

Elsevier Editorial System(tm) for Plasmid
Manuscript Draft

Manuscript Number:

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

Article Type: Special Issue: ISPB 2012

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

Corresponding Author: Prof. Manuel Espinosa, PhD

Corresponding Author's Institution: Centro de Investigaciones Biológicas

First Author: Cris Fernández-López

Order of Authors: Cris Fernández-López; Fabián Lorenzo-Díaz; Rosa Pérez-Luque; Lorena Rodríguez-González; Roeland Boer; Rudi Lurz; Alicia Bravo; Miquel Coll; Manuel Espinosa

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.

Suggested Reviewers:

Opposed Reviewers:

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.

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

3

4 Cris Fernández-López^{1*}, Fabián Lorenzo-Díaz^{2*}, Rosa Pérez-Luque^{3,4}, Lorena
5 Rodríguez-González¹, Roeland Boer^{3,4}, Rudi Lurz⁵, Alicia Bravo¹, Miquel Coll^{3,4} and
6 Manuel Espinosa^{1§}

7 *¹Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones*
8 *Científicas, Ramiro de Maeztu, 9, 28040 Madrid (Spain); ²Instituto Universitario de*
9 *Enfermedades Tropicales y Salud Pública de Canarias, Astrofísico Francisco*
10 *Sánchez, s/n, 38203 La Laguna, Tenerife (Spain); ³Institute for Research in*
11 *Biomedicine and, ⁴Institut de Biologia Molecular de Barcelona (IBMB-CSIC), Parc*
12 *Científic de Barcelona, Baldiri Reixac 10-12, 08028 Barcelona (Spain); ⁵Max-Planck*
13 *Institute für molekulare Genetik, Ihnestrasse 63, Berlin (Germany)*

14

15

16

17 §Correspondence: mespinosa@cib.csic.es; Fax: (+34) 915360432

18 * Equal contribution

19

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

22 *Running title:* MobM relaxase of pMV158

23 *Category:* Plasmid functions

24 *Special issue*

25

26

27 **Abstract**

28 The MobM relaxase (494 amino acids) encoded by the promiscuous
29 streptococcal plasmid pMV158 recognizes the plasmid origin of transfer, *oriT*_{pMV158},
30 and converts supercoiled pMV158 DNA into relaxed molecules by cleavage of the
31 phosphodiester bond of a specific dinucleotide within the sequence 5'-GTGTG/TT-3'
32 ("*/*" being the nick site). After cleavage, the protein remains stably bound to the 5'-
33 end of the nick site. Band-shift assays with single-stranded oligonucleotides and size-
34 exclusion chromatography allowed us to show that MobM was able to generate
35 specific complexes with one of the inverted repeats of the *oriT*_{pMV158}, presumably
36 extruded as stem-loop structure. A number of tests have been performed to attain a
37 better characterization of the nicking activity of MobM and its linkage with its target
38 DNA. The optimal pH for DNA relaxation was found to be 6.5. Upon nicking, gel
39 retardation assays showed that MobM formed stable complexes with its target DNA.
40 Moreover, MobM bound to relaxed pMV158 molecules were visualized by electron
41 microscopy. The staphylococcal plasmids pUB110 and pE194, and the streptococcal
42 plasmid pDL287 harbour putative *oriTs* and may encode Mob proteins homologous
43 to MobM. The *oriT*_{pUB110}, *oriT*_{pDL287}, and *oriT*_{pE194} sequences share 100%, 70%, and
44 67% (in a 43-nucleotide stretch and allowing a 3-bp gap) identity to *oriT*_{pMV158},
45 respectively. Nicking assays using supercoiled DNAs from pUB110, pDL287, and
46 pE194 showed that MobM was able to relax, to a different degree, all plasmid DNAs.
47 Our results suggest that cross-recognition of heterologous *oriTs* by Mob proteins
48 could play an important role in the plasmid spreading between bacteria.

49

50

51 1. Introduction

52 Bacterial plasmids are genetic entities that replicate in an independent and
53 controlled manner from their hosts (del Solar et al., 1998). Plasmids can be
54 transferred among bacteria mostly by conjugation (self-transmissible plasmids) or
55 mobilization (mobilizable plasmids). The former encode all functions needed for their
56 transfer, whereas the latter make use of functions provided by other (auxiliary)
57 plasmids (Garcillán-Barcia et al., 2011; Grohmann et al., 2003; Smillie et al., 2010).
58 Conjugative and mobilizable plasmids, as well as other self-transmissible DNA
59 elements, like integrative-conjugative elements and phage-like sequences, constitute
60 the mobilome and they play a key role in the spread of genetic information among
61 bacteria by horizontal gene transfer. The mobilome strongly influences the co-
62 evolution of the bacterium-plasmid pair and represents a reservoir of DNA (up to 25%
63 of the total bacterial DNA) that is shared among bacterial species (Thomas, 2000).
64 Transfer requires the assembly of plasmid-encoded proteins on a specific plasmid
65 DNA region, the origin of transfer (*oriT*) to generate a macromolecular complex, the
66 relaxosome (Lanka and Wilkins, 1995). The key player is the plasmid-encoded
67 nicking-closing protein: the DNA relaxase (Francia et al., 2004; Garcillán-Barcia et
68 al., 2009). This protein initiates transfer by cleaving the phosphodiester bond at a
69 specific dinucleotide located within the *oriT* (Datta et al., 2003; Guasch et al., 2003),
70 generating an aminoacyl-DNA adduct that is experimentally recognizable because
71 the 5'-end of the cleaved DNA will be occluded, whereas its 3'-end will be accessible
72 to enzymes (Pansegrau and Lanka, 1996a). The DNA-relaxase complex would be
73 transferred to the recipient cell by means of the coupling protein and the
74 macromolecular protein complex, the transferosome, that constitute a type IV
75 secretion system (Gomis-Rüth and Coll, 2006; Gomis-Ruth et al., 2002; Llosa et al.,
76 2002). Once into the recipient, the relaxase–DNA intermediate would restore the
77 original circular plasmid molecule after termination of transfer through a reversion of
78 the strand transfer reaction, in a mechanism that is similar to that described for
79 termination of rolling circle replication (RCR) (Novick, 1998; Pansegrau and Lanka,
80 1996b). Finally, conversion of single-stranded DNA molecules into double-stranded
81 plasmid forms in the recipient cells is carried by conjugative replication that initiates
82 from a lagging-strand origin (Lorenzo-Díaz and Espinosa, 2009a; Parker and Meyer,
83 2005).

84

85 Large plasmids may be self-transmissible because they encode the entire
86 machinery for their transfer, even though a large number of plasmids seem to be
87 non-mobilizable (Smillie et al., 2010). However, many small-sized plasmids that
88 replicate by the rolling-circle mechanism (RCR-plasmids) contain a single gene
89 cassette, composed by a gene encoding the *trans*-acting Mob relaxase and a *cis*-
90 acting *oriT*, that allows these plasmids to be transferred by means of helper plasmids
91 (Grohmann et al., 2003). In the case of the streptococcal plasmid pMV158 (Burdett,
92 1980), the relaxase MobM was purified and several of its interactions with its cognate
93 origin of transfer, *oriT*_{pMV158}, were characterized *in vitro* (de Antonio et al., 2004;
94 Grohmann et al., 1999; Guzmán and Espinosa, 1997). Transfer of pMV158 mediated
95 by plasmids of the Inc18 and IncP α families was shown to occur between different
96 bacterial species in which the plasmid replicates (Farías and Espinosa, 2000;
97 Lorenzo-Díaz and Espinosa, 2009b). The relaxase domain of MobM was further
98 characterized by purification of a truncated protein, MobMN199, which contains the
99 first 199 residues of MobM. Contrary to the dimeric configuration of the native MobM,
100 MobMN199 was shown to be a monomer, so that we could map the dimerization
101 domain as located in the C-terminal moiety of the protein (Lorenzo-Díaz et al., 2011).

102 In the present work, we have analyzed the interactions of the full length MobM
103 protein with its cognate *oriT*_{pMV158} and the stability of the linkage between the protein
104 and its target DNA after cleavage the strand to be transferred by gel-shift assays and
105 electron microscopy. Attempts at optimizing the nicking reaction showed that the
106 optimum pH was 6.5, while the temperature (30 °C), time of reaction (20 min) and
107 divalent cations (8 mM Mn²⁺) were assessed previously (de Antonio et al., 2004;
108 Guzmán and Espinosa, 1997; Lorenzo-Díaz et al., 2011). Under these optimal
109 conditions, maximum cleavage reached up to ~65% of the total input DNA.
110 Furthermore, we present results showing that MobM protein was able to relax
111 supercoiled DNAs from RCR-plasmids with *oriT*s that share total (plasmid pUB110)
112 or partial (plasmids pDL287 and pE194) homology with the *oriT*_{pMV158}.

113 **2. Materials and Methods**

114 *2.1. Bacterial strains, plasmids, and culture conditions*

115 *Streptococcus pneumoniae* 708 (*end-1, exo-2, trt-1, hex-4, malM594*; (Lacks,
116 1968) was used as host for plasmids pMV158 and pDL287. The *Bacillus subtilis* 168
117 MB56 strain (*trpC2*, lab collection (Espinosa et al., 1982) was used as host for
118 plasmids pUB110 and pE194. The RCR-streptococcal plasmids pMV158 (Burdett,
119 1980) and pDL287 (Le Blanc et al., 1992; LeBlanc et al., 1993; a gift of D. Galli) and
120 the RCR-staphylococcal plasmids pUB110 (McKenzie et al., 1986) and pE194
121 (Horinouchi and Weisblum, 1982) were used for nicking assays. *Escherichia coli*
122 BL21 (DE3) (*r_B m_B, gal, ompT, int::P_{lacUV5}-T7 gene 1 imm 21 nin5*; a gift of F.W.
123 Studier) was used for purification of the full length MobM protein. This strain harbours
124 a single copy of the T7 RNA polymerase gene integrated into the chromosome,
125 under the control of the inducible *lacUV5* promoter (Studier et al., 1990). For over-
126 expression of the *mobM* gene, plasmid pLGM2 was used; it contains two copies of
127 the *mobM* gene under the control of the ϕ 10 promoter of phage T7 (Guzmán and
128 Espinosa, 1997).

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

136

137 2.2. Purification of plasmid DNA

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

146

147 2.3. Overproduction and purification of MobM

148 MobM was overproduced and purified essentially as described (de Antonio et
149 al., 2004), but with some modifications that allowed us to obtain a higher protein yield
150 (Lorenzo-Díaz et al., 2011). Briefly, cell pellets from 4l-cultures were concentrated
151 (100x) in buffer A (20 mM Tris-HCl pH 7.6, 1 mM EDTA, 1 mM dithiothreitol, 5%
152 (v/v) glycerol) supplemented with 1 M NaCl and a tablet of a protease inhibitor
153 cocktail (Roche Applied Science). Cells were disrupted by passage through a French
154 pressure cell, the whole-cell extract was centrifuged to remove cell debris, and
155 protein MobM was purified essentially as described (Lorenzo-Díaz et al., 2011).
156 Protein concentration was determined with a NanoDrop ND-1000
157 Spectrophotometer, and the final yield was of 8-10 mg of purified MobM. Protein
158 preparations were stored in buffer A supplemented with 500 mM NaCl and kept at -
159 80 °C. Analyses of the MobM protein hydrodynamic parameters were done by
160 analytical ultracentrifugation and determination of the molecular mass of the
161 protomer was performed by mass spectrometry (MALDI-TOF-TOF) under the
162 reported conditions (Lorenzo-Díaz et al., 2011; Moreno-Córdoba et al., 2012).

163

164 2.4. Nicking activity of MobM

165 Nicking assays were performed essentially as reported (Guzmán and
166 Espinosa, 1997; Lorenzo-Díaz et al., 2011). Usually the standard reaction mixtures
167 (20 µl) contained 25 mM Tris-HCl pH 7.6, 0.1 mM EDTA, 10% glycerol (v/v), 1 mM
168 dithiothreitol, NaCl 50mM, 8 mM MnCl₂ and purified protein (240 nM). Mixtures were
169 incubated in the reaction buffer for 5 min at room temperature before the addition of
170 supercoiled plasmid DNA. The amount of plasmid DNA used was 500 ng, except for
171 the heterologous *oriT* recognition assays, in which 100 ng of each supercoiled DNA
172 was used. Unless otherwise stated, the reaction mixtures were incubated at 30 °C,
173 20 min, and reactions were stopped by treatment with SDS (0.5%) and proteinase K
174 (1 mg/ml) followed by incubation at 37 °C, 30 min. Generation of open circular forms
175 (forms FII) was monitored by agarose (1%) gel electrophoresis. Gels were stained
176 with ethidium bromide (EtBr, 1 µg/ml) and DNA was visualized using a Gel-Doc
177 system (BioRad). The intensity of the bands was quantified using the QuantityOne
178 software. To calculate the percentage of open circular forms, the intensity of the
179 bands corresponding to supercoiled and relaxed forms (FI and FII, respectively) was
180 quantified in the absence and in the presence of MobM.

181

182 2.5. Electron microscopy

183 Reaction mixtures (20 μ l) contained 20 mM Tris-HCl pH 7.0, 1 mM
184 dithiothreitol, 1 mM EDTA, 1% glycerol, 50 mM NaCl, 15 mM MgCl₂, pMV158 DNA (8
185 nM) and MobM protein (375 nM). After 30 min at 30 °C, MobM-DNA complexes were
186 fixed with 0.3% glutaraldehyde, 15 min at the same temperature. Then, reactions
187 were diluted 10-fold in buffer GA (10 mM triethanolamine chloride, pH 7.5, 10 mM
188 MgCl₂), adsorbed onto freshly cleaved mica, positively stained with 2% uranyl
189 acetate, rotary shadowed with Pt/Ir, and covered with a carbon film as described
190 previously (Spiess and Lurz, 1988). Micrographs of the carbon film replica were
191 taken using a Philips CM100 (FEI Company, Hillsboro, Oregon) electron microscope
192 at 100 kV on 35-mm film.

193

194 2.6. Electrophoretic mobility shift assay (EMSA)

195 To analyse complex formation between MobM and its target DNA by EMSA,
196 single-stranded or supercoiled DNA harbouring a part of, or the entire *oriT*_{pMV158}
197 sequence, were used as substrates. In the first case, oligonucleotides harbouring
198 either the inverted repeat 2 or the inverted repeat 3 (IR2 and IR3, respectively) of the
199 *oriT*_{pMV158} were employed. Both oligonucleotides were labelled with the fluorophore
200 Cy5 either at their 3' or 5'-ends. Thus, oligonucleotide IR2 was
201 TAAAGTATAGTGTG/TTATACTTTA-Cy5 (coordinates 3582-3605 of pMV158,
202 accession number [X15669](#)), and oligonucleotide IR3 was Cy5-
203 GCACACACTTTATGAATATAAAGTATAGTGTG/ (coordinates 3564-3595).
204 Oligonucleotides were heated (80 °C, 15 min), after which the solution was flash-
205 cooled to 4 °C. Different amounts of MobM were incubated (20 min, 24 °C) with 2 nM
206 of each oligonucleotide in buffer containing 20 mM Tris-HCl pH 7.6, 1 mM EDTA, 1
207 mM dithiothreitol, 5% (v/v) glycerol and 300 mM NaCl. Protein-DNA complexes were
208 separated by electrophoresis on 10% native polyacrylamide gels as described
209 (Lorenzo-Díaz et al., 2011). EMSA assays with supercoiled DNA were performed in
210 the same buffer but supplemented with 50 mM NaCl and 8 mM Mn⁺². Mixtures
211 containing 8 nM of pMV158 DNA were incubated with 240 nM of MobM, 20 min, 30
212 °C. Then, reactions were treated or not with SDS (0.5%) and proteinase K (1 mg/ml)
213 followed by incubation at 37 °C, 30 min. Samples were analyzed in agarose (1%)
214 gels and stained with EtBr (1 μ g/ml). In some cases, as indicated in the Results,
215 reaction mixtures were not treated with proteinase K but were subjected to restriction

216 with *EcoRI* and *AflIII* enzymes (New England Biolabs). The resulting DNA fragments
217 were separated by electrophoresis on 5% polyacrylamide gels and stained with EtBr
218 (1µg/ml).

219

220 *2.7. Size exclusion chromatography*

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

231

232 *2.8. Bioinformatics resources*

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

244

245 **3. Results and Discussion**

246 *3.1. Features of the MobM relaxase*

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

250 plasmid has been established in nearly twenty bacterial species, and dissection of its
251 functions has shown that it is composed by three main genetic modules (Fig. 1A): i)
252 LIC, which harbours genes and loci involved in leading strand replication and control
253 (del Solar et al., 2002), ii) DET, including a *tetL* gene determinant that provides
254 resistance to tetracycline (Lacks et al., 1986), and iii) MOB, harbouring the relaxase-
255 encoding *mobM* gene and the *oriT*_{PMV158} (Guzmán and Espinosa, 1997; Lorenzo-Díaz
256 et al., 2011; Priebe and Lacks, 1989). In addition, the plasmid harbours two lagging-
257 strand origins, *ssoU* and *ssoA*, the latter used for replication and transfer between
258 streptococcal species, whereas the former seems to be involved in interspecies
259 transfer, most likely being the responsible for the promiscuity of the replicon (Kramer
260 et al., 1999; Lorenzo-Díaz and Espinosa, 2009a).

261 The MobM protein (Fig. 1B and Table 1) is a DNA relaxase of 494 amino acid
262 residues, with a theoretical mass of 57,874 Da per protomer, and a determined value
263 of 57,900 Da as determined by MALDI-TOF-TOF. Analytical ultracentrifugation of the
264 purified protein showed a molecular mass of $115,650 \pm 1,800$ Da, and a frictional
265 ratio of $f/f_0 = 1.65$, indicative of a dimeric protein with an ellipsoid shape (Table 1).
266 These results agree with our previous determinations (de Antonio et al., 2004) but
267 with a higher accuracy. The same applies for the MALDI-TOF-TOF experiments done
268 with the new protein preparations, which gave a value of 57,900 Da for the mass of
269 the MobM protomer, being 57,707 Da the expected value if we reduce 149 Da for the
270 mass of the first Met residue which was removed (determined by N-terminal
271 sequencing of the protein) and 18 Da for the peptide bond (Table 1). Protein MobM
272 was shown to cleave supercoiled DNAs at the specific sequence 5'-TAGTGTG/TTA-
273 3' ("/" being the phosphodiester bond cleaved by the protein) (de Antonio et al., 2004;
274 Guzmán and Espinosa, 1997; Lorenzo-Díaz et al., 2011). Two main regions (Fig. 1B)
275 have been defined in the full length MobM (Lorenzo-Díaz et al., 2011). The N-
276 terminal moiety (roughly the first 200 residues) contains the catalytic domain with
277 three conserved motifs: i) HxxR (x, any amino acid) of unknown function yet; ii)
278 NYEL, which has been predicted to harbour the catalytic tyrosine, Y44 (Francia et al.,
279 2004; Grohmann et al., 1999), and iii) HxDE...PHUH (U, a hydrophobic residue),
280 which is present in the Rep-initiator proteins of the plasmids of the pMV158 family
281 and which should contain the metal coordination motif (Ilyina and Koonin, 1992;
282 Koonin and Ilyina, 1993). This N-terminal domain is contained within the truncated
283 version of the full length MobM, the so-called MobMN199 protein which has been

284 previously characterized in terms of cleavage of supercoiled DNA and binding affinity
285 for several single-stranded DNAs (Lorenzo-Díaz et al., 2011). The C-terminal moiety
286 of MobM harbours the functions related to: i) dimerization, since MobMN199 was
287 monomeric in solution (Lorenzo-Díaz et al., 2011); we attribute this fact to the
288 presence of a putative leucine-zipper motif that we have located between residues
289 317 and 338 (Fig. 1C); ii) association to the cell membrane that was disrupted by a
290 quadruple mutation in one of the predicted coiled coil regions of MobM: the COILS
291 software predicts a high content of coiled coils between residues 420 and 465.
292 Specifically, changes R421P, L423P, L425P, and L427P resulted in a MobM mutant
293 protein that was unable to associate to the cell membrane (de Antonio et al., 2004). A
294 pMV158-derivative harbouring the four mutations was unable to be transferred
295 between pneumococcal strains (not shown). Further mutagenesis studies are needed
296 to refine the distribution of sub-domains within the C-terminal region of MobM.

297

298 *3.2. Nicking activity of MobM*

299 We have reported that the optimal temperature for MobM-mediated DNA
300 cleavage was 30 °C, being Mn²⁺ the preferred cation, although it could be substituted
301 by Mg²⁺, and partially by Ca²⁺ (de Antonio et al., 2004; Guzmán and Espinosa, 1997).
302 We have also discovered that the presence of Mn²⁺ ions greatly augmented the
303 thermal stability of the protein, so that we modified the nicking conditions (Lorenzo-
304 Díaz et al., 2011). Here we wanted to analyze another parameter, not tested before,
305 which was the effect of the pH on the nicking reaction. For this, we used a MobM
306 protein preparation that was purified following a new protocol that increased the yield
307 and purity of the protein (Lorenzo-Díaz et al., 2011). When supercoiled pMV158 DNA
308 (forms FI) was incubated with this protein preparation in the presence of 8 mM Mn²⁺,
309 30 °C, 20 min, and pH 7.2, we found that ~55% of the supercoiled pMV158
310 molecules were relaxed (not shown). We next analysed the influence of the pH on
311 the nicking activity of MobM in presence of 8 mM Mn²⁺ at 30 °C, 20 min (Fig. 2). The
312 highest nicking activity (~65% of FII forms) was observed when the pH was 6.5. Such
313 an activity was even higher than that reported previously (~50% of relaxed
314 molecules) not only for MobM (Guzmán and Espinosa, 1997) but also for other
315 relaxases, like those encoded by plasmids RP4 (Pansegrau et al., 1990), R388
316 (Llosa et al., 1995), and F (Matson et al., 1993).

317 We next performed a MobM concentration-dependent relaxation assay
318 incubating supercoiled pMV158 DNA with increasing concentrations of MobM (Fig.
319 3A). The reaction mixtures were incubated under the above optimal conditions (30
320 °C, 20 min, pH 6.5, and 8 mM Mn²⁺). Then, they were treated with proteinase K to
321 remove the MobM protein that remains stably bound to the 5'-end generated by its
322 nicking activity. The FI and FII plasmid forms were separated by electrophoresis on
323 an agarose gel (Fig. 3A). At 175 nM of MobM, 63% of the supercoiled molecules (FI)
324 were relaxed (FII), and such a percentage did not increase at higher MobM
325 concentrations. The appearance of open circular molecules (forms FII) was also
326 monitored by electron microscopy. To this end, pMV158 supercoiled DNA (5540-bp)
327 was incubated with MobM under nicking conditions. Samples were further fixed with
328 glutaraldehyde and prepared for electron microscopy. Under these conditions,
329 supercoiled and relaxed plasmid molecules bound to MobM were visualized. An
330 electron micrograph of this experiment is shown in Figure 3B. To verify the specificity
331 of the MobM-pMV158 complexes, samples were digested with *StuI* after the fixation
332 with glutaraldehyde and prior to their preparation for electron microscopy. Plasmid
333 pMV158 has a single *StuI* site (coordinate 4626). Linear DNA molecules bound to
334 MobM were then visualized. The contour lengths of the DNA regions between
335 complexes and DNA ends were measured, and the MobM binding site was
336 determined. The majority (65%) of the 50 complexes examined had MobM positioned
337 around coordinates 3585-3596, which fit well with the position of the nicking site
338 mapped previously between coordinates 3595 and 3596 (Guzmán and Espinosa,
339 1997). From the above analyses, we may conclude that the supercoiled closed forms
340 ('ready to replicate') of pMV158 would be in equilibrium with the forms relaxed by
341 MobM at *oriT*_{pMV158} ('ready to go'). This equilibrium would account for the RC-
342 mechanism of replication of pMV158: once the RepB initiator cleaves the plasmid
343 supercoiled forms at the double-stranded replication origin (*dso* in Fig. 1A) to start
344 replication, FII molecules would be unsuitable to be transferred because MobM also
345 needs a supercoiled substrate to initiate conjugative plasmid transfer. Conversely,
346 plasmid molecules relaxed by MobM at initiation of transfer would be unable to
347 replicate. Run-off assays developed to detect strand discontinuities in growing cells
348 harbouring pMV158 allowed us to map RepB-relaxed and MobM-relaxed forms *in*
349 *vivo* (Grohmann et al., 1997; Zechner et al., 1997), thus perhaps both types of
350 relaxed molecules could co-exist within the same cell. The requirement of Mn²⁺ (not

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

356

357 3.3. Complex formation between MobM and *oriT*_{pMV158}-mimicking oligonucleotides

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

384

385 3.4. Stable linkage of MobM to its target DNA

386 One experimental approach to show that a relaxase is able to generate stable
387 complexes with its target DNA after nicking is to precipitate the protein-DNA
388 complexes with SDS and KCl and analyze the DNA content in the pellet and in the
389 supernatant by native PAGE, as shown earlier for topoisomerases (Trask et al.,
390 1984), and later on for several relaxases, MobM among them (Guzmán and
391 Espinosa, 1997; Matson et al., 1993; Pansegrau et al., 1990; Szpirer et al., 2001).
392 Here we have developed a simpler approach in which the protein stable linkage to a
393 region of the DNA is tested by DNA-relaxation and restriction analyses and the
394 products separated and analyzed by native PAGE. This would result in the
395 appearance of either a fragment with anomalous migration or a 'missing' fragment
396 due to DNA-protein complex retained in the well. First, and to estimate whether this
397 approach was feasible for MobM, supercoiled pMV158 DNA was incubated with
398 MobM under optimal nicking conditions. Next, samples were treated or not with
399 proteinase K and loaded on an agarose gel. As shown in Figure 5A, the FII forms
400 generated by the nicking activity of MobM entered the agarose gel only after
401 treatment with proteinase K. The Y44 amino acid residue has been proposed as the
402 catalytic residue of MobM (Francia et al., 2004; Garcillán-Barcia et al., 2009;
403 Grohmann et al., 1999). *If this was the case, the predicted covalent adduct after a*
404 *total proteinase K digestion of MobM-pMV158 complexes would be the DNA*
405 *attached to a dipeptide (Asn-Tyr). In a second experiment, pMV158 was incubated or*
406 *not with MobM under optimal nicking conditions, and subsequently it was restricted*
407 *simultaneously with EcoRI and AflII. The expected products are schematized in Fig.*
408 *5B, and the resulting DNA fragments were subsequently separated on native 5%*
409 *polyacrylamide gels (Fig. 5C). In the absence of MobM, the four expected restriction*
410 *fragments were visualized (3556, 852, 587, and 545 bp). However, in the MobM-*
411 *treated samples, the oriT-containing DNA fragment (545-bp, coordinates 3170-3715)*
412 *was shifted and part of the MobM-uncleaved DNA (some 35% of the input DNA) was*
413 *not detected at the expected position, most likely due to its retention in the position of*
414 *the other fragments (note, for instance, the 'fuzziness' in the 587-DNA band). These*
415 *results supported that MobM formed stable complexes with the relaxed plasmid*
416 *molecules generated by its nicking activity, and provide a fast and reliable procedure*
417 *to detect stable association of a DNA-relaxing enzyme (initiator of RCR or relaxase)*
418 *with its target DNA and to map the DNA region targeted by the enzyme.*

419

420 3.5. Recognition of heterologous *oriT*s by MobM protein

421 Bioinformatics approaches performed among all RCR-plasmids so far
422 described that could encode a MOB cassette similar to the one present in pMV158
423 allowed the definition of the MOB_V family of relaxases (Francia et al., 2004; Garcillán-
424 Barcia et al., 2009). In the case of pMV158, its *oriT*_{pMV158} contains three partially
425 overlapping IR that could generate three alternative stem-loop structures in which the
426 position of the dinucleotide cleaved by MobM would be placed in different positions
427 (Espinosa, 2013; Lorenzo-Díaz et al., 2011; Fig. 6B). Due to their structure,
428 generation of one of the hairpins would hinder the formation of the other two,
429 indicating perhaps that the accessibility of the relaxase to its target could depend on
430 the superhelicity of the plasmid DNA. To test whether MobM could recognize
431 heterologous *oriT*s from RCR-plasmids belonging to the MOB_{V1} subfamily, we
432 selected, in addition to pMV158, three more plasmids: the streptococcal plasmid
433 pDL287 that is a derivative of pVA380-1 (Le Blanc et al., 1992) and that was shown
434 to be relaxed by MobM (Grohmann et al., 1999), and the staphylococcal plasmids
435 pUB110 (McKenzie et al., 1986), and pE194 (Horinouchi and Weisblum, 1982).
436 These latter plasmids were hosted by *B. subtilis* because we have been unable to
437 transfer them to *S. pneumoniae*, most likely because of the different degree of
438 supercoiling of the plasmid DNAs. In fact, we showed that the initiator of replication
439 RepB from pMV158 was able to nick DNA from pE194 (with which there is homology
440 at the *dso*) only when the supercoiling of pE194 was altered (Moscoso et al., 1995).
441 The putative Mob proteins encoded by pDL287, pUB110, and pE194 (herein named
442 MobL, MobU, and MobE, respectively) showed homology to MobM (Fig. 6A).
443 EMBOSS needle global sequence alignment (Rice et al., 2000) of MobM (494
444 residues), MobU (420 residues), MobL (430 residues), and MobE (403 residues)
445 revealed various degrees of homologies among the full length proteins. The first 200
446 residues, harbouring the relaxase domain, showed the highest homology, reaching
447 up to 96.5% identity in the pair MobM/MobL (Table 2). Concerning the *oriT*s, it was
448 observed that *oriT*_{pUB110}, *oriT*_{pDL287}, and *oriT*_{pE194} sequences share 100%, 70%, and
449 67% (in a 43-nucleotide stretch and allowing one 3 bp-gap) identity to *oriT*_{pMV158},
450 respectively. Western blot assays using cell extracts from *S. pneumoniae* harbouring
451 either pMV158 or pDL287 and cell extracts from *B. subtilis* carrying pUB110 showed

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

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

463 It is interesting to point out that some RCR-plasmids exhibit an 'orphan' *oriT*,
464 since they have a region whose nucleotide sequence is very similar to that of well
465 characterized *oriTs* but lack a cognate putative relaxase gene; such is the case of
466 plasmids pA1 from *Lactobacillus plantarum* (Vujcic and Topisirovic, 1993) and
467 pCl411 from *Leuconoctoc lactis* (Coffey et al., 1994). This, in conjunction to the *oriTs*
468 of some RCR-plasmids being defined as site-specific plasmid recombination (Pre)
469 regions (Gennaro et al., 1987), raise the question of whether cross-recognition of
470 heterologous *oriTs* by Mob proteins could play a role in the plasmid cassettes
471 shuffling, perhaps facilitating the generation of new MOB modules and, as a
472 consequence, spreading between bacterial species.

473

474 **Acknowledgements**

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

480

481

482 **References**

- 483 Burdett, V., 1980. Identification of tetracycline-resistant R-plasmids in *Streptococcus*
484 *agalactiae* (group B). *Antimicrob. Agents Chemother.* 18, 753-760.
- 485 Coffey, A., et al., 1994. Nucleotide sequence and structural organization of the small
486 broad-host-range plasmid pCI411 from *Leuconostoc lactis*. *Microbiology.* 140,
487 2263-2269.
- 488 Datta, S., et al., 2003. Structural insights into single-stranded DNA binding and
489 cleavage by F factor Tral. *Structure.* 11, 1369-1379.
- 490 de Antonio, C., et al., 2004. Features of the plasmid pMV158-encoded MobM, a
491 protein involved in its mobilization. *J. Mol. Biol.* 335, 733-743.
- 492 del Solar, G., et al., 1987. Replication of the streptococcal plasmid pMV158 and
493 derivatives in cell-free extracts of *Escherichia coli*. *Mol. Gen. Genet.* 206, 428-
494 435.
- 495 del Solar, G., et al., 1998. Replication and control of circular bacterial plasmids.
496 *Microbiol. Mol. Biol. Rev.* 62, 434-464.
- 497 del Solar, G., et al., 2002. A genetically economical family of plasmid-encoded
498 transcriptional repressors in control of plasmid copy number. *J. Bacteriol.* 184,
499 4943-4951.
- 500 Espinosa, M., 2013. Plasmids as models to study macromolecular interactions: the
501 pMV158 paradigm. *Res. Microbiol.* doi: 10.1016/j.resmic.2013.01.006
- 502 Espinosa, M., et al., 1982. Interspecific plasmid transfer between *Streptococcus*
503 *pneumoniae* and *Bacillus subtilis*. *Mol. Gen. Genet.*, 195-201.
- 504 Farías, M. E., Espinosa, M., 2000. Conjugal transfer of plasmid pMV158: uncoupling
505 of the pMV158 origin of transfer from the mobilization gene *mobM*, and
506 modulation of pMV158 transfer in *Escherichia coli* mediated by IncP plasmids.
507 *Microbiology.* 146, 2259-2265.
- 508 Francia, M. V., et al., 2004. A classification scheme for mobilization regions of
509 bacterial plasmids. *FEMS Microbiol. Rev.* 28, 79-100.
- 510 Garcillán-Barcia, M. P., et al., 2011. Identification of bacterial plasmids based on
511 mobility and plasmid population biology. *FEMS Microbiology Reviews.* 35,
512 936-956.
- 513 Garcillán-Barcia, M. P., et al., 2009. The diversity of conjugative relaxases and its
514 application in plasmid classification. *FEMS Microbiol. Rev.* 33, 657-687.

515 Gennaro, M. L., et al., 1987. A site-specific recombination function in *Staphylococcus*
516 *aureus* plasmids. J. Bacteriol. 169, 2601-2610.

517 Gomis-Rüth, F. X., Coll, M., 2006. Cut and move: protein machinery for DNA
518 processing in bacterial conjugation. Curr. Opin. Struct. Biol. 16, 744-752.

519 Gomis-Ruth, F. X., et al., 2002. Conjugative plasmid protein TrwB, an integral
520 membrane type IV secretion system coupling protein. Detailed structural
521 features and mapping of the active site cleft. J Biol Chem. 277, 7556-66.

522 Grohmann, E., et al., 1999. Mobilisation of the streptococcal plasmid pMV158:
523 interactions of MobM protein with its cognate *oriT* DNA region. Mol. Gen.
524 Genet. 261, 707-715.

525 Grohmann, E., et al., 2003. Conjugative plasmid transfer in Gram-positive bacteria.
526 Microbiol. Mol. Biol. Rev. 67, 277-301.

527 Grohmann, E., et al., 1997. Determination of specific DNA strand discontinuities with
528 nucleotide resolution in exponentially growing bacteria harbouring rolling
529 circle-replicating plasmids. FEMS Microbiol. Lett. 152, 363-369.

530 Guasch, A., et al., 2003. Recognition and processing of the origin of transfer DNA by
531 conjugative relaxase TrwC. Nat. Struct. Biol. 10, 1002-1010.

532 Guzmán, L., Espinosa, M., 1997. The mobilization protein, MobM, of the
533 streptococcal plasmid pMV158 specifically cleaves supercoiled DNA at the
534 plasmid *oriT*. J. Mol. Biol. 266, 688-702.

535 Horinouchi, S., Weisblum, B., 1982. Nucleotide sequence and functional map of
536 pE194, a plasmid that specifies inducible resistance to macrolide, lincosamine,
537 and streptogramin type B antibiotics. J. Bacteriol. 150, 804-814.

538 Ilyina, T., Koonin, E., 1992. Conserved sequence motifs in the initiator proteins for
539 rolling circle DNA replication encoded by diverse replicons from eubacteria,
540 eukaryotes and archaeobacteria. Nucl. Acids Res. 20, 3279-3285.

541 Koonin, E. V., Ilyina, T. W., 1993. Computer-assisted dissection of rolling circle DNA
542 replication. BioSystems. 30, 241-268.

543 Kramer, M. G., et al., 1999. Characterization of a single-strand origin, *ssou*, required
544 for broad host range replication of rolling-circle plasmids. Mol. Microbiol. 33,
545 466-475.

546 Lacks, S., 1966. Integration efficiency and genetic recombination in pneumococcal
547 transformation. Genetics. 53, 207-235.

548 Lacks, S. A., 1968. Genetic regulation of maltosaccharide utilization in
549 *Pneumococcus*. *Genetics*. 60, 685-706.

550 Lacks, S. A., et al., 1986. Identification and analysis of genes for tetracycline
551 resistance and replication functions in the broad-host-range plasmid pLS1. *J.*
552 *Mol. Biol.* 192, 753-765.

553 Lanka, E., Wilkins, B. M., 1995. DNA processing reactions in bacterial conjugation.
554 *Annu. Rev. Biochem.* 64, 141-169.

555 Le Blanc, D. J., et al., 1992. Molecular, genetic, and functional analysis of the basic
556 replicon of pVA380-1, a plasmid of oral streptococcal origin. *Plasmid*. 28, 130-
557 145.

558 LeBlanc, D. J., et al., 1993. Identification and characterization of a mobilization gene
559 in the streptococcal plasmid pVA380-1. *Plasmid*. 30, 296-302.

560 Lorenzo-Díaz, F., et al., 2011. The MobM-relaxase domain of plasmid pMV158:
561 thermal stability and activity upon Mn²⁺-and DNA specific-binding. *Nucl. Acids*
562 *Res.* 39, 4315-4329.

563 Lorenzo-Díaz, F., Espinosa, M., 2009a. Lagging strand DNA replication origins are
564 required for conjugal transfer of the promiscuous plasmid pMV158. *J.*
565 *Bacteriol.* 191, 720–727.

566 Lorenzo-Díaz, F., Espinosa, M., 2009b. Large-scale filter mating assay for intra- and
567 inter-specific conjugal transfer of the promiscuous plasmid pMV158 in Gram-
568 positive bacteria. *Plasmid*. 61, 65-70.

569 Llosa, M., et al., 2002. Bacterial conjugation: a two-step mechanism for DNA
570 transport. *Mol. Microbiol.* 45, 1-8.

571 Llosa, M., et al., 1995. Nicking activity of TrwC directed against the origin of transfer
572 of the IncW plasmid R388. *J. Mol. Biol.* 246, 54-62.

573 Matson, S. W., et al., 1993. Characterization of the reaction product of the *oriT*
574 nicking reaction catalyzed by *E. coli* DNA helicase I. *J. Bacteriol.* 175, 2599-
575 2606.

576 McKenzie, T., et al., 1986. The nucleotide sequence of pUB110: some salient
577 features in relation to replication and its regulation. *Plasmid*. 15, 93-103.

578 Moreno-Córdoba, I., et al., 2012. The toxin-antitoxin proteins RelBE2Spn of
579 *Streptococcus pneumoniae*: characterization and association to their DNA
580 target. *Proteins*. 80, 1834-1846.

581 Moscoso, M., et al., 1995. In vitro recognition of the replication origin of pLS1 and of
582 plasmids of the pLS1 family by the RepB initiator protein. J. Bacteriol. 177,
583 7041-7049.

584 Novick, R. P., 1998. Contrasting lifestyles of rolling-circle phages and plasmids.
585 Trends Biochem. Sci. 23, 434-438.

586 Pansegrau, W., et al., 1990. *In vitro* assembly of relaxosomes at the transfer origin of
587 plasmid RP4. Proc. Natl. Acad. Sci. USA. 87, 6555-6559.

588 Pansegrau, W., Lanka, E., 1996a. Enzymology of DNA strand transfer by conjugative
589 mechanisms. Prog. Nucl. Acid Res. Mol. Biol. 54, 197-251.

590 Pansegrau, W., Lanka, E., 1996b. Mechanisms of initiation and termination reactions
591 in conjugative DNA processing: independence of tight substrate binding and
592 catalytic activity of relaxase (Tral) of IncPa plasmid RP4. J. Biol. Chem. 271,
593 13068-13076.

594 Parker, C., Meyer, R., 2005. Mechanisms of strand replacement synthesis for
595 plasmid DNA transferred by conjugation. J. Bacteriol. 187, 3400-3406.

596 Priebe, S. D., Lacks, S. A., 1989. Region of the streptococcal plasmid pMV158
597 required for conjugative mobilization. J. Bacteriol. 171, 4778-4784.

598 Projan, S. J., Novick, R. P., 1988. Comparative analysis of five related
599 staphylococcal plasmids. Plasmid. 19, 203-221.

600 Rice, P., et al., 2000. EMBOSS: The European Molecular Biology Open Software
601 Suite Trends in Genetics. 16, 276-277.

602 Rosch, J. W., et al., 2009. Role of the manganese efflux system *mntE* for signalling
603 and pathogenesis in *Streptococcus pneumoniae*. Mol. Microbiol. 72, 12-25.

604 Ruiz-Cruz, S., et al., 2010. Novel plasmid-based genetic tools for the study of
605 promoters and terminators in *Streptococcus pneumoniae* and *Enterococcus*
606 *faecalis*. J. Microb. Meth. 83, 156-163.

607 Smillie, C., et al., 2010. Mobility of plasmids. Microbiol. Mol. Biol. Rev. 74, 434-452.

608 Spiess, E., Lurz, R., 1988. Electron microscopic analysis of nucleic acids and nucleic
609 acid-protein complexes. Methods Microbiol. 20, 293-323.

610 Stassi, D. L., et al., 1981. Cloning of chromosomal genes in *Streptococcus*
611 *pneumoniae*. Proc. Natl. Acad. Sci. USA. 78, 7028-7032.

612 Studier, F. W., et al., 1990. Use of T7 RNA polymerase to direct expression of cloned
613 genes. Meth. Enzymol. 185, 60-89.

614 Szpirer, C. Y., et al., 2001. Mobilization function of the pBHR1 plasmid, a derivative
615 of the broad-host-range plasmid pBBR1. J. Bacteriol. 183, 2101-2110.

616 Thomas, C. M., 2000. The horizontal gene pool. Harwood Academic Publishers,
617 Amsterdam, p. 419.

618 Trask, D. K., et al., 1984. Rapid detection and isolation of covalent DNA/protein
619 complexes: application to topoisomerase I and II. EMBO J. 3, 671-676.

620 Vujcic, M., Topisirovic, L., 1993. Molecular analysis of the rolling-circle replicating
621 plasmid pA1 of *Lactobacillus plantarum* A112. Appl. Environm. Microbiol., 274-
622 280.

623 Zechner, E. L., et al., 1997. Specific cleavage of chromosomal and plasmid DNA
624 strands in Gram-positive and Gram-negative bacteria can be detected with
625 nucleotide resolution. Proc. Natl. Acad. Sci. USA. 94, 7435-7440.

626

627

628 **Figure legends**

629

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

646 **Fig. 2.** Effect of pH on the nicking activity of MobM. Before addition of pMV158 DNA
647 (8 nM), MobM (240 nM) was kept in the reaction buffer for 5 min at room
648 temperature, and the cleavage reactions were done under standard conditions.
649 Supercoiled (FI forms) and relaxed (FII forms) plasmid molecules were separated by
650 agarose (1%) gel electrophoresis and the percentage of relaxed molecules
651 (efficiency of cleavage) at the different pHs was calculated. The results are an
652 average of three independent experiments.

653 **Fig. 3.** Nicking activity of purified MobM protein. **A.** Plasmid pMV158 DNA relaxation
654 mediated by increasing concentrations of MobM. Supercoiled pMV158 DNA (8 nM)
655 was incubated with the indicated concentration of MobM under standard conditions.
656 After proteinase K treatment, supercoiled (FI) and relaxed (FII) plasmid molecules
657 were separated by agarose (1%) gel electrophoresis. The gel was stained with EtBr
658 (1 µg/ml). **B.** Electron micrograph showing a supercoiled pMV158 molecule and a

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

661

662 **Fig. 4.** Binding of MobM to oligonucleotides containing the inverted repeats IR-2 and
663 IR-1 of the *oriT*_{pMV158}. **A.** Cy5-labelled oligonucleotides IR-2 and IR-3 were incubated
664 (24 °C, 20 min, no Mn²⁺) with increasing amounts of MobM protein (0, 10, 50, 250,
665 and 1000 nM, from left to right). DNA-protein complexes were separated by
666 electrophoresis on a 5% native polyacrylamide gel and visualized by the use of a
667 Phosphorimager. **B.** Size exclusion elution profile of mixtures of MobM with
668 oligonucleotides IR-2 and IR-3 monitored by absorbance of the eluate at 260/280 nm.
669 The elution profile of MobM with IR-2 (straight line) and IR-3 (dashed line)
670 oligonucleotides was obtained using a Superdex 200 10/300 column. The vertical
671 dotted lines indicate the theoretical elution volumes of the three species (MobM, IR-2
672 and IR-3) based on the size standards of the column calibration.

673

674 **Fig. 5.** MobM remains stably bound to the relaxed pMV158 DNA. **A.** DNA from
675 pMV158 (8 nM) was incubated with MobM (240 nM) 20 min, 30°C, in the presence of
676 8 mM Mn²⁺. Samples were treated (+) or not (-) with proteinase K and the resulting
677 plasmid forms were separated by electrophoresis on an agarose gel (1%). The
678 arrowhead on the left of the gel indicates the position of the wells. **B.** Schematic
679 restriction map of pMV158 indicating the relative positions of the cleavage sites for
680 *EcoRI* and *AflIII* (in brackets), and the sizes (in bp) of the DNA fragments generated
681 by these enzymes (coordinates of pMV158 in parenthesis). The *oriT* region (grey
682 box) and nick site (arrowhead) are also depicted. **C.** Reaction mixtures (treated as in
683 panel **A** but undigested with proteinase K) were digested simultaneously with *EcoRI*
684 and *AflIII* and analyzed by electrophoresis on a native 5% polyacrylamide gel. The
685 expected sizes (in bp) of the pMV158 DNA fragments generated (panel **B**) are
686 indicated to the right. After electrophoresis, gels were stained with EtBr (1 µg/ml).

687

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

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

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

702

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 ("I")	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 °C	(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

[Click here to download high resolution image](#)

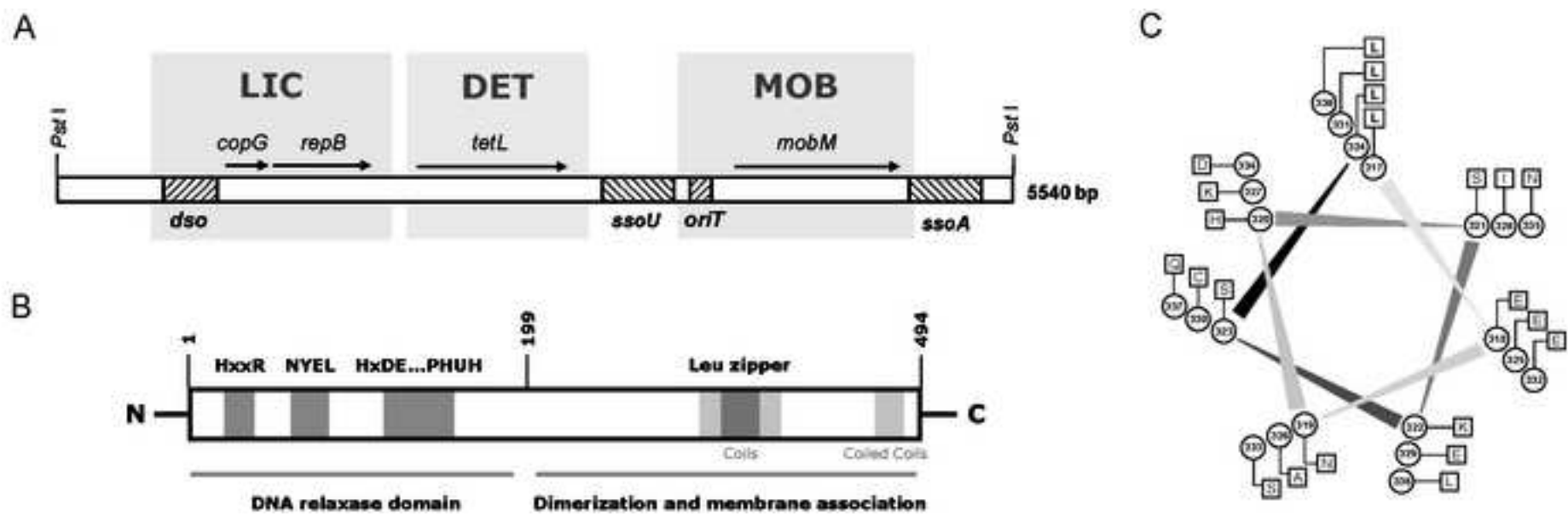
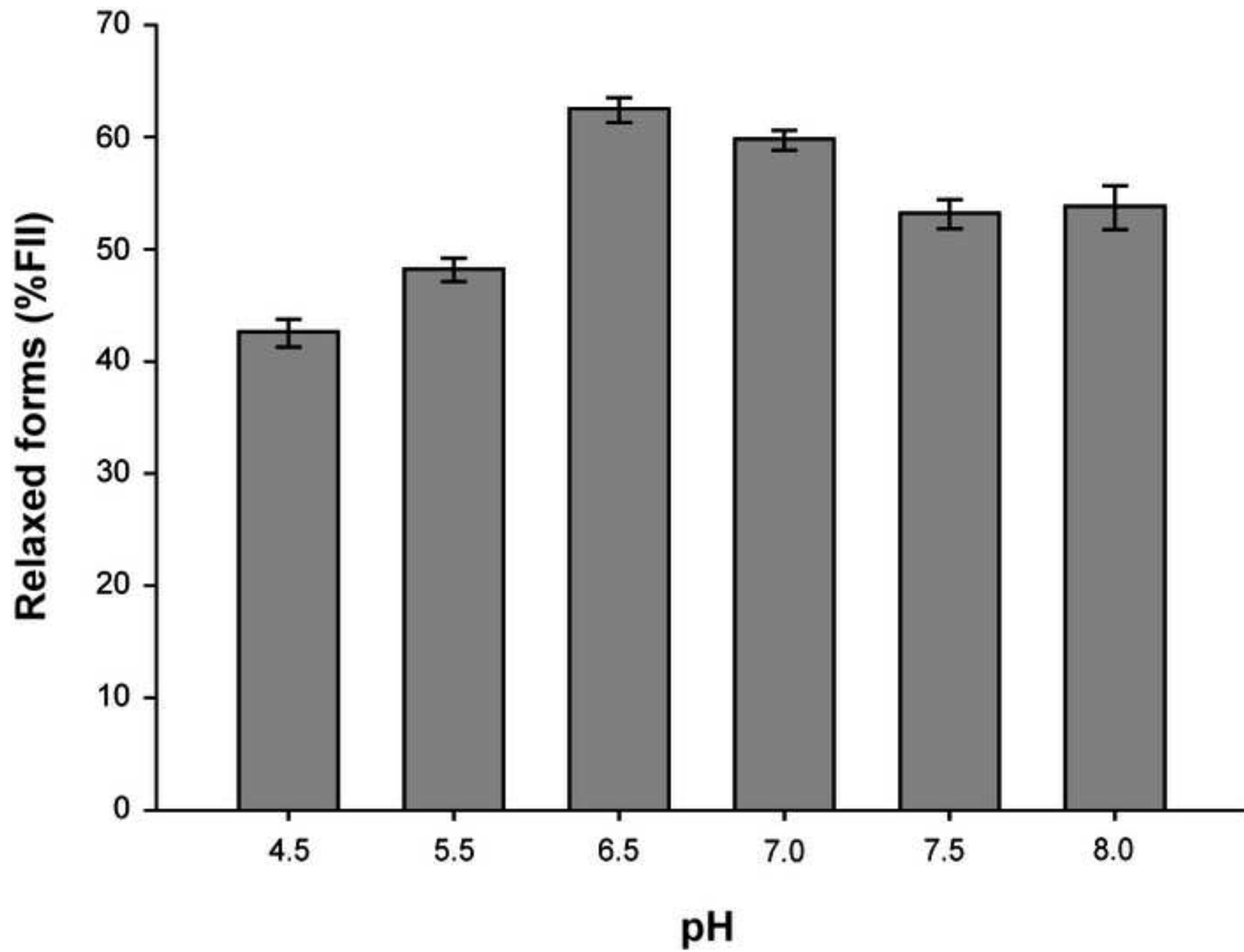


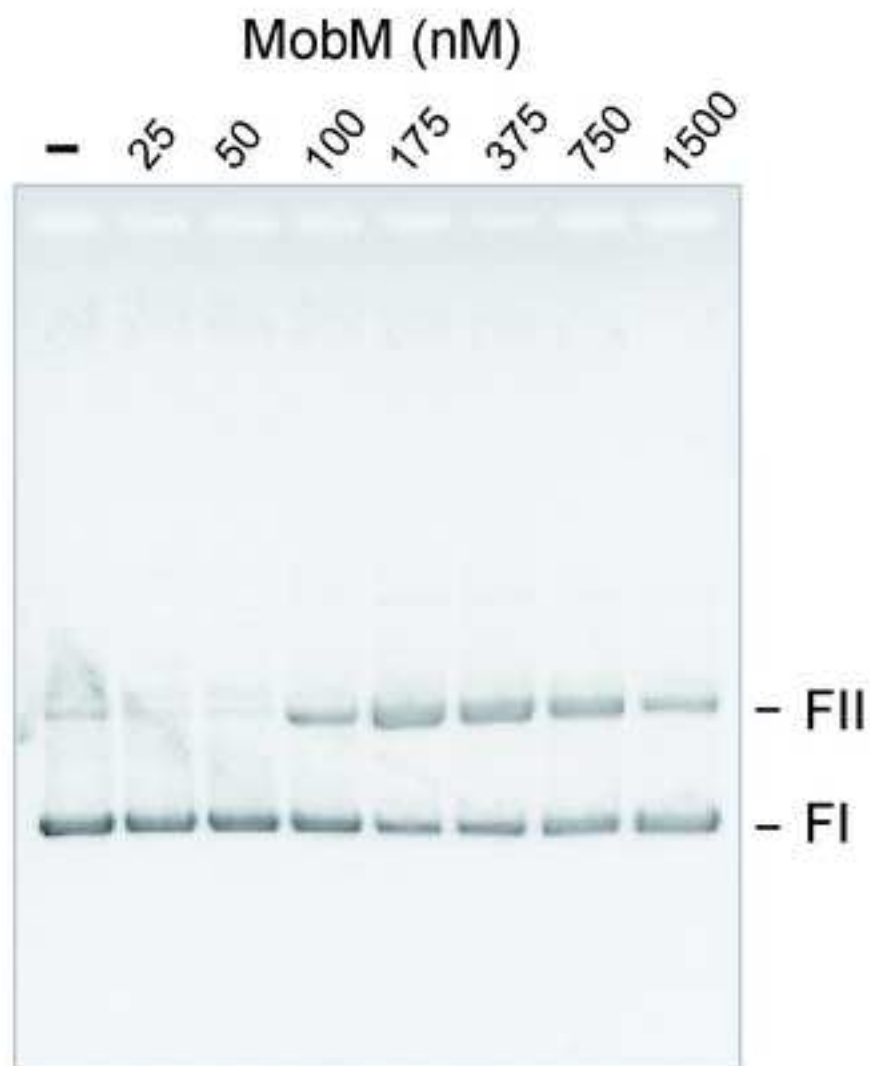
Figure
[Click here to download high resolution image](#)



Figure

[Click here to download high resolution image](#)

A



B

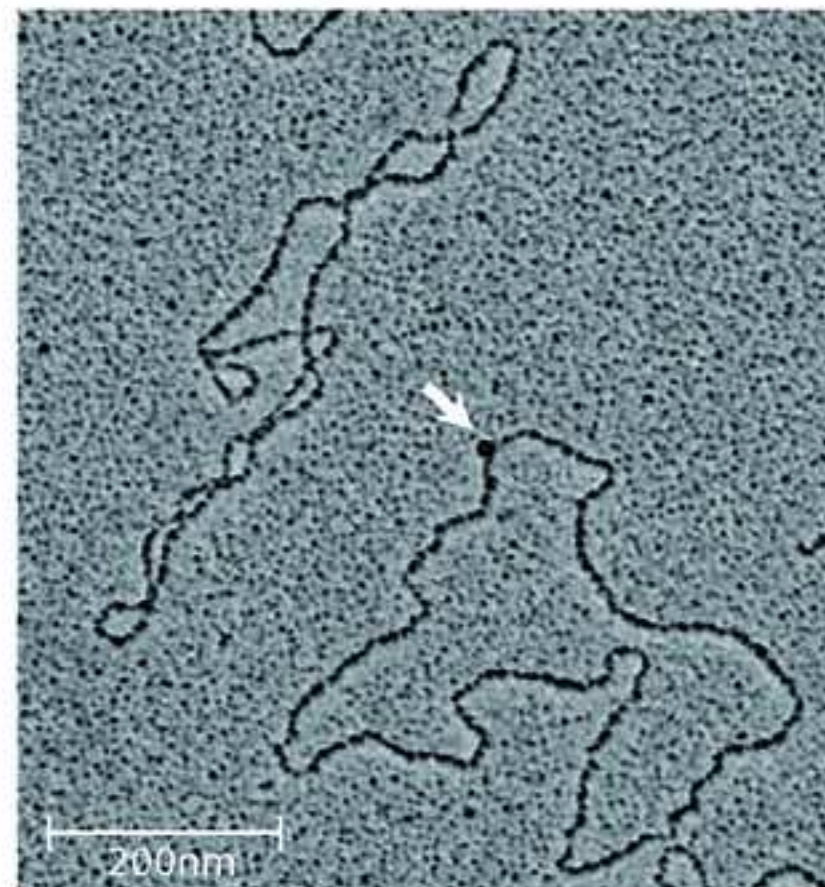
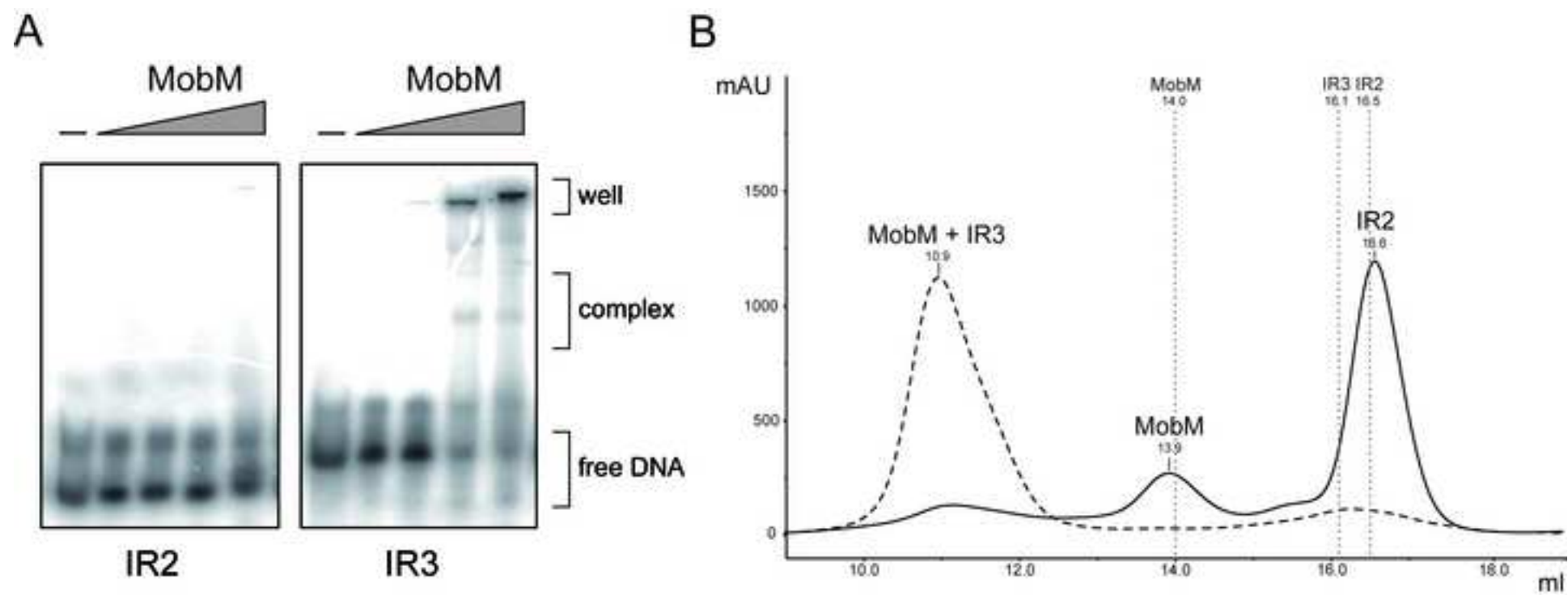
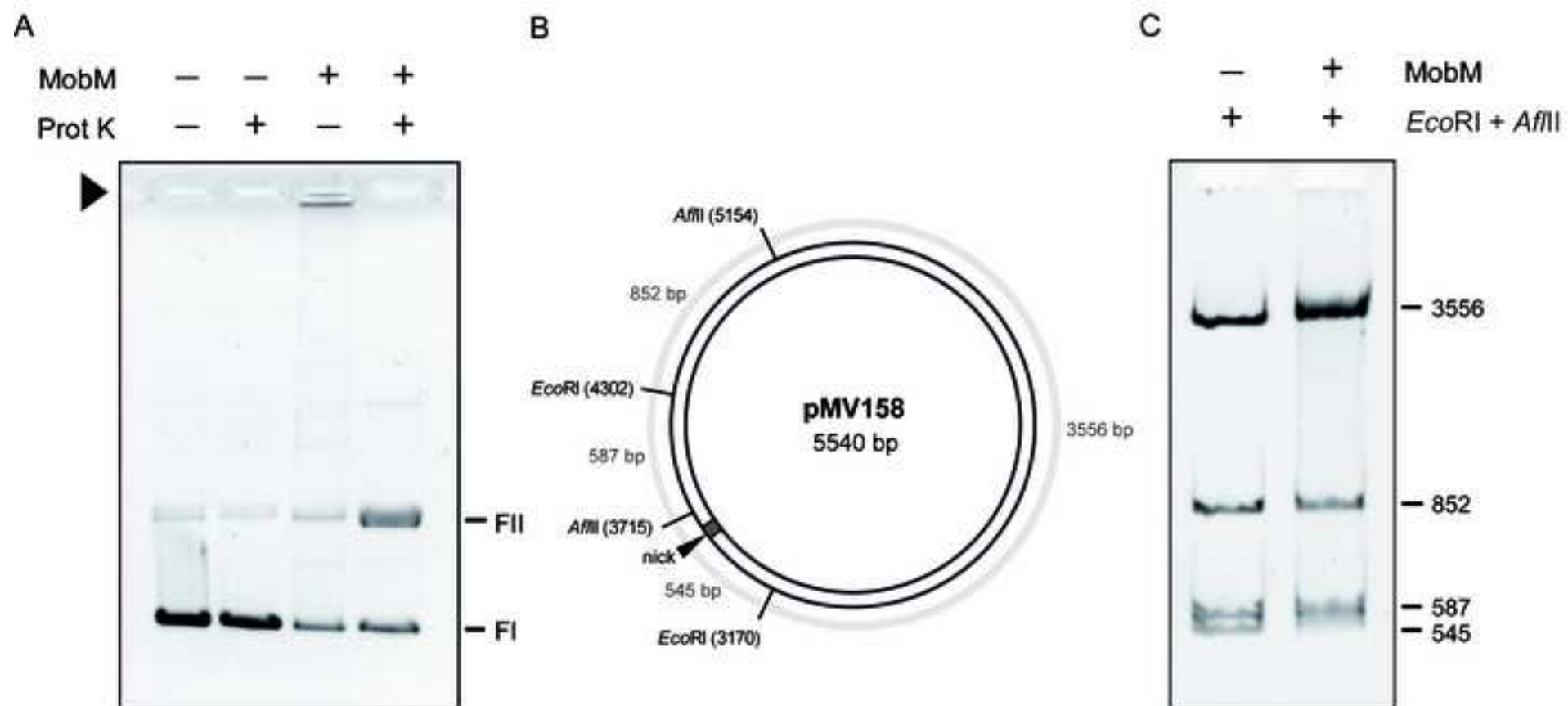


Figure
[Click here to download high resolution image](#)



Figure

[Click here to download high resolution image](#)



A

		20	I	40	II	60	80		
MobM	MSYVMARMQKMF	-AGNLGGAFKINER	VFETHSNKDI	NPSRSHLN	YESTDRDRSVS	YEKOIKDYV	NENKVSNT	AIRKDAVLCDE 82	
MobL	MSYVMARMQKMF	-AGNLGGAFKINER	VFETHSNKDI	DPSRSHLN	YESTDRDRSVS	YEKOIKDYV	NENKVSNT	AIRKDAVLCDE 83	
MobU	MSYAVCRMQKVF	-SAGLKGMOFHNOR	ERKSR	TDDIDH	ERTRENYDL	KN-DKNIDY	NERVKEI	IESOKTGT-RKTRKDAVLCDE 81	
MobE	MSHSILVARV	GGSSNTN	IQRN	QENKNY	NKINHEE	TYKND	LN-AQNI	KDKKIDETIDENYSYSGK-RKTRSDAIRHVD 82	
		100		120	III	140	160		
MobM	WIITSDKQFF	EQLDEEQ	TRTFETAK	NYFAENY	GESNIAYAS	VMILDS	TPHM	MGVVHFE-NGKLESSAMFD-REELKHICEDL 164	
MobL	WIITSDKQFF	EQLDEEQ	TRTFETAK	NYFAENY	GESNIAYAS	VMILDS	STPHM	MGVVHFE-NGKLESSAMFD-REELKHICEDL 165	
MobU	LLVTSDRDFF	EQLDPGEQ	KRFPEESY	KLESERY	GKQNIAYAT	VHND	QTPHML	LGVVHMR-DGKIQGNVEN-ROELLWLQDKF 163	
MobE	GLVTSKQDF	DDSGEIER	EIKDSLE	FLENEY	GKEMLYAT	VHLL	ERVHMI	FFVH	LTEDERLSAKEQLGNKKDFTQLQDRF 166
		180		200		220	240		
MobM	PRYMSDHGF	FELEERG	KLNSEAK	HKTVAE	FKRAMAD	MELKEEL	LEKYHAP	PVVDERTGELNNDTEAFWHEKEFADMFEVQSPIRET 248	
MobL	PRYMSDHGF	FELEERG	KLNSEAK	HKTVAE	FKRAMAD	MELKEEL	LEKYHAP	PLFVDERTGELNNDTEAFWHEKEFADMFEVQSPIRET 249	
MobU	BEHMKKQGF	FELEERG	KLNSEAK	HKTVAE	FKRAMAD	MELKEEL	LEKYHAP	PLFVDERTGELNNDTEAFWHEKEFADMFEVQSPIRET 243	
MobE	NEFVNEKGY	FELEERG	KLNSEAK	HKTVAE	FKRAMAD	MELKEEL	LEKYHAP	PLFVDERTGELNNDTEAFWHEKEFADMFEVQSPIRET 242	
		260		280		300	320		
MobM	TNOEKMDWL	RKQYQEL	LKKLESS	KKPLEDD	LSHLELL	DKTKKEY	IKIDSE	ASERASEISKAEGYINTLNHKSLEAKICLE 332	
MobL	TNREKMDWL	RKQYQEL	LKKLESS	KKPLEDD	LSHLELL	DKTKKEY	IKIDSE	TSERLSEISKAEGYINTLNHKSLEAKIERLE 333	
MobU	SMFGLGKEI	MTEKKPT	KNVVIS	ERDYKN	LVTAARD	NDRLKQ	HVRNLM	STDMAREYKMLSEHG--QVKIKYSGLVERFNEVNVN 325	
MobE	-----	LFSGR	ETGRK	ILTAD	EFERLQ	ETISSA	ERIVDD	YENIKSTDYTTENQELKRRRE--SLKVVNTWKGYHLEKSK 315	
		340		360		380	400		
MobM	SDNLOLEK	QKATKLE	AKALNE	SELREL	KPKKNF	LKGEHY	ELSP	QFEGLEAEVYRSRTILHHKDIELEQAKROVSLRASKNYFT 416	
MobL	REGLKLEK	LKTQIAD	LKIMSE	KELAAIT	PKKGVF	GKEYVEL	TKEQ	FEFKGLIYRSRNLVHOKELENEQLRRIVPLRRSK-RFE 416	
MobU	DYNELLE	ENKSL	-----	LKSKI	SDLKRD	VSLIYES	TKEFL	KERTDGLKAFKNVFKGFVDKVKDKTAQFOEKHDLEPKKNEFE 401	
MobE	EVNKLK	RENDS	-----	INEQL	NVSEK	FQASTV	TLYRA	ARANFPGEFKGFNRLEKEKFFND-SKFEIVGQFMDVVQDNV 386	
		420		440		460	480		
MobM	ASLEBAKE	AKGESI	DRLKSEI	KRLKN	ENSILRQ	ONDKML	GKLREL	MPDKAFKNLLSELKAIKPIVNIKKKAIKSLF 494	
MobL	ASWNE	LKKV	RERA	-----	-----	-----	-----	----- 430	
MobU	LTHN	REVK	ERSR	DQMSL	-----	-----	-----	----- 420	
MobE	QKVDR	KREK	ORTD	DDLEM	-----	-----	-----	----- 403	

B

pMV158 | 5' -GCACACACTTTATGAATATAAA---GTATAGTGTG/TTATACTTTAC-3'
 pUB110 | 5' -GaACgCACcTTtactATgTAAA---GgcTAGTAcG/TTAcACTTTAC-3'
 pDL287 | 5' -aCgacttaaTTAcGAA-gTAAAtaaGTcTAGTGTG/TTAgACTTTAt-3'
 pE194 | 5' -aCgacttaaTTAcGAA-gTAAAtaaGTcTAGTGTG/TTAgACTTTAt-3'

Figure

[Click here to download high resolution image](#)

