Auto-regulation of the Synthesis of the MobM Relaxase Encoded by the Promiscuous Plasmid pMV158

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Abbreviations used are: bp, base pair(s); EMSA, electrophoretic mobility shift assays; G+, Gram-positive; G-, Gram-negative; HGT, horizontal gene transfer; ICEs, integrative and conjugative elements; MU, Miller units; nt, nucleotide(s); RNAP, RNA polymerase; ss, single-stranded

The streptococcal promiscuous plasmid pMV158 (5540-bp) replicates by the rolling circle mechanism and can be mobilized among a wide number of Gram-positive and –negative bacteria. The plasmid region involved in its conjugative transfer includes the *mobM* gene, which encodes the MobM relaxase, and the *cis*-acting origin of transfer (*oriT*). MobM initiates transfer by cleavage of supercoiled pMV158 DNA at a specific dinucleotide within *oriT*. In the present work, we have performed a detailed transcriptional analysis to assess the role of MobM in the control of its own gene expression. By *in vivo* and *in vitro* approaches, we demonstrated that *mobM* transcription in *Escherichia coli* was mostly initiated from a promoter (*Pmob2*) different from the one (*Pmob1*) used in *Lactococcus lactis*. Whereas promoter *Pmob1* was embedded within the *oriT* sequence, promoter *Pmob2* was placed apart but adjacent to *oriT*. Further, MobM was able to repress the expression of its own gene from both promoters. Given the promiscuity of pMV158, the organization of the *mobM* promoter region suggests a strategy of the plasmid to cope with different transcription machineries of the hosts it colonizes.

Bacterial horizontal gene transfer (HGT) is mediated by mobile genetic elements of which plasmids, bacteriophages, and the phage-related chromosomally-encoded integrative and conjugative elements (ICEs) constitute the vast majority (14, 53). An increasing number of these mobile elements (the so-called bacterial mobilome) have been found as the number of totally sequenced genomes is increasing. There are nearly 2,000 complete plasmid genomes available at the GeneBank but their transferability has been tested only for a limited number of them, mostly replicons isolated from Gram-negative (G-) bacteria; much less attention has been given to plasmids from Gram-positive (G+) hosts (16, 41). Initiation of conjugative plasmid transfer involves the relaxation of the supercoiled DNA by a plasmid-encoded protein (the relaxase). This protein cleaves a specific phosphodiester bond (the nick site) of the strand to be transferred and remains covalently bound to its 5'-end. Nicking would be followed by a rolling-circle replication-like process in which the relaxase-single-stranded (ss) DNA complex is piloted to the cell membrane where the coupling protein and the Type IV Secretion System would pump the relaxase-ssDNA complex to the recipient cell (30). Once in the recipient, the cell machinery would recognize a single-strand origin of replication generated in the incoming ssDNA to perform the synthesis of the complementary DNA strand by a mechanism of lagging strand replication (4, 29, 55).

Important features in HGT are the signals that trigger the process, which are largely unknown for many plasmids other than those requiring quorum-sensing signals (13, 16). Accordingly, another key feature is the control of the intracellular levels of the relaxase (and hence of the relaxosome formation). In some plasmid transfer systems, this process seems to be exerted by ribbon-helix-helix accessory proteins that play a

role in regulating DNA relaxation by inducing bends in the DNA and/or in controlling synthesis of the relaxase by binding to DNA regions close to (or included into) its gene promoter. These are the cases of TraY of F (34), TrwA of R388 (33), MbeC of ColE1 (49), and MobC of pC221 (42). In other cases, like TraI_F, TraA_pIP501, and Mob_pBBR1 (19, 22, 47), it has been shown that expression of the relaxase gene is negatively regulated at the transcriptional level by the activity of the relaxase protein itself. However, the interactions between the relaxase and the host RNA polymerase (RNAP) at the plasmid origin of transfer (*oriT*) remain to be investigated.

The bacterial RNA polymerase (RNAP) holoenzyme is a complex of six subunits ($\alpha_2\beta\beta'\omega\sigma$). In general, bacterial genomes encode diverse forms of the σ-factor, and each of them confers promoter specificity to the RNAP (17, 51). Most transcription in exponentially growing bacterial cells is initiated by RNAPs that carry a housekeeping σ-factor similar to the *E. coli* σ^{70}. Promoters recognized by these RNAPs are characterized by two main sequence elements, the –35 (consensus 5´-TTGACA-3´) and –10 (consensus 5´-TATAAT-3´) hexamers (reviewed in (20)). Additionally, some of these promoters contain an extended –10 element that is located one nucleotide upstream of the –10 hexamer. This element is more conserved in G+ bacteria (5´-TRTG-3´motif) than in *E. coli* (5´-TG-3´motif) (32, 38, 50). The two conserved hexamers are separated by a region, termed 'spacer', which has no consensus sequence but has a structure that is important for σ^{70} recognition and activity of the promoters (40).

The promiscuous streptococcal plasmid pMV158 (5540-bp) represents one of the simplest systems for an efficient DNA transfer among different bacterial species, G+ and G-. It has a genetic organization such as all genes that encode proteins are placed

94 in the same orientation (Fig. 1A). In addition to the genes and loci required for its
95 leading-strand rolling-circle replication (*repB*, *copG*, and the double-stranded origin,
96 *dso*), two other cassettes are present in the plasmid, namely an antibiotic resistance
97 marker (a *tetL*-type determinant) and a mobilization cassette. The latter includes the
98 *mobM* gene, which encodes the MobM relaxase protein (494 residues), and the *oriT*.
99 The MobM protein is the representative of the MOB_V family of relaxases, constituted by
100 more than 100 members so far (14). It has been shown that MobM cleaves supercoiled
101 or ssDNA at a specific dinucleotide (coordinates 3595 and 3596; *nic*) within the *oriT*
102 sequence (18) (Fig. 1B). The *oriT* (coordinates 3564-3606) is unique in the sense that it
103 has three inverted repeats, IR (IR1, IR2, IR3; Fig. 1C) rather than the common single IR
104 found in most of the studied plasmid *oriT*s (55). We have shown that IR1 and IR3 are
105 preferentially recognized by protein MobM on ssDNA substrates, at least *in vitro* (28).
106 The role of IR2, if any, is presently unknown but the conservation of the *oriT* sequence
107 among the MOB_V plasmid family suggests that anyone of the three IRs could be
108 involved in the recognition of *oriT* by MobM at the initiation of relaxosome formation in
109 the donor cell and/or at the termination reaction to close the transferred strand within
110 the recipient cell. A DNase I footprinting assay showed that MobM binds, although very
111 poorly, to linear double-stranded DNA fragments containing the *oriT* sequence (15); the
112 protein is, however, unable to cleave linear dsDNA (18). Specifically, MobM protected a
113 region between coordinates 3582 and 3605, which includes IR2, although the exact
114 upstream border of the footprint remained unclear (Fig. 1B). Recently, using ssDNAs
115 and a truncated MobM protein (MobMN199), the minimal *oriT* sequence was delimited

to a stretch of 26 nucleotides (coordinates 3570-3595) that is located just upstream of the nick site. This minimal origin includes IR1 (28).

In the present work, we have performed an in-depth *in vitro* and *in vivo* transcriptional analysis of the pMV158-encoded MobM relaxase. We demonstrate that the major promoter governing transcription of the *mobM* gene in *E. coli* is close to, but different from, the one used in the G+ *Lactococcus lactis* (12). Whereas the latter promoter is located within the *oriT* sequence (Fig. 1B), the newly identified promoter is placed just downstream of it. This organization would provide a unique example of the versatility of the transfer system of a plasmid that can be mobilized among many different bacterial species. In addition, we demonstrate that the relaxase MobM is able to negatively regulate its own synthesis, which adds another level of complexity to the compact region of the plasmid spanning its *oriT*.

MATERIALS AND METHODS

Bacterial strains, plasmids and oligonucleotides. The *E. coli* TOP10 (Invitrogen) and JM109 (54) strains were used as hosts for the plasmids used in this work. *E. coli* JM109(DE3) (Promega) was employed for the β-galactosidase assays; this strain is a JM109-derivative in which the DE3 lysogen (46) provides the T7 RNA polymerase gene fused to the *lac*UV5 promoter and the *lac*I^q repressor gene. Thus, expression of the T7 RNA polymerase is induced by IPTG. In addition to the plasmids constructed in this work (see below), we used the following *E. coli* plasmids: i) vector pET5 (Novagen); ii) pLGM2, which is a pET5-derivative that carries the *mobM* gene

under control of the ϕ10 promoter of phage T7 (18), and iii) pMP220, which carries a promoterless *lacZ* gene (44). For small-scale preparations of plasmid DNA, the High Pure Plasmid Isolation Kit (Roche Applied Science) was used. Plasmid DNA from pMV158 (25) was purified from *S. pneumoniae* 708 (*trt-1*, *hex-4*, *end-1*, *exo-2*, *malM594*) (24) by two consecutive CsCl gradients as described (7). Oligonucleotides used in this work are listed in Table 1.

Growth and transformation of bacteria. *E. coli* cells were grown in TY medium (Pronadisa) at 37ºC. In the case of plasmid-harbouring cells, the media were supplemented with tetracycline (5 µg/ml and 2 µg/ml for pMV158 and pMP220-derivatives, respectively) or 100 µg/ml ampicillin (plasmids pET5 and pLGM2). *S. pneumoniae* cells harbouring pMV158 were grown in medium AGCH (23), supplemented with 0.2% yeast extract, 0.3% sucrose, and 1 µg/ml tetracycline as detailed (37). The protocol used to transform *E. coli* by electroporation was described previously (10).

Polymerase chain reaction (PCR) conditions. Phusion High-Fidelity DNA Polymerase (Finnzymes) was used for all PCR applications. The reaction mixtures (50 µl) contained 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, pH 8.8, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Roche Applied Science), 0.4 µM of each primer, 1 ng of template DNA and 0.65 units of DNA polymerase. An initial denaturation step was performed at 98ºC for 30 sec, followed by 30 cycles that included the next steps: (i) denaturation at 98ºC for 10 sec, (ii) annealing at around 55ºC (depending on the primer Tm) for 20 sec, and (iii) extension at 72ºC for 30-60 sec A final extension step was performed at 72ºC for 10 min. PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN).

RNA isolation and primer extension assays. JM109 cells harbouring pMV158 and JM109(DE3) cells harbouring the combinations of pLGM2 and pORI-P, or pLGM2 and pIR2-P were exponentially grown at 37ºC to an optical density at 600 nm (OD_{600}) of 0.4. The JM109(DE3) cells harbouring plasmids were divided into 10 ml aliquots. Each aliquot was supplemented with IPTG (1 mM final concentration) and incubated for the indicated time at the same temperature. As controls, 10-ml cultures incubated without IPTG were used. The Aurum Total RNA Mini Kit (BioRad) was used to isolate total RNA. Cultures were processed as specified by the supplier, and analyses of RNAs and primer extension assays were performed as reported (5). Dideoxy-mediated chain termination sequencing reactions were run in the same gel. Labelled products were visualized using the FUJIFILM Image Analyzer FLA-3000.

Electrophoretic mobility shift assays (EMSA). A 437-bp fragment (coordinates 3371-3807 of pMV158) was generated by PCR using the DraF and DraR primers (Table 1). The DraR primer included a *Dra*I restriction site. Digestion of the 437-bp DNA with *Dra*I generated a 362-bp DNA fragment (coordinates 3429-3790). Reaction mixtures (20 µl) contained 40 mM Tris-HCl, pH 7.5, 150 mM KCl, 10 mM $MgCl_2$, 0.01% Triton X-100, 120 nM of *E. coli* RNAP (Epicentre) and 5 nM of the 362-bp DNA fragment. After 30 min at 37ºC, RNAP-DNA complexes were treated with heparin (0.25-100 µg/ml final concentration) for 5 min at the same temperature. Electrophoresis condition and analyses of the retarded bands were performed as reported (21).

Electron microscopy. DNA fragments from pMV158 of two sizes, namely 2375-bp (coordinates 3121-5495) and 699-bp (coordinates 3121-3819) were obtained by PCR using pMV158 DNA as template and the ssoUF/ssoAR and ssoUF/ssoUR

185 oligonucleotides (Table 1), respectively, as primers. Reaction mixtures (10 µl) contained
186 40 mM Tris-HCl, pH 7.5, 150 mM KCl, 10 mM $MgCl_2$, 0.01% Triton X-100, 120 nM of *E.*
187 *coli* RNAP (Epicentre) and 5-20 nM of the 2375- or 699-bp DNA fragments. After 15 min
188 at 37ºC, RNAP-DNA complexes were fixed with 0.3% glutaraldehyde for 15 min at the
189 same temperature. Then, reactions were diluted 10-fold in buffer GA (10 mM
190 triethanolamine chloride, pH 7.5, 10 mM $MgCl_2$), adsorbed onto freshly cleaved mica,
191 positively stained with 2% uranyl acetate, rotary shadowed with Pt/Ir, and covered with
192 a carbon film as described (45). Micrographs of the carbon film replica were taken using
193 a Philips CM100 (FEI Company, Hillsboro, Oregon) electron microscope at 100 kV on
194 35-mm film. The contour length of the RNAP-DNA complexes and the positions on the
195 DNA fragments were measured on projections of 35-mm negatives using a digitizer
196 (LM4, Brühl, Nüremberg, Germany).

197 **DNase I footprinting assays.** A 162-bp DNA fragment (coordinates 3527-
198 3688 of pMV158) was generated by PCR using the P-116 and P+46 primers
199 (Table 1). To label this fragment at the 5'-end of a particular strand, the
200 corresponding primer was treated with T4 polynucleotide kinase and [γ-^{32}P]-ATP
201 (3000 Ci/mmol; Hartmann) before performing the amplification reaction. DNase I
202 footprintings were performed essentially as reported earlier (21), with the
203 concentration of RNAP as indicated in results. Dideoxy-mediated chain
204 termination sequencing reactions using pMV158 and either the P-116 or the
205 P+46 oligonucleotide were run in the same gel.

206 **In vitro transcription analysis.** *In vitro* transcription reactions were carried out
207 under multiple-round conditions. Reactions (50 µl) contained 45 mM Tris-HCl, pH 7.5,

150 mM KCl, 45 mM NaCl, 10 mM $MgCl_2$, 2 mM DTT, 0.01% Triton X-100, 1.5% glycerol, 10-30 nM of PCR-amplified linear DNA, 250 μM of each NTP (Promega), 10 μCi of [α-^{32}P]-UTP (3000 Ci/mmol, GE Healthcare), 10 units of SUPERase·In (Ambion), and 24 nM of the *E. coli* RNAP (Epicentre). After incubation at 37ºC, 30 min, non-incorporated nucleotide was removed using MicroSpin G-25 columns (GE Healthcare). Samples were then dried in a SpeedVac, dissolved in RNA loading buffer (80% formamide, 10 mM EDTA, pH 8.0, 0.1% bromophenol blue, 0.1% xylene cyanol), heated at 85ºC, 5 min, and subjected to electrophoresis in 8 M urea-6% polyacrylamide gels. Dideoxy-mediated chain termination sequencing reactions were run in the same gel. Specifically, the Sequenase Quick-Denature Plasmid Sequencing Kit (USB), pUC19 DNA, the UC–50 primer (Table 1) and [α-^{32}P]-dATP (6000 Ci/mmol, GE Healthcare) were used. Following electrophoresis, the gel was exposed to X-ray films.

Construction of plasmids pORI-P and pIR2-P. The IncP broad host range vector pMP220 has single restriction sites for *Eco*RI and *Pst*I. These sites are located upstream of the promoterless *lacZ* gene (44). This plasmid vector was used to evaluate the promoter activity of pMV158 DNA regions by measuring β-galactosidase activity. To construct plasmid pORI-P, a 150-bp region (coordinates 3541-3690 of pMV158) was amplified by PCR with the ORI and MR oligonucleotides (Table 1). These oligonucleotides include single *Eco*RI and *Pst*I restriction sites, respectively. After *Eco*RI and *Pst*I digestion of the PCR-amplified DNA, the generated product (coordinates 3555-3676) was mixed with pMP220 DNA digested with both enzymes. The mixture was then treated with T4 DNA ligase (New England Biolabs). For the construction of plasmid pIR2-P, a 123-bp region of pMV158 (coordinates 3568-3690) was amplified using the

IR2 and MR primers. The IR2 oligonucleotide included an *Eco*RI site. After *Eco*RI and *Pst*I treatment of the PCR-amplified DNA, the digestion product (coordinates 3583-3676) was inserted into the pMP220 vector. Ligation mixtures were used to transform *E. coli* TOP10 cells. Transformants were selected for resistance to 2 µg/ml tetracycline at 37ºC. Plasmid DNA was isolated and analyzed by agarose gel mobility. To confirm the constructions, the inserted fragment and the regions of pMP220 that are flanking the insert were sequenced. Dye-terminator sequencing was carried out at Secugen (Centro de Investigaciones Biológicas, Madrid).

β-galactosidase activity measurements. *E. coli* JM109(DE3) cells carrying the indicated plasmids were grown at 37ºC as indicated above to middle-exponential phase (OD_{600} ~0.4). Then, the cultures were divided in two. IPTG (1 mM final concentration) was added to one half of the cultures to induce the expression of the *mobM* gene from plasmid pLGM2. As controls, the other half of the cultures did not received IPTG. Measurement of the activity was performed as reported (3), but the samples (200 µl) were dispensed in a 96-well microplate and absorbance data were collected with a VarioskanFlash reader (ThermoScientific). The β-galactosidase specific activities were calculated in Miller units (MU) (31).

RESULTS

Initiation of *mobM* transcription in *E. coli* cells. The pMV158_*mobM* cassette is flanked by two lagging strand origins of replication, *ssoU* and *ssoA* (Fig. 1A). It was shown that whereas either the *ssoA* or the *ssoU* could be used for intraspecific transfer

254 in *S. pneumoniae*, interspecific transfer required an intact *ssoU* (29). Thus, this latter
255 origin appeared to be required for the extraordinary promiscuity of pMV158 (8). Plasmid
256 pMV158 has been mobilized between different G+ bacteria, like *L. lactis* (12) and
257 *Enterococcus faecalis* (29, 35), using pAMβ1 as auxiliary plasmid. In the former
258 bacterium, it was shown that transcription of the *mobM* gene started at coordinate 3609
259 of pMV158 (12). Thus, the lactococcal RNAP appeared to recognize a promoter
260 sequence, named *Pmob1* (Fig. 1B). This promoter has a near-consensus –10 hexamer
261 (5´-TATAcT-3´), a consensus –10 extension (5´-TGTG-3´), and shows a 3/6 match at
262 the –35 element (5´-aTGAat-3´). The extension of the -10 sequence, also termed the -
263 15 motif (9), seems to be particularly important for promoters of G+ bacteria (2, 38).
264 Moreover, the –10 and –35 elements of promoter *Pmob1* are separated by 16
265 nucleotides, and the –10 hexamer is located just downstream of the dinucleotide
266 cleaved by MobM (*nic*; Fig. 1B) (18), placing it within the minimal *oriT* sequence (28).
267 In addition to the G+ hosts, pMV158 was shown to replicate in the G- bacterium *E.*
268 *coli* (7). Mating experiments showed that the IncPα RP4 or the IncW R388 plasmids
269 were able to mobilize pMV158 between *E. coli* strains (11). In the case of RP4, it was
270 shown that transfer of pMV158 required the products of genes *traG* (coupling protein;
271 (1) and *traF* (mating pair formation; (27). It was thus interesting to explore in some
272 depth the transcriptional features of the pMV158 *mobM* gene in the G- host. To identify
273 the transcription initiation site of the *mobM* gene in *E. coli*, we carried out primer
274 extension experiments using the *mobM*-PE oligonucleotide as primer (coordinates
275 3748-3725; Table 1) and total RNA isolated from *E. coli* cells harbouring plasmid
276 pMV158. As shown in Figure 2, two cDNA extension products of 106 (major product)

277 and 140 (minor product) nucleotides were detected. The 140-nt cDNA revealed a
278 transcription initiation event at coordinate 3609 from the *Pmob1* promoter, whereas the
279 appearance of the 106-nt cDNA might correspond to: i) a degradation product from the
280 larger 140-nt cDNA; ii) a premature stop during retrotranscription; or iii) a transcription
281 initiation event at coordinate 3643 from a different promoter. Sequence analysis of the
282 region located just upstream of coordinate 3643 using the BPROM
283 (http://linux1.softberry.com/berry.phtml) prediction program supported the latter
284 hypothesis. It revealed the existence of an additional promoter sequence, herein termed
285 *Pmob2* (see Fig. 1B). The –10 element (5´-TAaAcT-3´) of this putative promoter is
286 located at the proper distance of 7 nucleotides from the transcription start site
287 (coordinate 3643). Moreover, the *Pmob2* promoter has a consensus –10 extension (5´-
288 TGTG-3´), and the –10 and –35 (5´-TgGAag-3´) sequence elements are separated by
289 17 nucleotides, which is the optimum spacer length for *E. coli* (20). Therefore, promoter
290 *Pmob2* is placed just downstream of the *oriT*, and it is close to, but not overlapping with
291 promoter *Pmob1* (Fig. 1B).
292
293 ***E. coli* RNAP binds to the *Pmob2* promoter *in vitro*.** The interaction of the *E.*
294 *coli* RNAP with the promoter region of the *mobM* gene was further studied by several *in*
295 *vitro* methods. In a first approach, we performed EMSA experiments using the *E. coli*
296 RNAP holoenzyme (σ^{70} factor) and a 362-bp linear DNA fragment (coordinates 3429-
297 3790) under conditions that favoured generation of open complexes (37ºC and in the
298 absence of NTP substrates). The DNA fragment contains the *Pmob1* and *Pmob2*
299 promoter sequences (see Fig. 1B). In these assays, heparin (0.25-100 µg/ml) was used

300 as competitor and the molar ratio of RNAP to DNA was 24:1 (Fig. 3). Without heparin,
301 aggregates of DNA-bound RNAP molecules, which did not enter the native gel, were
302 observed. After addition of heparin (5 μg/ml), RNAP-DNA complexes with much slower
303 electrophoretic mobility than the free DNA were detected, indicating that non-specifically
304 bound RNAP molecules were displaced by such a concentration of competitor. RNAP-
305 DNA interactions were disrupted at heparin concentrations above 10 μg/ml (Fig. 3).
306 Hence, the *E. coli* RNAP was able to interact with the promoter region of the *mobM*
307 gene forming complexes that were unstable to heparin challenge. It is worth pointing out
308 that RNAP does not generate stable complexes resistant to competitors in all promoters
309 (for an in-depth discussion, see ref 36).

310 To verify further the specificity of the complexes formed by the *E. coli* RNAP on
311 the *mobM* promoter region, we carried out electron microscopy assays. In a first
312 experiment, the *E. coli* RNAP was incubated with a 2375-bp linear pMV158 DNA
313 fragment (coordinates 3121-5495). This fragment contains the *ssoU* and *ssoA* single-
314 strand origins, the *oriT* sequence and the *mobM* gene (see Fig.1A). Then, RNAP-DNA
315 complexes were fixed with glutaraldehyde, prepared for electron microscopy and
316 visualized as described in Materials and Methods. Electron micrographs of RNAP-DNA
317 complexes are shown in Figure 4. To determine the RNAP binding site, the contour
318 lengths of the DNA regions between complexes and DNA ends were measured and the
319 position of the RNAP determined. Figure 4A shows the distribution of the RNAP
320 positions on the 2375-bp fragment. Of 174 complexes examined, the majority (73%)
321 showed a RNAP bound to one DNA region located at a maximum distance of 503-bp
322 from one DNA end and of 1872-bp from the other end, respectively. This indicated that

the *E. coli* RNAP binds specifically either around coordinate 3624 (*Pmob2* promoter region) or around coordinate 4992 (MobM-coding region) (see Fig. 1). No clear indication of complexes of RNAP at *Pmob1* region was observed. To define precisely the RNAP recognition site, we performed similar electron microscopy assays but this time using a 699-bp DNA fragment spanning coordinates 3121 to 3819. The majority (75%) of the 105 complexes analyzed showed RNAP binding in a peak about 187-bp from the nearest DNA end (Fig. 4B). This result positioned the RNAP binding site around the coordinate 3632 (*Pmob2* promoter region) or 3308. Collectively taken, the above results showed that the *E. coli* RNAP binds specifically around the coordinates 3624 and 3632, occupying the –10 sequence element of the *Pmob2* promoter.

To define accurately the position of the *E. coli* RNAP on the *mobM* promoter region, DNase I footprinting assays were performed using a 162-bp DNA fragment (coordinates 3527-3688), which contains the *Pmob1* and *Pmob2* promoters (see Fig. 1). Such a fragment was radioactively labelled either at the 5´-end of the coding strand (Fig. 5A) or at the 5´-end of the non-coding strand (Fig. 5B). On the coding strand, the region spanning the –43 and –15 positions relative to the transcription start site of the *Pmob2* promoter was protected against DNase I digestion. Changes in the DNase I sensitivity (diminished cleavages) were also observed at positions of adjacent regions (from –52 to –48 and from –13 to +20). In the case of the non-coding strand, RNAP-mediated protections were observed from –45 to around +21. Therefore, these results demonstrated that the *E. coli* RNAP recognizes *in vitro* the *Pmob2* promoter rather than the *Pmob1* promoter, even though this latter promoter was recognized, albeit weakly, *in vivo* (Fig. 2).

***E. coli* RNAP initiates *mobM* transcription from the *Pmob2* promoter *in vitro*.** We next investigated whether *E. coli* RNAP was able to transcribe the *mobM* gene from the *Pmob2* promoter *in vitro*. To this end, *in vitro* transcription assays under multiple-round conditions were carried out (Fig. 6). Two linear DNA fragments of 362-bp (coordinates 3429-3790) and 699-bp (coordinates 3121-3819) were used as templates. These fragments, which contain the *Pmob1* and *Pmob2* promoters, were the same used in the EMSA and electron microscopy studies (see above). Transcription from the *Pmob2* promoter should generate run-off transcripts of 148-nt or 177-nt using the 362-bp or 699-bp templates, respectively. However, transcription from the *Pmob1* promoter should produce run-off transcripts of 182-nt or 211-nt with the 362-bp or 699-bp DNAs, respectively. The *in vitro* transcription products were resolved on denaturing gels, and their size was estimated by comparison with the size of DNA fragments generated by dideoxy-mediated chain-termination sequencing reactions (Fig. 6). When the 362-bp DNA was used as template (lane 1), an RNA product that comigrated with a 158-nt DNA was detected. In the case of the 699-bp template (lane 2), the main RNA product observed comigrated with a 188-nt DNA. Since RNA runs about 5-10% slower than DNA of the same size in the sequencing gels (39), we conclude that the major RNA products correspond to run-off transcripts synthesized by recognition of the *Pmob2* promoter. Hence, *in vitro* as well as *in vivo*, the *E. coli* RNAP transcribes the *mobM* gene preferentially from the *Pmob2* promoter.

Auto-regulation of *mobM* gene expression in *E. coli*. Using linear double-stranded DNA fragments from pMV158 and DNase I footprinting techniques, we showed previously that purified MobM protein was able to protect a region spanning coordinates 3582 and 3605, although it was needed a vast excess of MobM because of the poor binding of the protein to linear dsDNA (our unpublished observations). Such a region included the IR2 element of *oriT* (15) (see Fig. 1B). Now, we have shown that *E. coli* RNAP binds preferentially to the *Pmob2* promoter both *in vivo* and *in vitro* (see above). Specifically, it protected about 66-bp on the non-coding strand, from –45 (coordinate 3598) to about +21 (coordinate 3663) relative to the transcription start point of the *Pmob2* promoter (Fig. 5). These results suggested that the binding of MobM to *oriT* should prevent RNAP from gaining access to the *Pmob2* promoter and, consequently, it should reduce expression of the *mobM* gene. First, we tested this prediction by performing *in vitro* transcription experiments with the *E. coli* RNAP in the presence of MobM. Repression of the *Pmob2* promoter was observed but, as stated above, a large amount of MobM protein was required (not shown). Subsequently, we designed an *in vivo trans*-complementation assay based on the use of two compatible plasmids: pLGM2 (18) and pORI-P (this work). Plasmid pLGM2 is a pET5-derivative that carries the *mobM* gene under control of the T7 Φ10 promoter. Plasmid pORI-P is a pMP220-derivative that carries the 3555-3676 region of pMV158 inserted upstream of the promoterless *lacZ* gene. This region, in addition to the *Pmob1* and *Pmob2* promoters, contains the three IRs of *oriT* (Fig. 1B). Both plasmids were introduced into JM109(DE3), in which expression of the T7 RNAP is inducible by IPTG. Thus, JM109(DE3) cells harbouring pLGM2 and pORI-P synthesize MobM only when they are

391 grown in the presence of IPTG. Measurement of *lacZ* expression under these conditions
392 showed that β-galactosidase activity decreased nearly two-fold as compared to the
393 activity detected in cells grown in the absence of IPTG (Fig. 7). On the contrary, no
394 changes in *lacZ* expression were detected in JM109(DE3) cells harbouring pET5, which
395 lacks the *mobM* gene, and pORI-P (control strain). The MobM-mediated repression was
396 moderate albeit statistically significant. Similar repression levels were observed for the
397 pMV158-*repB* gene using a construct similar to the one employed here (6). Whether
398 these effects are due to the employment of the T7 Φ10 promoter to direct synthesis of
399 the protein or to an intrinsic property of the pneumococcal sequences is not known at
400 present. We can conclude that MobM reduced the activity of the *Pmob1* and/or *Pmob2*
401 promoters *in vivo*.

402 The above interpretation was further confirmed by primer extension experiments
403 using total RNA isolated from JM109(DE3) cells carrying both plasmids, pLGM2 and
404 pORI-P. As primers, a mixture of the 5'-labelled *lacZ*-PE and *tetA*-PE oligonucleotides
405 (Table 1) was used. They anneal to the *lacZ* and *tetA* (tetracycline resistance)
406 transcripts of the pORI-P plasmid, respectively. With the primer *lacZ*-PE and in the
407 absence of IPTG (Fig. 8, lane 3), a minor cDNA product of 131-nt (promoter *Pmob1*)
408 and a major cDNA product of 97-nt (promoter *Pmob2*) were detected. The amount of
409 both products decreased when bacteria were grown in the presence of IPTG for 30 min
410 (lane 4) or 120 min (lane 5). With the primer *tetA*-PE, used as internal control, a major
411 cDNA product of 85-nt (promoter *PtetA*) was detected in the absence of IPTG (lane 3).
412 However, the amount of such a product did not change in the presence of IPTG (lanes 4
413 and 5). We can conclude that MobM was able to repress *in vivo* not only the minor

414 transcription initiated from promoter *Pmob1* but also the major transcription initiated
415 from promoter *Pmob2*.

416 We next analyzed whether the IR1/IR3 inverted repeats of the pMV158-*oriT* were
417 required for such MobM-mediated repression. To this end, we constructed plasmid
418 pIR2-P, which is a pMP220-derivative that carries the 3583-3676 region of pMV158
419 inserted upstream of the promoterless *lacZ* gene (Fig. 7). The –35 hexamer of the
420 *Pmob1* promoter (3575-3580 coordinates) was regenerated as a result of the cloning.
421 Thus, plasmid pIR2-P carries an intact IR2 but lacks the left arm of IR1/IR3 (Fig. 1B).
422 Expression of *lacZ* in JM109(DE3) cells harbouring both plasmids, pLGM2 and pIR2-P,
423 decreased nearly two-fold when they were grown in the presence of IPTG (synthesis of
424 MobM; Fig. 7). No changes in *lacZ* expression were observed in the control strain
425 (JM109(DE3) carrying both pET5 and pIR2-P). MobM-mediated repression was further
426 confirmed by primer extension on total RNA using a mixture of the 5'-labelled
427 oligonucleotides *lacZ*-PE and *tetA*-PE as primers (not shown).

428 Taken all the above results together, we can conclude that MobM is able to
429 repress transcription from the *Pmob2* promoter in *E. coli*, and thus regulate its own
430 synthesis. This repression does not require the left arm of IR1/IR3, although we have to
431 consider that the right arm of IR1/IR3 and the left arm of IR2 overlap (Fig. 1B).

432

433 **DISCUSSION**

434 The promiscuous plasmid pMV158 represents one of the simplest systems for an
435 efficient DNA transfer among different bacterial species, G+ and G-. Its MobM relaxase
436 is the representative of the MOB_V family of relaxases, constituted by more than 100

437 members (14), representing a wealth of genetic information that merits to be explored in
438 depth. The results presented here demonstrate that the pMV158 *mobM* gene is
439 transcribed by the *E. coli* RNAP from a promoter (*Pmob2*) that is different from the one
440 (*Pmob1*) previously shown to be used in the G+ bacterium *L. lactis* (12). The DNA
441 region protected by RNAP extends up to the +20 position relative to the *Pmob2*
442 transcription start site (Figs. 5 and 9A). This is indicative of the formation of an open
443 complex, as should be expected under the experimental conditions employed.
444 Inspection of the DNA sequence around the pMV158-*oriT* showed a high degree of
445 conservation among many different plasmids from G+ bacteria (18). As the number of
446 sequenced replicons increased, we have found now that a few streptococcal plasmids
447 maintain the structure of the two *Pmob1* and *Pmob2* promoters (Fig. 9B). This is the
448 case of plasmids pER13 and pSMQ172 from *S. thermophilus* (43, 48), pVA380-1 from
449 *S. ferus* (26), and pRW35 from *S. pyogenes* (52). Cross-recognition of the pVA380-
450 1_*oriT* by the pMV158_MobM was previously demonstrated (15). The genetic structure
451 of the *oriT*s and promoter(s) in the mobilization region of these plasmids suggests that it
452 is not per chance that pMV158 exhibit two promoters differentially used in G+ or G-
453 bacteria, and allows us to postulate that they could also be transferred to *E. coli*.

454 Typically for a small mobilizable plasmid, pMV158 encodes only those conjugative
455 functions required for DNA processing: the *cis*-acting *oriT* and the DNA relaxase MobM.
456 Our *in vivo* transcriptional studies showed that: i) synthesis of the pMV158-relaxase
457 MobM is auto-regulated, and regulation of the *mobM* gene appears to be regulated by
458 MobM solely, since we have not found any indication of the presence of a regulatory
459 antisense RNA in the entire *mob* cassette (our unpublished results); ii) the region of *oriT*

involved in auto-regulation is represented by IR2, and iii) auto-regulation does not require the presence of intact IR1/IR3 elements (Fig. 1C). Furthermore, a DNase I footprinting assay using *oriT*-containing linear double-stranded DNA fragments and a vast excess of purified protein, showed that MobM protected IR2 (15). Whether MobM has two modes of binding to *oriT*, one for relaxosome formation (IR1/IR3) and another for auto-regulation (IR2) is presently unknown, although it seems likely under the light of our present results.

In summary, our data demonstrate that binding of MobM to its cognate *oriT* is required not only to initiate plasmid transfer but also to control *mobM* gene expression, as previously hypothesized on the basis of the structure of the DNA region surrounding the pMV158-*oriT* (28-29). Auto-regulation of the synthesis of a relaxase of the MOB_V family was previously shown for the *E. coli* plasmid pBBR1, although it was based only on transcriptional fusions (47). Similarly, mutational analyses indicated that TraI of plasmid F also self-regulates its own synthesis. However in neither case, a detailed investigation of the transcriptional structure of the plasmid *oriT* was performed. To our knowledge, this is the first in-depth transcriptional study performed on a conjugative relaxase from G+ bacteria. Our results show that the *mobM* gene can be transcribed from two different promoters, which may be used depending upon the host in which the plasmid establishes. While promoter *Pmob1* is located within *oriT*, promoter *Pmob2* is adjacent to it (Figs. 1B and 9A), and yet both promoters are subjected to self-regulation. Given the promiscuity of pMV158, such genetic organization suggests a strategy of the plasmid to cope with the different transcription machinery of G+ and G- bacteria.

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662

663 TABLE 1. Oligonucleotides used in this work

Name	Sequence (5´ to 3´)[a]	Coordinates[b]
DraF	GGTGGAGATTTTTTGAGTG	3371-3389
DraR	CACGTTCATTATGC**TTTAAA**GCTCCTCCC	3807-3779
ssoUF	GGGATCAACTTTGGGAGAGA	3121-3140
ssoUR	GCGTCTCAAAAACACGTTCA	3819-3800
ssoAR	TCACAACGCTCACCTCCA	5495-5478
P-116	TTATGGTTTTGGTCGGCACT	3527-3546
P+46	CACGAGCCGACACAGTCTATT	3688-3668
ORI	GGCACTGCC**GAATTC**CTCGCAGAGC	3541-3565
IR2	ACACTTTAT**GAATTC**AAAGTATAGTGT	3568-3594
MR	CAGACGAGCCG**CTGCAG**TCTATTGCT	3690-3665
mobM-PE	GCAACCATGTAACTCATAGATTTC	3748-3725
lacZ-PE	GTGATTTTTTTCTCCATTTTAGC	-----
tetA-PE	GGGGTATGTTGGGTTTCACGTCTG	-----
UC-50	TTGTGAGCGGATAACAATTTC	-----

664 [a]Base changes that generate restriction sites (in bold) are underlined

665 [b]Coordinates are given with respect to pMV158 sequence (Acc. no. NC_010096)

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669

FIGURE LEGENDS

FIG. 1. (A) Genetic map of pMV158. Only relevant features are indicated. Genes are depicted as arrows pointing in the direction of transcription. *copG* and *repB* genes are involved in plasmid DNA replication. The position of the origins for leading (*dso*) and lagging (*ssoU* and *ssoA*) strand synthesis is indicated. The *tetL* gene confers resistance to tetracycline in both G+ and G- bacteria. The origin of transfer (*oriT*) and the *mobM* gene are involved in conjugative mobilization. P: *Pst*I, N: *Nco*I, E: *Eco*RI. Coordinates are given in parentheses. (B) Nucleotide sequence of the pMV158 region spanning coordinates 3564 and 3650. This region includes the *oriT* and the promoter region of the *mobM* gene. The three overlapping inverted repeats (IR1, IR2, IR3) (28) and the nicking site (*nic*) (18) conforming the *oriT* are indicated. The minimal *oriT* sequence (coordinates 3570-3595) on ssDNA is boxed (28). The shadowed sequence (coordinates 3582-3605) denotes the MobM binding site defined by DNase I footprinting assays although the precise upstream boundary of protection against DNase I remained unclear (15). The main sequence elements of the *Pmob1* (12) and *Pmob2* (this work) promoters are underlined in grey or black, respectively. The transcription start site for each promoter is indicated with an arrow. The position of the translation start codon (ATG) of the *mobM* gene is also shown. (C) Scheme showing the three possible stem-loop structures that could adopt the *oriT* sequence on ss-DNAs. The arrowhead indicates the *nic* position.

692 FIG. 2. Primer extension on total RNA isolated from *E. coli* cells carrying plasmid
693 pMV158. The primer (*mobM*-PE; Table 1) annealed to the transcripts in the region
694 corresponding to coordinates 3725-3748 of pMV158. The size expected for the cDNAs
695 if transcription initiation occurs at coordinates 3609 (*Pmob1*) or 3643 (*Pmob2*) is
696 indicated below the gel. Primer extension products were resolved on denaturing gels (7
697 M urea, 8% polyacrylamide). As DNA size markers, dideoxy-mediated chain-termination
698 sequencing reactions using pMV158 DNA and the *mobM*-PE primer were run in the
699 same gel (lanes A, G, T, C). The size of the cDNA extension products (lane P) is
700 indicated on the right in nucleotides.
701
702 FIG. 3. EMSA analysis of RNAP-DNA complexes. The *E. coli* RNAP (120 nM) was
703 incubated with the 362-bp DNA (5 nM) (coordinates 3429-3790) at 37ºC for 30 min.
704 Then, heparin was added at the indicated concentrations. After 5 min, the reaction
705 mixtures were loaded onto a native gel (5% polyacrylamide). The gel was stained with
706 ethidium bromide. Bands corresponding to free DNA (F) and to specific RNAP-DNA
707 complexes (C) are indicated.
708
709 FIG. 4. Electron micrographs of RNAP-DNA complexes. The *E. coli* RNAP (120 nM)
710 was incubated with (A) the 2375-pb fragment (5 nM) (coordinates 3121-5495) or with
711 (B) the 699-bp fragment (20 nM) (coordinates 3121-3819). After 15 min at 37ºC,
712 complexes were fixed with glutaraldehyde (0.3%) for 15 min at the same temperature.
713 The distribution of the RNAP positions on both DNA fragments is shown. Bars on the
714 electron micrographs denote 500-bp.

715

716 FIG. 5. DNase I footprints of RNAP-DNA complexes. The 162-bp DNA fragment
717 (coordinates 3527-3688) was labelled at the 5´-end of either the coding (A) or the non-
718 coding (B) strand. Then, the labelled DNA (2.6 nM) was incubated (lane 2) or not (lane
719 1) with the RNAP (10 nM). Dideoxy-mediated chain-termination sequencing reactions
720 were run in the same gel (lanes A, C, G, T). The main sequence elements of the *Pmob1*
721 and *Pmob2* promoters and the RNAP-protected regions are indicated with brackets.
722 The indicated positions are relative to the transcription start point of the *Pmob2*
723 promoter.

724

725 FIG. 6. *In vitro* transcription assays. Linear DNA fragments of 362-bp (lane 1) and 699-
726 bp (lane 2) were used as templates. The coordinates of both templates are indicated
727 below the gel. The transcription start site of the *Pmob1* and *Pmob2* promoters is shown
728 in grey and black, respectively. Reactions were initiated by the addition of *E. coli* RNAP.
729 A denaturing gel (8 M urea, 6% polyacrylamide) was used for resolving transcripts.
730 Dideoxy-mediated chain-termination sequencing reactions using pUC19 plasmid DNA
731 (54) and the UC–50 primer (Table 1) were run in the same gel (lanes A, C, G, T). The
732 size of the DNA fragments that comigrate with the run-off transcripts is indicated on both
733 sides of the gel in nucleotides.

734

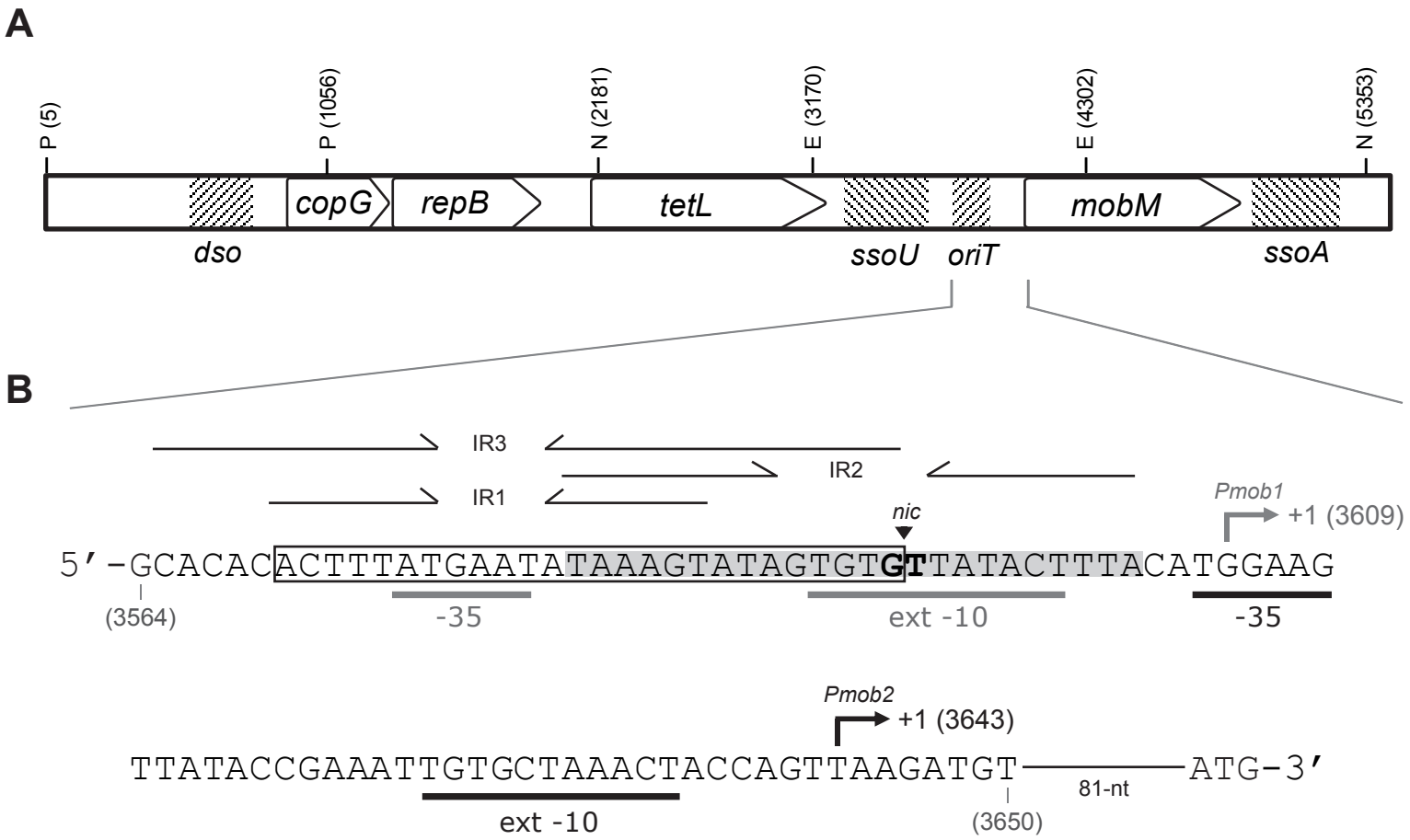
735 FIG. 7. *β*-galactosidase assays. Relevant features of the pORI-P and pIR2-P plasmids
736 are indicated. The *Pmob2* (P2) promoter is located just downstream of the right arm of
737 the IR2 inverted repeat (see Fig. 1B). Unlike pORI-P, pIR2-P lacks the left arm of the

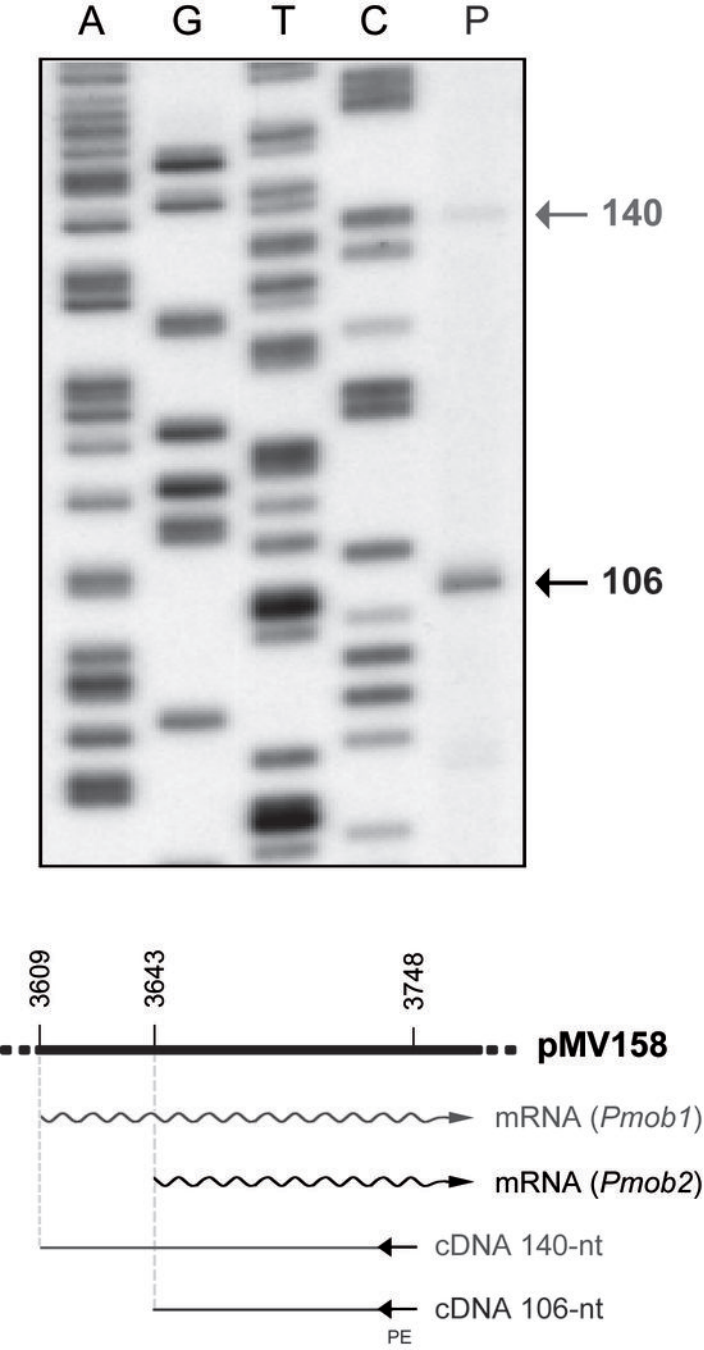
IR1/IR3 inverted repeats (see Fig. 1 B). Thus, pORI-P and pIR2-P differ in the nucleotide sequence just upstream of the –35 element of the *Pmob1* (P1) promoter, which was regenerated due to the cloning. Each plasmid was introduced into the *E. coli* JM109(DE3) strain harbouring either pLGM2 (IPTG inducible expression of the *mobM* gene) (18) or the pET5 vector (lacking the *mobM* gene). β- galactosidase activity (Miller units) was measured in bacteria growing in the absence (-) or in the presence (+) of IPTG. Each result represents the mean of three independent experiments (standard deviation is given).

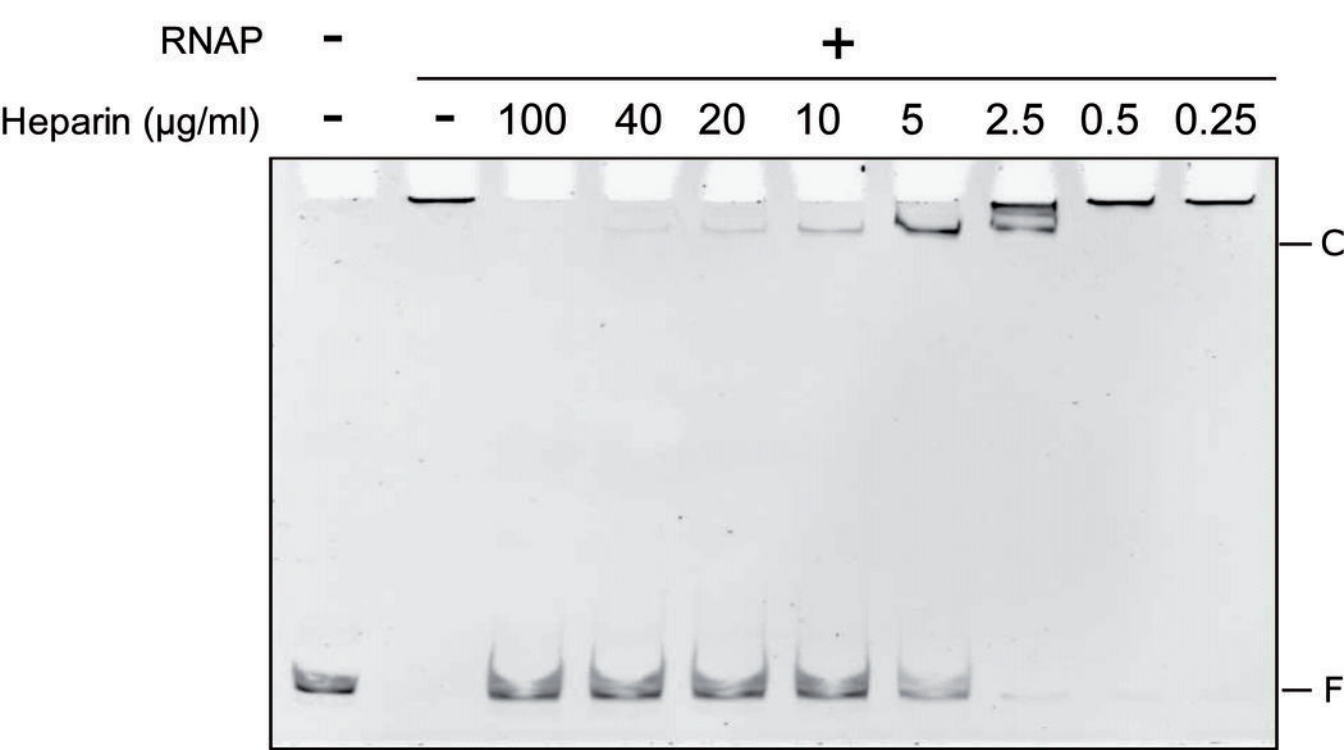
FIG. 8. Primer extension on total RNA isolated from JM109(DE3)/pLGM2/pORI-P cells. When the culture reached an OD_{600} of 0.4, five aliquots (10 ml) were withdrawn and incubated without IPTG for 30 min (lanes 1, 2, 3) or with IPTG for 30 min (lane 4) or 120 min (lane 5). The *lacZ*-PE primer (lanes 1, 3, 4, 5) and/or the *tetA*-PE primer (lanes 2, 3, 4, 5) were used. Primer extension products were analyzed by 8 M urea / 6% polyacrylamide gel electrophoresis. Dideoxy-mediated chain termination sequencing reactions using pORI-P DNA and the *lacZ*-PE oligonucleotide were run in the same gel. A longer exposition of the upper part of the gel (lanes 3, 4, 5) is shown. The size of the cDNA extension products is indicated on the right in nucleotides.

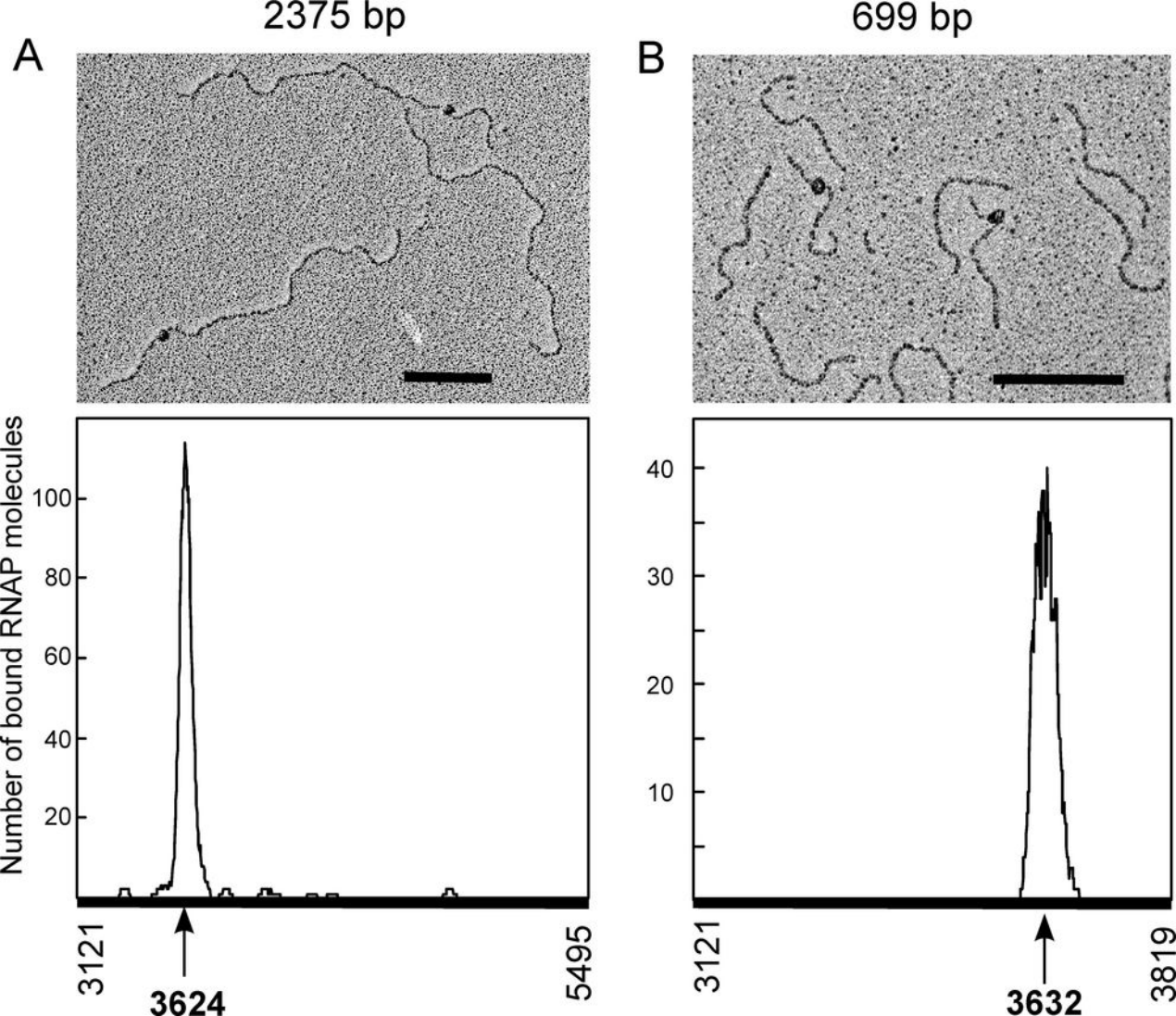
FIG. 9. (A) Nucleotide sequence of the pMV158 region that includes the *Pmob1* (in red) and *Pmob2* (in blue) promoters. Transcription start sites are indicated with arrows. The regions protected by the *E. coli* RNAP against DNase I digestion (this work) are indicated with brackets. The encircled sequence denotes the MobM binding site defined

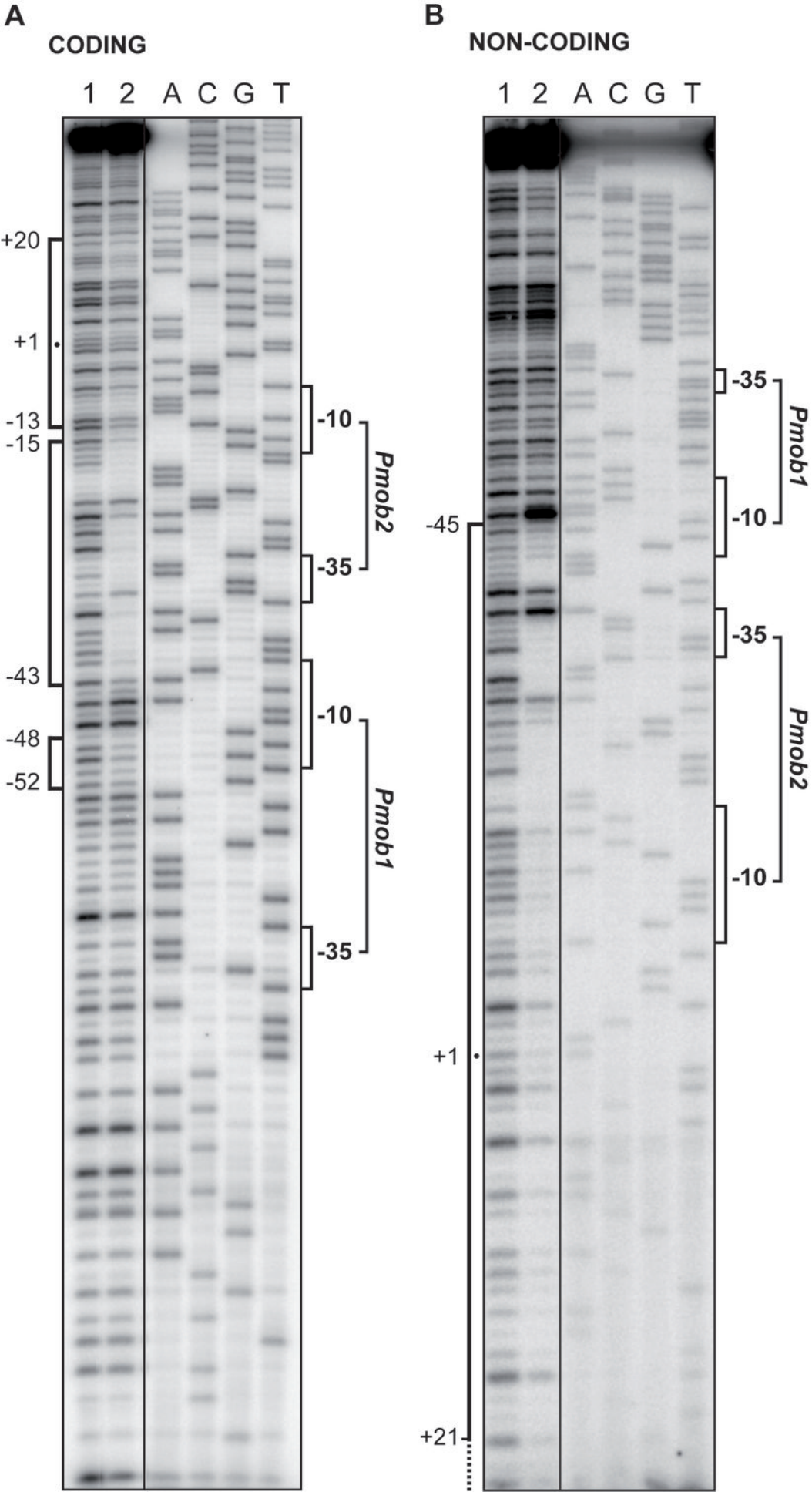
761 by DNase I footprinting assays, although the precise upstream boundary of protection
762 remained unclear (15). (B) Sequence alignment of the *mobM* promoter region from
763 streptococcal plasmids closely related to pMV158: pER13 and pSMQ172 from *S.*
764 *thermophilus*; pVA380-1 from *S. ferus*; and pRW35 from *S. pyogenes*. The nick site ("/")
765 and the main elements of the *Pmob1* and *Pmob2* promoters are indicated.
766

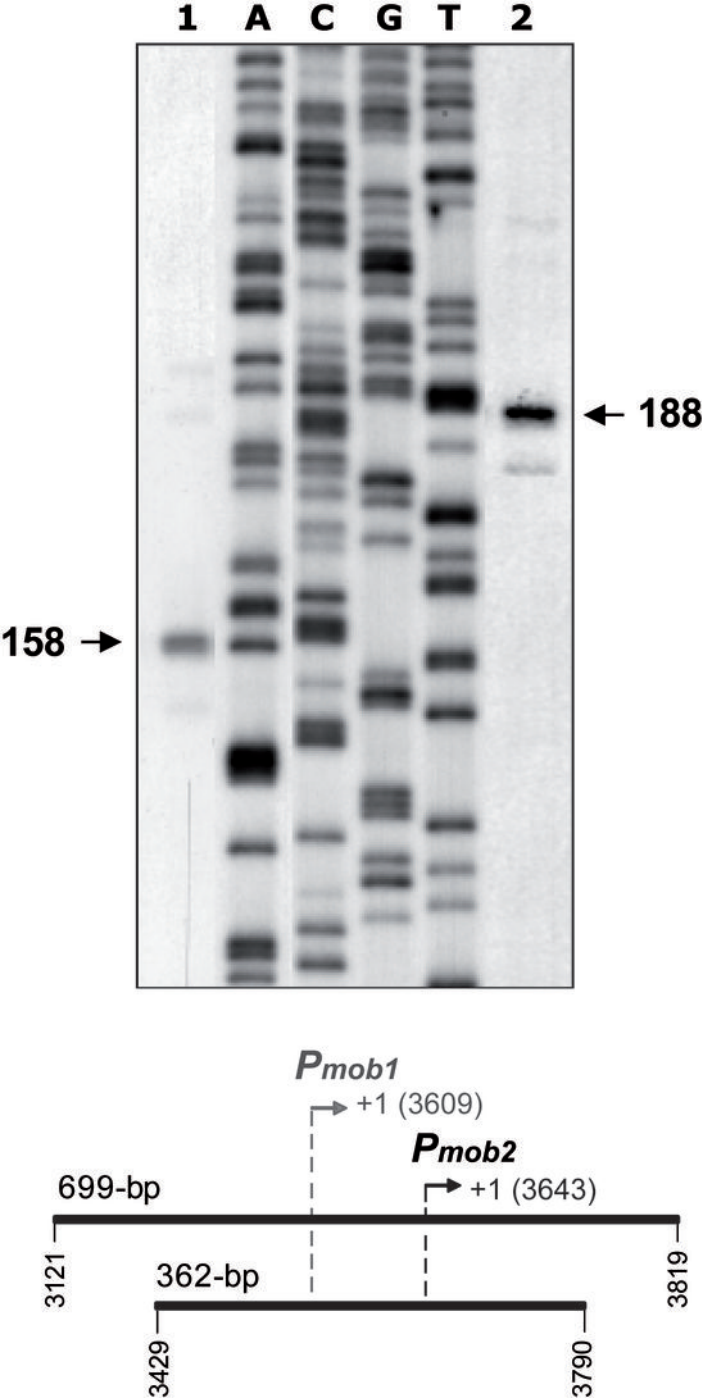

A
P (5)
P (1056)
N (2181)
E (3170)
E (4302)
N (5353)
copG
repB
tetL
mobM
dso
ssoU
oriT
ssoA
B
IR3
IR2
IR1
nic
Pmob1
+1 (3609)
5′-GCACAC ACTTTATGAATATAAAGTATAGTGTGTTATACTTTACATGGAAG
(3564)
-35
ext -10
-35
Pmob2
+1 (3643)
TTATACCGAAATTGTGCTAAACTACCAGTTAAGATGT
81-nt
ATG-3′
ext -10
(3650)
C
IR3
IR1
IR2

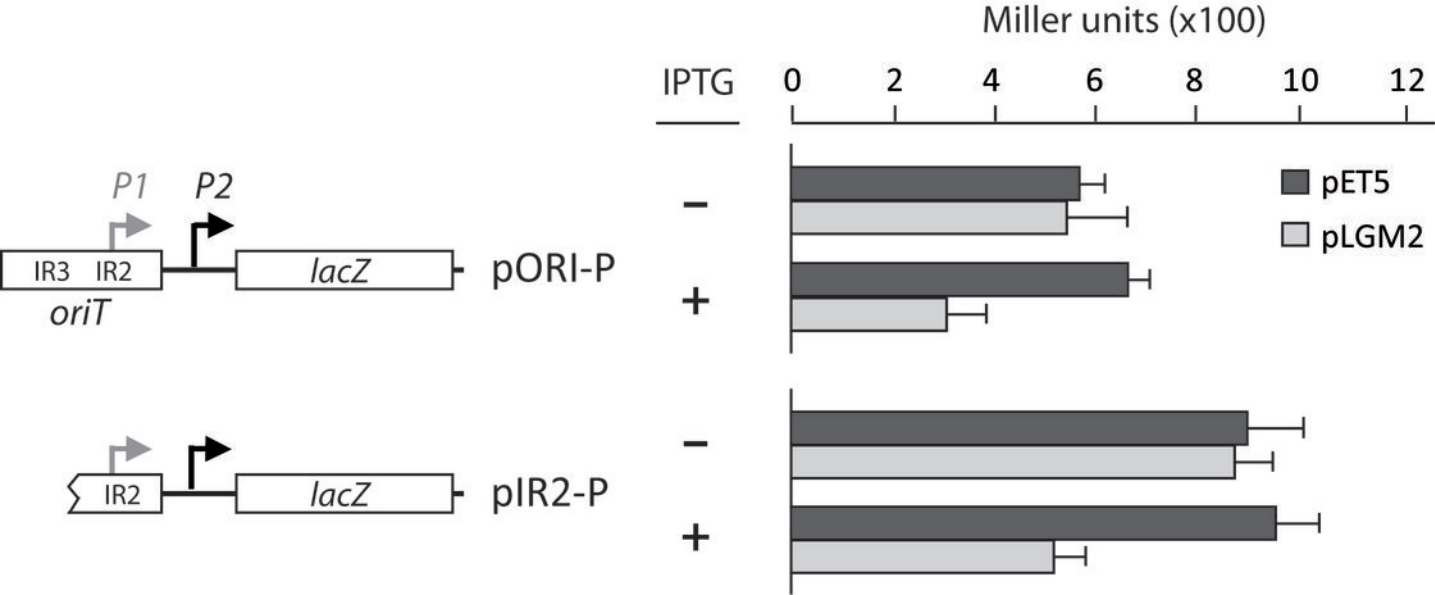

A
G
T
C
P
140
106
3609
3643
3748
pMV158
mRNA (Pmob1)
mRNA (Pmob2)
cDNA 140-nt
cDNA 106-nt
PE

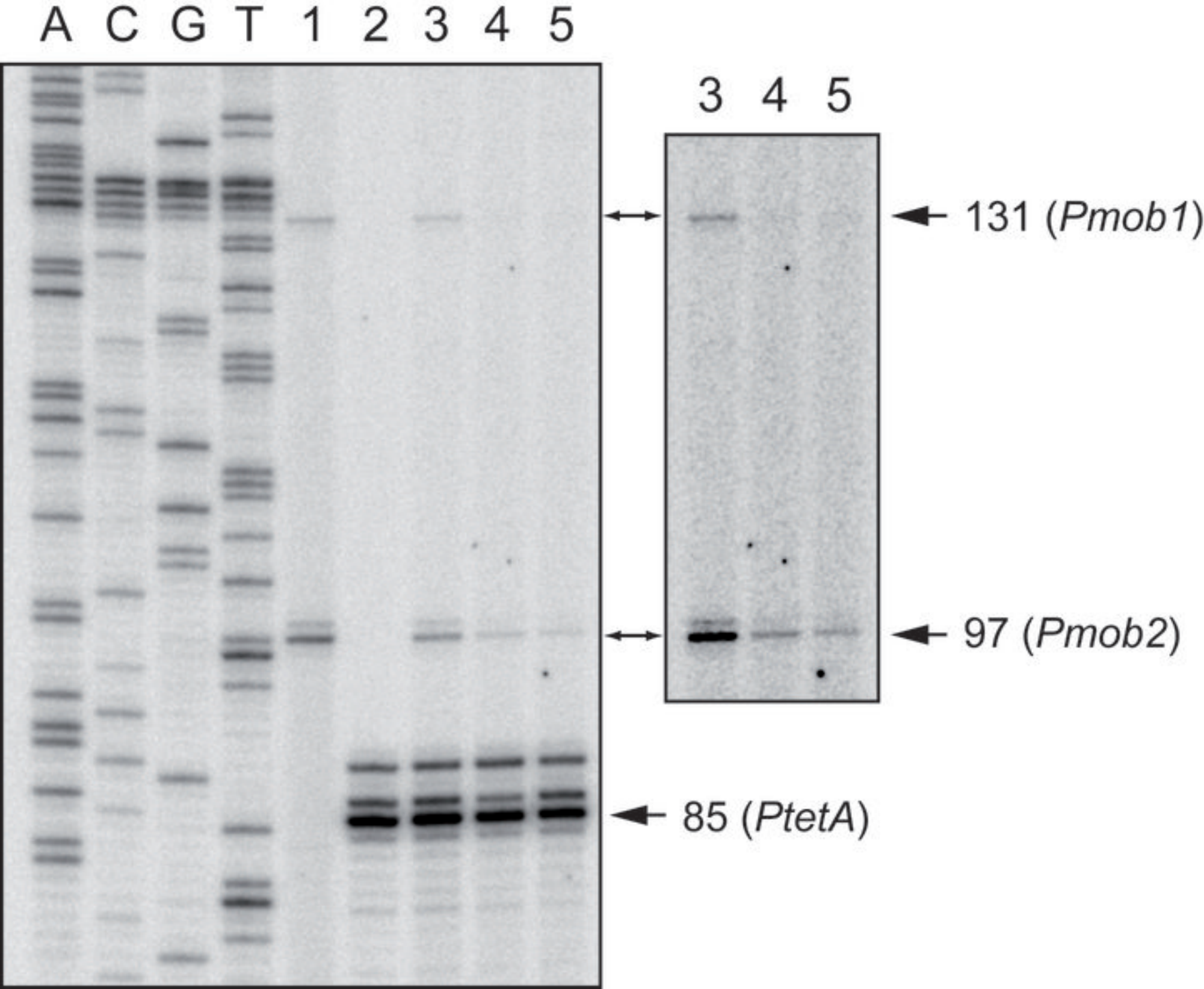

RNAP
-
+
Heparin (μg/ml)
-
-
100
40
20
10
5
2.5
0.5
0.25
C
F

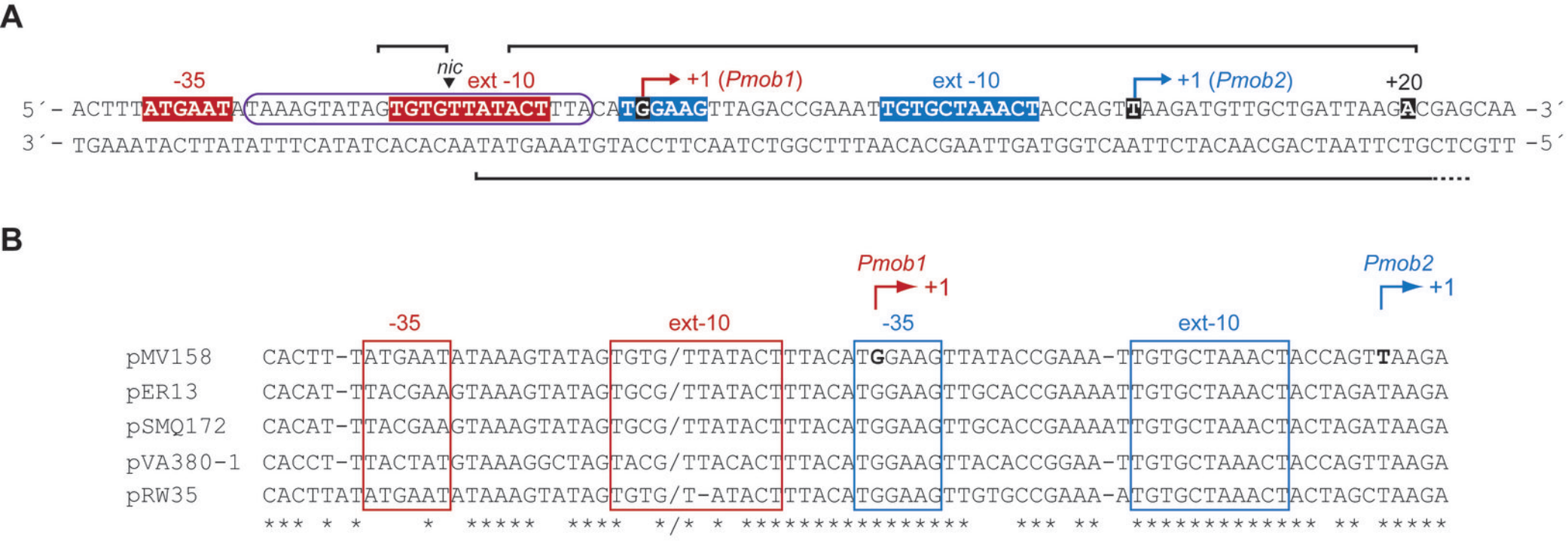

2375 bp
699 bp
A
B
Number of bound RNAP molecules
100
80
60
40
20
40
30
20
10
3121
3624
5495
3121
3632
3819


A
CODING
1 2 A C G T
+20
+1
-13
-15
-43
-48
-52
-10
-35
Pmob2
-10
-35
Pmob1
B
NON-CODING
1 2 A C G T
-45
+1
+21
-35
-10
Pmob1
-35
-10
Pmob2


1
A
C
G
T
2
188
158
Pmob1
+1 (3609)
Pmob2
+1 (3643)
699-bp
3121
3819
362-bp
3429
3790


Miller units (x100)
IPTG
0
2
4
6
8
10
12
P1
P2
IR3
IR2
lacZ
oriT
pORI-P
IR2
lacZ
pIR2-P
–
+
–
+
pET5
pLGM2


A C G T 1 2 3 4 5
3 4 5
131 (Pmob1)
97 (Pmob2)
85 (PtetA)


A
-35
nic
ext -10
+1 (Pmob1)
ext -10
+1 (Pmob2)
+20
5´- ACTTTATGAATATAAAGTATAGTGTGTTATACTTTACATGGAAGTTAGACCGAAATTGTGCTAAACTACCAGTTAAGATGTTGCTGATTAAGACGAGCAA -3´
3´- TGAAATACTTATATTTCATATCACACAATATGAAATGTACCTTCAATCTGGCTTTAACACGAATTGATGGTCAATTCTACAACGACTAATTCTGCTCGTT -5´
B
Pmob1
+1
Pmob2
+1
-35
ext-10
-35
ext-10
pMV158 CACTT-TATGAATATAAAGTATAGTGTG/TTATACTTTACATGGAAGTTATACCGAAA-TTGTGCTAAACTACCAGTTAAGA
pER13 CACAT-TTACGAAGTAAAGTATAGTGCG/TTATACTTTACATGGAAGTTGCACCGAAAATTGTGCTAAACTACTAGATAAGA
pSMQ172 CACAT-TTACGAAGTAAAGTATAGTGCG/TTATACTTTACATGGAAGTTGCACCGAAAATTGTGCTAAACTACTAGATAAGA
pVA380-1 CACCT-TTACTATGTAAAGGCTAGTACG/TTACACTTTACATGGAAGTTACACCGGAA-TTGTGCTAAACTACCAGTTAAGA
pRW35 CACTTATATGAATATAAAGTATAGTGTG/T-ATACTTTACATGGAAGTTGTGCCGAAA-ATGTGCTAAACTACTAGCTAAGA