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The heat-shock DnaK protein is required for plasmid R1 replication and it is dispensable for plasmid ColE1 replication

Rafael Giraldo-Suárez, Elena Fernández-Tresguerres, Ramón Díaz-Orejas*, Abderrahim Malki[1] and Masamichi Kohiyama[1]

Centro de Investigaciones Biolgicas (CSIC), C/ Velázquez 144, 28006 Madrid, Spain and [1]Institut Jacques Monod (CNRS), Université Paris VII-Tour 43, 2 Place Jussieu, 75251 Paris Cedex 05, France

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ABSTRACT

Plasmid R1 replication *in vitro* is inactive in extracts prepared from a *dnaK756* strain but is restored to normal levels upon addition of purified DnaK protein. Replication of R1 in extracts of a *dnaKwt* strain can be specifically inhibited with polyclonal antibodies against DnaK. RepA-dependent replication of R1 in *dnaK756* extracts supplemented with DnaKwt protein at maximum concentration is partially inhibited by rifampicin and it is severely inhibited at sub-optimal concentrations of DnaK protein. The copy number of a run-away R1 vector is reduced in a *dnaK756* background at 30°C and at 42°C the amplification of the run-away R1 vector is prevented. However a run-away R1 vector containing *dnaK* gene allows the amplification of the plasmid at high temperature. These data indicate that DnaK is required for both *in vitro* and *in vivo* replication of plasmid R1 and show a partial compensation for the low level of DnaK by RNA polymerase. In contrast ColE1 replication is not affected by DnaK as indicated by the fact that ColE1 replicates with the same efficiency in extracts from *dnaKwt* and *dnaK756* strains.

INTRODUCTION

Heat-shock proteins of *E.coli* are a group of proteins subject to *hptR*-dependent induction upon shifting to high temperature (1). DnaK is a major heat-shock protein in *E.coli*, and is the prokaryotic analogue of the eukaryotic Hsp70 protein (2). DnaK, either alone or in combination with two other heat-shock proteins, DnaJ and GrpE, is involved in a variety of important cell functions, including DNA replication (1). The implication of DnaK in the replication of extrachromosomal elements has been analyzed in detail in *E.coli* using bacteriophage λ DNA, plasmids P1 or F.

During λ phage DNA replication the lambda P protein brings the DnaB helicase to the origin of replication but prevents its activity until DnaK releases the P protein from the preinitiation complex (3, 4, 5, 6). During P1 replication DnaK acts by converting a dimeric inactive form of the initiation protein RepA into a monomeric active form that efficiently binds to the origin of replication (7, 8). The DnaK protein is also required for activation of the plasmid F RepE initiation protein prior or during its binding to the replication origin (9, 10). DnaK can also physically interact with the DnaA protein (11, 12) and promotes the conversion of an inactive agregate of the protein into an active DnaA form containing ATP (13, 14).

R1 is an antibiotic resistant plasmid of gram-negative bacteria that, unlike lambda, P1 and F replicons, does not contain iterons in its minimal origin of replication, oriR (15). Initiation of R1 replication requires a plasmid specific protein, RepA (16, 17), that interacts with a 100 bp region within oriR and promotes the sequence-specific binding of DnaA to a binding site (dnaA box) present in oriR (16, 18). The initial RepA–oriR interactions, that leads to DNA looping, may involve a dimeric or tetrameric form of RepA (19). *In vitro* replication of plasmid R1 is DnaA-dependent (16, 20) but, in contrast with oriC, the ATP form of this protein is not required for R1 replication (21). *In vivo* replication of plasmid R1 can occur in the absence of the DnaA protein. This DnaA independent replication, is very inefficient and requires RepA and oriR (22, 23). The requirement for DnaC and DnaB in R1 replication (16, 20) suggests that DnaB is transfered to *oriR* as a DnaC–DnaB complex. Thus, replication of plasmid R1 differs at the initiation stage from lambda DNA, plasmids P1, F, or chromosomal (oriC) replication. In contrast to the replication of the above elements, replication of ColE1 is not promoted by any plasmid-encoded protein. ColE1 replication can occur efficiently in the absence of DnaA (24) and requires DnaB and DnaC proteins at an early stage (25, 26). This makes improbable a role of DnaK in ColE1 replication. DnaK is not required in the reconstituted *in vitro* replication system of plasmid ColE1 (27).

Using DnaK protein-deficient extracts and *in vitro* replication systems we demonstrate in this report that DnaK is required for

* To whom correspondence should be addressed

replication of plasmid R1 and confirm its dispensability for ColE1 replication. Furthermore we present evidence for the involvement of DnaK in plasmid R1 replication *in vivo*.

MATERIAL AND METHODS

Bacterial strains and plasmids

E.coli K12 strains C600 (wild-type reference) (28) and WM1288 (*dnak756* thermosensitive mutant) (29) were used in all experiments. The following plasmids were used: *copA* mini-R1 plasmid pKN177 (30), *copB* mini-R1 plasmid pET80 (31), R1 run-away vector pMOB45 (32) and its pMOB45-*dnaKwt* recombinant (33), ColE1 plasmid (34), and the λdv derivative pCB44 (35).

Purification of the RepA and DnaK proteins

Purification of RepA protein of plasmid R1 was performed as described by Giraldo and Díaz (19). The RepA preparation used contained 1.5 μ/μl of protein and was 80% pure. Purification of DnaK protein was done as described (33). The preparation of DnaK protein was >90% pure; the stock solution was at a concentration of 5 μg of DnaK/μl.

Antibodies against RepA, DnaK or DnaA proteins

Polyclonal antibodies against RepA were obtained following the method of Tjian *et al.* (36) using a band of RepA obtained after PAGE of a partially purified sample of protein as immunogen. The immune serum obtained was purified from other *E.coli* contaminating antigens by immunoprecipitation with a crude extract of *E.coli* C600, made in 10 mM Tris−HCl pH=7.5, 140 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1mM p-aminobenzamidine, 1% Triton X-100, 1% Na-deoxycholate, 0.025% Na-azide and 1% BSA, (1:1 ratio). The RepA specificity of the resulting antisera was tested by Western-blot analyses performed according to the specifications of Bio-Rad (Bio-Rad Laboratories). The samples were stored at −70°C.

The preparation of both anti-DnaA and anti-DnaK polyclonal antisera was performed as described (11).

In vitro replication assays

Extract type I was prepared from bacteria grown at 30°C by freezing and thawing (37, 38). Extracts type II were prepared from extracts type I by precipitation of the proteins with 60% or 30% saturation in ammonium sulphate, followed by dialysis against 500 times volume of 50mM HEPES pH 8.0, 50 mM KCl, 1mM EDTA, 1 mM DTT. Extracts type I or type II were stored frozen at −70°C. *In vitro* replication assays of R1 were carried out for 60 minutes at 30°C, in the presence of purified RepA protein, using as DNA template a supercoiled R1 miniplasmid. The composition of the assay mixtures have been described (20, 30). *In vitro* replication of plasmid R1 requires the RepA initiator protein. Extracts type I can synthesize proteins in a coupled transcription−translation reaction; the RepA protein is synthesized *de novo* in these extracts and used to replicate the R1 template. Extracts type II are deficient in synthesis of proteins and replication of plasmid R1 in these extracts requires the addition of purified RepA protein to the assay mixtures. When antisera against DnaK, RepA or DnaA were used, the complete replication mixtures were preincubated for 20 minutes on ice with an appropriate dilution of the antibodies before incubation at 30°C. The *in vitro* replication reactions were evaluated by quantitating the amount of radioactive ^{3}H-dCTP incorporated into acid insoluble material.

Determination of plasmid copy number

Whole cell lysates were prepared as described (39) from cells containing plasmid grown at 30°C or shifted from 30°C to 42°C for two and a half hours. The samples were electrophoresed on 0.8% agarose-TAE gels at low voltage as described (40). The amount of plasmid and chromosomal DNA in each lane was evaluated by densitometry analysis (LKB Ultroscan) and the plasmid copy number per genome equivalent was calculated as described (41).

Transformation

Transformation was performed by the $CaCl_2$ method, according to Lederberg and Cohen (42).

RESULTS

In vitro replication studies: DnaK is required for plasmid R1 replication and is dispensable for replication of ColE1 plasmid

In vitro replication of plasmid ColE1 or R1 was first assayed in type I extracts prepared from *E.coli* WM1288 (*dnaK756*) or from *E.coli* C600 (*dnaKwt*). The mutation *dnaK756*, that confers thermosensitivity to the cells, was selected because purified DnaK756 protein has been shown to be unable to release λ P protein from the preinitiation complex (43). Our results demonstrated that type I extracts prepared from the *dnaKwt* strain were active in the *in vitro* replication of both ColE1 and R1, whereas extracts prepared from the *dnaK756* strain, efficiently replicated ColE1 but not R1 (Fig. 1A and 1D).

All attempts to restore replication of plasmid R1 in type I extracts from *dnaK756* strains by addition of purified DnaK protein were unsuccessful. Nor could replication of plasmid R1 be obtained in 60% ammonium sulphate fraction of the above extract in the presence of purified RepA and DnaK proteins. As previously reported (20), the 60% ammonium sulphate fraction of the wild-type extract replicates efficiently R1 in the presence of RepA (data not shown). This suggested that in the absence of active DnaK the extracts may accumulate an inhibitor of R1 replication. We therefore attempted to remove this inhibitor by ammonium sulphate fractionation. To this aim we prepared a 30% ammonium sulphate fraction of type I extracts from the *dnaK756* strain. As a control a 30% ammonium sulphate fraction was also prepared from type I extract of the wild-type strain. The 30% ammonium sulphate fraction corresponding to the wild- type strain supported *in vitro* replication of plasmid R1 when supplemented with RepA protein (Fig. 1E). The corresponding fraction from the *dnaK756* strain was unable to promote replication of plasmid R1 in the presence of RepA. However, addition of RepA and DnaK proteins to this extract promoted efficient replication of plasmid R1. Up to 4.0 μg of purified DnaK protein per assay give an optimal complementation and addition of higher amounts of DnaK reduced the efficiency of replication (Fig. 1B). These results clearly indicated that R1 replication *in vitro* was dependent on DnaK. This conclusion was further supported by the fact that RepA dependent replication of plasmid R1 promoted by the 30% ammonium sulphate fraction corresponding to the wild-type strain was specifically inhibited by addition of polyclonal antibodies against DnaK protein (Fig. 1E).

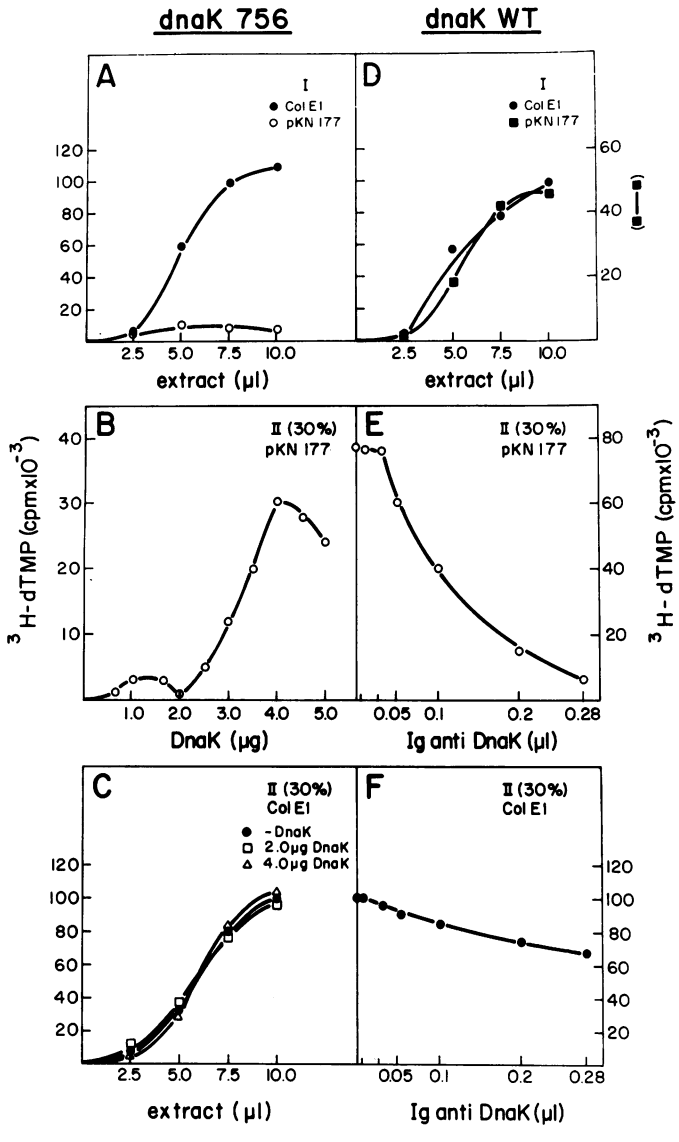

Figure 1. Requirement for DnaK in replication of mini-plasmid R1 pKN177 *in vitro* and dispensability of this protein for the replication of ColE1 *in vitro*. A–C, assays carried out with extracts from the *dnaK756* strain: **A)** *In vitro* replication of ColE1 and pKN177 with increasing amounts of extract type I, **B)** complementation of pKN177 replication in 30% ammonium sulphate fraction (7.5 µl extract) with increasing amounts of DnaK. **C)** Effect of DnaK protein on the replication of ColE1 titrated with 30% ammonium sulphate fraction. D–F, *in vitro* replication assays carried out with extract from the *dnaKwt* strain: **D)** *in vitro* replication of pKN177 and ColE1 plasmids at increasing amounts of type I extracts. **E)** Effect of polyclonal antibodies against DnaK on the *in vitro* replication of pKN177 in 30% ammonium sulphate fraction. **F)** Effects of polyclonal antibodies against DnaK on the *in vitro* replication of ColE1 plasmid in 30% ammonium sulphate fraction. The type of extract used in each assay is indicated on the upper-right region of the graphic: I stands for extracts type I and II (30%) indicates extracts type II, 30% ammonium sulphate fraction.

In contrast to R1 replication, ColE1 *in vitro* replication was equally efficient in the presence of type I extracts corresponding either to *dnaKwt* or *dnak756* strains or in 30% ammonium sulphate fractions of these extracts. Addition of DnaK (2 or 4 µg/assay) had no effect on ColE1 replication as tested using various amounts of the 30% ammonium sulphate fraction corresponding to the *dnaK756* strain (Fig. 1C). Finally, replication of ColE1 in extracts from the *dnaKwt* strain (30% ammonium sulphate fraction) was not specifically inhibited by polyclonal antibodies against DnaK (Fig. 1F). These results indicated that the replication of ColE1 *in vitro* was independent of DnaK.

Table I. Characterization of R1 replication in the type II, 30% ammonium sulphate fraction from a *dnaK756* strain supplemented with different amounts of DnaK

Addition (+)/omission (−)	R1 replication (%) 4.0 µg DnaK	2.5 µg DnaK
None	100[a]	100[b]
+ Ig anti-DnaA	7	nd[c]
+ Ig anti-RepA	5	nd
− RepA	5	nd
+ Novobiocin 4	nd	nd
+ Rifampicin	40	20

The R1 miniplasmid used as DNA substrate in the assays was pKN177. a: 100% R1 replication corresponds to 72 pMols of ^{3}H-dCMP incorporated into pKN177 DNA. b: 100% R1 replication corresponds to 10 pMols of ^{3}H-dCMP incorporated into pKN177 DNA. c: not determined.

Characterization of the DnaK-dependent replication of plasmid R1

Replication of plasmid R1 in *dnaK756* extracts (30% ammonium sulphate fraction), supplemented with optimal amounts of DnaK and RepA proteins, was dependent on the DnaA protein, as indicated by the specific inhibition of DNA synthesis observed when the extracts were incubated with polyclonal antibodies against DnaA protein (Table I). Data shown in Table I also indicated that this replication was strictly dependent on the addition of RepA to the assays and that in the presence of this protein replication was inhibited by polyclonal antibodies against RepA. *In vitro* replication of plasmid R1 in these extracts was inhibited to background levels by novobiocin (25 µg/ml), an antibiotic that inhibits DNA gyrase. This indicated that initiation of R1 replication required negative supertwists in the template. All these features indicated that the complementation observed corresponded to specific and semiconservative R1 replication. Similar results were obtained when the assays were carried out with the 30% ammonium sulphate fraction of cultures from wild-type strain (not shown). However inhibition of RNA polymerase by rifampicin had different effects on R1 replication in *dnaKwt* and *dnaK756* extracts (30% ammonium sulphate fractions): RepA-dependent replication of plasmid R1 was resistant to 25 µg/ml rifampicin in *dnaKwt* extracts whereas the same concentration of the antibiotic inhibited more than 60% R1 replication in *dnaK756* extracts supplemented with an optimal amount of DnaK protein (4.0 µg per assay). This inhibition increased to 80% when the assay was done with sub-optimal amounts of DnaK (2.5 µg). These results suggested that transcription by RNA polymerase played an important role in replication of plasmid R1 in DnaK deficient conditions.

In vivo amplification of R1 DNA at high temperature is DnaK-dependent

The involvement of DnaK on the *in vivo* replication of plasmid R1 was examined by using a temperature dependent copy mutant of R1, pMOB45, and its derivative containing the *dnaK* gene. A shift of temperature from 30°C to 42°C results in amplification of this copy mutant (32). If DnaK is required for R1 replication it is expected that pMOB45 would not be amplified in the *dnaK756* background at 42°C. However, the pMOB45-*dnaKwt*

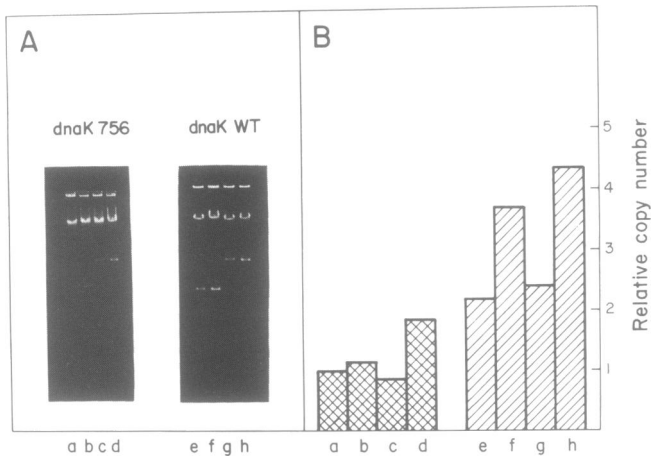

Figure 2. Replication of the pMOB45 vector and pMOB45-dnaKwt recombinant at 30°C and 42°C in cultures of *dnaKwt* or *dnaK756* strains. **A)** Gel electrophoresis analysis developed with ethidium-bromide of total lysates of cultures containing the pMOB45 vector (tracks a, b, e, f) or the pMOB45-*dnaKwt* recombinant (tracks c, d, g, h) that were grown at 30°C throughout (tracks a, c, e, g) or shifted for two and a half hours to 42°C (tracks b, d, f, h). **B)** Ratio between plasmid and chromosomal DNA (relative copy number) obtained from densitometric analyses of samples a–h shown in section A. Value 1 was given arbitrarily to the relative copy number corresponding to sample a.

Table II. Transformation frequencies of R1 miniplasmid pET80, pBR322 and ldv plasmids pCB44 in *dnaKwt* or *dnaK756* backgrounds

Strain	Transformation frequency		
	pET80	pBR322	pCB44
C600 (*dnaKwt*)	$1{,}4.10^{-4}$	$2{,}2.10^{-4}$	$1{,}1.10^{-4}$
WM1288 (*dnaK756*)	$0{,}8.10^{-4}$	$1{,}0.10^{-4}$	$<1{,}6.10^{-6}$

The number of transformants per viable cell corresponding to 11 fmols of DNA is shown. The temperature used to grow the cells and to select the transformants was in all cases 30°C.

recombinant should complement the *dnaK756* mutation and this complementation should allow amplification of this recombinant at high temperature. The results obtained (Fig. 2) confirm this hypothesis: the pMOB45 vector and the pMOB45-*dnaK* recombinant are amplified at 42°C in the *dnaKwt* background, but in the *dnaK756* background only the pMOB45-*dnaK* recombinant is amplified at the non-permissive temperature. These results underline the requirement of DnaK for *in vivo* replication of plasmid R1 at high temperature. This experiment also shows that, at 30°C, the copy number of the R1-miniplasmid pMOB45 is higher in the *dnaKwt* background than in the *dnaK756* background (Fig. 2B). This indicated that at 30°C the *dnaK756* mutation is already limiting the availability of DnaK for R1 replication and suggests that, *in vivo*, and in the absence of thermal stress, DnaK is also involved in the replication of plasmid R1.

Differential effects of the dnak756 allele on the efficiencies of transformation between mini-R1 and λdv DNAs

Further evidence for the DnaK requirement for *in vivo* replication of plasmid R1 was obtained by comparing the efficiencies of transformation of the R1-miniplasmid pET80 in *dnaKwt* to those in *dnaK756* background. These efficiencies were also compared to those obtained with similar DNA amounts from the λdv derivative pCB44, a DnaK dependent replicon, and from the DnaK independent replicon pBR322. Because the *dnak756* allele confers thermosensitivity to the host, expression of the transformation mixtures and selection of the transformants were done at 30°C. The results obtained (Table II) show that while the R1 miniplasmid transforms with similar efficiencies the *dnaKwt* and the *dnaK756* strains, the λdv plasmid transforms at least 100 fold more efficiently the *dnaKwt* background than the *dnaK756* background. This indicates that, at 30°C, the *dnak756* mutation reduces the level of active DnaK protein below that required for efficient establishment of the λ replicon, and that these reduced levels do not appear to be limiting the establishment of the R1 mini-plasmid by transformation.

DISCUSSION

In this communication we have shown that the DnaK protein participates in R1 replication both *in vitro* and *in vivo*, and that DnaK is dispensable for ColE1 replication. *In vitro* replication studies have demonstrated that replication of plasmid R1 can not be observed in *dnaK756* extracts, and that addition of purified DnaK protein to the replication assay restores R1 replication. This result clearly indicates the requirement of DnaK for R1 replication. In contrast, *in vitro* replication of ColE1 is equally efficient in extracts prepared from the *dnaK756* strain and in extracts prepared from the *dnaKwt* strain and is not affected by the presence of purified DnaK protein in these assays. This shows clearly that the enzymatic machinery involved in ColE1 replication (26), most of which is used for R1 replication (16, 17), does not require activation by DnaK and is consistent with the observation that the reconstituted ColE1 system does not require DnaK (27).

The mechanism of action of DnaK protein in R1 replication remains to be elucidated. However there are several data that suggest possible roles for DnaK in R1 replication: i) The replication protein RepA of R1 tends to aggregate as an inactive form, due to hydrophobic interactions (19). A possible role for DnaK in R1 replication could be to prevent the aggregation of the RepA protein. In fact aggregated forms of the RepA protein obtained after over-expression of the *repA* gene fail to bind to oriR sequences *in vitro* although they can promote initiation of R1 replication in extracts of *dnaKwt* strains (20). This suggests that the DnaK protein present in these extracts is able to disassemble some RepA aggregates and to assist in proper refolding the protein into an active form. This would resemble the role played by DnaK in P1 replication (7, 8). ii) The lack of complementation by DnaK of R1 replication in the 60% ammonium sulphate fraction corresponding to the *dnaK756* strain and the effective replication promoted by this protein in the 30% ammonium sulphate fraction of the same strain, indicate that in the absence of the DnaK protein, an inhibitor is formed that can be removed by ammonium sulphate fractionation. Note that this inhibitor affects R1 but not ColE1 replication. However, it is not known if this inhibitor is also formed *in vivo*. iii) In 30% ammonium sulphate fractions prepared from *dnaK756* strain, and under sub-optimal complementation with DnaK, RepA-dependent replication is sensitive to rifampicin. This sensitivity was not observed when the assay was performed with the equivalent wild-type extracts. The increased sensitivity to rifampicin in the DnaK deficient extract could well indicate that in the presence of limiting levels of DnaK, there is a requirement for transcriptional

'clearing' of the origin from unwanted protein–DNA complexes; it could also reflect an RNA polymerase deficiency in *dnaK756*, as suggested elsewhere (44).

To evaluate the requirement for DnaK in the *in vivo* replication of plasmid R1, we took advantage of the temperature-dependent amplification of mini-plasmid vector pMOB45, a run-away copy mutant of plasmid R1 (32). These analyses indicated that in *dnaK756* background at high temperature, the vector was not amplified but that a *dnaKwt* recombinant, pMOB45-*dnaKwt*, that complements the *dnaK756* mutation, was amplified. This clearly suggested that *in vivo* DnaK is needed for replication of plasmid R1 at high temperature. A second possible alternative is the suppression of the DnaK deficiency by an excess of the initiator protein. This seems to be the case when the replication protein of plasmid F is overproduced (9,10). However, this explanation can be ruled out because the shift to restrictive temperature of *dnaK756* host strains containing either the pMOB45 or the pMOB45-*dnaKwt* recombinants, that might increase the RepA levels in both cases, only results in amplification of the pMOB45-*dnaKwt* recombinant. In addition, the reduction of copy number of pMOB45 in the *dnaK756* background at 30°C, indicates that this protein is also involved in replication of plasmid R1 under non thermal-stress conditions. However, this involvement is clearly less strict for R1 than for λdv replication: data presented in this work have shown that at 30°C the efficiencies of transformation of a λdv recombinant in a *dnaKwt* strain is at least two orders of magnitude higher than in a *dnaK756* background while they are similar for an R1 miniplasmid. An involvement of DnaK in replication of plasmids F and P1 under conditions of no thermal-stress has been also suggested to explain the increased instability of these replicons in a *dnaKts* background at the permissive temperature (45).

We have recently observed that replication of plasmid R1 *in vitro* is less efficient in extracts prepared from a *dnaJts* strain than in extracts prepared from a *dnaJwt* strain, while replication of ColE1 plasmid is equally efficient in these extracts (our unpublished data). These preliminary data suggest a possible role for DnaJ in the replication of plasmid R1 *in vitro*.

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