bound to MobM were then visualized. The contour lengths of the DNA regions between complexes and DNA ends were measured, and the MobM binding site was determined. The majority (65%) of the 50 complexes examined had MobM positioned around coordinates 3585-3596, which fit well with the position of the nicking site mapped previously between coordinates 3595 and 3596 (Guzmán and Espinosa, 1997). From the above analyses, we may conclude that the supercoiled closed forms ('ready to replicate') of pMV158 would be in equilibrium with the forms relaxed by MobM at $oriT_{pMV158}$ ('ready to go'). This equilibrium would account for the RC-mechanism of replication of pMV158: once the RepB initiator cleaves the plasmid supercoiled forms at the double-stranded replication origin (*dso* in Fig. 1A) to start replication, FII molecules would be unsuitable to be transferred because MobM also needs a supercoiled substrate to initiate conjugative plasmid transfer. Conversely, plasmid molecules relaxed by MobM at initiation of transfer would be unable to replicate. Run-off assays developed to detect strand discontinuities in growing cells harbouring pMV158 allowed us to map RepB-relaxed and MobM-relaxed forms *in vivo* (Grohmann et al., 1997; Zechner et al., 1997), thus perhaps both types of relaxed molecules could co-exist within the same cell. The requirement of Mn^{2+} (not

only for MobM but also for the RepB initiator), and the relatively low pH for optimum MobM-mediated relaxation *in vitro* could be explained by the involvement of this cation in pneumococcal virulence (Rosch et al., 2009), and for the decrease in the pH of the medium when culturing *S. pneumoniae* and related streptococci and lactococci.

3.3. Complex formation between MobM and oriT$_{pMV158}$-mimicking oligonucleotides

We have previously shown that the MobMN199 truncated protein containing the relaxase domain of MobM was unable to bind to oligonucleotides that contain the IR2 of the plasmid *oriT* (Lorenzo-Díaz et al., 2011). This was unexpected since the right arm of IR2 is one of the conserved elements of the origins of transfer of plasmids of the MOB$_V$ family (Garcillán-Barcia et al., 2009). To test whether a similar behaviour applied to the full length MobM, experiments were performed and formation of MobM-ssDNA complexes was tested by two methods, namely EMSA and size exclusion gel chromatography using in both cases oligonucleotides harbouring either the IR2 or the IR3 (Fig. 4). EMSA tests (Fig. 4A) showed that, indeed, the affinity of MobM for IR2 was very low, if any, whereas the protein showed great affinity for the IR3 oligonucleotide. Size exclusion gel chromatography using the full length MobM confirmed these results (Fig. 4B). The elution profiles of the respective mixtures of MobM with the two oligonucleotides showed that MobM could not form stable complexes with the oligonucleotide mimicking IR2, since the eluted peaks were consistent with pure protein (13.9 ml) and free DNA (16.6 ml). A complex of the full-length MobM and the IR2 sequence was therefore not observed. However, when the oligonucleotide employed mimicked IR3, no signal was detected in the expected elution volumes for free protein and DNA, but a single peak corresponding to MobM-DNA complexes was observed (10.9 ml, Fig. 4B). Differences in binding can be explained either by the extended stem-loop structure of IR3 with respect to that of IR2 or by the differences in the position of the nick site: IR3 would have the nick site placed in the base of the hairpin, whereas IR2 would have it placed in the loop (see Fig. 6B). Inspection of known structures indicates that the sequence following the hairpin exhibited more interaction with the protein, which suggested to us that this part is the more important determinant for recognition of the target DNA by the relaxase.

3.4. Stable linkage of MobM to its target DNA

One experimental approach to show that a relaxase is able to generate stable complexes with its target DNA after nicking is to precipitate the protein-DNA complexes with SDS and KCl and analyze the DNA content in the pellet and in the supernatant by native PAGE, as shown earlier for topoisomerases (Trask et al., 1984), and later on for several relaxases, MobM among them (Guzmán and Espinosa, 1997; Matson et al., 1993; Pansegrau et al., 1990; Szpirer et al., 2001). Here we have developed a simpler approach in which the protein stable linkage to a region of the DNA is tested by DNA-relaxation and restriction analyses and the products separated and analyzed by native PAGE. This would result in the appearance of either a fragment with anomalous migration or a 'missing' fragment due to DNA-protein complex retained in the well. First, and to estimate whether this approach was feasible for MobM, supercoiled pMV158 DNA was incubated with MobM under optimal nicking conditions. Next, samples were treated or not with proteinase K and loaded on an agarose gel. As shown in Figure 5A, the FII forms generated by the nicking activity of MobM entered the agarose gel only after treatment with proteinase K. The Y44 amino acid residue has been proposed as the catalytic residue of MobM (Francia et al., 2004; Garcillán-Barcia et al., 2009; Grohmann et al., 1999). If this was the case, the predicted covalent adduct after a total proteinase K digestion of MobM-pMV158 complexes would be the DNA attached to a dipeptide (Asn-Tyr). In a second experiment, pMV158 was incubated or not with MobM under optimal nicking conditions, and subsequently it was restricted simultaneously with *Eco*RI and *Afl*II. The expected products are schematized in Fig. 5B, and the resulting DNA fragments were subsequently separated on native 5% polyacrylamide gels (Fig. 5C). In the absence of MobM, the four expected restriction fragments were visualized (3556, 852, 587, and 545 bp). However, in the MobM-treated samples, the *oriT*-containing DNA fragment (545-bp, coordinates 3170-3715) was shifted and part of the MobM-uncleaved DNA (some 35% of the input DNA) was not detected at the expected position, most likely due to its retention in the position of the other fragments (note, for instance, the 'fuzziness' in the 587-DNA band). These results supported that MobM formed stable complexes with the relaxed plasmid molecules generated by its nicking activity, and provide a fast and reliable procedure to detect stable association of a DNA-relaxing enzyme (initiator of RCR or relaxase) with its target DNA and to map the DNA region targeted by the enzyme.

3.5. Recognition of heterologous oriTs by MobM protein

Bioinformatics approaches performed among all RCR-plasmids so far described that could encode a MOB cassette similar to the one present in pMV158 allowed the definition of the MOB_V family of relaxases (Francia et al., 2004; Garcillán-Barcia et al., 2009). In the case of pMV158, its *oriT*$_{pMV158}$ contains three partially overlapping IR that could generate three alternative stem-loop structures in which the position of the dinucleotide cleaved by MobM would be placed in different positions (Espinosa, 2013; Lorenzo-Díaz et al., 2011; Fig. 6B). Due to their structure, generation of one of the hairpins would hinder the formation of the other two, indicating perhaps that the accessibility of the relaxase to its target could depend on the superhelicity of the plasmid DNA. To test whether MobM could recognize heterologous *oriT*s from RCR-plasmids belonging to the MOB_{V1} subfamily, we selected, in addition to pMV158, three more plasmids: the streptococcal plasmid pDL287 that is a derivative of pVA380-1 (Le Blanc et al., 1992) and that was shown to be relaxed by MobM (Grohmann et al., 1999), and the staphylococcal plasmids pUB110 (McKenzie et al., 1986), and pE194 (Horinouchi and Weisblum, 1982). These latter plasmids were hosted by *B. subtilis* because we have been unable to transfer them to *S. pneumoniae*, most likely because of the different degree of supercoiling of the plasmid DNAs. In fact, we showed that the initiator of replication RepB from pMV158 was able to nick DNA from pE194 (with which there is homology at the *dso*) only when the supercoiling of pE194 was altered (Moscoso et al., 1995). The putative Mob proteins encoded by pDL287, pUB110, and pE194 (herein named MobL, MobU, and MobE, respectively) showed homology to MobM (Fig. 6A). EMBOSS needle global sequence alignment (Rice et al., 2000) of MobM (494 residues), MobU (420 residues), MobL (430 residues), and MobE (403 residues) revealed various degrees of homologies among the full length proteins. The first 200 residues, harbouring the relaxase domain, showed the highest homology, reaching up to 96.5% identity in the pair MobM/MobL (Table 2). Concerning the *oriT*s, it was observed that *oriT*$_{pUB110}$, *oriT*$_{pDL287}$, and *oriT*$_{pE194}$ sequences share 100%, 70%, and 67% (in a 43-nucleotide stretch and allowing one 3 bp-gap) identity to *oriT*$_{pMV158}$, respectively. Western blot assays using cell extracts from *S. pneumoniae* harbouring either pMV158 or pDL287 and cell extracts from *B. subtilis* carrying pUB110 showed

that antibodies anti-MobM recognized MobL, but not MobU (not shown) in agreement with the highest homologies found between MobM and MobL (Fig. 6A and Table 2).

To study whether MobM was able to recognize the heterologous *oriT*s, nicking experiments were performed under standard conditions. As shown in Figure 7, MobM was able to convert supercoiled FI DNA forms into FII forms not only from pUB110 (identical *oriT*s) but also from pDL287 and pE194. The efficiency of MobM-mediated cleavage was similar for pMV158 and pUB110 (~50% of nicking), whereas it was lower for plasmid pDL287 and pE194, 40% and 30% of nicking, respectively. This set of results shows the possibility of cross-recognition of heterologous *oriT* sequences by Mob relaxases encoded by RCR-plasmids, thus opening the possibility of creating new relaxase chimaeras.

It is interesting to point out that some RCR-plasmids exhibit an 'orphan' *oriT*, since they have a region whose nucleotide sequence is very similar to that of well characterized *oriT*s but lack a cognate putative relaxase gene; such is the case of plasmids pA1 from *Lactobacillus plantarum* (Vujcic and Topisirovic, 1993) and pCI411 from *Leuconoctoc lactis* (Coffey et al., 1994). This, in conjunction to the *oriT*s of some RCR-plasmids being defined as site-specific plasmid recombination (Pre) regions (Gennaro et al., 1987), raise the question of whether cross-recognition of heterologous *oriT*s by Mob proteins could play a role in the plasmid cassettes shuffling, perhaps facilitating the generation of new MOB modules and, as a consequence, spreading between bacterial species.

Acknowledgements

Research financed by the Spanish Ministry of Economy and Competitiveness (grants CSD-2008-00013-INTERMODS and BFU2010-19597 to M.E., BFU2009-11868 to A.B., and BFU2008-02372/BMC, CONSOLIDER CSD 2006-23, and BFU2011-22588 to M.C), the Generalitat de Catalunya (SGR2009-1309 to M.C.), and the European Commission (FP7 Cooperation Project SILVER - GA No. 260644, to M.C.).

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Figure legends

Fig. 1. Relevant features of pMV158 plasmid and the MobM relaxase. **A**. Genetic map of pMV158 showing the relative positions of the plasmid modules, of genes encoding proteins (arrows) and origins of replication and transfer (stripped rectangles). Genes *copG* and *repB* are involved in plasmid DNA replication. Replication of the leading strand initiates at the double-stranded origin (*dso*), whereas lagging strand synthesis starts at either one of the two single-stranded origins (*ssoU*, *ssoA*). The *tetL* gene confers resistance to tetracycline. The *mobM* gene and the origin of transfer (*oriT*) are involved in conjugative mobilization. **B**. Proposed domains of MobM. The three conserved motifs indicated within the N-terminal moiety derived from a group of mobilization proteins obtained from the ProDom program. Such proteins constitute a family known as Pre/Mob enzymes, since they were firstly reported to be involved in plasmid recombination (Projan and Novick, 1988). The C-terminal moiety harbours regions likely involved in dimerization and interaction with the cell membrane (de Antonio et al., 2004). **C**. Helical wheel projection of residues Leu317 to Leu338 of the MobM sequence (Leu-zipper motif prediction), which we propose would be involved in MobM dimerization.

Fig. 2. Effect of pH on the nicking activity of MobM. Before addition of pMV158 DNA (8 nM), MobM (240 nM) was kept in the reaction buffer for 5 min at room temperature, and the cleavage reactions were done under standard conditions. Supercoiled (FI forms) and relaxed (FII forms) plasmid molecules were separated by agarose (1%) gel electrophoresis and the percentage of relaxed molecules (efficiency of cleavage) at the different pHs was calculated. The results are an average of three independent experiments.

Fig. 3. Nicking activity of purified MobM protein. **A**. Plasmid pMV158 DNA relaxation mediated by increasing concentrations of MobM. Supercoiled pMV158 DNA (8 nM) was incubated with the indicated concentration of MobM under standard conditions. After proteinase K treatment, supercoiled (FI) and relaxed (FII) plasmid molecules were separated by agarose (1%) gel electrophoresis. The gel was stained with EtBr (1 µg/ml). **B**. Electron micrograph showing a supercoiled pMV158 molecule and a

pMV158 molecule relaxed by MobM. MobM bound to the relaxed plasmid DNA is indicated with an arrow.

Fig. 4. Binding of MobM to oligonucleotides containing the inverted repeats IR-2 and IR-1 of the *oriT*$_{pMV158}$. **A.** Cy5-labelled oligonucleotides IR-2 and IR-3 were incubated (24 ºC, 20 min, no Mn^{2+}) with increasing amounts of MobM protein (0, 10, 50, 250, and 1000 nM, from left to right). DNA-protein complexes were separated by electrophoresis on a 5% native polyacrylamide gel and visualized by the use of a Phosphorimager. **B.** Size exclusion elution profile of mixtures of MobM with oligonucleotides IR-2 and IR-3 monitored by absorbance of the eluate at 260/280 nm. The elution profile of MobM with IR-2 (straight line) and IR-3 (dashed line) oligonucleotides was obtained using a Superdex 200 10/300 column. The vertical dotted lines indicate the theoretical elution volumes of the three species (MobM, IR-2 and IR-3) based on the size standards of the column calibration.

Fig. 5. MobM remains stably bound to the relaxed pMV158 DNA. **A**. DNA from pMV158 (8 nM) was incubated with MobM (240 nM) 20 min, 30ºC, in the presence of 8 mM Mn^{2+}. Samples were treated (+) or not (-) with proteinase K and the resulting plasmid forms were separated by electrophoresis on an agarose gel (1%). The arrowhead on the left of the gel indicates the position of the wells. **B.** Schematic restriction map of pMV158 indicating the relative positions of the cleavage sites for *Eco*RI and *Afl*II (in brackets), and the sizes (in bp) of the DNA fragments generated by these enzymes (coordinates of pMV158 in parenthesis). The *oriT* region (grey box) and nick site (arrowhead) are also depicted. **C.** Reaction mixtures (treated as in panel **A** but undigested with proteinase K) were digested simultaneously with *Eco*RI and *Afl*II and analyzed by electrophoresis on a native 5% polyacrylamide gel. The expected sizes (in bp) of the pMV158 DNA fragments generated (panel **B**) are indicated to the right. After electrophoresis, gels were stained with EtBr (1 µg/ml).

Fig. 6. Putative Mob proteins and *oriT*s sequences from plasmids pMV158 (MobM), pDL287 (MobL), pUB110 (MobU), and pE194 (MobE). **A**. Sequence alignment of the four relaxases. The three conserved motifs (I, II and III) of the Pre/Mob family of enzymes are indicated (de Antonio et al., 2004; Francia et al., 2004; Garcillán-Barcia

et al., 2009; Guzmán and Espinosa, 1997). **B**. Sequence alignment of the *oriT*s. Lower case letters indicate nucleotide differences from $oriT_{pMV158}$. The three overlapping inverted repeats (IR1, IR2 and IR3) and the nicking site (G/T) are indicated. The shadowed sequences could lead to the formation of three alternative stem-loop structures.

Fig. 7. *In vitro* relaxation of homologous (pMV158) and heterologous (pUB110, pDL287, and pE194) supercoiled DNAs by purified MobM. Supercoiled plasmid DNAs (100 ng, forms FI) were incubated or not with MobM (240 nM) under optimal conditions. Generation of relaxed molecules (FII) was analyzed by electrophoresis on agarose gels (1%).

Table 1 Properties of MobM protein[a]

Feature	Description	Reference
Size	494 residues	(Priebe and Lacks, 1989)
Molecular mass (Da)		
- Derived from DNA sequence	57,874	(Priebe and Lacks, 1989)
- Denaturing SDS gels	58,000	(Guzmán and Espinosa, 1997)
- MALDI-TOF	57,900	This work
- Analytical ultracentrifugation	115,650 ± 1,800	This work
Native configuration	Homodimer	(de Antonio et al., 2004); this work
Frictional ratio (f/f_0)	1.65	This work
Shape	Prolate ellipsoid dimer	This work
N-terminal amino acid sequence		
- Expected	MSYMVARMQKM	(Priebe and Lacks, 1989)
- Determined	SYMVARMQKM	(Lorenzo-Díaz et al., 2011)
Secondary (Circular Dichroism)	60% α-helix, 4% β, 36% random coils	(de Antonio et al., 2004; Lorenzo-Díaz et al., 2011)
Fluorescence	Two Trp residues in hydrophobic pocket	(de Antonio et al., 2004)
Prediction of C-terminal domain	Mostly coiled coils	This work
Enzymatic activity	DNA relaxase	(Guzmán and Espinosa, 1997)
DNA substrate	Supercoiled or single-stranded	(Guzmán and Espinosa, 1997)
Cleavage site (“/”)	5’-TAGTGTG/TTA-3’	(Guzmán and Espinosa, 1997)
Minimal DNA binding target	5’-CTTTATGAATATAAAGTATAGTGTG-3’	(Lorenzo-Díaz et al., 2011); this work
Relaxation conditions		
- Temperature	30 ºC	(de Antonio et al., 2004)
- pH	6.5	This work

- Time	20 min	(Guzmán and Espinosa, 1997)
- Cations	Mn^{2+} (8 mM)	(Lorenzo-Díaz et al., 2011)
Maximum cleavage	~65% of supercoiled substrate DNA	This work
Other co-factors	Not appear to be needed	
Association to cell membrane	Yes	(de Antonio et al., 2004)
Other plasmid-encoded proteins	Not known	

[a] (the appropriate references are given in brackets)

Table 2. Homologies between Mob proteins of the four analyzed plasmids

	Full length (FL)		N-terminal domain (N-200)	
	Identity (%)	Similarity (%)	Identity (%)	Similarity (%)
MobM/MobL	73.3	77.8	96.5	98
MobU/MobL	33.6	53.6	45.5	67.8
MobU/MobE	31.4	53	42.9	65.4
MobM/MobU	29	46.3	45.3	66.7
MobL/MobE	26.2	44.4	34.6	61.4
MobM/MobE	24.4	42.2	35.1	61.5

Figure

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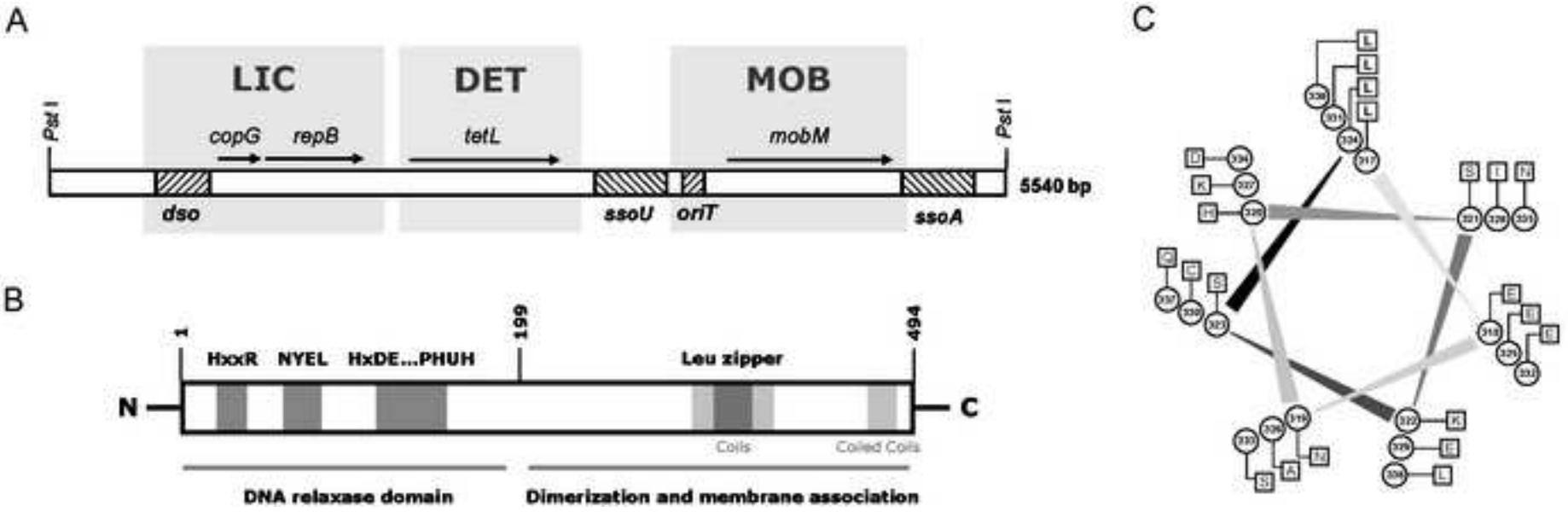

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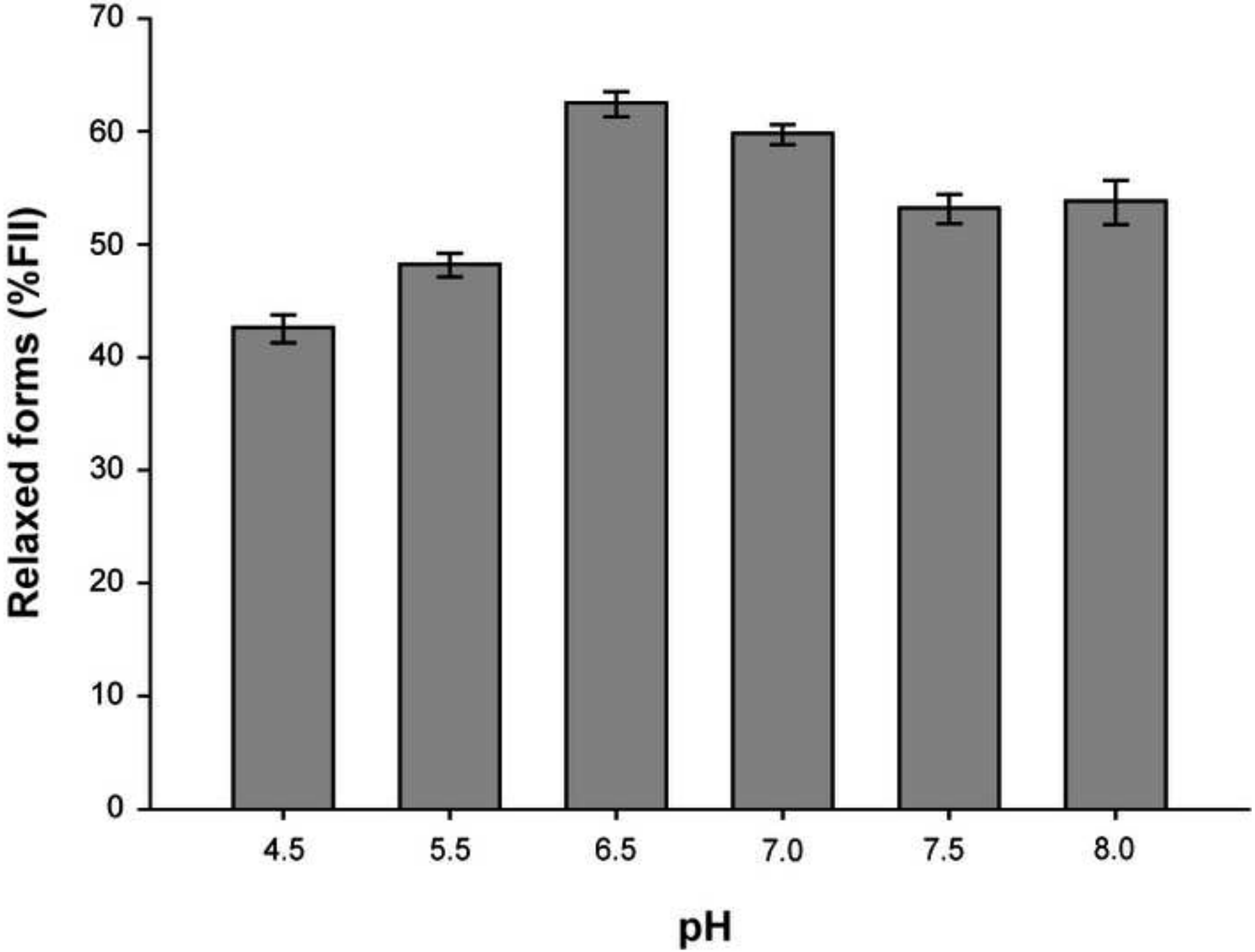

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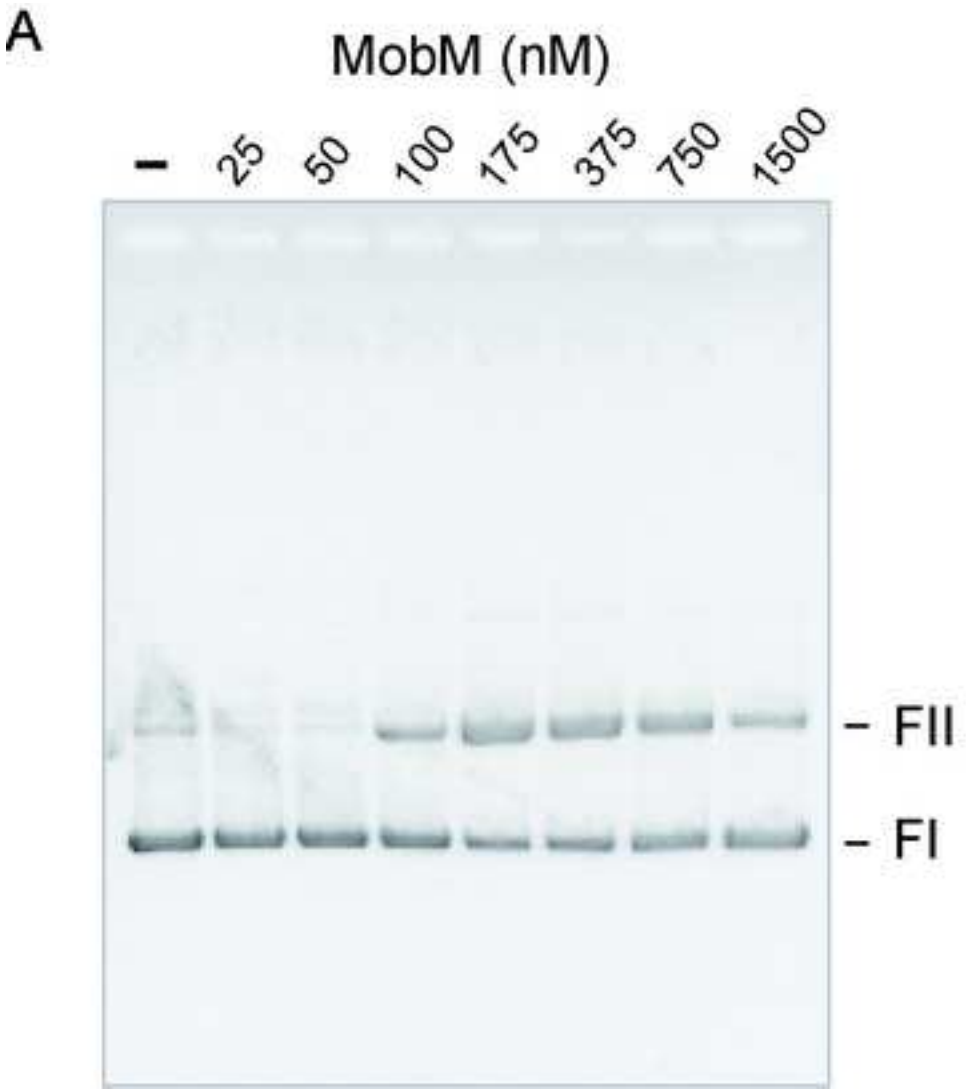

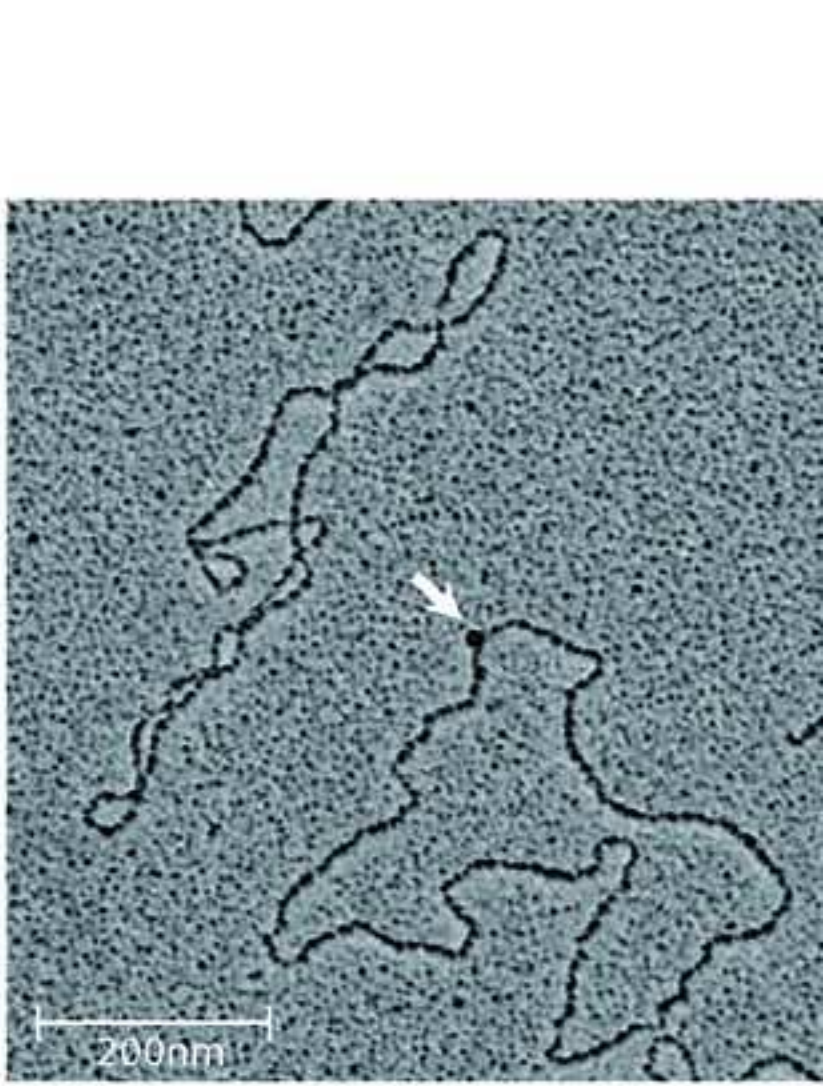

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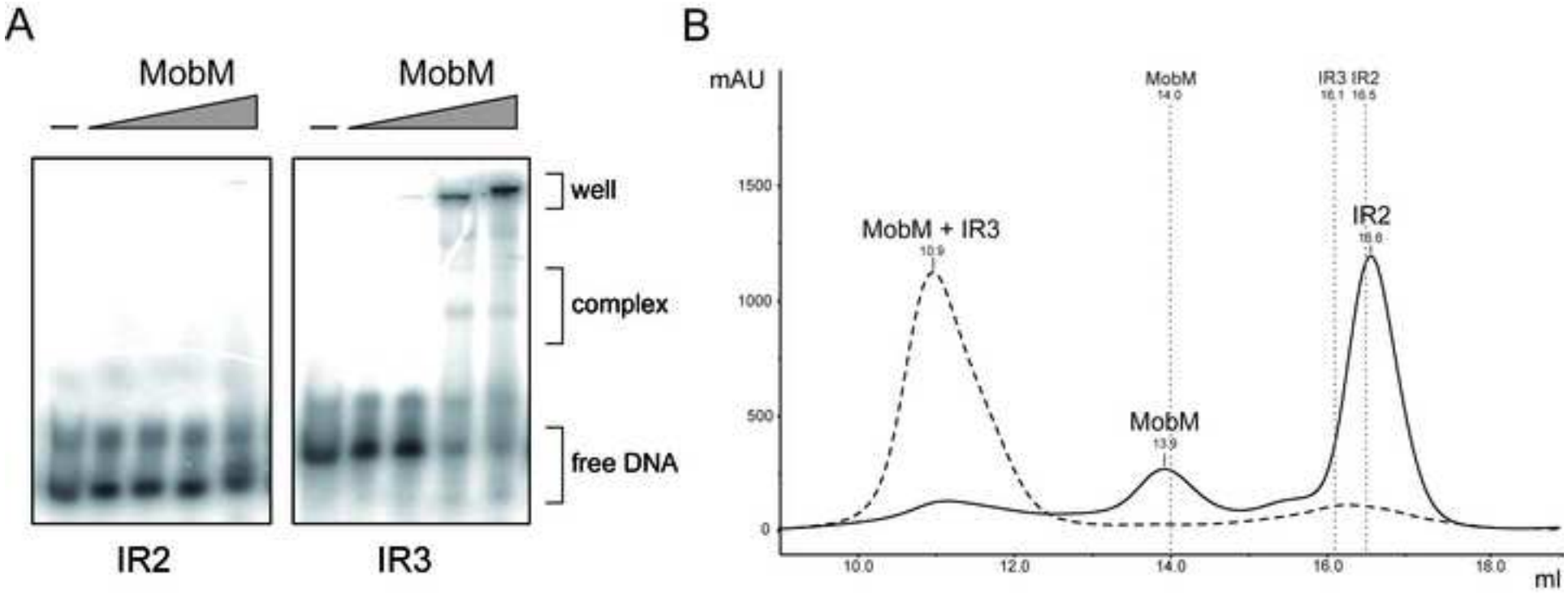

Figure
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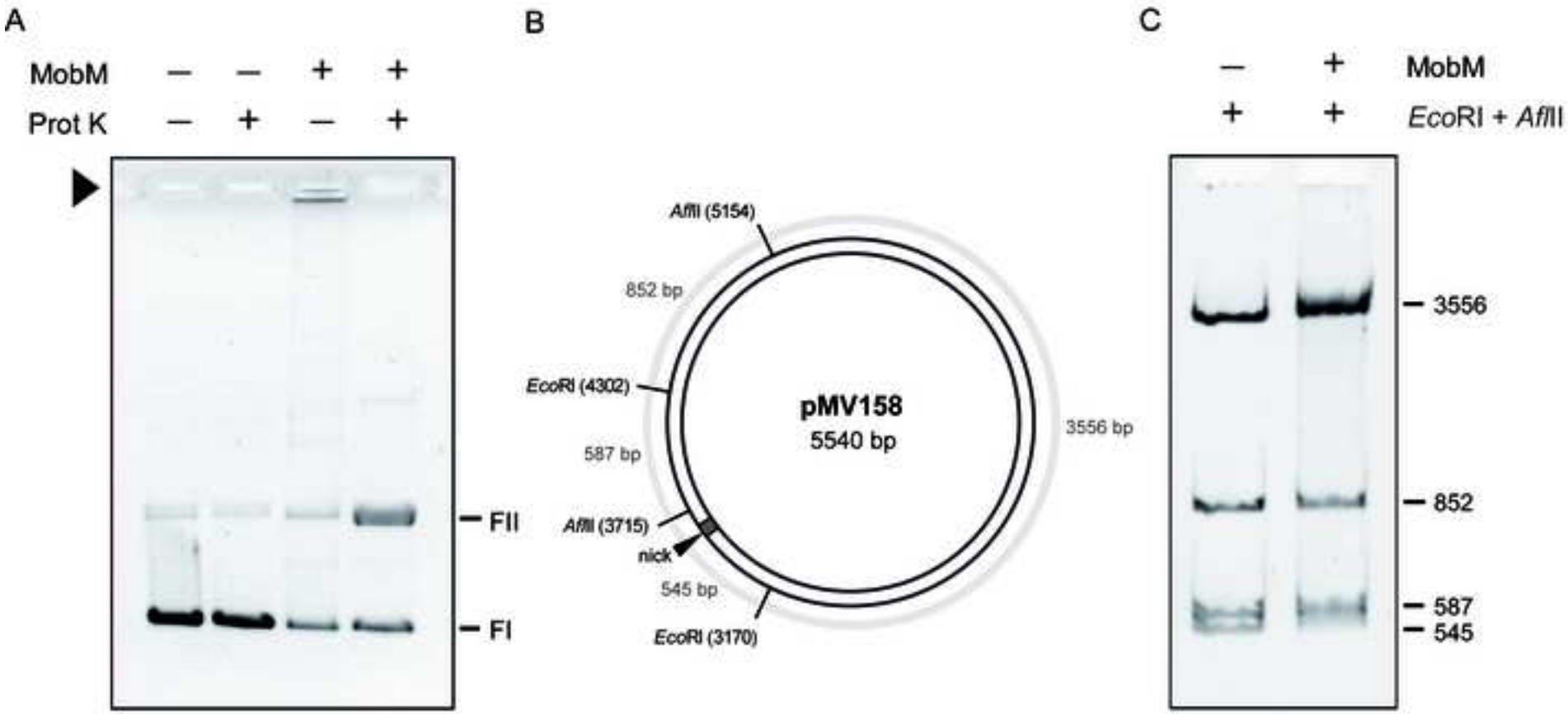

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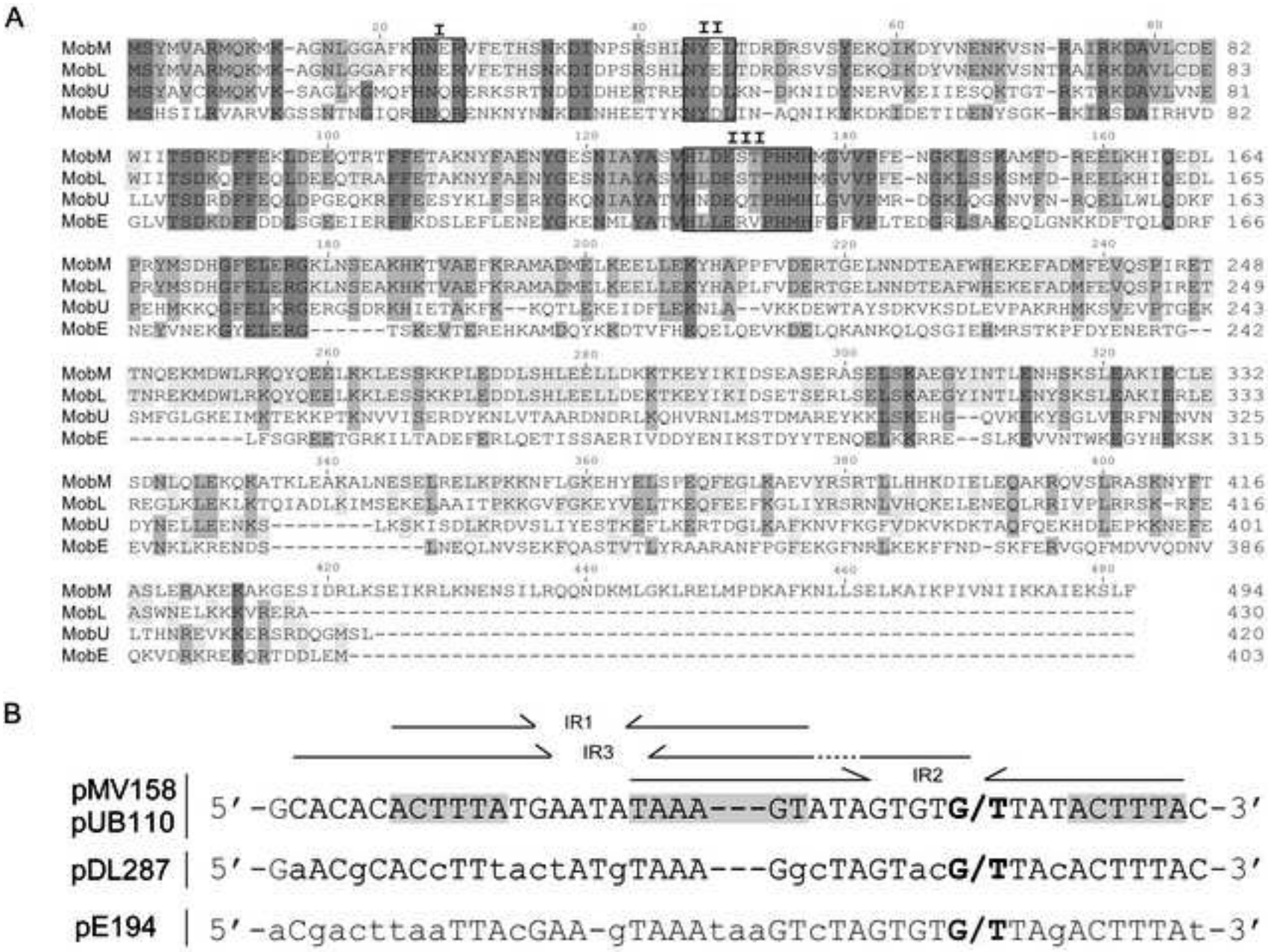

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