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Modulation of pPS10 Host Range by Plasmid-Encoded RepA Initiator Protein

***Running title*: Modulation of pPS10 host range by RepA.**

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ABSTRACT

We report here the isolation and analysis of novel *repA* host range mutants of pPS10, a plasmid originally found in *Pseudomonas savastanoi*. Upon hydroxylamine treatment, five plasmid mutants were selected for their establishment in *Escherichia coli* at 37ºC, temperature conditions in which the wild-type form cannot be established. The mutations were located in different functional regions of the plasmid RepA initiation protein and the mutants differ in their stable maintenance, copy number and ability to interact with sequences of the basic replicon. Four of them have broadened their host range and one of them, unable to replicate in *Pseudomonas,* has therefore changed its host range. Moreover, the mutants also increase their replication efficiency in strains other than *E. coli* such as *Pseudomonas putida* and *Alcaligenes faecalis*. None of these mutations changed drastically the structure or thermal stability of the wild-type RepA protein, but in all cases an enhanced interaction with host-encoded DnaA protein was detected by gel-filtration chromatography. The effects of the mutations on the functionality of RepA protein are discussed in the framework of a three-dimensional model of the protein. We propose possible explanations for the host range effect of the different *repA* mutants, including the improvement of limiting interactions of RepA with specific host replication factors such as DnaA.

INTRODUCTION

Although many plasmids are only able to replicate in their original host and related species, some of them have an extended host range and can be established in several different bacteria. The study of plasmid host range determinants has been addressed by focusing on broad host range replicons (see 3 and 44 for reviews), mainly RSF1010 and RK2. Plasmid RSF1010 is a broad host range replicon that codes for its own replication initiator proteins allowing a host-independent initiation replication mechanism (6,39). The broad host range replicon RK2 uses a different strategy: it synthesizes two variants of the TrfA initiation protein at the *oriV* region, and the initiation of replication needs the participation of one or the other of these TrfA proteins, depending on the host (10,11,41). Moreover, the number, sequence and spacing of its several DnaA boxes also influence the replication in RK2 (7,8,40). Genetic analysis suggests that interactions between the C-terminal region of TrfA and host replication factor(s) can specifically affect plasmid host range (2). Broad host range plasmids replicating by rolling-circle mechanism have also been identified, a classic example of which is pLS1 (3, 4).

Narrow host range plasmids can also be used to identify host range determinants. A genetic approach consists in the isolation of plasmid mutants that allow their establishment in a new host. This approach has been followed with pPS10, a plasmid that can be efficiently established in *Pseudomonas* at 30ºC and 37ºC but not in *Escherichia coli* (33). Transformation of *E. coli* with pPS10 at 37ºC does not yield visible colonies on solid medium, whereas at 30ºC only minute colonies that are not viable in subsequent cultures are seen (13). These characteristics make pPS10 a "borderline" plasmid in terms of efficient replication in *E. coli* and opens up the possibility of transfering it from *Pseudomonas savastanoi,* a bacteria pathogenic to the olive tree, to a human enteric bacteria such as *E. coli*.

The minimal replicon of pPS10 contains the origin of replication (*oriV*), including four 22-bp iterons, followed by two 8 bp inverse repeats overlapping the -35 and -10 sequences of the *repA* promoter, and the *repA* gene coding for the initiation replication protein RepA, of 230 amino acids (see scheme in Ref. 30). Analysis of the primary structure of this protein, together with other biochemical and biophysical studies (20) indicates that RepA is built up from a leucine-zipper motif, involved in dimerization (16,22), followed by two winged-helix domains (amino acids 42-113 and 135-221) connected by a flexible linker. The second domain contains a putative helix-turn-helix (HTH) motif for DNA binding (18). The RepA protein of pPS10 interacts as a monomer with the iterons of *oriV* and, jointly with the DnaA protein of the host, promotes the initiation of pPS10 replication (13,16,21,34). On the other hand, the RepA protein, as a dimer, binds to the inverted repeats and represses its own transcription (16,17)

In a previous work (13) we reported the isolation of a pPS10 mutant, pMM141, that was readily established as an autonomous replicon in *E. coli* and in the original host. The mutant contains a single amino acid change (A31V) in the leucine-zipper region of the RepA (13,21). The mutation does not alter the dimerization state of RepA nor its capability to interact with the iterons of the origin or the *repA* operator (16) suggesting that it could have affected interactions with critical host replication factor(s). In this sense, we have recently reported one DnaA mutant that allows the efficient establishment of wild-type pPS10 in *E. coli* (30). The genetic study of this mutant suggests that concerted interactions between RepA and DnaA play a relevant role in this establishment.

In this work we report the isolation and characterization of new host range mutations in RepA obtained by *in vitro* mutagenesis of the pPS10 replicon. The study of the pPS10 derivatives

show a variety of phenotypic effects and points to the improvement of interactions between RepA and host factors such as DnaA as a key determinant of its host range.

MATERIALS AND METHODS.

Bacterial strains and plasmids. *E. coli* K-12 strains used were: CC118 (24), C600EL (*hsdR*) (kindly supplied by K. Nordström) and BT1000 (*pol*A1) (46). Other strains: *Pseudomonas aeruginosa* PAO1024 (r^-m^+) (33); *Pseudomonas putida* KT2440 (r^-m^+) (1,32); *Agrobacterium tumefaciens* DSM30510 (5), and *Alcaligenes faecalis* (German Research Center for Biotechnology).

Plasmids used were: pRG9B, a pPS10-pBR322 derived shuttle vector (13); pMAL-c2 (New England Biolabs), an expression vector to fuse proteins to maltose binding protein (MBP); pUCProm and pUCIt are pUC18Not derivatives that include the *repA* operator and the *oriV* iteron sequences of pPS10 respectively (18).

Media and growth conditions. Cultures were usually grown in LB medium according to Sambrook *et al.* (38) at 30ºC or 37ºC (*E. coli*) or 30ºC (rest of strains). When required, media were supplemented with antibiotics to select for plasmids confering resistance to them: ampicillin (Ap) (100 μg ml^{-1}), and kanamycin (Km) (50 μg ml^{-1} for *E. coli*, *A. tumefaciens* and *A. faecalis*; 75 μg ml^{-1} for *Pseudomonas*). *A. tumefaciens* was grown in Ty medium, according to Díaz *et al.* (5) supplemented with rifampicin 50 μg ml^{-1}. Growth of cells for the MBPRepA expression was carried out in 2xYT medium (38) supplemented with glucose 0.2%.

General methods. Plasmid DNA was purified using the QIAGEN miniprep kit. Restriction enzymes were from New England BioLabs, Roche Diagnostics and Amersham Biosciences. Cloning procedures, gel electophoresis of DNA and proteins and radioactive labeling of DNA were performed as described by Sambrook *et al.* (38). Primer oligonucleotides were synthesized in a Beckman DNA synthesizer 1000M, desalted, and used without futher purification. DNA sequencing

was carried out in a Perkin-Elmer ABI-Prism 377 DNA sequencer using fluorescent chain terminators.

Bacteria transformation and electroporation. *E. coli* transformation was carried out as described by Lederberg and Cohen (29). In the case of *Pseudomonas*, we used the method described by Bagdasarian and Timmis (1). Electroporation competent cells of *E. coli*, *A. faecalis*, and *A. tumefaciens* were prepared according to Dower *et al.* (9). Competent cells, with the exception of *A. tumefaciens*, were kept overnight at 4ºC in order to increase its competence. Electroporation competent cells of *Pseudomonas* were prepared as described by Smith and Iglewski (42). In all the cases one pulse of 25μF, 200Ω, 2.5KV was delivered with the Gene-Pulser (Bio-Rad). Immediately after electroporation, cells were suspended in 2 ml SOC medium (38) and incubated for one hour at 30ºC. Cells were then plated at appropriate dilutions, and the number of transformants, grown at 30ºC, calculated with respect to viable cells.

DNA mutagenesis. Hydroxylamine mutagenesis was carried out by the protocol of Humphreys *et al* (25). Twenty μl of plasmid DNA (0.2 mg ml^{-1}) were mixed with 100 μl of 0.1 M sodium phosphate pH 6.0 containing 1 mM EDTA and 80 μl of 1 M hydroxylamine at pH 6.0 (Sigma). Upon incubation at 75ºC for 30 minutes, the sample was extensively dialyzed against TE buffer (Tris 10mM, EDTA 1 mM, pH 8.0).

Plasmid copy number. Cell lysates were obtained from exponential phase cultures as described by Projan *et al.* (36). DNA was transferred onto nitrocellulose membranes according to Southern (43) using the protocol described in Sambrook *et al.* (38). The 964-bp *Eco*RI fragment from plasmid pRG9B was labelled with (α-^{32}P)-dCTP and used as the hybridization probe. Total amount of

plasmid and chromosome DNA were evaluated by densitometry using the Molecular Dynamics densitometer fitted with the analysis program Image Quant.

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Plasmid stability. In order to check the segregational stability of pPS10 derivatives, a culture was grown at 30ºC in Km-containing LB medium to an optical density at 600 nm of 0.4. Then, 100 µl of a 10^{-5} dilution were plated on medium without antibiotic. After overnight incubation at 30ºC, one hundred independent colonies were patched on LB and LB + Km plates to determine the number of cells that retained the plasmid after the incubation. Colonies growing on non-selective medium were pooled together, and individual colonies were again isolated on LB plates. One hundred of these colonies were patched on LB and LB + Km plates to determine the percentage of plasmid containing cells after a second propagation on non selective medium at 30ºC. The procedure was reiterated a third and fourth time when needed. These values correspond to the stability of the plasmid after approximately 30, 60, 90 and 120 generations without selection, as thirty generations is roughly the time needed to form a clearly visible colony (13).

Construction of pMalRepA plasmids. The ATG-*Eco*RI fragment of pSBM plasmids (nucleotides 577-1500 according to Nieto *et al.*, 34) was amplified by PCR using *Pfu* polymerase (Stratagene) and the oligonucleotides 5'-ATGGTCGAGAACAAAGTCACG-3' (ATG initiation site underlined) and 5'-CCGGAATTCTCTGTGCCATAGC-3' (*Eco*RI site underlined). The former oligonucleotide was phosphorilated by T4 polynucleotide kinase so that the PCR product, once digested with *Eco*RI, was cloned into the *Xmn*I-*Eco*RI sites of pMAL-c2. Integrity of the constructions was verified by DNA sequencing.

Purification of MBPRepA proteins. *E. coli* CC118 cells harbouring the pMalRepA plasmids were grown to mid-exponential phase. MBPRepA expression was then induced with 10 μM isopropyl-β-D-galactopyranoside (IPTG), and incubation proceeded overnight. Cell extracts were applied to an amylose column equilibrated in a buffer containing 10 mM Tris, pH 7.4, 500 mM NaCl, 1 mM EDTA and 1 mM dithiotreitol (DTT), and eluted with the same buffer plus 20 mM maltose. Purity of the samples was checked by SDS-PAGE electrophoresis (28). Protein concentration was evaluated by absorption spectroscopy using an extinction coefficient ε_{280} = 78678 M^{-1} cm^{-1}, calculated with the parameters of Fasman (12) making use of the known aromatic amino acid content of the fusion protein.

Circular dichroism spectroscopy (CD). Circular dichroism experiments were carried out in a Jasco J-720 spectropolarimeter fitted with a thermostated cell holder and interfaced with a Neslab RTE-110 water bath. Isothermal wavelength spectra were acquired at a scan speed of 20 nm min^{-1} with a response time of 2 seconds and averaged over at least 6 scans at 5ºC. The protein was previously dialyzed against a buffer containing 20 mM sodium phosphate, pH 6.5, 1 mM dithiotreitol, 1 mM EDTA and 500 mM NaCl. Protein concentration was 2.5 μM and the cuvette pathlength was 1 or 2 mm. Thermal denaturation experiments were performed using a heating rate of 50ºC h^{-1} and a response time of 2 seconds. Scans were collected in 1 mm cuvettes and using a protein concentration of 2.5 μM. To avoid evaporation, samples were overlaid with mineral oil (Sigma). Data were analyzed according to a sequential three-state denaturation mechanism involving sequential unfolding of MBP and RepA moieties.

Electrophoretic mobility shift assays (EMSA). EMSA experiments were carried out based on the procedure by Fried and Crothers (14). For the operator binding assays, the radioactive probe used was the 90-bp *Not*I fragment of pUCProm, whereas for the iterons binding assays, the probe was the

440-bp *Not*I fragment of pUCIt (18). Both fragments were labelled with (α-^{32}P)-dCTP and 1 unit of Klenow enzyme. Probe quantitation was carried out by agarose gel electrophoresis, measuring the fluorescence emited by the sample stained with ethidium bromide, using the BioRad Gel Doc system and the Molecular Analyst program. The RepA-DNA binding reaction took place in a final volume of 20 µl containing 25 mM Hepes-KOH buffer, pH 8.0, plus 1 mM EDTA, 4% saccharose, 50 mg ml^{-1} bovine serum albumin, 4 mM DTT, 50 mM KCl, 100 ng phenolized and sonicated calf thymus DNA, either 24.5 fmol of iteron probe or 8.6 fmol of promoter probe and increasing concentrations of MBPRepA protein. Reactions were assembled on ice and then transferred to room temperature for 30 minutes. Then 5 µl of loading buffer were added to the samples and loaded into a pre-run non-denaturing 5% polyacrylamide gel (29:1, in TBE). Electrophoresis was carried out in 0.5x-TBE at 200 V keeping the temperature at 4ºC for 7 hours (iterons binding assays) or 4 hours (operator binding assays). Finally, gels were dried at 80ºC and autoradiographed.

Modelling of RepA structure. RepA structure was modelled using the Swiss-Model threading utility (http://www.expasy.ch/spdbv/) (23) using the published structure of RepE as a template (27). The rough model was subsequently refined by steepest descent energy minimization.

Gel filtration analysis of the interaction between Escherichia coli DnaA and MBPRepA variants. Seventy five µl of a 25-fold concentrated extract of a stationary phase *E. coli* LE392 [pUC392] culture (O.D.$_{600}$ = 3.5) (30) grown at 30 ºC was mixed with either 25 µl of an extract of a 2.5-fold concentrated extract of a stationary phase culture of *E. coli* CC118 strain harboring the different pMALRepA constructs grown at 37 ºC to an O.D.$_{600}$ of 2.2, or with 25 µl of sodium phosphate buffer 20mM pH 7.0 plus 150 mM NaCl (control experiment). The mixtures were kept on ice for 30 min and subsequently loaded onto a Sephadex G-75 column (26 x 0.9 cm) equilibrated in sodium phosphate buffer 20mM pH 7.0 plus 150 mM NaCl and run with the same buffer at a

flow rate of 1 ml min^{-1} and at 20ºC. Exclusion (6.0 ml) and total (21.0 ml) volumes were determined with dextran blue and potassium dichromate, respectively. 200 µl fractions were collected, and 100 µl of each fraction was subjected to a dot-blot analysis using the ECL kit (Amersham Biosciences) and rabbit anti-DnaA antibodies in a 1:25000 dilution (kindly supplied by Drs. W. Messer and H. Seitz, Max-Planck-Institut für molekulare Genetik, Berlin-Dahlem, Germany).

RESULTS

Isolation of new pPS10 derivative mutants able to replicate in *E. coli* at 37ºC. The plasmid chosen for this work was pRG9B (13), a co-integrate containing the pMB9 (from pBR322 plasmid) and pPS10 replicons (Fig. 1). Using pRG9B has two major advantages: *i*) it allows to purify a high amount of plasmid DNA in *pol*A$^+$ *E. coli* strains, where the pMB9 replicon directs the replication of the co-integrate, and *ii*) the pPS10-based replication can be analyzed in *pol*A *E. coli* strains (such as BT1000), where the pMB9 replicon is inactive (this strain, deficient in DNA polymerase I, cannot sustain replication of ColE1-type replicons). A solution of pRG9B DNA was treated with hydroxylamine, a mutagenic agent that fundamentally induces C→T transitions (35), and the reaction mixture was used to transform *E. coli* BT1000. Since the wild-type pPS10 replicon cannot be established in *E. coli* at 37ºC, suitable plasmid mutants were directly selected by plating at this temperature.

In order to map the mutations induced by hydroxylamine, and to discard other transitions induced by the reagent outside the *repA* gene, we replaced the 964-bp *Eco*RI fragment of pRG9B by the corresponding fragments of the mutants, yielding the pSBM plasmid series (Table 1). All the recombinant plasmids conserved the ability to replicate in *E. coli* BT1000 at 37ºC. Moreover, pSBM plasmids were also able to transform *P. aeruginosa* with the exception of pSBM135 (data not shown). Therefore, the latter is a case of host range modification instead of host range broadening.

The 964-bp *Eco*RI fragments in which the mutations were located were sequenced. Five different single-base mutations were found, leading to single amino acid changes in the RepA proteins (Table 1). pSBM31 plasmid posseses the same mutation than the host range plasmid

pMM141, previously described in our laboratory (13), *i.e*. the A31V change in the leucine-zipper region of the RepA protein. pSBM44, pSBM47 and pSBM93 present their mutations (G44S, T47I and R93C, respectively) within the first of the two proposed winged-helix domains of the protein (20). Finally, pSBM135 contains a G135S change at the beginning of the second winged-helix domain (20). It should be noted that no mutations in the Shine-Dalgarno region of *repA* gene were found.

In order to check the establishment of the pPS10 derivatives in *E. coli pol*A$^+$ strains (such as C600EL), as well as in other Gram-negative bacteria, pSBM plasmids deleted of the pMB9 replicon were constructed (pBM series). This was carried out by digestion with *Pst*I (see Fig. 1) followed by intramolecular religation of the plasmid and transformation in the *P. aeruginosa* PAO1024 strain. Plasmids pBM31, pBM44, pBM47 and pBM93 were thus obtained from the corresponding pSBM plasmids. pBM9B plasmid was obtained from non-mutated pRG9B. All pBM plasmids but pBM9B were also able to efficiently transform BT1000.

Since pSBM135 cannot replicate in *Pseudomonas*, transformation to obtain the pBM135 construct was carried out directly in the *E. coli* BT1000 strain at 30ºC. Km^r transformants formed larger colonies than those obtained with pRG9B and were viable in subsequent cultures in the presence of antibiotic selection, but it was not possible to detect plasmid DNA from them by standard purification procedures. For this reason, we did not carry out any studies on pBM135, using pSBM135 instead. This should be taken into account, especially when comparisons are carried out with the pBM derivatives of the other *repA* mutants (see below).

Stability of the host range mutants. Although wild-type pPS10 can replicate in *E. coli* at 30ºC, establishment in this host is very inefficient, and the plasmid is rapidly lost in the absence of

selection (13). Table 2 shows the segregational stability of both the parental plasmid and the pBM mutants in *P. aeruginosa* and *E. coli*. In all cases the stability approaches 100% in *P. aeruginosa* at 30ºC after 120 generations, whereas in *E. coli* C600EL the stability is different among the mutants: pBM44 and pBM47 are slightly more stable than the wild-type, whereas pBM31 and pBM93 turned out to be the most stable ones. Plasmid pSBM135 is very unstable in *E. coli* BT1000 and, as described above, has lost its capacity to replicate in *P. aeruginosa*. Whereas pBM plasmids represent a host range broadening case, pSBM135 seems to be an example of a shift in host range, somehow imperfect as the establishment in *E. coli* is only marginal.

It is noteworthy that the pMM141 host range mutant of pPS10 presents a better stability in *E. coli* than pBM31, in spite of sharing the same *repA* mutation (13). This can be attributed to a second mutation detected outside the *rep*A gene in pMM141, that is currently under investigation in our laboratory.

Copy number of the host range mutants. The copy number of pPS10 in *E. coli* must be very low even at 30ºC. Km^r colonies obtained at 30ºC after transformation are very small and grow poorly either on plates or in liquid media. Moreover, we were unable to detect pPS10 in such transformants by standard DNA isolation procedures. However, its residual presence in *E. coli* is demonstrated by the ability of such preparations to transform the *Pseudomonas* strain PAO1024 (13). Taking this into account, it was important to test whether an increase in copy number could be a factor contributing to the establishment of pPS10 in *E. coli*.

Densitometry of Southern hybridization gels using the pRG9B 964-bp *Eco*RI fragment as a probe (Fig. 2) clearly shows an increase in copy number for pBM31 and pBM93 in *P. aeruginosa* (4- and 8-fold, respectively), relative to the wild-type pBM9B plasmid. pBM44 and pBM47 are

found in roughly the same copy number than the wild-type derivative pBM9B. pSBM135, as described above, is unable to be established in *P. aeruginosa*. It is noteworthy the presence of multimers in the case of pBM31 and pBM93 (Fig. 2).

Copy number in *E. coli* was studied in the *polA*$^+$ and *polA* strains C600EL and BT1000 respectively (data not shown). We could not calculate the copy number of the mutants relative to wild-type plasmid due to the marginal establishment in *E. coli* of the latter. As in *P. aeruginosa*, pBM93 displayed the highest copy number in both strains, followed by pBM31, pBM44 and pBM47. In BT1000, pBM47 and pSBM135 must be present in a very low copy number because it was necessary a long exposition of the autoradiography to detect the signal. In addition, replication efficiency was always higher in C600EL than in BT1000, suggesting that the *pol*A1 mutation may influence the efficiency of pPS10 plasmid replication even though it should be, in principle, independent of DNA polymerase I.

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Mutant establishment in other bacterial strains. It was of interest to study the establishment of the mutated plasmids in different bacterial strains other than *P. aeruginosa* and *E. coli* and belonging to different Proteobacteria subdivisions (47). Accordingly we determined the transformation efficiency and the stability of the pBM plasmids in *Pseudomonas putida* (γ-subdivision), *Alcaligenes faecalis* (β-subdivision) and *Agrobacterium tumefaciens* (α-subdivision), at 30ºC.

Results are shown in Tables 3 and 4. Transformation efficiencies after electroporation of the mutant pBM plasmids in *P. aeruginosa*, *E. coli* and *A. faecalis* were not significantly different to

that of the wild-type (Table 3). In *P. putida*, 100-1000 times more transformants were obtained with the mutants than with pBM9B. Neither the wild-type nor the mutants are able to transform *A. tumefaciens*. Stability of the wild-type and mutant plasmids is high in both species of *Pseudomonas* (>90%) (Table 4). On the other hand, the mutant plasmids are more stable than the wild-type in *E. coli* and *A. faecalis*, in accordance with their increased host range phenotype. In both organisms, pBM31 and pBM93 show the highest stability values (Table 4).

Structural characterization of MBPRepA proteins. In order to determine how the mutations might affect the protein structure, stability or functionality, we carried out the purification of RepA as a chimera with the maltose binding protein (MBPRepA). Details of the construction and purification of MBPRepA fusions are given in the Materials and Methods section. Attempts to release the MBP part to yield the RepA moiety by factor Xa protease digestion failed due to low digestion rates and the low solubility of the RepA protein (data not shown). In any case, MBPRepA fusions are soluble and have been previously shown to conserve their regulatory activity *in vivo* (15), and their DNA-binding functionality *in vitro* (18).

In view of the RepA mutations reported in Table 1, which include some non-conservative changes, the possibility existed that they could alter the structure of RepA, inducing a conformational change that might affect to its functionality. However, circular dichroism spectra show no significative changes between mutant and wild-type MBPRepA proteins (data not shown), strongly suggesting that the mutations do not affect the secondary stucture to a significant extent. Another possibility could be that the structure was unaffected but the stability had change, maybe accounting for an increased or decreased *in vivo* stability of the complexes with the DNA or with the host range factors involved in the initiation of replication. For this reason, we performed thermal stability experiments by following the change of CD signal with temperature. Typical traces were biphasic,

with two transitions corresponding to the independent unfolding of MBP and RepA domains, and no significative differences were found in the denaturation temperatures of the wild-type and the mutant RepA moieties (data not shown), demonstrating that the thermal stability of all RepA variants is comparable.

Further structural information can be deduced from the high sequence similarity between RepA and the replication protein RepE from the F plasmid. The three-dimensional structure of the monomeric form of RepE bound to iterons has been recently solved (27). This allows the modelling of RepA using the structure of RepE as a template, by threading procedures using the Swiss-Model utilities included in the Swiss-PDB viewer (23). Fig. 3 shows a model of wild-type RepA, and highlighted are the proposed structural domains and the positions mutated in this work. Based in this model, it is noteworthy that all the mutations accumulate in regions far away from the DNA recognition patches and that they are very buried in the protein except Arg93 and, in much less degree, Thr47. Calculated accesibilities for Arg93, Thr47, Gly135, Ala31 and Gly44 are, respectively, 50%, 17%, 11%, 6% and <5%.

Binding of RepA to the iterons of the replication origin. One of the first steps in plasmid replication, is the binding of the initiator protein to the origin of replication. We have previously shown that wild-type MBPRepA binds to the four iterons present in the origin (16, 18). The efficiency of mutant MBPRepA proteins in binding to pPS10 iterons was analysed by EMSA experiments using a DNA probe containing the 4 iterons and an increasing amount of MBPRepA. The results show that the binding of MBPRepA(A31V), MBPRepA(G44S) and MBPRepA(T47I) is very similar to MBPRepA as roughly the same amount of protein is necessary to deplete the free probe (Fig. 4). On the other hand, both MBPRepA(R93C) and MBPRepA(G135S) show an increased binding efficiency to the same probe, and also display an increased cooperativity in

binding, as high molecular weight complexes (arising from binding to multiple iterons) are formed with much less protein than wild-type or the three other MBPRepA mutants (Fig. 4).

Binding of RepA to the inverted repeats of the *repA* gene operator. The RepA-mediated initiation of replication is also dependent on the intracellular levels of the monomeric form. To check whether the highest interaction of MBPRepA(R93C) and MBPRepA(G135S) with the iterons of the origin might reside in an alteration of the dimerization constant K_d of RepA mutants, we performed EMSA experiments using a DNA probe containing the inverted repeats of the *repA* operator. As the dimeric form is required for RepA binding to this probe, the assay should reveal the possible effect of the mutations on the dimer/monomer ratio.

Similarly to what was observed with the iterons, the strongest DNA binding corresponds to MBPRepA(R93C) and MBPRepA(G135S), whereas the differences between the rest of the mutants and the wild-type are hardly noticeable (Fig. 5). This makes unlikely the hypothesis of a better interaction just due to an alteration in K_d of the RepA mutants. In addition, the result indicates that these mutations affect positively a common element involved in the binding of RepA to both the iterons of the origin and the *repA* operator region.

Binding of RepA to *Escherichia coli* DnaA protein. In a previous work (30) we have characterized an *E. coli* chromosomal mutant (LE403) that allows the efficient replication of wild-type pPS10. The LE403 strain codes for a mutant DnaA protein (DnaA403) which seems the main responsible for the establishment of the plasmid. This DnaA mutant forms a complex with wild-type MBPRepA that can be detected by gel filtration chromatography (30). To check whether the mutant MBPRepA proteins reported in this work were capable of interacting with wild-type DnaA, we performed a similar chromatography experiment, the result of which is shown in Fig. 6. Visible

complexes are detected in the exclusion volume of a Sephadex G-75 column for all MBPRepA mutants, but not for the wild-type, which displays a similar pattern than the control lane without MBPRepA. Although the intensities of the wells, developed by Western blot analysis, are variable between mutants, we do not think that they can be correlated with the respective protein-protein affinities, but rather they are consequence of the technical limitations of the procedure used. An accurate affinity quantitation must wait for a complete biophysical study. Even so, the signal corresponding to MBPRepA(G135S) is very faint, and, given the peculiar characteristics of the protein and the marginal stability of the pSBM135 mutant (see above), this could reflect the formation of a relatively unstable RepA-DnaA complex compared to the rest of the mutants. In any case, this result suggests that mutations have all strengthened a RepA-DnaA interaction that is very weak in the case of wild-type RepA.

DISCUSSION

In this work we have attempted to gain deeper insights into plasmid host range searching for mutants of the *Pseudomonas* plasmid pPS10 that can be established in *E. coli* at 30 or 37ºC. All the mutants tested presented a single mutation in the *rep*A gene, coding for the replication initiator RepA, clearly pointing to this protein as a major host range determinant factor, as previously proposed (13). We have investigated several properties of the mutants, namely stability, copy number in *P. aeruginosa* and *E. coli*, establishment in other species, structural integrity of RepA proteins, DNA binding efficiency, and interaction with host-encoded DnaA protein. Most of these parameters do not change regularly upon mutation, when compared to wild-type RepA, with the exception of an improved interaction with DnaA that is displayed by all mutants and not by the wild-type protein (Fig. 6). This result, together with the fact that a similar RepA-DnaA enhanced interaction has been recently described in the case of an *E. coli* mutant that allows the establishment of wild-type pPS10 (30) prompts us to hypothesize that an effective communication between the replication protein RepA and other host factors such as DnaA is a crucial determinant of the host range of the plasmid.

The replication initiation complex involves the simultaneous action of three participants: RepA, DNA (iterons, DnaA box, A+T-rich region, etc) and host factor(s). Consequently, several different mechanisms for the host range change mediated by RepA can be envisaged. Although some of these mechanisms might explain the establishment of some of the mutants, they do not constitute a general explanation. For example:

i) A copy-up phenotype would ensure a plasmid stable inheritance. This could apply for pBM93 and pBM31, the mutants in highest copy number that also show the highest segregational

stability in *E. coli* (Fig. 2, Table 2). The reasons for this copy-up phenotype are unknown, but they must be due to an effect specifically involving RepA. In fact, the influence of Rep proteins on the copy-up phenotype has been described in plasmids such as P1 (44), F (26) and R6K (49). It is noteworthy that in the case of the mutant Q96R of the replication protein of pSC101 (50) this residue could correspond to Arg 93 in pPS10 RepA (4). Many of these mutants seem to be affected in Rep-Rep interactions responsible for coupling plasmid molecules at their specific origins preventing them from replication in conditions of high Rep protein concentration (the so-called "handcuffing" mechanism, 31). Nevertheless, the fact that the copy number of pBM44 and pBM47 is certainly very similar to pBM9B and yet they are more efficiently established in *E. coli* than the latter plasmid, discard copy number increase as a general explanation for the broadening of the host range in our pPS10 mutants.

ii) An enhanced binding of RepA mutants to iterons could increase the general stability of the initiation complex, like MBPRepA(R93C), encoded by pBM93 (Fig. 4). However, the efficiency of binding of MBPRepA(A31V), MBPRepA(G44S) and MBPRepA(T47I) is indistinguishable from that of wild-type MBPRepA and, paradoxically, the highest efficiency of binding is displayed by MBPRepA(G135S), corresponding to pSBM135, the mutant that presents the worst establishment in *E. coli* and the only one unable to replicate in *P. aeruginosa*. One possible reason explaining the marginal replication efficiency of pSBM135 might reside in the fact that MBPRepA(G135S) binds strongly to the operator sequence (Fig. 5) and its expression may be repressed to such low levels that replication of pSBM135 is hampered (*E. coli*) or even abolished (*P. aeruginosa*). However, MBPRepA(R93C) also binds tightly to the operator (Fig. 5) and, instead, induces a copy-up phenotype in both species. Although, due to steric hindrance, the MBP tag could somehow affect the cooperativity of the binding, it is a common part in all MBPRepA proteins (mutant and wild-type), so that its possible influence is cancelled out when a comparative analysis

between MBPRepA fusions is made. Furthermore, MBPRepA fusions are fully functional *in vivo* (15) and also we have found by thermal denaturation experiments (data not shown) that the MBP moiety constitutes a structural entity independent of the RepA part.

iii) Another possible mechanism might involve a higher RepA monomer concentration induced by a decrease in the dimerization constant (k_d) of the mutant proteins, and leading to a lower repression of *repA* transcription by dimers. However, an increased binding to iterons by MBPRepA(R93C) and MBPRepA(G135S) (Fig. 4) is also linked to an enhanced binding to the operator sequences (Fig. 5). If K_d had decreased, as it happens with the RepALZ12 mutant (16), it should be expected that the protein would bind less strongly to the operator as a consequence of the decrease in RepA dimers. On the other hand, we have previously seen that in the mutant pMM141, which carries the same *repA* mutation than pBM31, the dimerization constant of the protein is not significatively altered (16).

From the above considerations, it follows that copy number or enhanced iteron binding are accompanying effects arising from an underlying common mechanism, *i.e*. the improvement of the interaction between RepA and host factors. A similar situation seems to apply in RK2, where different forms of the TrfA protein bound to the origin iterons may also interact with DnaA bound at the *dna*A boxes in order to transfer this protein at the replication fork. Different molecular interaction patterns based in these basic components activate the initiation of RK2 replication in different hosts (2,7,8).

Inspection of the RepA protein at the structural level would be highly valuable to explain how the mutations have altered the interactions with other molecules, although the three-dimensional structure of the protein has unfortunately not yet been solved. According to the

mechanism proposed by Giraldo *et al.* (20), RepA monomers display in solution an elongated conformation with the leucine-zipper and the two winged-helix domains loosely packed between them. This conformation allows the two domains of the monomer to interact with the DNA. Although the host range mutations shown here do not seem to induce large structural changes in the secondary structure of the unbound protein (data not shown), they might be of more importance once the complex with DNA or with host factors is formed. In this work we have made a comparative analysis with the recently solved structure of the monomeric form of the replication protein RepE from the F plasmid, bound to iterons (27). The high sequence similarity between RepE and RepA allows modelling of the latter using the structure of the former as a template. Fig. 3 shows a model of wild-type RepA. The positions mutated are localized far from the DNA-binding region and appear, in general, buried in the core of the protein, but the effects of mutations may be transmitted to this or other functional parts of the protein. It is possible that substitution of glycines 44 and 135 for serine in the RepA(G44S) and RepA(G135S) proteins modify the flexibility of these regions since glicine is an amino acid that allows a great conformational freedom. The position 135 is especially important since it is located at the end of the flexible linker between the two winged-helix domains, and loss of conformational fluctuations may help to fix the complex of the protein with DNA, causing an enhancement in binding of MBPRepA4(G135S) as shown in Figs. 4 and 5. On the other hand, replacement of the polar amino acids Arg93 and Thr47 by hydrophobic residues (Cys and Ile, respectively) is a more drastic event. Due to their solvent-exposed situation far from the DNA binding site, they may be involved in protein-protein interactions with host factors or with another RepA molecule. It is noteworthy that Arg93 is located in the model very close to Val98, the counterpart of which is Arg118 in RepE, a residue involved in RepE-RepE interactions (27).

An examination of the RepE-based RepA model also suggests interesting functionalities for the leucine-zipper region. This sequence is unequivocally involved in RepA dimerization (16). The

fact that the A31V is a host range mutation prompted us to speculate about direct interactions between the zipper and the host factors (16,20). However, as shown in Fig. 3, if RepA behaves like RepE, the leucine-zipper might internalize in the protein when bound to iterons, establishing many hydrophobic contacts within the protein core. Since the A31V mutation does not affect K_d significantly (16), it could be possible that this mutation only exerts its effect when the protein forms the initiation complex through inner conformational changes that are transmitted to other parts of the protein, *i.e.*, the ones involved in truly RepA-host factor interactions.

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REFERENCES

1.-Bagdasarian, M.M. and Timmis, K.N. 1982. Host: vector systems for gene cloning in *Pseudomonas*. Curr. Topics in Microbiol. and Immunol. **96**: 47-67.

2.-Cereghino, J.L. and Helinski, D.R. 1993. Essentiality of the three carboxyl-terminal amino acids of the plasmid RK2 replication initiation protein TrfA for DNA binding and replication activity in gram-negative bacteria. J. Biol. Chem. **268**: 24926-24932.

3.-del Solar, G., Alonso, J.C., Espinosa, M. and Díaz-Orejas, R. 1996. Broad-host-range plasmid replication: an open question. Mol. Microbiol. **21**: 661-666.

4.-del Solar G., Giraldo R., Ruiz-Echevarria M.J., Espinosa M. and Díaz-Orejas R. 1998. Replication and control of circular bacterial plasmids. Microbiol. Mol. Biol. Rev. **62**:434-464.

5.-Díaz, E., Munthali, M., de Lorenzo, V. and Timmis, K.N. 1994. Universal barrier to lateral spread of specific genes among microorganisms. Mol. Microbiol. **13**: 855-861.

6.-Diaz,R. and Staudenbauer ,W.L. 1982. Replication of the broad host range plasmid RSF1010 in cell-free extracts of *Escherichia coli* and *Pseudomonas aeruginosa*. Nucleic Acids Res. **10**:4678-4702

7.-Doran, K.S., Helinski, D.R. and Konieczny, I. 1999. Host-dependent requirement for specific DnaA boxes for plasmid RK2 replication. Mol. Microbiol. **33**: 490-498

8.-Doran, K.S., Konieczny, I. and Helinski, D.R. 1998. Replication origin of the broad host range plasmid RK2. Positioning of various motifs is critical for initiation of replication. J. Biol. Chem. **273**: 8447-8453.

9.-Dower, W.I., Miller, J.F. and Ragsdale, C.W. 1988. High efficiency transformation of *E. coli* by high voltage electroporation. Nucl. Acid. Res. **16**: 6127-6145.

10.-Durland, R.H. and Helinski, D.R. 1987. The sequence encoding the 43-kilodalton TrfA protein is required for efficient replication or maintenance of minimal RK2 replicons in *Pseudomonas aeruginosa*. Plasmid **18**: 164-169.

11.-Fang, F.C. and Helinski, D.R. 1991. Broad-host-range properties of plasmid RK2: importance of overlapping genes encoding the plasmid replication initiation protein TrfA. J. Bacteriol. **173**: 5861-5868.

12.-Fasman, G. 1976. In *Handbook of Biochemistry and Molecular Biology*. Vol III. 3ª Ed. CRC Press, Cleveland, OH.

13.-Fernández-Tresguerres, E., Martín, M., García de Viedma, D., Giraldo, R. and Díaz-Orejas, R. 1995. Host growth temperature and a conservative amino acid substitution in the replication protein of pPS10 influence plasmid host range. J. Bacteriol. **177**: 4377-4384.

14.-Fried, M. and Crothers, D.M. 1981. Equilibria and kinetics of *lac* repressor-operator interactions by polyacrylamide gel electrophoresis. Nucl. Acid. Res. **9**: 6505-6525.

15.-García de Viedma, D. 1994. Análisis de la proteína RepA del plásmido pPS10: Interacciones y motivos implicados en autorregulacion e inicio de replicación. Doctoral Thesis. Universidad de Alcalá de Henares. (Madrid).

16.-García de Viedma, D., Giraldo, R., Rivas, G., Fernández-Tresguerres, E. and Díaz-Orejas, R. 1996. A leucine-zipper motif determines different functions in a DNA replication protein. EMBO J. **15**: 925-934.

17.-García de Viedma, D., Giraldo, R., Ruíz-Echevarría, M.J., Lurz, R. and Díaz-Orejas, R. 1995. Transcription of *repA*, the gene of the initiation protein of the *Pseudomonas* plasmid pPS10, is autoregulated by interactions of the RepA protein at a symmetrical operator. J. Mol. Biol. **247**: 211-223.

18.-García de Viedma, D., Serrano-López, A. and Díaz-Orejas, R. 1995. Specific binding of the replication protein of plasmid pPS10 to direct and inverted repeats is mediated by an HTH motif. Nucl. Acid. Res. **23**: 5048-5054.

19.-DELETED XXX

20.-Giraldo, R., Andreu, J.M. and Díaz-Orejas, R. 1998. Protein domains and conformational changes in the activation of RepA, a DNA replication initiator. EMBO J. **17**: 4511-4526.

21.-Giraldo, R., Martín, M., Fernández-Tresguerres, M.E., Nieto, C. and Díaz, R. 1992. Mutations within the minimal replicon of plasmid pPS10 increase its host range. In: DNA Replication: the regulatory mechanisms. Hughes, P., Fanning, E., and Kohiyama, M (eds). Berlín:

Springer-Verlag, pp 225-237

22.-**Giraldo, R., Nieto, C., Fernández-Tresguerres, E. and Díaz, R**. 1989. Bacterial zipper. Nature **342**: 866.

23.-Guex, N. and Peitsch, M.C. 1997. SWISS-MODEL and the Swiss-PdbViewer: An environment for comparative protein modeling. Electrophoresis **18**: 2714-2723.

24.-Herrero, H., De Lorenzo, V. and Timmis, K.N. 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol. **172**: 6557-6567.

25.-Humphreys G.O., Willshaw G.A., Smith H.R. and Anderson E.S. 1976. Mutagenesis of plasmid DNA with hydroxylamine: isolation of mutants of multi-copy plasmids. Mol. Gen. Genet. **145**: 101-108.

26.-Kawasaki, Y., Wada, C. and Yura, T. 1991. Mini-F plasmid mutants able to replicate in the absence of σ32: mutations in the *repE* coding region producing hyperactive initiatior protein. J. Bacteriol. **173**:1064-1072

27.-Komori, H., Matsunaga, F., Higuchi, Y., Ishiai, M., Wada, Y. and Miki, K. 1999. Crystal structure of a prokaryotic replication initiator protein bound to DNA at 2.6 Å resolution. EMBO J. **18**: 4597-4607.

28.-Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**: 680-685.

29.-Lederberg, E.M. and Cohen, S.N. 1974. Transformation of *Salmonella typhimurium* by plasmid deoxyribonucleic acid. J. Bacteriol. **119**: 1072-1074.

30.-Maestro, B., Sanz, J.M., Faelen, M., Couturier, M., Diaz-Orejas, R. and Fernandez-Tresguerres, E. 2002. Modulation of pPS10 host range by DnaA. Mol. Microbiol. **46**, 223-234.

31.-McEachern, M.J., Bott, M.A., Tooker, P.A. and Helinski, D.R. 1989. Negative control of plasmid R6K replication: possible role of intermolecular coupling of replication origins. Proc Natl Acad Sci U S A. **86**:7942-6.

32.-Nieto, C. 1988. Desarrollo de vectores de clonaje a partir del plásmido pPS10 de *Pseudomonas savastanoi* y caracterización de su replicón básico. Doctoral Thesis. Universidad Autónoma de Madrid.

33.-Nieto, C., Fernández-Tresguerres, E., Sánchez, N., Vicente, M. and Díaz, R. 1990. Cloning vectors, derived from a naturally occurring plasmid of *Pseudomonas savastanoi*, specifically tailored for genetic manipulations in *Pseudomonas*. Gene **87**: 145-149.

34.-Nieto, C., Giraldo, R., Fernández-Tresguerres, E. and Díaz, R. 1992. Genetic and functional analysis of the basic replicon of pPS10, a plasmid specific for *Pseudomonas* isolated from *Pseudomonas syringae* patovar *savastanoi*. J. Mol. Biol. **223**: 415-426.

35.-Phillips, J.H. and Brown, D.M. 1967. The mutagenic action of hydroxylamine. Prog. Nucl. Acid. Res. Mol. Biol. **7**: 349-368.

36.-Projan, S.J., Carleton, S. and Novick, R.P. 1983. Determination of plasmid copy number by fluorescence densitometry. Plasmid **9**: 182-190.

37.-DELETED XXX

38.-Sambrook, J., Frisch, E.F. and Maniatis, T. 1989. In: Molecular cloning, a laboratory manual. 2nd Ed. Cold Spring Harbor. New York.

39.-Scherzinger, E., Haring, V., Lurz, R. and Otto, S. 1991. Plasmid RSF1010 DNA replication *in vitro* promoted by purified RSF1010 RepA, RepB and RepC proteins. Nucl. Acid. Res. **19**: 1203-1211.

40.-Shah, D.S., Cross, M.A., Porter, D. and Thomas, C.M. 1995. Dissection of the core and auxiliary sequences in the vegetative replication origin of promiscuous plasmid RK2. J. Mol. Biol. **254**: 608-622.

41.-Shingler, V. and Thomas, C.M. 1984. Analysis of the *trf*A region of broad-host-range plasmid RK2 by transposon mutagenesis and identification of polypeptide products. J. Mol. Biol. **175**: 229-249.

42.-Smith, A.W. and Iglewski, B.H. 1989. Transformation of *Pseudomonas aeruginosa* by electroporation. Nucleic. Acids. Res. **17**: 10509.

43.-Southern, E. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**: 503-521

44.-Sozhamannan, S. and Chattoraj, D.K. 1993. Heat shock proteins DnaJ, DnaK and GrpE stimulate P1 plasmid replication by promoting initiation binding to the origin. J. Bacteriol. **175**:3546-3555

45.-Thomas, C.M. ed. 1989. Promiscuous plasmids of gram-negative bacteria. Academic Press, London.

46.-Wechsler, J.A., Nusslein, V., Otto, B., Klein, A., Bonhoeffer, F., Hezzman, R., Gloger, L.S. and Schaller, H. 1973. Isolation and characterization of thermosensitive *Escherichia coli* mutants defective in deoxyribonucleic acid replication. J. Bacteriol **113**: 1381-1388.

47.-Woese, C.R. 1987. Bacterial evolution. Microbiological Reviews **51**: 221-271.

48.-Woody, R.W. 1995. Circular Dichroism. Methods Enzymol. **246**: 34-71.

49.-York, D. and Filutowicz, M. 1993**.** Autoregulation-deficient mutants of the plasmid R6K-encoded π protein distinguish between palindromic and nonpalindromic binding sites. J. Biol. Chem. **268**: 21854-21861.

50.-Xia, G., Manem, D., Yu, Y. and Caro, L. 1993. In vivo and in vitro studies of a copy number mutation of the RepA replication protein of plasmid pSC101. J. Bacteriol. **175**:4165-4175.

TABLE 1. Mutations in the RepA protein that amplify or modify the host range of pPS10 plasmid.

Plasmid	**Amino acid change**	**Protein**
pSBM31	Ala31 → Val (A31V)	RepA(A31V)
pSBM44	Gly44 → Ser (G44S)	RepA(G44S)
pSBM47	Thr47 → Ile (T47I)	RepA(T47I)
pSBM93	Arg93 → Cys (R93C)	RepA(R93C)
pSBM135	Gly135 → Ser (G135S)	RepA(G135S)

TABLE 2. Stability of mini-pPS10 host range mutants in *E. coli* and *P. aeruginosa*.

	Percentage of plasmid containing cells								
	E. coli C600EL				*P.aeruginosa* PAO 1024				
Plasmid	**Cycle[a]**	**1**	**2**	**3**	**4**	**Cycle**	**1**	**2**	**3**	**4**
pBM9B		<1	—	—	—		>99	>99	>99	>99
pBM31		69	34	7	3		>99	>99	>99	>99
pBM44		23	<1	—	—		>99	>99	>99	>99
pBM47		19	<1	—	—		>99	>99	>99	>99
pBM93		78	53	37	21		>99	>99	>99	>99
pSBM135[b]		<1	—	—	—		N.D.	N.D.	N.D.	N.D.

[a] Cycles of propagation in the absence of selection. One cycle corresponds to the number of generations (about 30) needed to form a colony from a single cell (12).

[b] The stability is determined in the *pol*A1 BT1000 *E. coli* strain.

N.D. Not determined, as pSBM135 cannot be established in *P. aeruginosa*.

TABLE 3. Electroporation efficiency at 30ºC of different Gram-negative bacteria with the pBM variants.

	Strain				
Plasmid	***P. aeruginosa***	***E. coli***	***P. putida***	***A. faecalis***	***A. tumefaciens***
pBM9B	0.6[a]	9.7	$3.9\ 10^{-2}$	16	$<10^{-4}$
pBM31	2.9	0.9	3.0	5.3	$<10^{-4}$
pBM44	3.8	1.6	18	24	$<10^{-4}$
pBM47	0.8	2.6	12	7.8	$<10^{-4}$
pBM93	3.1	6.3	6.0	21	$<10^{-4}$

[a] Values are shown as number of transformants per viable cell and ng of DNA.

TABLE 4. Stability of pBM variants in different bacterial strains at 30ºC.

	Strain				
Plasmid	***P. aeruginosa***	***E. coli***	***P. putida***	***A. faecalis***	***A. tumefaciens***
pBM9B	>99[a]	<1	92	<1	—
pBM31	>99	69	>99	25	—
pBM44	>99	23	>99	6	—
pBM47	>99	19	>99	3	—
pBM93	>99	78	>99	15	—

[a] Percentage of cells that contain the plasmid after 30 generations in the absence of selection. Approximately 30 generations are needed to form a colony from a single cell (12).

FIGURE LEGENDS

FIG. 1. Circular map of plasmid pRG9B. It contains the basic replicon of plasmids pPS10 (black) and pBR322 (dotted) and the kanamycin resistant gene from pKT231 (white). Most relevant restriction endonucleases sites are also shown.

FIG. 2. Copy number of the host range mutants in *P. aeruginosa*. *A***,** autoradiograph from total lysates of *P. aeruginosa* containing wild-type pBM9B and mutated pBM plasmids hybridized to a *repA* DNA probe. *B***,** copy number of pBM mutants relative to pBM9B. Values are the media obtained from five different experiments, and were obtained by autoradiographs densitometry corrected to the same amount of chromosomal DNA in all the samples. pSBM135 is not included, as it cannot be established in *P. aeruginosa*.

FIG. 3. Two views (differing in 180º rotation) of the modelled three dimensional structure of pPS10 RepA protein based on the amino acid similarity with the RepE initiation replication protein of F plasmid, showing the mutated positions. The picture shows the N- and C-terminal winged-helix domains in white and grey, respectively, as well as the connecting turn (red), and the LZ motif (blue). DNA is only shown for presentation purposes. Drawing was performed using RASMOL (http://www.umass.edu/microbio/rasmol/index2.htm).

FIG. 4. Binding of MBPRepA proteins to the *oriV* region. Increasing quantities of protein (0, 0.43, 0.72, 1.44, 2.88, 4.32, 5.76, 7.20, 8.65, 10.09, 11.53, 12.97, 14.41, 21.61, 28.82 pmol) were incubated with 24.5 fmol of the labelled origin probe.

FIG. 5. Binding of MBPRepA proteins to the promoter operator region. Increasing quantities of protein (0, 0.07, 0.14, 0.29, 0.43, 0.57, 0.72, 0.86, 1.01, 1.29, 1.44, 1.80, 2.16, 2.52, 2.88 pmol) were incubated with 8.6 fmol of the labelled operator probe.

FIG. 6. Dot-blot analysis of the interaction of DnaA with wild-type and mutant MBPRepA fusions by gel-filtration chromatography. The exclusion volume of the column is 6.0 ml. Extracts were obtained from *E. coli* LE392 [pUC392] and CC118 [pMALRepA]. Lane 1, DnaA + MBPRepA wild-type; lane 2, DnaA + MBPRepA(G135S); lane 3, DnaA + MBPRepA(G44S); lane 4, DnaA + MBPRepA(R93C); lane 5, DnaA + MBPRepA(T47I); lane 6, DnaA + MBPRepA(A31V).

Fig. 1 (Maestro, B., Sanz, J.M., Díaz-Orejas, R. and Fernández-Tresguerres, E.)

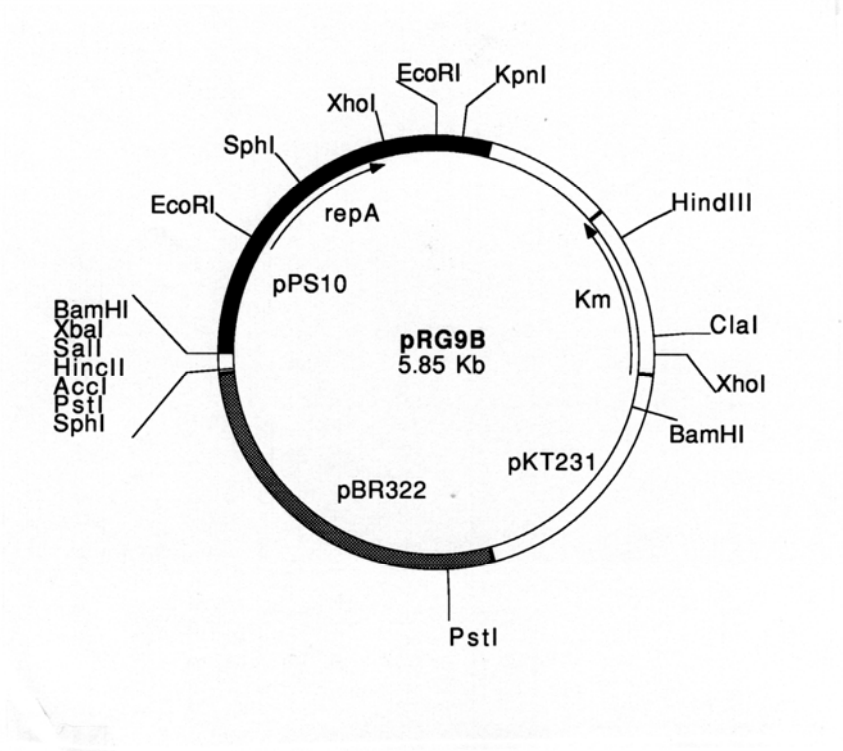
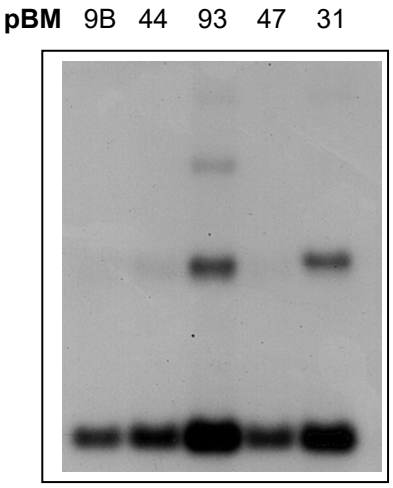


Fig. 2 (Maestro, B., Sanz, J.M., Díaz-Orejas, R. and Fernández-Tresguerres, E.)

A



B

Plasmid	Copy number in *P. aeruginosa* PAO1024
pBM9B	1
pBM31	3.70 ± 0.45
pBM44	1.70 ± 0.26
pBM47	1.40 ± 0.21
pBM93	7.40 ± 1.54

Fig. 3 (Maestro, B., Sanz, J.M., Díaz-Orejas, R. and Fernández-Tresguerres, E.)

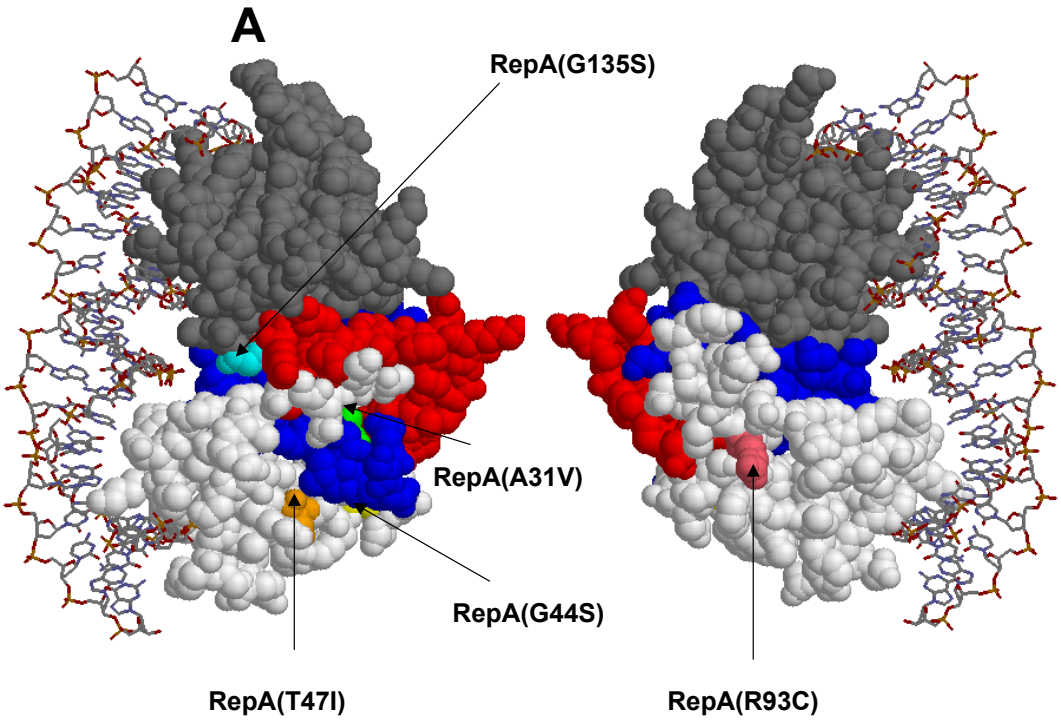


Fig. 4 (Maestro, B., Sanz, J.M., Díaz-Orejas, R. and Fernández-Tresguerres, E.)

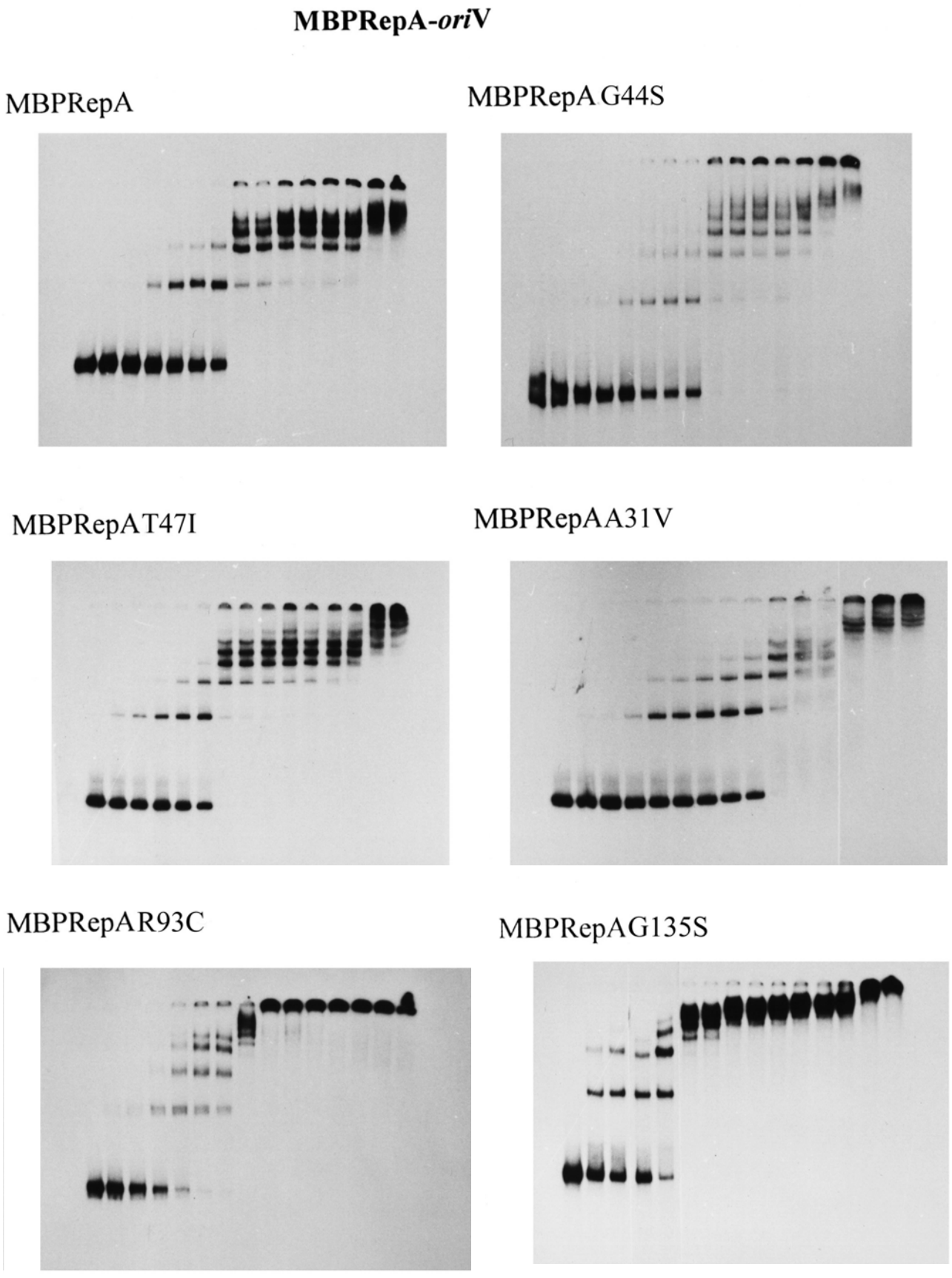


Fig. 5 (Maestro, B., Sanz, J.M., Díaz-Orejas, R. and Fernández-Tresguerres, E.)

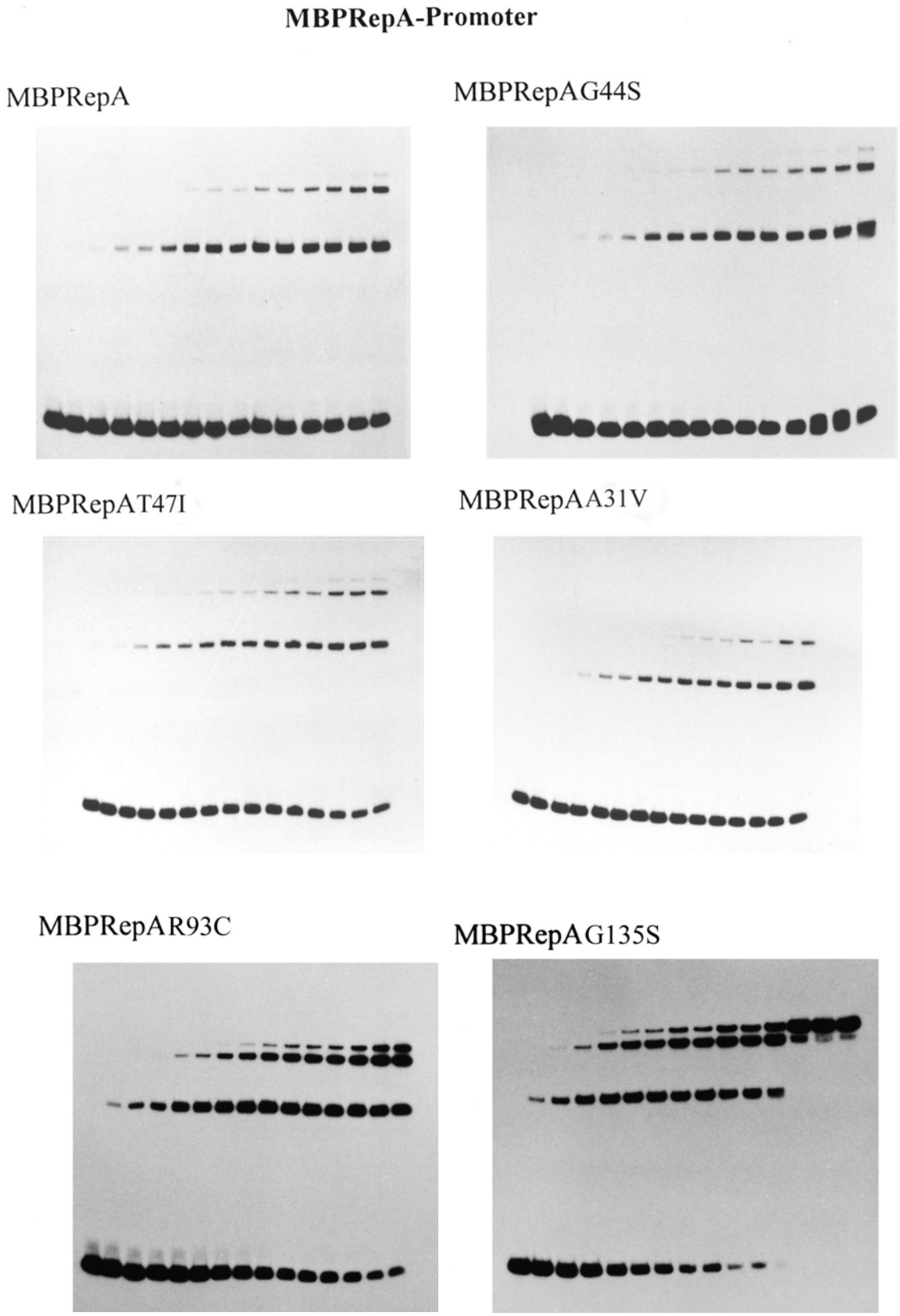


Fig. 6 (Maestro, B., Sanz, J.M., Díaz-Orejas, R. and Fernández-Tresguerres, E.)