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Modulation of pPS10 host range by DnaA

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Summary

Narrow-host-range plasmid pPS10, originally found in *Pseudomonas savastanoi*, is unable to replicate in other strains such as *Escherichia coli*. Here, we report that the establishment of pPS10 in *E. coli* can be achieved by a triple mutation in the *dnaA* gene of *E. coli* (*dna*A403), leading to Q14amber, P297S and A412V changes in the DnaA host replication protein (DnaA403 mutant). As the *E. coli* strain used contained double amber suppressor mutations (*sup*E, *sup*F), the amber codon in *dnaA*403 can be translated into glutamine or tyrosine. Genetic analysis of DnaA proteins containing either the individual changes or their different combinations suggests that the P297S mutation is crucial for the establishment of the pPS10 replicon in *E. coli.* The data also indicate that the P297S change is toxic to the cell and that the additional mutations in DnaA403 could contribute to neutralize this toxicity. To our knowledge, this work reports the first chromosome mutant described in the literature that allows the host range broadening of a plasmid, highlights the essential role played by DnaA in the establishment of pPS10 replicon in *E. coli* and provides support for the hypothesis that interactions between RepA and DnaA modulate the establishment of pPS10 in that bacteria and probably in other species.

Introduction

The establishment of a plasmid replicon in a new host depends on interactions occurring at the initiation of DNA replication. Plasmids use different strategies to increase their host range: (i) initiation independent of the host factors, as in the case of RSF1010 plasmid (Díaz and Staudenbauer, 1982; Scherzinger *et al*., 1991); (ii) versatile interaction of the initiator proteins with the origin of replication, as in the case of RK2 (Pasengrau *et al*., 1994; Doran *et al*., 1999); and (iii) improved communication between plasmid initiator proteins and host replication factors (Fernández-Tresguerres *et al*., 1995).

pPS10 is a plasmid first isolated from *Pseudomonas savastanoi* (Nieto *et al*., 1990). The 1823 bp basic replicon contains an origin of replication (*oriV*) and a gene (*rep*A) that codes for the replication initiator protein RepA of 26.7 kDa (230 amino acids) (Nieto *et al*., 1992). In the origin of replication, there are four 22-bp-long direct repeats (iterons) flanked by a DnaA box and an A+T-rich region (Fig. 1). The same configuration was reported previously for the origin of replication of plasmids that depend on the DnaA protein of the host (Bramhill and Kornberg, 1988a). Accordingly, there is a requirement of DnaA for the replication of pPS10, as has been demonstrated both *in vivo* and *in vitro* (Giraldo *et al*., 1992; Fernández-Tresguerres *et al*., 1995). On the other hand, the concomitant interaction of monomers of the RepA protein with the iterons of the origin triggers the initiation of plasmid replication (García de Viedma *et al*., 1996).

In vivo and *in vitro* experiments have revealed that pPS10 replication is dependent on host proteins such as DnaA, DnaB, DnaC or DnaK (Giraldo *et al*., 1992; Fernández-Tresguerres *et al*., 1995). Among them, DnaA is an essential protein for the initiation of prokaryotic chromosomal replication (for a review see Skarstad and Boye, 1994; Kaguni, 1997). During the replication initiation process at *ori*C, DnaA is involved in several steps (Messer *et al*., 2001): (i) recognition and binding to the origin (Fuller *et al*., 1984; Speck and Messer, 2001); (ii) opening of the DNA helix (Bramhill and Kornberg, 1988b; Gille and Messer, 1991); and (iii) recruitment of the DnaB helicase to the origin (Marszalek and Kaguni, 1994; Sutton *et al*., 1998; Seitz *et al*., 2000). In *Escherichia coli*, the *dna*A gene is located at the 83.63 minute of the chromosome, and its expression is self-regulated at the transcriptional level from two *dna*A promoters, *dna*Ap1 and *dna*Ap2 (Atlung *et al*., 1985), located 230 bp and 150 bp upstream from the transcription start codon respectively (Hansen *et al*., 1982).

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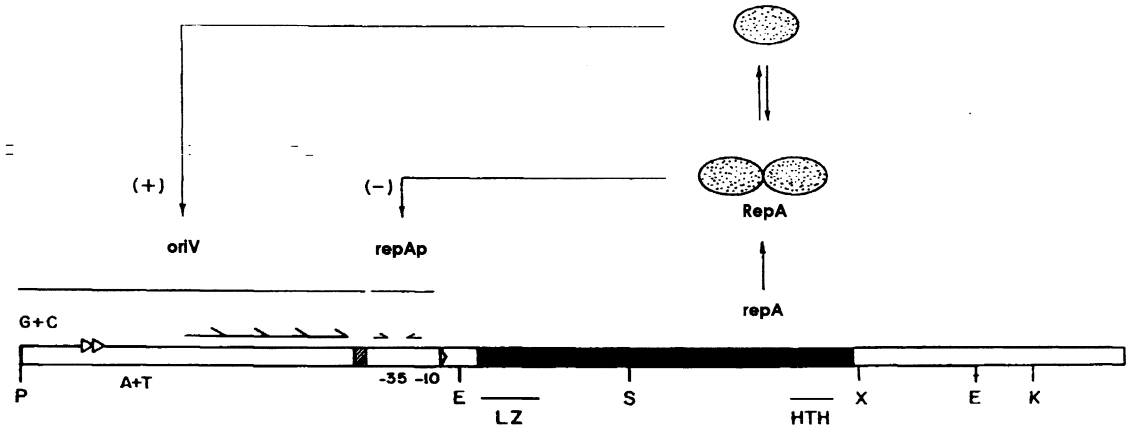

Fig. 1. Schematic representation of plasmid pPS10 minimal origin of replication. The *ori*V region contains a *dna*A box (dashed box), four identical iterons of 22 bp (→), an A+T-rich region (A+T) containing two 11-mer (>) and a G+C region (G+C). Two inverted 8-mer repeats of (→←) flank the −35 region of the promoter for *rep*A, which presents a region that codes for a leucine zipper motif (LZ) and another coding for an helix–turn–helix motif (HTH). Interactions of dimers with operator and monomers with iterons are also shown. Restriction enzymes relevant to this work: P, *Pvu*II; E, *Eco*RI; S, *Sph*I; X, *Xho*I and K, *Kpn*I.

DnaB helicase is also essential for replication initiation. This protein forms a complex with DnaC, which is loaded into the unwound *ori*C by DnaA. DnaB is also known to interact with plasmid initiation replication proteins from R6K and pSC101. Domains of interaction with all these proteins are known (Seitz *et al*., 2000).

DnaA recognizes and binds specifically to 9 bp sequences named DnaA boxes, with the stricter consensus sequence 5′-TT(A/T)TNCACA-3′ (Schaper and Messer, 1995). These sequences have been detected in the origin of replication of several prokaryotic chromosomes (Fujita *et al*., 1989; Fukuoka *et al*., 1990; Yee and Smith, 1990) and plasmids (Bramhill and Kornberg, 1988a), and their structural properties can be determinant for the plasmid host range (Shah *et al*., 1995; Doran *et al*., 1998; 1999). The concerted participation of DnaA and plasmid-encoded replication proteins (Rep) has been described in many plasmids, and its exact role in the formation of the open initiation complex depends on each replicon (Helinski *et al*., 1996). On the other hand, direct implication of DnaA as a host range determinant has been described in the case of the broad-host-range plasmid RK2: binding properties of DnaA proteins from different bacteria to the DnaA boxes in plasmid *ori*V discriminate the ability of the plasmid to replicate in such bacteria (Caspi *et al*., 2000).

pPS10 is able to replicate efficiently in *Pseudomonas aeruginosa* and *Pseudomonas putida*, but attempts to establish this plasmid in *E. coli* result in small colonies that lose viability rapidly, particularly at temperatures above 30°C (Giraldo *et al*., 1992; Nieto *et al*., 1992). This inefficient propagation has been named ‘abortive establishment’ (Giraldo *et al*., 1992). *In vivo*, neither the biosynthesis *in trans* of *Pseudomonas* DnaA protein nor the adjustment of the DnaA box of pPS10 to the consensus DnaA box sequence of *E. coli* is able to improve the marginal establishment of pPS10 in this host significantly (Giraldo *et al*., 1992; Nieto *et al*., 1992).

In a previous report, we showed that a mutation in the leucine zipper region of the pPS10 RepA protein improves the establishment of the plasmid in *E. coli* (Fernández-Tresguerres *et al*., 1995), suggesting that an appropriate interaction between RepA and host replication factors could be at the basis of the plasmid host range. Here, we show that a triple mutant DnaA protein of *E. coli* promotes the efficient establishment of the wild-type pPS10 replicon in this host. We propose that interactions between these two initiator proteins modulate the establishment of pPS10 in *E. coli* and probably in other hosts.

Results

Isolation of an E. coli *mutant that allows the establishment of the wild-type replicon pPS10*

To identify possible host factors playing a role in the establishment of pPS10 in *E. coli*, we mutagenized the strain LE392 with ethylmethanesulphonate (EMS), as described in *Experimental procedures*, and selected chromosomal mutants by their ability to be transformed efficiently with the mini-pPS10 plasmid pCN38.

EMS-treated cells were electroporated with pCN38 and plated at 30°C, 37°C and 42°C. We checked three different temperatures in order to increase the possibility of finding mutants. In many cases, we isolated colonies that grew normally in liquid medium with and without kanamycin selection, and with an apparent plasmid stability >99%, but from which we could not detect extrachromosomal DNA by the alkaline lysis method. This could be

explained if plasmid DNA had integrated in the chromosome as a result of the selective pressure and the inability of the plasmid to replicate efficiently in an autonomous way. This hypothesis was confirmed by Southern hybridization of total DNA using a probe containing the *rep*A gene (data not shown).

From the numerous colonies analysed in different series of EMS mutagenesis (>200 experiments), only one clone was found (at 30°C) from which we could isolate pCN38 by standard procedures. Moreover, the plasmid turned out to be very stable in solid medium at 30°C after 30 generations without selection: 89% of the cells maintained the plasmid under these conditions. This percentage decreased to 30% at 37°C.

Plasmid DNA extracted from that clone was used to electroporate the wild-type LE392 strain. Even after a 48 h incubation at 30°C, 37°C or 42°C, Km^r transformants formed very small colonies, as expected for the defective establishment of the wild-type plasmid. Therefore, the mutant phenotype does not result from a spontaneous mutation in the pCN38 plasmid, but from a mutation in the LE392 chromosome. This was confirmed by the reproduction of the pCN38 stabilization phenotype after transformation in a plasmid-cured clone of the mutant strain. The new strain was named LE403.

Location of the mutated gene(s) in LE403 responsible of the establishment of pPS10 replicon

pPS10 requires different host proteins to start its replication (Giraldo *et al*., 1992; Fernández-Tresguerres *et al*., 1995). Among them, DnaA and DnaB are necessary at the beginning of the replicative process of theta-type replicons and have been reported to be indispensable for pPS10 (Giraldo *et al*., 1992; Fernández-Tresguerres *et al*., 1995). It is possible that the establishment of pCN38 in LE403 is caused by a mutation in the gene coding for one of these proteins, leading to a better interaction with RepA or with the pPS10 origin of replication. Therefore, we first directed our attention to the chromosomal regions containing the *dna*A or *dna*B genes.

Bacterial mating experiments

To localize the mutations, we first performed interrupted mating experiments with different types of Hfr strains carrying a selectable marker (tetracycline resistance gene) at a known position (Fig. 2) and LE403 containing pCN38 and pBBR1CM. Crosses were made as described in *Experimental procedures* at 30°C as pCN38 is frequently lost in LE403 at higher temperatures (see above). The transfer of the wild-type locus results in the loss of pCN38. The results are shown in Table 1, and their analysis suggests that the mutated locus of LE403 lies between minutes 76 and 84 of the *E. coli* chromosome.

P1 generalized transduction

Within the chromosomal region mapped by mating, there are several genes (*gyr*B, *dna*N, *rec*F, *gid*A, *dna*A, etc.)

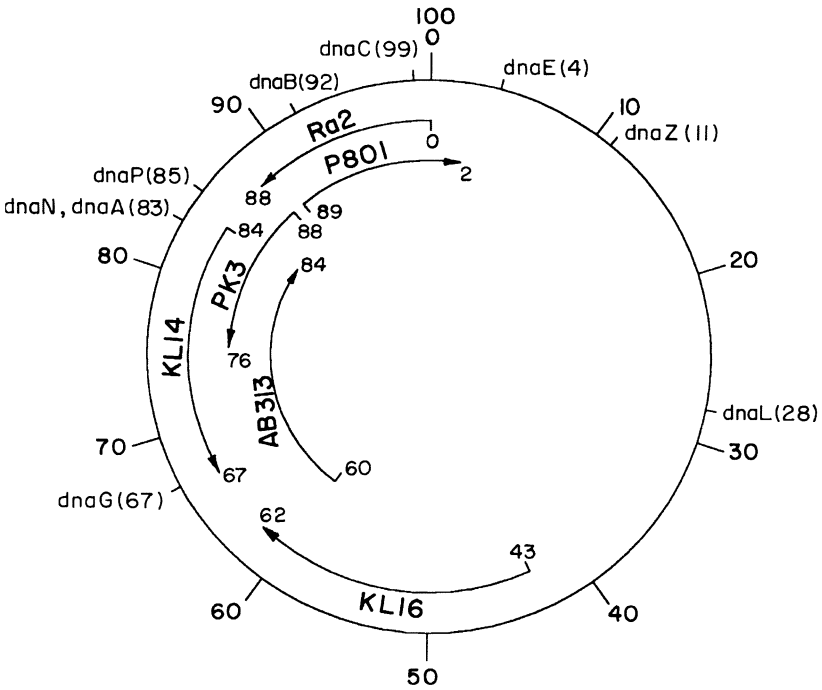

Fig. 2. *E. coli* chromosome map and Hfr strains used in mating experiments. The origin of DNA transfer and the position of the tetracycline marker injected by the Hfr are indicated. Numbers refer to the minute of the chromosome according to Messer and Weigel (1996).

Table 1. Percentage of pCN38 containing transconjugants at 30°C after mating experiments of LE403[pCN38, pBBR1CM] with different Hfr strains.

Hfr strain	Transfer		Tn*10* position (min)	% of LE403 cells that maintain pCN38 after mating
	Polarity	Origin (min)		
Control (without donor cells)				89
Ra-2	CW	88	0	82
KL16	CCW	62	43	81
P801	CCW	2	89	72
AB313	CCW	84	60	48
PK3	CW	76	88	41
KL14	CW	67	84	40

CW, clockwise; CCW, counterclockwise.

involved in replication or recombination processes that could be responsible for the mutant phenotype of LE403. To refine the mapping of the mutated gene(s), we carried out a P1 generalized transduction experiment, focusing our attention on the *dna*A gene as the main candidate. For this purpose, the P1 transducing lysate was obtained on a donor strain containing a Tn*10* transposon (Tc^r) very close to the wild-type *dna*A gene. Less than 1% of the Tc^r transductants were Km^r (i.e. retained the pPS10 plasmid), which suggests that the mutated gene(s) conferring maintenance and stability to this replicon are located very close to the tetracycline marker of CAG18499, and therefore to the *dna*A and *gyr*B genes.

Sequencing of dnaA *gene of LE403 strain*

The *dna*A gene of LE403, together with its two promoters, *dna*Ap1 and *dna*Ap2, was cloned in the pUC18 vector as described in *Experimental procedures*, yielding the pUC403 plasmid. DNA sequencing of the cloned region revealed three mutations in the coding region of *dna*A: (i) C→T (nucleotide 40). This transition generates an amber codon (UAG) that leads to the synthesis of a truncated protein in the absence of an amber suppressor. However, LE403 contains two amber suppressors (*sup*E, *sup*F), so that the UAG codon can be translated into glutamine or tyrosine respectively. This gives rise to three variants in the 14 position of the protein: Gln-14→Stop (Q14Stop), Gln-14→Tyr (Q14Y) and Gln-14→Gln (Q14Q), i.e. restoring the original amino acid. (ii) C→T (nucleotide 889). It leads to a change Pro-297→Ser (P297S). (iii) C→T (nucleotide 1235), yielding the Ala-412→Val (A412V) modification.

No mutations were found in the promoter region. As the cloning method involved the use of the polymerase chain reaction (PCR) technique, which can introduce undesired mutations in the amplified gene, cloning and sequencing of the LE403 *dna*A gene was performed again with DNA amplified from a second, independent PCR experiment, obtaining the same result. Moreover, as a control, we cloned and sequenced the *dna*A gene and its two promoters, *dna*Ap1 and *dna*Ap2, from the LE392 strain using the same protocol (plasmid pUC392), finding no mutations in the cloned gene.

Therefore, the results show that LE403 contains a triple mutant *dna*A gene (named *dna*A403) coding for a generic protein (DnaA403) that, in fact, constitutes an assembly of three different proteins, the levels of which depend on the degree of suppression at amino acid position 14 (see below).

Complementation studies

To confirm that the establishment of pCN38 in LE403 resulted from a mutant DnaA protein, we carried out complementation studies using both the wild-type and the mutant strain, and the plasmids pUC392 and pUC403, in which the expression of the genes *dna*A and *dna*A403 is directed by their own promoters. Plasmid pUC18 was used as a control of no DnaA synthesized *in trans*. It should be pointed out that pPS10 and pUC replicons are compatible, and that the stability of pCN38 in LE403 is not affected by the presence of pUC18 (Table 2). As a control, we checked the effect of pUC18, pUC392 and pUC403 on the behaviour of LE392 and LE403, without finding major differences in growth on solid or liquid medium (data not shown).

The smallest colonies correspond, as expected, to the inefficient establishment of pCN38 in LE392[pUC18, pCN38] (Fig. 3A), which is in accordance with the segregational instability of pCN38 plasmid (Table 2). The synthesis of extrachromosomal wild-type DnaA in LE392[pUC392, pCN38] induces a significant increase in colony size (Fig. 3B), even though no improvement in pCN38 stability is detected by the method used (Table 2). On the other hand, the colony size of LE392[pUC403, pCN38], in which the protein synthesized *in trans* is DnaA403 (Fig. 3C), is similar to the control LE403[pUC18, pCN38] (data not shown), although the colonies developed fully only after 36 h incubation instead of 24 h. The

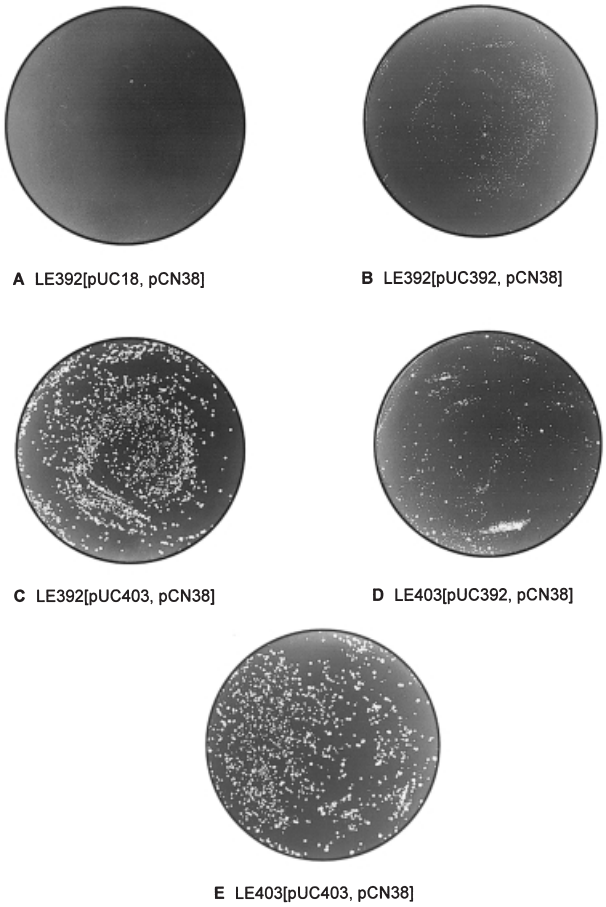

Fig. 3. Complementation experiment. Plates were photographed after 36 h of incubation at 30°C.

stability of pCN38 in the LE392[pUC403, pCN38] system reaches 20% (Table 2). This result suggests that DnaA403 is capable of emulating the phenotype of LE403 by means of complementation experiments, and that the DnaA403 protein synthesized from the pUC derivative (pUC403) exerts a 'dominant' effect over the chromosome-encoded DnaA.

Wild-type DnaA supplied by pUC392 greatly disturbs the establishment of pCN38 in LE403, as deduced by the smaller size of the clones (Fig. 3D) and the low stability of the pCN38 plasmid in these conditions (Table 2). This confirms the 'dominant' role of plasmid-encoded DnaA. Finally, it is noteworthy that an extra supply of DnaA403 to LE403 in turn produces a decrease in the segregational stability of pCN38 (from 89% to 25%). This suggests a negative effect concerning the establishment of pCN38 in LE403 arising from a possible excess of the mutant protein.

Functional analysis of the mutations found in DnaA403

Site-directed mutagenesis of dnaA gene. In order to evaluate the relative importance of the three mutations found in DnaA403 in the pCN38 establishment, we devised a site-directed mutagenesis experiment to isolate the 11 possible combinations of mutations. An ochre codon (UAA) was introduced rather than an amber codon to prevent suppression by *sup*E or *sup*F in the LE392 strain. It should be noted that an in frame ATG codon is located 23 nucleotides downstream from the TAA mutation and could lead to N-terminal truncated DnaA proteins holding the remaining mutations.

We obtained six of the plasmids listed in Table 3. The remaining mutant plasmids could not be isolated despite multiple attempts. Interestingly, all the candidates carrying a *dna*A gene with the P297S mutation and most of those with the Q14Y mutation also contained deletions or frameshifts unless, in the case of P297S, the mutation was accompanied by the Q14Ochre mutation (leading to a truncated and, probably inactive, protein). All these data suggest that, in the system used, extrachromosomal DnaA proteins displaying only the P297S or Q14Y mutations might be toxic to the cell. However, it should be taken

Table 2. Complementation experiment of *dna*A403.

Strain and plasmids	Chromosomal DnaA variant	Extrachromosomal DnaA variant	Colony size[a]	pCN38 segregational stability[b]
LE392[pUC18, pCN38]	Wild type	None	+	<1%
LE392[pUC392, pCN38]	Wild type	Wild type	++	<1%
LE392[pUC403, pCN38]	Wild type	DnaA403	++++	20%
LE403[pUC18, pCN38]	DnaA403	None	++++[c]	86%
LE403[pUC392, pCN38]	DnaA403	Wild type	+++	<1%
LE403[pUC403, pCN38]	DnaA403	DnaA403	++++	25%

a. LE392 and LE403 containing pUC392 or pUC403 were transformed with pCN38, and the colony size (represented by crosses) was investigated after 36 h of incubation at 30°C by visual inspection (Fig. 2). (+) means abortive establishment, whereas (++++) corresponds to growth of LE403[pUC18, pCN38], as a reference of maximum size.
b. Percentage of cells harbouring pCN38 after 30 generations without selection. The plasmid stability was checked as described in *Experimental procedures*.
c. Incubation time 24 h.

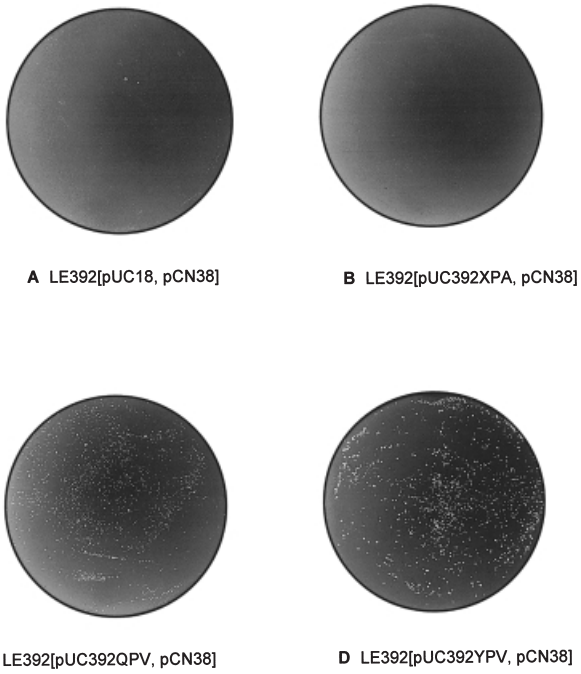

Fig. 4. Functional analysis of DnaA403 mutations by complementation. Plates were photographed after 36 h of incubation at 30°C. Only one ochre mutant is shown (Plate B) as a representative sample of the rest.

into account that cell toxicity of the triple mutant DnaA403 can be ruled out, as LE392 or LE403 cells (in the absence of pCN38) are able to grow at the same rate harbouring the pUC403 plasmid or not (data not shown).

Complementation assays with the single and multiple mutant DnaA variants. To determine the possible implication of each of the DnaA mutations on pPS10 replication in LE403, complementation experiments were carried out with each of the plasmids listed in Table 3. Figure 4 shows the morphology of the colonies at 30°C, and the stability of pCN38 in the presence of the different DnaA mutants is reflected in Table 4. The truncated DnaA(Q14Ochre) proteins (only one of which is shown as an example in Fig. 4B) are either not expressed or unable to improve the establishment of pCN38. As the results also show that neither DnaA(A412V) nor DnaA(Q14Y, A412V) is able to emulate the role of DnaA403 (Fig. 4C and D; Table 4), the P297S mutation seems to be essential for the establishment of pCN38 in the LE403 mutant.

Evidence of interaction between DnaA403 and RepA

The results shown here could be explained by a direct effect of the mutations on a possible RepA–DnaA interaction. In order to obtain an experimental basis giving support to a discussion of the results in these terms, we monitored the formation of DnaA–RepA complexes by gel filtration chromatography in Sephadex G-75, keeping track of DnaA by Western analysis. Figure 5 (top) shows that DnaA403 elutes in a volume higher than the column exclusion volume, which is in accordance with its molecular mass (51.4 kDa), below the exclusion size of the resin (≈ 75 kDa). On the other hand (Fig. 5, bottom), some DnaA403 is detected in the exclusion volume (6 ml) when mixed with RepA fused to the maltose-binding protein (MBPRepA protein, 69.4 kDa). Detection of DnaA403 in ml 6.0–6.5 occurs concomitantly with a decrease in DnaA403 protein in ml 7.5–8.0 when compared with the control (top lane). This result strongly suggests the constitution of a high-molecular-mass DnaA403–MBPRepA complex that does not dissociate during the characteristic time of chromatography.

Table 3. Site-directed mutagenesis for functional analysis of DnaA403 mutations.

Mutations in DnaA			
Amino acid 14	Amino acid 297	Amino acid 412	Plasmid obtained
Gln	Pro	Ala	pUC392 (wild type)
Amber	*Ser*	*Val*	pUC403
Ochre	Pro	Ala	pUC392XPA
Tyr	Pro	Ala	NO
Gln	*Ser*	Ala	NO
Gln	Pro	*Val*	pUC392QPV
Ochre	*Ser*	Ala	pUC392XSA
Tyr	*Ser*	Ala	NO
Gln	*Ser*	*Val*	NO
Ochre	Pro	*Val*	pUC392XPV
Tyr	Pro	*Val*	pUC392YPV
Ochre	*Ser*	*Val*	pUC392XSV
Tyr	*Ser*	*Val*	NO

Introduced mutations are shown in italics.
NO, plasmid that could not be obtained.

Table 4. Complementation experiment using DnaA mutants.

Strain and plasmids	Chromosomal DnaA variant	Extrachromosomal DnaA variant	Colony size[a]	pCN38 segregational stability[b]
LE392 [pUC18, pCN38]	Wild type	None	+	<1%
LE392 [pUC392XPA, pCN38]	Wild type	DnaA(Q14Ochre)	+	<1%
LE392 [pUC392QPV, pCN38]	Wild type	DnaA(A412V)	++	<1%
LE392 [pUC392YPV, pCN38]	Wild type	DnaA(Q14Y, A412V)	+++	<1%
LE403 [pUC18, pCN38]	DnaA403	None	++++[c]	86%

a. LE392 containing each of the pUC plasmids obtained by site-directed mutagenesis were transformed with pCN38, and the colony size (represented by crosses) was investigated after 36 h of incubation at 30°C by visual inspection (Fig. 3). (+) means abortive establishment, whereas (++++) correspond to LE403[pUC18, pCN38], as a reference of maximum size.
b. Percentage of cells harbouring pCN38 after 30 generations without selection. The plasmid stability was checked as described in *Experimental procedures*.
c. Incubation time 24 h.

Discussion

In this work, we have aimed to determine whether host factors can modulate the pPS10 host range, reporting the isolation of an *E. coli* mutant (LE403) in which the wild-type plasmid can propagate efficiently. Genetic studies strongly suggested that DnaA is responsible for the efficient initiation of replication of the mini-pPS10 plasmid pCN38 in LE403 and, in fact, DNA sequencing of the LE403 *dna*A gene revealed the presence of three changes in the related protein (Q14Amber, P297S and A412V). To our knowledge, this is the first chromosome mutant described in the literature that allows host range broadening of a plasmid.

In a strict sense, DnaA403 must be considered as a heterogeneous population of three variants when expressed in the double suppressor LE strains: (i) a truncated protein (amber codon recognized as a stop codon). Here, the presence of an in frame ATG codon 23 nucleotides downstream from the mutation might lead to a 24-amino-acid N-terminal shortened version of the mutant DnaA. (ii) A double mutant P297S/A412V (amber codon translated into wild-type glutamine by *sup*E). (iii) A triple mutant Q14Y/P297S/A412V (amber codon translated into tyrosine by *sup*F). The relative levels of each of these variants are very difficult to predict, as the levels of suppression by *sup*E and *sup*F might be variable, around 32–60% for *sup*E and 11–100% for *sup*F (Miller, 1991).

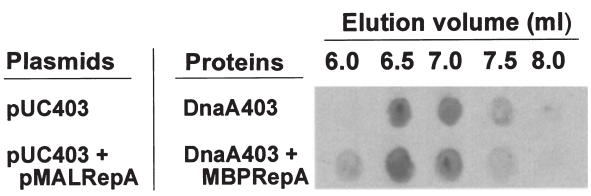

Fig. 5. Dot-blot analysis of the interaction of DnaA403 with wild-type MBPRepA by gel filtration chromatography. The exclusion volume of the column is 6.0 ml. Extracts were obtained from *E. coli* LE403 [pUC403] and CC118 [pMALRepA].

Complementation studies confirm that DnaA403 protein is responsible for efficient pPS10 propagation in LE403. When supplied *in trans* as part of a multicopy vector, the *dna*A403 allele, but not the wild type, allows the establishment and maintenance of the pCN38 replicon in the parental LE392 strain (Fig. 3B,C; Table 2). This points to a specific effect of the DnaA403 protein.

The 'dominance' effects observed when the mutated or wild-type protein is supplied *in trans* in heteroallelic situations (Fig. 3C and D, Table 2) probably reflect protein overexpression and/or repression of the chromosomal-encoded DnaA403 or DnaA protein, respectively, by the excess of heteroallelic protein. It can be argued that plasmid-encoded DnaA is not necessarily overexpressed compared with the chromosome-encoded protein, as the *dna*A and *dna*A403 genes are cloned opposite to the *lac* promoter in pUC392 and pUC403 and are supposedly only under the control of their natural promoters *dna*Ap1 and *dna*Ap2. However, the *dna*A gene with its own promoters has already been cloned in two middle-copy-number plasmids, pACYC184 (Sutton and Kaguni, 1995) and pBR322 (von Meyenburg and Hansen, 1987), and the concentration of DnaA protein was found to increase twofold and fivefold respectively. Furthermore, Braun *et al*. (1985) demonstrated that DnaA protein expressed from the chromosome is reduced in the presence of multiple copies of extrachromosomal DnaA.

The presence of plasmid-encoded DnaA403 also reduces the effect of chromosomal DnaA403, as the stability of pCN38 decreases to 25% under such circumstances (Table 2). The possible surplus of DnaA403 is therefore disadvantageous for pCN38. This result is in

accordance with the fact that LE392[pUC403, pCN38] fails to emulate the pCN38 stability values of the chromosomal mutant LE403[pUC18, pCN38] (Table 2).

Functional analysis of the DnaA403 mutations suggest that P297S is essential for pCN38 establishment in LE403

The fact that we could not isolate a DnaA variant exclusively carrying the P297S change by site-directed mutagenesis suggests that this mutation, in the absence of the other two, is toxic for the cell. This gives a clue about the striking isolation of a unique triple *E. coli* mutant containing an amber codon: the presence of such a codon could be a rather rudimentary way of controlling the DnaA403 protein levels and the type of variants synthesized. Furthermore, this analysis identified the P297S mutation as the most important for the establishment of pPS10 replicon in LE403, as neither DnaA(A412V) nor DnaA(Q14Y, A412V) emulates the role of DnaA403 (Fig. 4, Table 4). In any case, the additional participation of the other two mutations is probably also needed, as the isolation of a triple mutant as the only species is unlikely to happen fortuitously.

In our laboratory, we have isolated RepA mutants that allow the replication of pPS10 in this host without the *ori*V recognition being affected (Fernández-Tresguerres *et al*., 1995; B. Maestro *et al*., unpublished results). These data suggested that the entry of DnaA into the initiation complex might be mediated by RepA–DnaA interactions. In this sense, Fig. 5 shows the formation of a stable RepA–DnaA403 complex that can be detected by gel filtration chromatography. However, we cannot use the same approach to show differences with a putative wild-type DnaA–RepA complex, mainly because DnaA403, as described above, is not a single protein but a mixture of different species, the relative population of which is unknown and largely depends on the degree of amber suppression by the strain. These species may also display different affinities with RepA and/or different autoregulation properties. Therefore, a comparison with wild-type DnaA cannot be carried out unless single mutants are obtained, a result that has proved to be elusive in the case of the key mutant P297S (Table 3).

Glutamine 14 is located in the first of the four proposed domains for DnaA, more precisely in a domain involved in DnaA oligomerization and DnaB binding (Messer *et al*., 1999). On the other hand, proline 297 is found in the third functional domain of DnaA protein (Messer *et al*., 1999), within the region postulated as an interaction surface between DnaA and the RepA protein of pSC101 plasmid (Sutton and Kaguni, 1997). This residue is highly conserved between different species (Sutton and Kaguni, 1997), including *P. aeruginosa* (Stover *et al*., 2000). This fact explains how deleterious any mutation in this position may be, especially taking into account the rigidity induced in a polypeptide chain by this amino acid that can hardly be emulated by other residues, and suggests an alternative mechanism of participation in pPS10 replication of DnaA mutants containing the P297S change. Finally, alanine 412 is located in one of the two recognition helices of the fourth functional domain of DnaA, described as the DNA-binding region (Roth and Messer, 1995; Schaper and Messer, 1997). Many changes have been detected in this region that are affected in DNA binding (Sutton and Kaguni, 1997); for example, the A412P mutation leads to an important reduction in DNA binding affinity. It could be possible that DnaA403 protein, by means of the A412V mutation, is affected in DNA binding, and so in replication initiation and/or in autoregulation. In any case, it is difficult to envisage the effects of the P297S and A412V mutations at the molecular level until the three-dimensional structures of DnaA and DnaA complexes with other proteins and with the DNA are solved.

Although the hypothesis of a DnaA–RepA interaction altered by mutation is attractive, we cannot rule out the effect on other events taking place in the initiation complex. For example, a weakened DnaA–DnaB helicase interaction might in turn promote the loading of increased amounts of unbound DnaB into the complex by RepA.

In summary, this work reveals the important participation of DnaA protein in the pPS10 host range extension to *E. coli*, probably favouring the formation of a productive initiation complex with the plasmid replication protein RepA, and indicates that the DnaA-mediated establishment of a replicon can take place by alternative mechanisms depending on the species. The biochemical analysis of these interactions remains to be elucidated, but the availability of RepA and DnaA mutants favouring the establishment of pPS10 in *E. coli* gives important clues and now makes such analysis feasible.

Experimental procedures

Bacterial strains and plasmids

Escherichia coli K-12 strains used were: LE392 (F^-, *mcr*A, *met*B1, *trp*R55, *sup*E44, *sup*F58, *lac*Y1, *galKam*2, *gal*T22, *hsd*R514; Borck *et al*., 1976); BW6175 [HfrPK3 (PO 131), *thr*-1, *leu*B6, *azi*-15, *ton*A21, *lac*Y1, *sup*E44, λ^-, *arg*E::Tn*10*, *thi*-1; Wanner, 1986]; BW6169 [HfrAB313 (PO 13), *leu*B6, *lac*Y1, *lac*Z4, *sup*E44, *gal*-6, λ^-, *arg*A::Tn*10*, *thi*-1; Wanner, 1986]; BW6165 [Hfr801 (PO 120), *ara*-41, *lac*Y1, *lac*Y40, λ^{ind-}, *xyl*-7, *mtl*-2, *arg*E::Tn*10*; Wanner, 1986]; BW6164 [HfrRa-2 (PO 48), *mal*-28, *sfa*-4, *sup*E42, λ^-, *thr*-43::Tn*10*; Wanner, 1986]; BW6159 [HfrKL14 (PO 68), *rel*A1, *thi*-1, *spo*T1, λ^-, *ilv*$^-$::Tn*10*; Wanner, 1986]; BW6163 [HfrKL16 (PO 45), *rel*A1, *thi*-1, *spo*T1, λ^-, *zed*-977::Tn*10*; Wanner, 1986]; and CAG18499 (F^-, *rph*-1, zid-501::Tn*10*; Singer *et al*., 1989). Plasmids used were: the mini-pPS10 derivative

pCN38, Km^r (Nieto *et al*., 1990), pBBR1CM, Cm^r (Antoine and Locht, 1992), pUC18, Ap^r (Yanisch-Perron *et al*., 1985) and pMALRepA, a derivative of pMAL-c2 (New England Biolabs) with the *rep*A gene inserted in an in frame fusion with the maltose-binding protein, coding for the hybrid protein MBPRepA. This fusion has been shown to retain the functionality of unfused RepA (García de Viedma *et al*., 1996)

Media and growth conditions

Cultures were grown in LB medium (Sambrook *et al*., 1989). When required, media were supplemented with antibiotics as follows: ampicillin (Ap) 100 μg ml^{-1}; chloramphenicol (Cm) 20 μg ml^{-1}; tetracycline (Tc) 10 μg ml^{-1}; and kanamycin (Km) 50 μg ml^{-1}. Soft agar was prepared as LB medium containing 0.7% agar. LB medium containing 2.5 mM sodium citrate is cited in the text as LBC.

General methods

Plasmid DNA was purified using the Qiagen miniprep kit. Transformation of *E. coli* cells was carried out by electroporation as described by Dower *et al*. (1988). Restriction enzymes were from New England BioLabs, Roche Diagnostics and Amersham Biosciences. Polymerase *Pfu* was from Stratagene. Cloning procedures and gel electrophoresis of DNA were performed as described by Sambrook *et al*. (1989). Primer oligonucleotides were synthesized in a Beckman DNA synthesizer 1000M, desalted and used without further purification. DNA sequencing was carried out in a Perkin-Elmer ABI-Prism 377 DNA sequencer using fluorescent chain terminators.

Chromosome mutagenesis

Random mutagenesis of LE392 chromosome was carried out by treatment with ethylmethanesulphonate (EMS). A stationary phase culture (100 μl) of the strain was mixed with 3 ml of soft agar and plated onto LB agar. Then, 10 μl of EMS (Sigma) was spotted in the centre of the plate. After overnight incubation at 30°C, 37°C or 42°C, bacteria from the halo surrounding the spot were scraped and cultured in 5 ml of LB medium, from which competent cells were prepared and transformed with pCN38. To test the mutant phenotype of each selected colony, the presence of plasmid DNA was checked by standard purification methods and its segregational stability verified as described below. The efficiency of the mutagenesis was tested by comparing the frequencies of colonies resistant to nalidixic acid (20 μg ml^{-1}) before and after treatment. Taking the frequency of appearance of spontaneous mutations as 5×10^{-9}, the EMS treatment was considered effective when the frequency of Nal^r mutants was increased by a factor of 10–100.

Plasmid construction

Plasmids pUC392 and pUC403 were constructed as follows: chromosomal DNA from the LE392 or LE403 strains was isolated by lysis of exponential phase cultures as described by Projan *et al*. (1983), followed by proteinase K digestion, phenol treatment and ethanol precipitation. The pellet was dissolved in water. The DNA fragment containing the *dna*A gene, the *dnaA*p1 and *dnaA*p2 promoters and a ribosome binding site (RBS) sequence was amplified by PCR using oligonucleotides 5′-TGGTCATTAAAGGATCCAATATGCGG CG-3′ and 5′-CTCACGTTCTACGGATCCTTTCATAGGT-3′ as primers (*Bam*HI restriction site is underlined). The PCR product was purified from agarose gels ('Geneclean', BIO-101), digested with *Bam*HI and cloned into the *Bam*HI site of the pUC18 plasmid. Integrity of the constructions was checked by DNA sequencing. It is noteworthy that, in all clones analysed, the fragments containing wild-type or mutant *dna*A gene were cloned opposite to the transcription direction from the *lac* promoter.

Site-directed mutagenesis

Oligonucleotide-directed mutagenesis was performed by the method of Deng and Nickoloff (1992) using the USE mutagenesis kit from Amersham Biosciences. The template was plasmid pUC392, and the oligonucleotides used were: 5′-CTTGCCCGATTGTAAGATGAGTTACC-3′ (mutation Q14Stop); 5′-CTTGCCCGATTGTACGATGAGTTACC-3′ (mutation Q14Y); 5′-CGATCGAACCGTCAGAGCTGGAAA CC-3′ (mutation P297S); 5′-AGATGGCGATGGTGCTGGCGAAA GA-3′ (mutation A412V).

In order to obtain double or triple mutants, either two or three of the oligonucleotides depicted above were hybridized simultaneously with the template, or one of the single or double mutants was hybridized with one of these three oligonucleotides.

Plasmid stability

In order to check the segregational stability of plasmid pCN38, the culture was grown in kanamycin-containing LB medium to an OD_{600} of 0.4. Then, 100 μl of a 10^{-5} dilution was spread on LB plates without antibiotic. After overnight incubation, 100 independent colonies were patched on LB and LB+kanamycin plates in order to determine the percentage of colonies that retain the plasmid after overnight incubation without selection (i.e. approximately 30 generations, Fernández-Tresguerres *et al*., 1995).

Mapping of the mutations

Interrupted mating. Nine hundred microlitres of an exponential phase culture of LE403 recipient strain containing the plasmids pCN38 (conferring resistance to kanamycin) and pBBR1CM (a compatible plasmid with a chloramphenicol resistance gene) were mixed with 100 μl of an exponential phase culture of one of the Hfr strains containing a Tn*10* (Tc^r marker). The cell mixture was incubated in a 25 ml flask without agitation for 1 h at 30°C. After this time, 5 ml of ice-cold 10 mM $MgSO_4$ was added, and the mixture was shaken vigorously in order to interrupt the mating. Upon centrifugation for 5 min at 3000 *g* at 4°C, the cell pellet was washed with 5 ml of 10 mM $MgSO_4$ and resuspended in 1 ml of the

same solution. One hundred microlitres of three different dilutions, mixed with 3 ml of melted soft agar, were spread onto LB plates containing chloramphenicol and tetracycline to select the Cm^r recipients that acquired the Tc^r marker from the donor. After incubation at 30°C for 24–48 h, 100 of the Cm^r Tc^r recombinants were patched on LB+Cm+Tc+Km, LB+Cm+Tc and LB plates in order to determine the percentage of transconjugants that maintain the pCN38 plasmid (Km^r).

As a control, the same assay was carried out with 900 μl of the recipient strain and 100 μl of LB. The percentage of Km^r colonies in this case was used as a reference for the experiments above.

P1-mediated generalized transduction. A P1 lysate was prepared on the *E. coli* strain CAG18499, which contains a Tc^r marker (*zid*-501::Tn*10*) that is 90% co-transducible with *dna*A and *gyr*B (Singer *et al*., 1989). A sample of 100 μl of a stationary phase culture of the donor strain was mixed with 4 ml of soft agar, 2.5 mM $CaCl_2$ and 100 μl of P1 phage (10^8 pfu ml^{-1}). The mixture was plated on LB containing 2.5 mM $CaCl_2$. After incubation at 37°C for 7 h, the soft agar layer was scraped into a centrifuge tube; 2 ml of LB was added, and the suspension was centrifuged at 7700 *g* for 15 min. The supernatant (P1 lysate with transducing particles) was recovered and stored with 25 μl of chloroform. Phage lysates with titres ranging from 10^9 to 10^{10} pfu ml^{-1} were used.

For the transduction assay, 4.7 ml of LB containing 2.5 mM $CaCl_2$ and 100 μl of the transduction lysate was mixed in a 25 ml flask and incubated at 37°C for 10 min in order to evaporate the chloroform. Then, 200 μl of a 10-fold concentrated stationary phase culture of the receptor strain containing the pCN38 plasmid was added, and the mixture was incubated at 30°C for 20 min. After adsorption, cells were pelleted and washed with 5 ml of ice-cold 50 mM sodium citrate in order to stop infection. Finally, cells were resuspended in 5 ml of LBC and incubated at 30°C for 45 min to allow the expression of the Tc^r determinant. After that time, cells were washed with 5 ml of ice-cold 50 mM sodium citrate and centrifuged for 5 min at 3000 *g*. The pellet was resuspended in 1 ml of the same solution, and 100 μl of 10^{-1} and 10^{-2} dilutions was plated on LBC+Tc plates. After incubation at 30°C for 24–48 h, 100 colonies were patched on LBC+Tc+Km plates, on LBC+Tc plates and on LBC in order to determine the number of colonies that maintain the pCN38 plasmid.

Gel filtration analysis of the interaction between DnaA403 and MBPRepA

A sample of 75 μl of a 25-fold concentrated extract of a stationary phase LE403 [pUC403] culture (OD_{600} = 3.5) grown at 30°C was mixed with either 25 μl of an extract of a 75-fold concentrated extract of a stationary phase CC118[pMALRepA] culture (OD = 2.2) grown at 37°C or 25 μl of 20 mM sodium phosphate buffer, 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 × 0.9 cm) equilibrated in 20 mM sodium phosphate buffer, pH 7.0, plus 150 mM NaCl and run with the same buffer at 20°C and a flow rate of 1 ml min^{-1}. Exclusion (6.0 ml) and total (21.0 ml) volumes were determined with dextran blue and potassium dichromate respectively. Fractions (500 μl) were collected, and 300 μl of each fraction was subjected to dot-blot analysis using the ECL kit (Amersham Biosciences) and rabbit anti-DnaA antibodies in a 1:25 000 dilution (kindly supplied by Drs W. Messer and H. Seitz, Max-Planck-Institut für Molekulare Genetik, Berlin-Dahlem, Germany).

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