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Host Growth Temperature and a Conservative Amino Acid Substitution in the Replication Protein of pPS10 Influence Plasmid Host Range

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pPS10 is a replicon isolated from *Pseudomonas syringae* pv. savastanoi that can be established at 37 C efficiently in *Pseudomonas aeruginosa* but very inefficiently in *Escherichia coli*. The establishment of the wild-type pPS10 replicon in *E. coli* is favored at low temperatures (30 C or below). RepA protein of pPS10 promotes in vitro plasmid replication in extracts from *E. coli*, and this replication depends on host proteins DnaA, DnaB, DnaG, and SSB. Mutant plasmids able to efficiently replicate in *E. coli* at 37 C were obtained. Three of four mutants whose mutations were mapped show a conservative Ala→Val change in the amino-terminal region of the replication protein RepA. Plasmids carrying this mutation maintain the capacity to replicate in *P. aeruginosa* and have a fourfold increase in copy number in this host. The mutation does not substantially alter the autoregulation mediated by RepA. These results show that the physiological conditions of the host as well as subtle changes in the plasmid replication protein can modulate the host range of the pPS10 replicon.

The analysis of the factors that determine plasmid host range is a topic of considerable interest because of its basic ecological and biotechnological implications and has been approached so far by studying broad-host-range replicons (34). Evaluation of the replication process in the broad-host-range replicons RSF1010, RK2, and pLS1 indicates the existence of different strategies to achieve promiscuous replication: in RSF1010, three plasmid proteins make the initiation process independent from host replication functions (31); in RK2, two differently processed forms (short and long) of the initiator allow different interactions with a host replication component(s) (29, 32); and in the rolling-circle-type pLS1 replicon, the efficiency of a minus origin influences plasmid establishment in different hosts (6). The analysis of plasmid host range determinants can also be done by using narrow-host-range plasmids. This approach has the advantage of direct selection that can be used to isolate mutations, in the plasmid or in the host, that change the host range of the plasmid. Conditions that affect the physiology of the host could also influence the propagation of a plasmid in a new host, and these conditions could also be explored by using narrow-host-range plasmids and direct selection.

pPS10 is a plasmid isolated from *Pseudomonas syringae* pv. savastanoi (25) that is particularly suited for this approach; it can replicate efficiently in several *Pseudomonas* spp., but it has a defective establishment in *Escherichia coli*. This favors the identification of conditions for mutants that overcome this defective establishment. Characterization of the possible plasmid host range mutants can be facilitated by the extensive knowledge about this replicon. The minimal replicon of pPS10, 1,267 bp in length, has been analyzed (27). This replicon can be divided into two functional regions: (i) a *cis*-acting region, *oriV*, that includes four 22-bp direct repeats flanked by a *dnaA* box and by an A+T-rich region containing two 11-mers and (ii) an adjacent region coding for a 26.7-kDa protein, RepA, which binds to *oriV* (9), promoting initiation of plasmid replication. The *repA* gene is transcribed from a σ^{70}-type promoter, and this transcription is autoregulated by interactions of RepA with a symmetric operator that overlaps the −35 box of the promoter (9b). RepA contains a leucine zipper (LZ)-like motif at its NH_2 end that is conserved in a few other plasmid replication proteins (12). We now have evidence that this sequence is a dimerization motif in RepA (9a), as was described for other LZs previously reported (16, 28). In a preliminary report (11) we showed that the inefficient establishment of the pPS10 replicon in *E. coli* was not due to poor expression of the RepA protein and could not be overcome by favoring interactions of the DnaA protein with the *dnaA* box adjacent to the iterons of the origin of replication. In the same communication we also showed that host range mutants of pPS10 can be easily obtained following in vitro mutagenesis of mini-pPS10 replicons with hydroxylamine. We now present evidence that the temperature of growth can influence the establishment of the plasmid in *E. coli* and that this establishment can also be achieved efficiently by a conservative change (Ala→Val) in the LZ motif of the replication protein. Evidence for the involvement of the replication protein in the host range of RK2 has been described recently (19), but in this case mutations in TrfA reduce the range of bacteria in which the plasmid can be established (3). Our results show that minor genetic changes in plasmid replication components can favor colonization of new hosts and underline the influence of the physiology of the host in this colonization. In addition, these results show the importance in replication of the LZ motif found in RepA.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used and their relevant characteristics are listed in Table 1. See also reference 27 for a complete description of the construction of plasmids pRG15 and pRG14. The minimal replicon of pPS10 is shown in Fig. 1.

Media and growth conditions. Bacterial cultures were grown in LBT (18) supplemented with thymine (20 μg/ml). Unless otherwise specified, all bacterial

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid (derivation)	Relevant genotype or characteristic	Source or reference
E. coli K-12		
C600EL	r^-/m^+	K. Nordström
BT1000	*polA1*	35
CSH50	Δ(*lac-pro*)	22
WM1502	*rnh-224*	W. Messer
WM1490	*rnh-224 dnaA850*::Tn*10*	W. Messer
P. aeruginosa PAO1024	r^-/m^+	K. Nordström
P. putida KT2440	r^-/m^+	25
E. aerogenes		S. Fernández
K. pneumoniae ma5		J. L. Garcia
P. denitrificans		S. Fernández
Plasmids		
pRG15 (pCN38)	Wild-type mini-pPS10	10
pRG14 (pCN38)	*dnaA* box adjusted to *E. coli* consensus	10
pMM141 (pRG14)	C-669→T transition in *repA*	11
pCN51 (pCN38-pBR322)	Recombinant of the two wild-type replicons	26
pRG9B (pRG14-pBR322)	pCN51/*dnaA* box of *E. coli*	10
pCN510 (pCN51)	*repA* gene deleted	10
pSB141 (pCN51)	C-669→T transition	11
RSF1010		25
M13mp19 (M13)	Cloning vector	21
pFusCE (pRS550)	*repA* promoter recombinant	9b
pMMB*repA* (pMMB67EH)	*repA* recombinant	25
pMMB*repA*141 (pMMB67EH)	*repA141* recombinant	9b

strains were grown at 30°C with good aeration. Kanamycin (25, 50, or 75 μg/ml) was used to select transformants of pPS10 miniderivatives in different bacteria.

Enzymes, chemicals, and radiochemicals. Restriction enzymes were obtained from New England Biolabs, Amersham, or Boehringer Mannheim. DNA ligase was obtained from Amersham; RNase A, lysozyme, and hydroxylamine were from Sigma. Products for electrophoresis, IPTG (isopropyl-β-D-thiogalactopyranoside), and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were from Bethesda Research Laboratories. Antibiotics were from Sigma and Boehringer Mannheim. DNA sequencing was performed by using a kit from Amersham. Elution of DNA fragments from agarose gels was performed by using the GeneClean kit of BIO101 Inc. Other chemicals were from Merck, Sigma, Probus, or Panreac. ^{35}S-dATP was obtained from Amersham. Film used for autoradiography was either Kodak X-OMAT or Hyper-film-β-max from Amersham; the nitrocellulose membranes used for DNA transfer were also from Amersham.

General methods. Mini- and midi-scale preparations of plasmid DNA were done by the alkaline lysis method (2). For large-scale preparations, clarified lysates were prepared as described, and the supercoiled plasmid DNA was separated from open-circle and linear DNA fragments by equilibrium centrifugation in CsCl-ethidium bromide gradients (4). Enzymatic treatment of DNA was done as described previously (20) with the modifications recommended by the suppliers. β-Galactosidase assays were performed with 1 mM IPTG as the inducer (22). Transformations of *E. coli* (17) and *P. aeruginosa* (1) were done as described previously.

Electroporation. Transformation of different bacteria by electroporation (8) was done with cell suspensions of 10^{10} to 10^{12} cells per ml in 10% glycerol. Forty microliters of the suspension was mixed with 1 to 5 μl of DNA (approximately 0.1 to 0.5 μg) in chilled electroporation cuvettes with a 0.2-cm electrode gap (Bio-Rad). Pulses of 12.5 kV/cm were delivered with the Gene Pulser and Pulser Controller apparatus (Bio-Rad). Immediately after electroporation, the cells were suspended in 1 ml of SOC broth (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 10 mM MgCl, 10 mM $MgSO_4$, and 20 mM glucose) and incubated at 30°C for one generation. Cells were then plated at appropriate dilutions with and without antibiotic selection, and the number of transformants, grown at 30°C, was calculated with respect to the number of viable cells.

Analysis of plasmid stability. pRG14 and pRG9B transformants of *E. coli* or *P. aeruginosa* were selected in LBT plus kanamycin. One hundred independent colonies from a single transformant plated in LBT were patched in LBT and LBT plus kanamycin to determine the number of kanamycin-sensitive colonies (segregants during colony formation in selective medium). The colonies growing in nonselective medium were pooled, and individual colonies were selected again in nonselective medium; 100 of these colonies were again patched in LBT and LBT plus kanamycin to determine the percentage of plasmid-free cells after the second propagation. When required, the procedure was reiterated a third time and the percentage of segregants was determined as described above; these values correspond to the stability of the plasmid after 0, 30, and 60 generations of growth in the absence of selection. Thirty generations is approximately the time needed to form a clearly visible colony.

In vitro replication. Type I extracts were prepared from *E. coli* C600EL cells

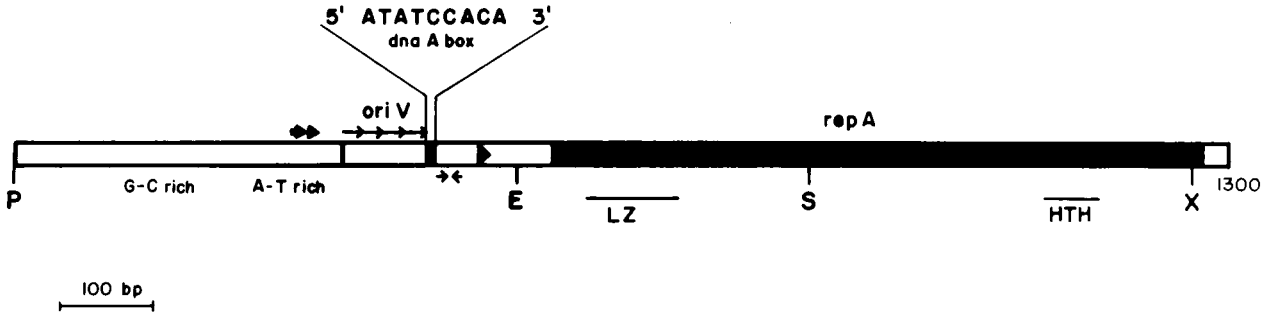

FIG. 1. Relevant regions in the basic replicon of pPS10. G-C- and A-T-rich regions are indicated. Thick arrows, 11-mer repeats; thin arrows, 22-bp direct repeats of the origin; →←, 8-bp inverted repeats in *repA* promoter. The *dnaA* box sequence and fragments within the *repA* gene coding for putative LZ and helix-turn-helix (HTH) motifs within the RepA protein are also shown. Restriction enzyme targets relevant for this work: P, *Pvu*II; E, *Eco*RI; S, *Sph*I; X, *Xho*I. There is another *Eco*RI site at bp 1500 which is shown in Fig. 3.

containing pCN51 or pSB141 by the freezing-and-thawing lysis method (33). Type II extracts were prepared from type I extracts in two stages: (i) removal of the endogenous DNA by precipitation with streptomycin sulfate and (ii) concentration of the proteins by ammonium sulfate precipitation (5). In vitro replication assays were also done as described previously (7). Polyclonal antisera against DnaA and DnaK were kind gifts from M. Kohiyama. Antisera against DnaB, DnaG, and SSB were kind gifts from E. Lanka.

Hydroxylamine mutagenic treatment (14). Twenty microliters of plasmid solution containing 4 μg of DNA was mixed with 100 μl of 0.1 M phosphate buffer (pH 6.0) containing 1 mM EDTA and 80 μl of 1 M NH_2OH in the same buffer. The mutagenesis mixture was incubated for 30 min at 75°C and was then dialyzed at 4°C against 500 ml of TE buffer (10 mM Tris-HCl [pH 7.5], 0.1 mM EDTA) for 2 h and then against another 500 ml of the same buffer for 12 h. One microgram of dialyzed DNA was used for transformation.

Transcriptional regulation of the *repA* gene. Expression of the *lacZ* gene under the control of the *repA* promoter was measured in the presence or absence of RepA protein as described previously (9b).

Determination of plasmid copy number. Total cell lysates were obtained from exponentially growing cultures of *P. aeruginosa* PAO1024, *E. coli* BT1000, or *E. coli* C600EL as described previously (30). The samples were electrophoresed on 0.8% agarose gels in TAE buffer (400 mM Tris base, 200 mM acetic acid, 20 mM EDTA) for 18 h at 20 V. The amounts of plasmid and chromosomal DNAs were evaluated by densitometric analysis (LKB Ultroscan), and relative plasmid copy numbers were calculated as described in the legend to Fig. 5.

RESULTS

Establishment of the pPS10 replicon in *E. coli* is temperature dependent. Preliminary observations showed that pPS10 derivatives were efficiently established in *P. aeruginosa* but not in *E. coli*: transformation of *E. coli* with mini-pPS10 at 37°C gives very small colonies which are not viable after being restreaked on antibiotic-containing plates (26). We found that the observed inefficient establishment of a mini-pPS10 replicon, pRG14, can be overcome by growing the transformants at a low temperature (30°C or below). This establishment is *polA* independent and can therefore be detected in an *E. coli polA1* background (BT1000), either with the pRG14 replicon or with pRG9B, a cointegrate of the pMB9 and pPS10 replicons (Table 2). In both cases transformants appeared in LBT after 72 h of incubation at 30°C or after 96 h at room temperature (20 to 24°C). On the other hand, colony formation was greatly reduced at 37°C and abolished at 42°C. By contrast, in *P. aeruginosa* PAO1024, transformants appeared after overnight incubation at 30 or 37°C. These results show that a low temperature allows the establishment of pPS10 in *E. coli*.

The copy number of mini-pPS10 must be very low in *E. coli*, as shown by the difficulty of detecting plasmid DNA in standard minipreparations. However, its presence was clearly indicated by the ability of the minipreparations to transform *P. aeruginosa* PAO1024 (data not shown). Since low-copy-number replicons are unstably maintained in growing cultures in the absence of active stability systems, we wanted to evaluate the stability of mini-pPS10 in *E. coli*. We measured the loss of pRG14 and pRG9B in cells growing at 30°C without selection. C600EL, a wild-type *E. coli* K-12 strain, and BT1000, a *polA* strain, were used as hosts in this analysis. Segregation of the plasmids was measured after 30, 60, and 90 generations of growth in the absence of selection. The results (Table 3) show that pRG14 is lost in C600EL and BT1000 after 30 generations, whereas pRG9B is lost only in BT1000, in which it replicates from the pPS10 replicon, and not in C600EL, in which the pMB9 replicon is functional. On the other hand, a copy-up mutant plasmid, pMM141 (see below), is stable in *E. coli* (Table 3). Analysis of stability in *P. aeruginosa* PAO1024 indicates that both pRG14 and pMM141 are stably inherited in this host, even after a third propagation in nonselective medium (Table 3). Taken together, these results indicate that at 30°C and in the presence of selection, the plasmid can be maintained in *E. coli* cells but that replication under these circumstances is inefficient and, as a consequence, the plasmid cannot be stably maintained in the absence of selection. In contrast, when the copy number is increased, the plasmid is stably inherited in the absence of selection in both *E. coli* and *P. aeruginosa*.

TABLE 2. Thermosensitivity of pPS10 establishment in *E. coli*

Plasmid	No. of transformants/μg of DNA				
	E. coli BT1000			*P. aeruginosa* PAO1024	
	30°C	37°C	42°C	30°C	37°C
pRG14	1,344[a]	10[a]	0	1,936	~10^4
pRG9B	1,172[a]	8[a]	0	868	~10^4

[a] Slowly growing cells.

TABLE 3. Stability of mini-pPS10 derivatives in *E. coli* and *P. aeruginosa*

Plasmid	% Plasmid-containing cells						
	E. coli				*P. aeruginosa* PAO1024		
	C600EL		BT1000				
	1 cycle[a]	2 cycles	1 cycle	2 cycles	1 cycle	2 cycles	3 cycles
pRG14	4	0	0	0	100	100	100
pRG9B	100	100	1	0	ND[b]	ND	ND
pMM141	100	100	96	68	100	100	100

[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.
[b] ND, not done.

In vitro replication of mini-pPS10 in cell extracts of *E. coli* promoted by the wild-type RepA protein. To characterize the replication requirements of pPS10 in *E. coli*, we prepared cell extracts from C600EL containing pCN51, a fused pPS10-pBR322 replicon, as a source of endogenous RepA protein. To assay pPS10 replication in the absence of pBR322 replication, we used extracts in which the endogenous pCN51 plasmid was removed by streptomycin sulfate precipitation (fraction II [see Materials and Methods]). These extracts were assayed by using pMM141, an *oriV* wild-type mini-pPS10 replicon, as a DNA substrate. In vitro replication of pMM141 promoted by the RepA wild-type protein yields supercoiled monomeric and dimeric forms of the plasmid, indicating that a complete cycle of replication can occur (Fig. 2). In vitro replication of pPS10 does not require transcription by host RNA polymerase, and it is inhibited by novobiocin, an inhibitor of DNA gyrase, which shows that supercoiled DNA is the substrate used in the process. In vitro replication of pPS10 is also inhibited by antibodies against DnaA, DnaB, DnaG, and SSB and is partially resistant to antibodies against DnaK. This partial inactivation could still indicate dependence on DnaK and could be due to the high levels of the DnaK protein in the extracts (1% of total protein) (13). Replication of pMM141 cannot be detected in extracts prepared from a pPS10-free replicon or in extracts of a strain containing pCN510, a pCN51 derivative with the *repA* gene deleted (data not shown), indicating that it requires RepA. This replication is replicon specific: the extract cannot activate replication of a mini-R1 plasmid (data not shown). These data show that replication of pPS10 in *E. coli* is dependent on RepA and *oriV* sequences and also requires host replication proteins acting at the initiation stage.

Isolation of pPS10 mutants able to replicate efficiently in *E.*

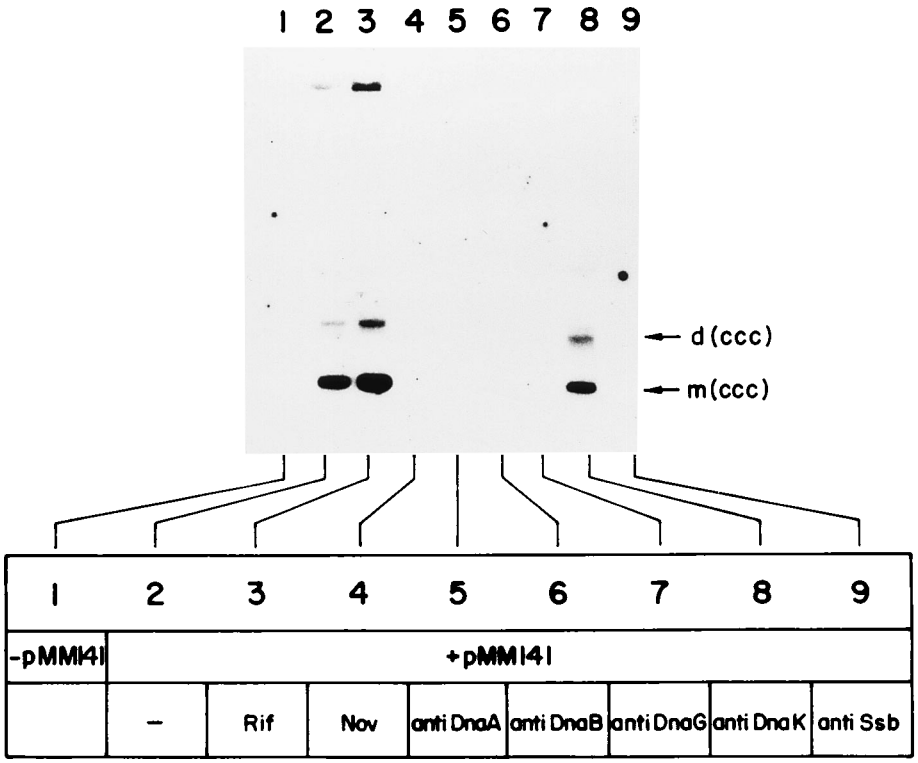

FIG. 2. In vitro replication of mini-pPS10 (pMM141). An autoradiograph of samples obtained as described in Materials and Methods is shown. Rif, rifampin; Nov, novobiocin. Lanes 5 to 9, antisera against the indicated host replication proteins were used. m and d, monomer and dimer plasmid DNA, respectively. ccc, covalently closed circular.

***coli* and mapping of their mutations.** The temperature dependence for the establishment of wild-type pPS10 in *E. coli* provides a method for direct selection of host range plasmid mutants. Plasmids able to transform *E. coli* at 37°C were obtained following in vitro mutagenesis with hydroxylamine of the pPS10 minireplicons pRG14 and pRG15.

The mutations present in three independently isolated pRG14 derivatives, pMM141, pMM142, and pMM146, and in one pRG15 derivative, pMM151, were mapped. In the first series of experiments, we took advantage of the fact that the mutants can replicate in a *polA1* strain. Fragments of the pBR322-pPS10 vector pCN51 were replaced by the equivalent fragments of the mini-pPS10 mutants, and the abilities of the recombinants to transform the *polA1* strain BT1000 were evaluated. Substitutions of either the 964-bp *Eco*RI fragment or the 853-bp *Pvu*II-*Sph*I fragment (Fig. 3) permit the establishment of reconstituted pCN51 derivatives in BT1000. This limits the location of the different mutations to the 317-bp *Eco*RI-*Sph*I region that includes the 5′ end of the *repA* gene. The location of the mutation present in pMM141 as a result of this reconstitution approach has been reported previously (11). Finally, the sequence of the *repA* gene included in the *Eco*RI-*Sph*I region was analyzed for the four mutants. All but pMM146 show a C→T transition at position 669 (Fig. 4). This mutation changes an alanine codon to a valine codon within the region of *repA* coding for LZ motif. This change is quite conservative, since alanine and valine are amino acids with hydrophobic side chains (short and medium, respectively), which suggests that, in principle, the mutation should not alter the secondary structure of the protein in this region. The mutation found in pMM146 is a G→A transition at position 707. This mutation is located downstream from and very close to the 3′ end of the *repA* region coding for the LZ motif and changes a glycine codon to a serine codon.

Requirements for pMM141 replication in *E. coli*. pMM141 was chosen for further study. The following results were obtained either with pMM141 itself or with pSB141, a pCN51 derivative carrying the *Eco*RI fragment of pMM141 which contains the mutant *repA* gene (Fig. 3). Replication of pMM141 in *E. coli* is dependent on RepA141 and on *oriV* sequences. This is indicated by the fact that pMM141 activates in *trans* replication of a pBR322-*oriV* recombinant in the *E. coli polA* strain BT1000 (data not shown). Further evidence of the involvement of RepA141 in replication of pMM141 in *E. coli* is given by the fact that replication of the fused pSB141 replicon in the *polA* background BT1000 is prevented by inversion or deletion of the fragment that contains the mutated *repA* gene (11). Replication of pMM141 in *E. coli* is dependent also on the DnaA protein: this is indicated by the failure of pMM141 to replicate in WM1490, a *dnaA* null strain (no transformants were obtained), but not in WM1502, its parental *dnaA*$^+$ strain ($>6 \times 10^3$ transformants per μg of DNA were obtained). In this experiment, transformation of the *dnaA*$^+$ and *dnaA* null strains by a DnaA-independent replicon, RSF1010, was used to evaluate the competence of the cultures ($>4 \times 10^3$ and $>6 \times 10^3$ transformants per μg of DNA, respectively, were obtained).

In vitro replication analysis of pMM141 with extracts containing RepA141 showed the same requirements described above for replication dependent on wild-type RepA protein (11) (Fig. 2).

From the in vivo and in vitro analyses it can be concluded that the phenotype of the pMM141 mutant is not due to in-

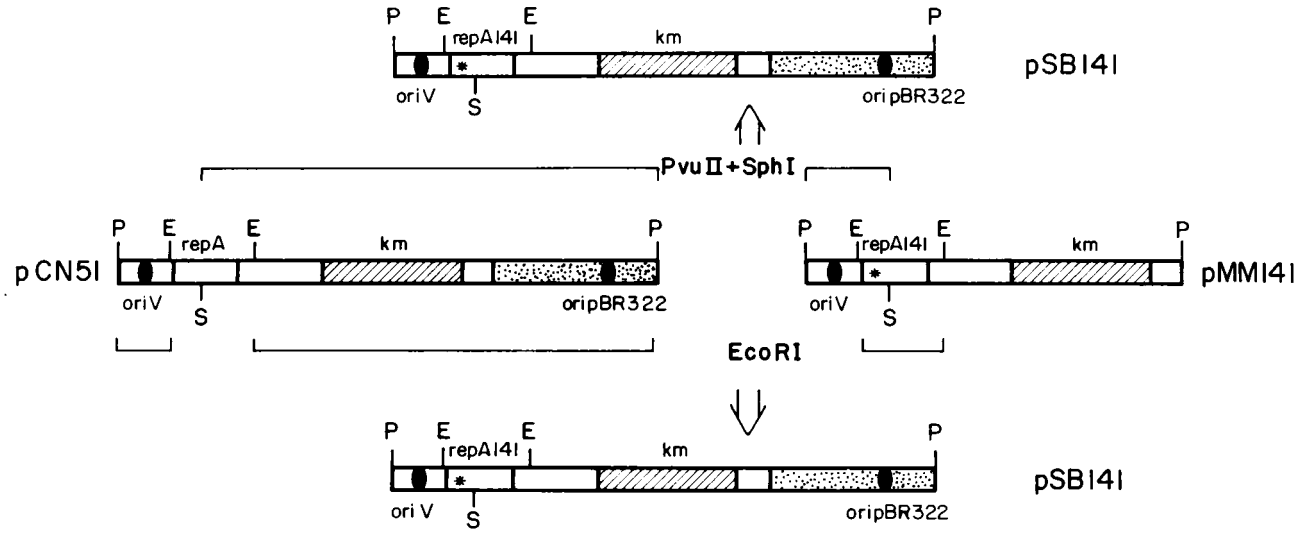

FIG. 3. Reconstitution experiments to locate the mutation in pMM141. Two different reconstitutions that result in the same pCN51 derivative, pSB141, containing the host range mutation present in pMM141 are indicated. These reconstitutions located the host range mutation within the small *Pvu*II-*Sph*I fragment (upper reconstruction) and the small *Eco*RI fragment (lower reconstruction) of pMM141, i.e., within the *Eco*RI-*Sph*I fragment of pMM141 that includes the 5′ end of *repA*. Schematic maps of pCN51 and pMM141 show the positions of restriction enzymes used. *oriV* and *repA* of pPS10, as well as *oriV* of pBR322, are indicated. *, location of the host range mutation; P, *Pvu*II; E, *Eco*RI; *S*, *Sph*I; Km, kanamycin resistance gene.

dependence from host replication factors involved in initiation of wild-type pPS10 replication.

pMM141 is a high-copy-number mutant in *P. aeruginosa*. Preliminary analysis indicated that the pMM141 mutant was a host range mutant because it could still replicate in *P. aeruginosa* (11). To analyze a possible effect of the mutation on this replication, we compared the copy numbers of pMM141 and pRG14, its parental replicon, in *P. aeruginosa*. This analysis showed that the copy number of pMM141 was fourfold higher than that of pRG14 (Fig. 5). Comparative analysis of the copy numbers of the mutant in *P. aeruginosa* and *E. coli* indicates that in *E. coli* the copy number of pMM141 is half of that found in *P. aeruginosa*. We conclude that the host range mutation also alters a negative regulatory loop, involving the LZ motif of RepA, that controls the copy number of the plasmid.

The RepA protein of pMM141 is able to autoregulate. RepA autoregulates its own synthesis at the transcriptional level by interactions with a symmetrical operator that overlaps the −35 box of the *repA* promoter (9b). To test whether the host range phenotype was correlated with an alteration in the repressor function of RepA141, we evaluated the ability of the mutant protein to repress in *trans* expression of a *lacZ* reporter gene fused transcriptionally to the *repA* promoter. Table 4 shows that high levels of RepA141 or RepA protein (with IPTG) inhibit to the same degree the activity of the *repA* promoter, as indicated by the reduction in the expression of the *lacZ* reporter gene. A small difference in repression modulated by wild-type RepA and RepA141 proteins can be detected only at low level of the proteins (without IPTG). This difference correlates with the qualitative evaluation of β-galactosidase production in Luria agar plates containing X-Gal: the expression of wild-type RepA or RepA141 protein renders white or blue fish-eye colonies, respectively, in the absence of IPTG; however, in the presence of the inducer, white colonies are formed in both cases. These results show that RepA141 conserves the

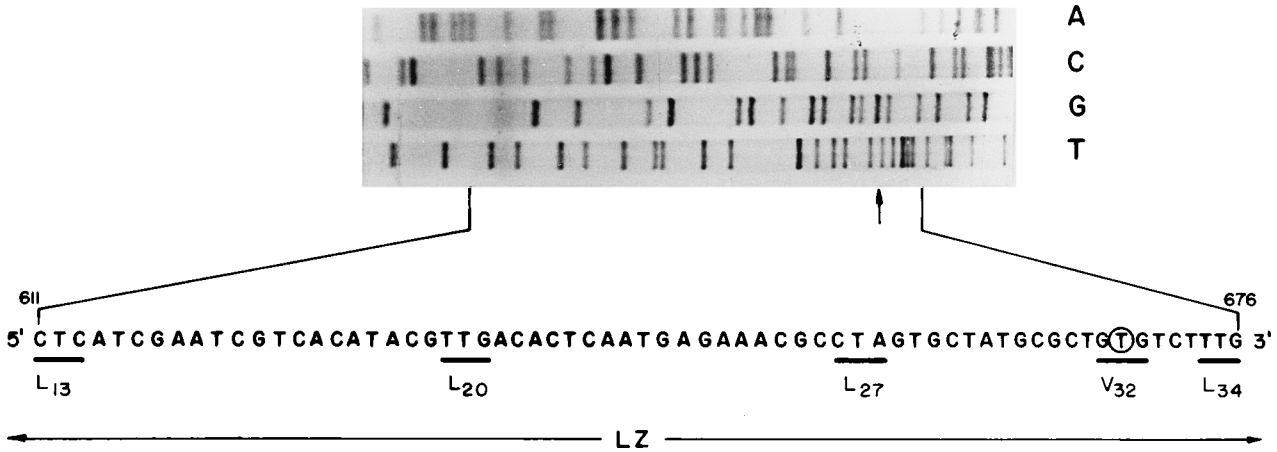

FIG. 4. DNA sequence of the coding region for the putative LZ in the *repA* gene of pMM141. The mutation introduces a C→T transition and changes the alanine codon at position 32 to a valine codon. The leucine codons in positions 13, 20, 27, and 34 that configure the motif are underlined.

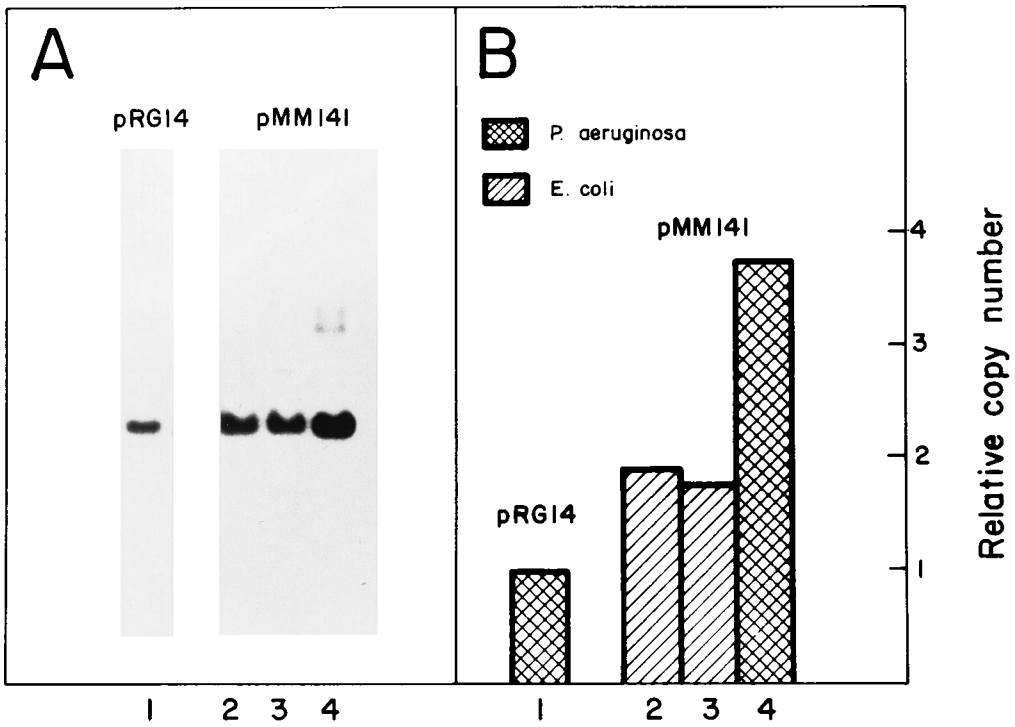

FIG. 5. Copy numbers of the host range mutant pMM141 in *P. aeruginosa* and *E. coli*. (A) Autoradiograph from total lysates of *P. aeruginosa* containing wild-type pRG14 (lane 1) or pMM141 (lane 4) and of *E. coli* BT1000 (lane 2) or C600EL (lane 3) containing pMM141. The hybridization probe was the 964-bp *Eco*RI fragment containing the *repA* gene (see Fig. 1 and 3). (B) Relative copy number of pMM141 with respect to pRG14 obtained by densitometry of the autoradiograph shown in panel A. Plasmid DNA values were corrected from chromosomal DNA variations that were estimated from the gels stained with ethidium bromide before hybridization. The relative copy number of pRG14 in *P. aeruginosa* was given a value of 1.

regulatory activity and also that the mutation has a minor effect on the repressor activity of the RepA141 protein.

Establishment of pMM141 and the wild-type pPS10 replicon in other gram-negative bacteria. pMM141 was isolated as a host range mutant able to establish efficiently in *E. coli*. To test whether pMM141 could be established in different hosts, we used electroporation as a wide-scope method to introduce plasmid DNA in bacteria. The results (Table 5) show that the pMM141 mutant could be established in *Pseudomonas putida*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, and *Paracoccus denitrificans* as well as in *E. coli*. Moreover, our results indicate that at 30°C, wild-type mini-pPS10 also can transform those bacteria, even though in some cases the efficiency is lower than that of pMM141.

TABLE 4. Repression of the *repA* promoter by RepA and Rep141

Plasmid[a] in CSH50	β-galactosidase activity (Miller units)		Inhibition (%)	
	Without IPTG	With IPTG[b]	Without IPTG	With IPTG
pFusCE	4,400			
pFusCE, pMMB*repA*	3,168	528	28	88
pFusCE, pMMB*repA*141	3,520	572	20	87

[a] pFusCE is a transcriptional fusion between the promoter of the *repA* gene and the *lacZ* reporter gene. pMMB*repA* and pMMB*repA*141 are RSF1010-based plasmids carrying the wild-type *repA* gene or the *repA* mutant gene, respectively, under the control of the ptac promoter.

[b] Expression of the proteins was induced with 1 mM IPTG.

DISCUSSION

The results presented in this paper show that the replication protein RepA of pPS10 plays a role in the determination of the plasmid host range. This is clearly indicated by the isolation of host range mutants with mutations that map in the *repA* gene. The mutation present in pMM141 introduces a conservative substitution (Ala→Val) within the LZ motif of the RepA protein. This indicates that minor genetic changes can favor the

TABLE 5. Electroporation of different bacteria with pPS10 derivatives

Bacterium	DNA (0.5 μg)	No. of transformants/viable cell
P. aeruginosa (PAO1024)	pRG14	6.7×10^{-4}
	pMM141	1.8×10^{-2}
P. putida (KT2440)	pRG14	$<1.3 \times 10^{-5}$
	pMM141	4.4×10^{-3}
E. coli (C600)	pRG14	2.0×10^{-2}
	pMM141	6.0×10^{-2}
E. aerogenes	pRG14	1.7×10^{-6}
	pMM141	2.0×10^{-5}
K. pneumoniae	pRG14	2.0×10^{-3}
	pMM141	7.4×10^{-3}
P. denitrificans	pRG14	5.0×10^{-7}
	pMM141	6.8×10^{-6}

establishment of the replicon in new hosts. The mutation also results in an increased copy number of pMM141, although autoregulation of RepA141 is largely unaffected (Table 4). On the other hand, recent gel retardation analysis indicates that the mutation has no detectable effect on the efficiency of interaction with the *oriV* region (9). These results indicate that both the increased copy number and the increased host range of pMM141 are due neither to greater expression of RepA nor to a better binding to *oriV*.

Our results show that the LZ motif of the RepA protein plays a role both in plasmid host range and in copy number control. Other data from our laboratory indicate that substitutions of Leu for Val in this motif have a deleterious effect on replication (10a), which confirms the importance of that motif in pPS10 replication. This is underlined by the fact that three independently isolated host range mutants had mutations that mapped in the same position (coordinate 669) (Fig. 4), and the fourth one mapped very close (coordinate 707) to the region coding for the motif. It will be interesting to explore whether the mutation prevents a potential inhibition of pPS10 replication modulated by RepA or improves interactions of RepA with specific host components which lead to a more efficient initiation of pPS10 replication. The LZ motif is partially conserved in the replication proteins of plasmids pSC101, R6K, and F (12). Data obtained with the pSC101 and R6K replicons confirm the role of this motif in replication. In plasmid pSC101 a mutation located in the proximity of the LZ motif of the replication protein increases the plasmid copy number (15). It remains to be tested whether this mutation affects interactions involving the LZ motif and whether it also increases the plasmid host range. In R6K a mutation in the putative LZ of the replication protein π interferes with π-π interactions that are required to form a DNA loop between the γ and β origins. That loop activates the replication of α and β origins and inactivates that of the γ origin (23). On the other hand, two different mutations of the π protein outside the LZ have recently been described as increasing the copy number by at least two different mechanisms most probably involving protein-protein interactions (24). The host ranges of all of these mutants have not been studied. Data recently obtained with the broad-host-range replicon RK2 show that genetic changes of the plasmid replication protein can also alter the host range of this replicon (3, 19).

We also have shown that the host range of the pPS10 replicon can be modified by the temperature at which the host is growing: at 30°C plasmid pPS10 can be established in a range of different gram-negative bacteria, implying that under appropriate conditions, more similar to natural environments, replication of pPS10 is not restricted to *Pseudomonas* hosts. This shows that the physiological conditions also play an important role in the establishment of plasmids in different hosts. Since interactions involving the RepA protein influence host range, it can be speculated that these interactions are prevented in *E. coli* at 37°C and favored at 30°C or below. These interactions should involve the *oriV* region and probably host replication factors.

The approach presented here can be extended to study whether barriers to the establishment of plasmid replicons in new hosts can be overcome by mutations in particular components of the host machinery involved in plasmid replication. Further analysis of broad-host-range mutants of pPS10 should also provide information on the involvement of other regions of RepA protein or *oriV* in the establishment of pPS10 in new replicons. On the whole, our data underline the genetic plasticity of the plasmid replication protein of pPS10 to accomplish particular interactions required for initiation of DNA replication in different hosts. They also underline the contribution of factors, such as temperature, that influence molecular interactions and also the physiology of the host.

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