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 \textit{mobM} gene, which encodes the MobM relaxase, and the \textit{cis}-acting origin of transfer (\textit{oriT}). MobM initiates transfer by cleavage of supercoiled pMV158 DNA at a specific dinucleotide within \textit{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 \textit{in vivo} and \textit{in vitro} approaches, we demonstrated that \textit{mobM} transcription in \textit{Escherichia coli} was mostly initiated from a promoter (\textit{Pmob2}) different from the one (\textit{Pmob1}) used in \textit{Lactococcus lactis}. Whereas promoter \textit{Pmob1} was embedded within the \textit{oriT} sequence, promoter \textit{Pmob2} was placed apart but adjacent to \textit{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 \textit{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
(α2ββ′ωσ). 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 σ70. 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 σ70 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
in the same orientation (Fig. 1A). In addition to the genes and loci required for its leading-strand rolling-circle replication (repB, copG, and the double-stranded origin, dso), two other cassettes are present in the plasmid, namely an antibiotic resistance marker (a tetL-type determinant) and a mobilization cassette. The latter includes the mobM gene, which encodes the MobM relaxase protein (494 residues), and the oriT. The MobM protein is the representative of the MOBv family of relaxases, constituted by more than 100 members so far (14). It has been shown that MobM cleaves supercoiled or ssDNA at a specific dinucleotide (coordinates 3595 and 3596; nic) within the oriT sequence (18) (Fig. 1B). The oriT (coordinates 3564-3606) is unique in the sense that it has three inverted repeats, IR (IR1, IR2, IR3; Fig. 1C) rather than the common single IR found in most of the studied plasmid oriTs (55). We have shown that IR1 and IR3 are preferentially recognized by protein MobM on ssDNA substrates, at least in vitro (28). The role of IR2, if any, is presently unknown but the conservation of the oriT sequence among the MOBv plasmid family suggests that anyone of the three IRs could be involved in the recognition of oriT by MobM at the initiation of relaxosome formation in the donor cell and/or at the termination reaction to close the transferred strand within the recipient cell. A DNase I footprinting assay showed that MobM binds, although very poorly, to linear double-stranded DNA fragments containing the oriT sequence (15); the protein is, however, unable to cleave linear dsDNA (18). Specifically, MobM protected a region between coordinates 3582 and 3605, which includes IR2, although the exact upstream border of the footprint remained unclear (Fig. 1B). Recently, using ssDNAs 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 \textit{in vitro} and \textit{in vivo}
transcriptional analysis of the pMV158-encoded MobM relaxase. We demonstrate that
the major promoter governing transcription of the \textit{mobM} gene in \textit{E. coli} is close to, but
different from, the one used in the G+ \textit{Lactococcus lactis} (12). Whereas the latter
promoter is located within the \textit{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 \textit{oriT}.

\textbf{MATERIALS AND METHODS}

\textbf{Bacterial strains, plasmids and oligonucleotides.} The \textit{E. coli} TOP10
(Invitrogen) and JM109 (54) strains were used as hosts for the plasmids used in this
work. \textit{E. coli} JM109(DE3) (Promega) was employed for the \(\beta\)-galactosidase assays; this
strain is a JM109-derivative in which the DE3 lysogen (46) provides the T7 RNA
polymerase gene fused to the \textit{lacUV5} promoter and the \textit{lacI}\(^q\) 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 \textit{E. coli} plasmids: i) vector
\textit{pET5} (Novagen); ii) \textit{pLG}M2, which is a \textit{pET5}-derivative that carries the \textit{mobM} gene
under control of the $\phi$10 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 37°C. 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$_4$)$_2$SO$_4$, 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 98°C for 30 sec, followed by 30 cycles that included the next steps: (i) denaturation at 98°C for 10 sec, (ii) annealing at around 55°C (depending on the primer Tm) for 20 sec, and (iii) extension at 72°C for 30-60 sec A final extension step was performed at 72°C 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 plR2-P were exponentially grown at 37°C 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 DraI restriction site. Digestion of the 437-bp DNA with DraI 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 MgCl2, 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 37°C, 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
oligonucleotides (Table 1), respectively, as primers. Reaction mixtures (10 µl) contained 40 mM Tris-HCl, pH 7.5, 150 mM KCl, 10 mM MgCl₂, 0.01% Triton X-100, 120 nM of E. coli RNAP (Epicentre) and 5-20 nM of the 2375- or 699-bp DNA fragments. After 15 min at 37ºC, RNAP-DNA complexes were fixed with 0.3% glutaraldehyde for 15 min at the same temperature. Then, reactions were diluted 10-fold in buffer GA (10 mM triethanolamine chloride, pH 7.5, 10 mM MgCl₂), adsorbed onto freshly cleaved mica, positively stained with 2% uranyl acetate, rotary shadowed with Pt/Ir, and covered with a carbon film as described (45). Micrographs of the carbon film replica were taken using a Philips CM100 (FEI Company, Hillsboro, Oregon) electron microscope at 100 kV on 35-mm film. The contour length of the RNAP-DNA complexes and the positions on the DNA fragments were measured on projections of 35-mm negatives using a digitizer (LM4, Brühl, Nüremberg, Germany).

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

**In vitro transcription analysis.** *In vitro* transcription reactions were carried out under multiple-round conditions. Reactions (50 µl) contained 45 mM Tris-HCl, pH 7.5,
150 mM KCl, 45 mM NaCl, 10 mM MgCl₂, 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-³²P]-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-³²P]-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 EcoRI and PstI. 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 EcoRI and PstI restriction sites, respectively. After EcoRI and PstI 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 EcoRI site. After EcoRI and
PstI 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
37°C. 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 37°C 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
in S. pneumoniae, interspecific transfer required an intact ssoU (29). Thus, this latter origin appeared to be required for the extraordinary promiscuity of pMV158 (8). Plasmid pMV158 has been mobilized between different G+ bacteria, like L. lactis (12) and Enterococcus faecalis (29, 35), using pAMβ1 as auxiliary plasmid. In the former bacterium, it was shown that transcription of the mobM gene started at coordinate 3609 of pMV158 (12). Thus, the lactococcal RNAP appeared to recognize a promoter sequence, named Pmob1 (Fig. 1B). This promoter has a near-consensus –10 hexamer (5´-TATAcT-3´), a consensus –10 extension (5´-TGTG-3´), and shows a 3/6 match at the –35 element (5´-aTGAat-3´). The extension of the -10 sequence, also termed the -15 motif (9), seems to be particularly important for promoters of G+ bacteria (2, 38). Moreover, the –10 and –35 elements of promoter Pmob1 are separated by 16 nucleotides, and the –10 hexamer is located just downstream of the dinucleotide cleaved by MobM (nic; Fig. 1B) (18), placing it within the minimal oriT sequence (28).

In addition to the G+ hosts, pMV158 was shown to replicate in the G- bacterium E. coli (7). Mating experiments showed that the IncPα RP4 or the IncW R388 plasmids were able to mobilize pMV158 between E. coli strains (11). In the case of RP4, it was shown that transfer of pMV158 required the products of genes traG (coupling protein; 1) and traF (mating pair formation; 27). It was thus interesting to explore in some depth the transcriptional features of the pMV158 mobM gene in the G- host. To identify the transcription initiation site of the mobM gene in E. coli, we carried out primer extension experiments using the mobM-PE oligonucleotide as primer (coordinates 3748-3725; Table 1) and total RNA isolated from E. coli cells harbouring plasmid pMV158. As shown in Figure 2, two cDNA extension products of 106 (major product)
and 140 (minor product) nucleotides were detected. The 140-nt cDNA revealed a transcription initiation event at coordinate 3609 from the \textit{Pmob1} promoter, whereas the appearance of the 106-nt cDNA might correspond to: i) a degradation product from the larger 140-nt cDNA; ii) a premature stop during retrotranscription; or iii) a transcription initiation event at coordinate 3643 from a different promoter. Sequence analysis of the region located just upstream of coordinate 3643 using the BPROM (http://linux1.softberry.com/berry.phtml) prediction program supported the latter hypothesis. It revealed the existence of an additional promoter sequence, herein termed \textit{Pmob2} (see Fig. 1B). The –10 element (5´-TAaAcT-3´) of this putative promoter is located at the proper distance of 7 nucleotides from the transcription start site (coordinate 3643). Moreover, the \textit{Pmob2} promoter has a consensus –10 extension (5´-TGTG-3´), and the –10 and –35 (5´-TgGAag-3´) sequence elements are separated by 17 nucleotides, which is the optimum spacer length for \textit{E. coli} (20). Therefore, promoter \textit{Pmob2} is placed just downstream of the \textit{oriT}, and it is close to, but not overlapping with promoter \textit{Pmob1} (Fig. 1B).

\textit{E. coli} RNAP binds to the \textit{Pmob2} promoter \textit{in vitro}. The interaction of the \textit{E. coli} RNAP with the promoter region of the \textit{mobM} gene was further studied by several \textit{in vitro} methods. In a first approach, we performed EMSA experiments using the \textit{E. coli} RNAP holoenzyme (σ\textsuperscript{70} factor) and a 362-bp linear DNA fragment (coordinates 3429-3790) under conditions that favoured generation of open complexes (37ºC and in the absence of NTP substrates). The DNA fragment contains the \textit{Pmob1} and \textit{Pmob2} promoter sequences (see Fig. 1B). In these assays, heparin (0.25-100 µg/ml) was used
as competitor and the molar ratio of RNAP to DNA was 24:1 (Fig. 3). Without heparin, aggregates of DNA-bound RNAP molecules, which did not enter the native gel, were observed. After addition of heparin (5 µg/ml), RNAP-DNA complexes with much slower electrophoretic mobility than the free DNA were detected, indicating that non-specifically bound RNAP molecules were displaced by such a concentration of competitor. RNAP-DNA interactions were disrupted at heparin concentrations above 10 µg/ml (Fig. 3). Hence, the *E. coli* RNAP was able to interact with the promoter region of the *mobM* gene forming complexes that were unstable to heparin challenge. It is worth pointing out that RNAP does not generate stable complexes resistant to competitors in all promoters (for an in-depth discussion, see ref 36).

To verify further the specificity of the complexes formed by the *E. coli* RNAP on the *mobM* promoter region, we carried out electron microscopy assays. In a first experiment, the *E. coli* RNAP was incubated with a 2375-bp linear pMV158 DNA fragment (coordinates 3121-5495). This fragment contains the *ssoU* and *ssoA* single-strand origins, the *oriT* sequence and the *mobM* gene (see Fig.1A). Then, RNAP-DNA complexes were fixed with glutaraldehyde, prepared for electron microscopy and visualized as described in Materials and Methods. Electron micrographs of RNAP-DNA complexes are shown in Figure 4. To determine the RNAP binding site, the contour lengths of the DNA regions between complexes and DNA ends were measured and the position of the RNAP determined. Figure 4A shows the distribution of the RNAP positions on the 2375-bp fragment. Of 174 complexes examined, the majority (73%) showed a RNAP bound to one DNA region located at a maximum distance of 503-bp 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
grown in the presence of IPTG. Measurement of lacZ expression under these conditions showed that β-galactosidase activity decreased nearly two-fold as compared to the activity detected in cells grown in the absence of IPTG (Fig. 7). On the contrary, no changes in lacZ expression were detected in JM109(DE3) cells harbouring pET5, which lacks the mobM gene, and pORI-P (control strain). The MobM-mediated repression was moderate albeit statistically significant. Similar repression levels were observed for the pMV158-repB gene using a construct similar to the one employed here (6). Whether these effects are due to the employment of the T7 Φ10 promoter to direct synthesis of the protein or to an intrinsic property of the pneumococcal sequences is not known at present. We can conclude that MobM reduced the activity of the Pmob1 and/or Pmob2 promoters in vivo.

The above interpretation was further confirmed by primer extension experiments using total RNA isolated from JM109(DE3) cells carrying both plasmids, pLGM2 and pORI-P. As primers, a mixture of the 5’-labelled lacZ-PE and tetA-PE oligonucleotides (Table 1) was used. They anneal to the lacZ and tetA (tetracycline resistance) transcripts of the pORI-P plasmid, respectively. With the primer lacZ-PE and in the absence of IPTG (Fig. 8, lane 3), a minor cDNA product of 131-nt (promoter Pmob1) and a major cDNA product of 97-nt (promoter Pmob2) were detected. The amount of both products decreased when bacteria were grown in the presence of IPTG for 30 min (lane 4) or 120 min (lane 5). With the primer tetA-PE, used as internal control, a major cDNA product of 85-nt (promoter PtetA) was detected in the absence of IPTG (lane 3). However, the amount of such a product did not change in the presence of IPTG (lanes 4 and 5). We can conclude that MobM was able to repress in vivo not only the minor
transcription initiated from promoter $P_{mob1}$ but also the major transcription initiated from promoter $P_{mob2}$.

We next analyzed whether the IR1/IR3 inverted repeats of the pMV158-oriT were required for such MobM-mediated repression. To this end, we constructed plasmid pIR2-P, which is a pMP220-derivative that carries the 3583-3676 region of pMV158 inserted upstream of the promoterless lacZ gene (Fig. 7). The –35 hexamer of the $P_{mob1}$ promoter (3575-3580 coordinates) was regenerated as a result of the cloning. Thus, plasmid pIR2-P carries an intact IR2 but lacks the left arm of IR1/IR3 (Fig. 1B). Expression of lacZ in JM109(DE3) cells harbouring both plasmids, pLGM2 and pIR2-P, decreased nearly two-fold when they were grown in the presence of IPTG (synthesis of MobM; Fig. 7). No changes in lacZ expression were observed in the control strain (JM109(DE3) carrying both pET5 and pIR2-P). MobM-mediated repression was further confirmed by primer extension on total RNA using a mixture of the 5'-labelled oligonucleotides lacZ-PE and tetA-PE as primers (not shown).

Taken all the above results together, we can conclude that MobM is able to repress transcription from the $P_{mob2}$ promoter in E. coli, and thus regulate its own synthesis. This repression does not require the left arm of IR1/IR3, although we have to consider that the right arm of IR1/IR3 and the left arm of IR2 overlap (Fig. 1B).

**DISCUSSION**

The promiscuous plasmid pMV158 represents one of the simplest systems for an efficient DNA transfer among different bacterial species, G+ and G-. Its MobM relaxase is the representative of the MOB$_V$ family of relaxases, constituted by more than 100
members (14), representing a wealth of genetic information that merits to be explored in depth. The results presented here demonstrate that the pMV158 mobM gene is transcribed by the E. coli RNAP from a promoter (Pmob2) that is different from the one (Pmob1) previously shown to be used in the G+ bacterium L. lactis (12). The DNA region protected by RNAP extends up to the +20 position relative to the Pmob2 transcription start site (Figs. 5 and 9A). This is indicative of the formation of an open complex, as should be expected under the experimental conditions employed. Inspection of the DNA sequence around the pMV158-oriT showed a high degree of conservation among many different plasmids from G+ bacteria (18). As the number of sequenced replicons increased, we have found now that a few streptococcal plasmids maintain the structure of the two Pmob1 and Pmob2 promoters (Fig. 9B). This is the case of plasmids pER13 and pSMQ172 from S. thermophilus (43, 48), pVA380-1 from S. ferus (26), and pRW35 from S. pyogenes (52). Cross-recognition of the pVA380-1_oriT by the pMV158_MobM was previously demonstrated (15). The genetic structure of the oriTs and promoter(s) in the mobilization region of these plasmids suggests that it is not per chance that pMV158 exhibit two promoters differentially used in G+ or G- bacteria, and allows us to postulate that they could also be transferred to E. coli.

Typically for a small mobilizable plasmid, pMV158 encodes only those conjugative functions required for DNA processing: the cis-acting oriT and the DNA relaxase MobM. Our in vivo transcriptional studies showed that: i) synthesis of the pMV158-relaxase MobM is auto-regulated, and regulation of the mobM gene appears to be regulated by MobM solely, since we have not found any indication of the presence of a regulatory 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 MOBv 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.
ACKNOWLEDGEMENTS

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^aBase changes that generate restriction sites (in bold) are underlined

^bCoordinates are given with respect to pMV158 sequence (Acc. no. NC_010096)
**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: *PstI*, N: *NcoI*, E: *EcoRI*. 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.
FIG. 2. Primer extension on total RNA isolated from *E. coli* cells carrying plasmid pMV158. The primer (*mobM*-PE; Table 1) annealed to the transcripts in the region corresponding to coordinates 3725-3748 of pMV158. The size expected for the cDNAs if transcription initiation occurs at coordinates 3609 (*Pmob1*) or 3643 (*Pmob2*) is indicated below the gel. Primer extension products were resolved on denaturing gels (7 M urea, 8% polyacrylamide). As DNA size markers, dideoxy-mediated chain-termination sequencing reactions using pMV158 DNA and the *mobM*-PE primer were run in the same gel (lanes A, G, T, C). The size of the cDNA extension products (lane P) is indicated on the right in nucleotides.

FIG. 3. EMSA analysis of RNAP-DNA complexes. The *E. coli* RNAP (120 nM) was incubated with the 362-bp DNA (5 nM) (coordinates 3429-3790) at 37°C for 30 min. Then, heparin was added at the indicated concentrations. After 5 min, the reaction mixtures were loaded onto a native gel (5% polyacrylamide). The gel was stained with ethidium bromide. Bands corresponding to free DNA (F) and to specific RNAP-DNA complexes (C) are indicated.

FIG. 4. Electron micrographs of RNAP-DNA complexes. The *E. coli* RNAP (120 nM) was incubated with (A) the 2375-pb fragment (5 nM) (coordinates 3121-5495) or with (B) the 699-bp fragment (20 nM) (coordinates 3121-3819). After 15 min at 37°C, complexes were fixed with glutaraldehyde (0.3%) for 15 min at the same temperature. The distribution of the RNAP positions on both DNA fragments is shown. Bars on the electron micrographs denote 500-bp.
FIG. 5. DNase I footprints of RNAP-DNA complexes. The 162-bp DNA fragment (coordinates 3527-3688) was labelled at the 5’-end of either the coding (A) or the non-coding (B) strand. Then, the labelled DNA (2.6 nM) was incubated (lane 2) or not (lane 1) with the RNAP (10 nM). Dideoxy-mediated chain-termination sequencing reactions were run in the same gel (lanes A, C, G, T). The main sequence elements of the \textit{Pmob1} and \textit{Pmob2} promoters and the RNAP-protected regions are indicated with brackets. The indicated positions are relative to the transcription start point of the \textit{Pmob2} promoter.

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

FIG. 7. \(\beta\)-galactosidase assays. Relevant features of the pORI-P and pIR2-P plasmids are indicated. The \textit{Pmob2} (P2) promoter is located just downstream of the right arm of 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 OD₆₀₀ 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
by DNase I footprinting assays, although the precise upstream boundary of protection remained unclear (15). (B) Sequence alignment of the \textit{mobM} promoter region from streptococcal plasmids closely related to pMV158: pER13 and pSMQ172 from \textit{S. thermophilus}; pVA380-1 from \textit{S. ferus}; and pRW35 from \textit{S. pyogenes}. The nick site ("/") and the main elements of the \textit{Pmob1} and \textit{Pmob2} promoters are indicated.
A

B

C

A

B

C