A dnaA box can functionally substitute for the priming signals in the *oriV* of the broad host-range plasmid RSF1010

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Abstract The initiation of replication from $oriV_{RSF1010}$, the replication origin of the broad host-range plasmid RSF1010, depends on RepA (helicase), RepB′ (primase), and RepC (intiator protein), encoded by RSF1010 itself, while this initiation event in *E. coli* is independent of *dnaA, dnaB, dnaC,* and *dnaG* [Scherzinger et al. (1984) Proc. Natl. Acad. Sci. USA 81, 654–658; Scholz et al. (1985) in: Plasmids in Bacteria, pp. 243–259, Plenum, New York; Haring and Scherzinger (1989) in: Promiscuous Plasmids of Gram-negative Bacteria, pp. 95–124, Academic Press, London; Scherzinger et al. (1991) Nucl. Acids Res. 19, 1203–1211]. We showed in this work that a newly constructed origin consisting of an $oriV_{RSF1010}$ and a DnaA protein binding site, the dnaA box, inserted near $oriV_{RSF1010}$ ($oriV_{RSF1010}$-dnaA box) could function without RepB′ primase, but required RepA and RepC. This $oriV_{RSF1010}$-dnaA box could not replicate in a *dnaA46* strain in which only RepA and RepC were supplied, even at a permissive temperature. These results indicate that an inserted dnaA box can functionally substitute for the RSF1010-specific *ssi* signals, the RepB′ dependent priming signals in $oriV_{RSF1010}$, and can direct a priming pathway different from the RSF1010-specific one, but related to DnaA protein.

Key words: DNA replication; Broad host range plasmid RSF1010; *ssi* signal; DnaA box

1. Introduction

RSF1010, an 8684-bp IncQ plasmid [5,6], can replicate in a wide variety of Gram-negative bacteria and in some Gram-positive bacteria [7,8]. This prominently broad host-range property is thought to be conferred by its unique initiation mechanism of DNA replication. RSF1010 encodes three kinds of replication protein: RepA (helicase), RepB′ (primase), and RepC (intiator protein) [1,3]. All these three proteins are essential for the initiation events in RSF1010 and make the replication of RSF1010 in *E. coli* independent of *dnaA, dnaB, dnaC,* and *dnaG* [1–4]. The DNA replication origin of RSF1010, $oriV_{RSF1010}$, consists of three and a half direct repeats (iterons) that are binding sites of RepC, the GC-rich region, the AT-rich region, and the two *ssi* signals: *ssiA* (RSF1010) on the *l*-strand and *ssiB* (RSF1010) on the *r*-strand [3,7,9]. These *ssiA* (RSF1010) and *ssiB* (RSF1010) are RepB′ dependent priming signals, each of which consists of a highly conserved 40-bp sequence [9,10]. Interestingly, when both *ssiA* and *ssiB* in an $oriV_{RSF1010}$ miniplasmid are replaced by primosome assembly sites from phage ΦX174 and plasmid pACYC184, and G-sites from phage G4 and plasmid pSY343, the obtained chimeric origins can function without RepB′, while these chimeric origins are still dependent on RepA and RepC [11,12]. This suggests that the priming in the $oriV_{RSF1010}$-dependent initiation processes is separated from the pre-priming events, such as origin recognition and duplex opening, for which RepA and RepC are responsible.

The DnaA protein binding sites, the dnaA boxes, exist in the *oriC* region of *E. coli* and in or near the replication origin of many plasmids [13], and DnaA protein binding to these dnaA boxes plays important roles in the initiation processes in each replicon [14]. It has been shown in the in vitro initiation of *oriC* of *E. coli* that DnaA protein promotes opening of the DNA strands and directs primosome assembly consisting of DnaB, DnaC, and DnaG onto the unwound region [15,16]. On the other hand, it has been shown in vitro that DnaA protein functions only in loading the primosome, binding to the dnaA box at an A-site isolated from plasmid R6K [17] or near the pBR322 replication origin [18].

By use of the unique initiation processes of RSF1010 replication, we tried to examine whether a dnaA box can be a substitute for *ssiA* (RSF1010) and *ssiB* (RSF1010) in vivo. In this paper, we report that a newly constructed origin consisting of an $oriV_{RSF1010}$ and a dnaA box inserted nearby $oriV_{RSF1010}$ ($oriV_{RSF1010}$-dnaA box) can function without RepB′, but requires RepA and RepC. The results suggest that an inserted dnaA box can functionally substitute for the RSF1010-specific *ssi* signals and direct a priming pathway different from the RSF1010-specific one, but related to DnaA protein.

2. Materials and methods

2.1. Bacterial strains

The *E. coli* strains used were JM109 (*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ (*lac-proAB*)), WM301 (*arg28 deoB23 gal11 his47 hsdSK12 lac11 leu19 mal met55 pro19 rbs rpsL56 sul1 thyA59 trp25*) [19], and WM448 (isogenic with WM301 except *dnaA46*) [19].

2.2. Plasmids

The helper plasmids pMMB2 (a ColD plasmid-based recombinant plasmid carrying *repA*, *repB′* and *repC* of RSF1010) and its deletion derivatives, pMMB2Δ5 (carrying *repC* only), pMMB2Δ67 (*repA* and *repC*), pMMB2ΔAE (*repB′*), pMMB2ΔSS (carrying no *rep* genes of RSF1010) have been described [1,10].

The $oriV_{RSF1010}$ miniplasmid pYT101Vα (1916 bp long) consists of the 444-bp *oriV* region of RSF1010 and the pBR322 *bla* (β-lactamase) gene, and has multi-cloning sites derived from pHSG399 (a derivative of pUC19 [20]) between *ssiA* of $oriV_{RSF1010}$ and *bla*. This miniplasmid was constructed with M13/YH101V47H, which is a derivative of M13/YH101VS [11], a recombinant M13 phage vector in which an $oriV_{RSF1010}$ was cloned. A *Hin*dIII linker was introduced between

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ssiA of $oriV_{RSF1010}$ and *bla* of M13/YH101V47H. To this *Hin*dIII site, a 57-bp *Hin*dIII fragment including multi-cloning sites were inserted to construct M13/YH101Vα. The 57-bp *Hin*dIII fragment had been constructed by inserting a *Hin*dIII linker into the unique *Eco*RI site in the multi-cloning sites derived from pHSG399. A 1916-bp fragment containing $oriV_{RSF1010}$, the multi-cloning sites, and *bla* was excised as a *Pvu*II fragment from replicative form (RF) DNA of M13/YH101Vα. Finally, a mini-RSF1010-plasmid, pYT101Vα, was obtained by self-ligation of this *Pvu*II fragment.

The plasmid pBXDnaA is a helper plasmid carrying the 1.7-kb *Bgl*II-*Xho*I fragment containing the coding region of $dnaA^+$ gene cloned in pHB10 [21]. The *Bgl*II-*Xho*I fragment containing the $dnaA^+$ gene was inserted into the *Bgl*II-*Xho*I site of a modifying pBR322-based plasmid, in which the *bla* gene was inactivated by deleting the 1129-bp *Dra*I-*Eco*RI (3230–4359, nucleotide numbers corresponding to [22]) segment including the whole of *bla*, to construct pBXDnaA. The unique *Bgl*II and *Xho*I site in the pBR322-based plasmid, into which the fragment containing the $dnaA^+$ gene was inserted, was constructed by insertion of a synthesized oligonucleotide linker into the *Dra*I-*Eco*RI site. It was confirmed that the replication inability of *dnaA* temperature-sensitive mutants at the restrictive temperature (42°C) could be rescued by the transformation of pBXDnaA into the mutants.

2.3. Oligonucleotides

The *Hin*dIII linker (5′ > CAAGCTTG < 3′) was obtained from Takara Shuzo Co., Ltd. The oligonucleotide containing the dnaA box (5′ > GTTATCCACAG < 3′) and the oligonucleotide linker for construction of pBXDnaA (5′ > AGATCTCTCGAGT < 3′) were synthesized in a DNA synthesizer (Applied Biosystems 394 DNA/RNA synthesizer).

2.4. DNA manipulation

Restriction endonucleases and DNA-modifying enzymes were purchased from Takara Shuzo Co., Ltd. and New England Biolabs, Inc. Plasmid DNA was extracted by the alkaline denaturation procedure [23]. Transformation was performed by the method of Chung et al. [24]. Concentrations of antibiotics in selective media were: ampicillin, 20 or 100 μg/ml; kanamycin, 100 μg/ml; streptomycin, 10 μg/ml; tetracycline, 40 μg/ml.

3. Results

3.1. The $oriV_{RSF1010}$-dnaA box can function without RepB′, but requires RepA and RepC

If a dnaA box could substitute for *ssi* signals in $oriV_{RSF1010}$, the origin that consists of $oriV_{RSF1010}$ and a dnaA box inserted near the $oriV_{RSF1010}$ should replicate without RepB′, but with RepA and RepC. Then, to construct the $oriV_{RSF1010}$-dnaA box, we inserted the synthetic dnaA box (11 bp containing a 9-bp consensus sequence for DnaA binding [13]) into the *Sma*I site between *ssiA* of $oriV_{RSF1010}$ and *bla* of the RSF1010-miniplasmid pYT101Vα (Fig. 1). The $oriV_{RSF1010}$-dnaA box-miniplasmids were used to transform JM109 harboring the helper plasmid pMMB2Δ67 (encoding RepA and C, but not RepB′), and the ampicillin (20 μg/ml) resistant transformants were selected to screen for the origin that could replicate with RepA and RepC but without RepB′.

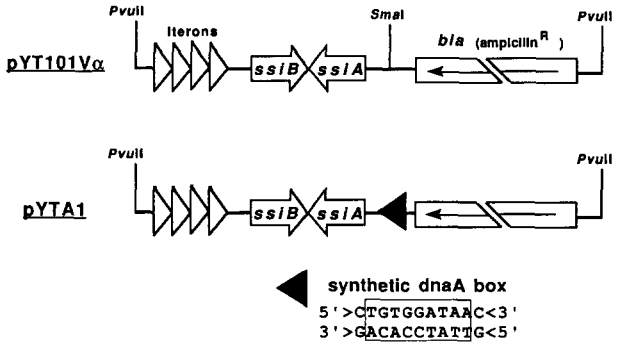

Fig. 1. Physical maps of the $oriV_{RSF1010}$ miniplasmid pYT101Vα and the $oriV_{RSF1010}$-dnaA box-miniplasmid, pYTA1. Physical maps of the miniplasmids linearised at the unique *Pvu*II site. Open arrows pointing leftward and rightward represent the *ssi* signals on the *l*-strand and the *r*-strand, respectively. Open triangles, iterons; open box, coding region of β-lactamase derived from pBR322 (arrow in the open box indicates the direction of transcription of β-lactamase). Closed triangle represents inserted synthetic dnaA box (5′ > GTTATCCACAG < 3′, in which 9 bases match the consensus sequence of the dnaA binding site) with the tip pointing toward 3′. The figure is not drawn to scale.

We could obtain three transformants which could replicate with only RepA and RepC, but without RepB′, and contained $oriV_{RSF1010}$-dnaA box-miniplasmids replicating autonomously from a single transformation. All of these $oriV_{RSF1010}$-dnaA box-miniplasmids, named pYTA1, contained only one dnaA box, inserted in one orientation, as shown in Fig. 1.

The miniplasmid pYTA1 was transformed into the strain JM109 harboring pMMB2 (encoding RepA, RepB′, and RepC) or one of its deletion derivatives, pMMB2Δ67, pMMB2Δ5 (encoding RepC only), pMMB2ΔAE (RepB′) or pMMB2ΔSS (encoding no RSF1010 Rep proteins), as a helper plasmid. Then, the numbers of ampicillin (100 μg/ml) resistant transformants were scored, to investigate the requirement of RSF1010 Rep functions for the replication of the $oriV_{RSF1010}$-dnaA box in pYTA1 (Table 1). pYTA1 could replicate in JM109 harboring pMMB2 or pMMB2Δ67, but not in JM109 harboring pMMB2Δ5, pMMB2ΔAE, or pMMB2ΔSS. pYTA1 could not replicate in these three strains even when the concentration of ampicillin for selection was reduced to 20 μg/ml (data not shown). This result indicates that RepB′ is not required for the replication of the $oriV_{RSF1010}$-dnaA box in pYTA1, but RepA and RepC are required for it. This result also suggests that the inserted dnaA box in pYTA1 dose not severely interfere the RepA, B′, C-dependent replication from $oriV_{RSF1010}$.

Table 1
Requirements of Rep functions for the replication of mini-plasmid pYTA1 containing the $oriV_{RSF1010}$-dnaA box

Host strain	Helper plasmid	*repA*	*repB′*	*repC*	Miniplasmid	
					pYT101Vα	pYTA1
JM109	pMMB2	+	+	+	8.2×10^2	5.4×10^2
	pMMB2Δ67	+	–	+	0	9.8×10^1
	pMMB2Δ5	+	–	–	0	0
	pMMB2ΔAE	–	+	–	0	0
	pMMB2ΔSS	–	–	–	0	0

The number of ampicillin (100 μg/ml) resistant transformants per 0.2 ng pYT101Vα and pYTA1 DNA is shown. The mini-plasmids pYT101Vα and pYTA1 were obtained by fractionation from the plasmid preparation from JM109 (pMMB2, pYT101Vα) and JM109 (pMMB2Δ67, pYTA1), respectively, by agarose gel electrophoresis. Results are shown as mean values of two determinations.

Table 2
Replication activity of pYTA1 in *dnaA* temperature-sensitive mutants

Host strain	Helper plasmid	*repA,C*	*repB'*	*dnaA*	Miniplasmid	
					pYT101Vα	pYTA1
WM301 (*dnaA*$^+$)	pMMB2	+	+	*dnaA*$^+$	1.2×10^3	1.1×10^3
	pMMB2Δ67	+	–	*dnaA*$^+$	0	3.2×10^1
	pMMB2Δ67+pBXDnaA	+	–	*dnaA*$^+$[*dnaA*$^+$]	0	6.3×10^1
WM448	pMMB2	+	+	*dnaA46*	6.3×10^2	6.5×10^2
	pMMB2Δ67	+	–	*dnaA46*	0	0
	pMMB2Δ67+pMMB2Δ67	+	–	*dnaA46*[*dnaA*$^+$]	0	4.0×10^1

The number of ampicillin (20 μg/ml) resistant transformants per 10 ng pYT101Vα and pYTA1 DNA is shown. Preparation of the mini-plasmid DNAs was as described in Table 1. Results are shown as mean values of two determinations.

3.2. *The replication activity of the ori$V_{RSF1010}$-dnaA box in dnaA temperature-sensitive mutants*

To determine whether DnaA protein is involved in the initiation events of the *ori$V_{RSF1010}$*-dnaA box in pYTA1, we examined the replication activity of pYTA1 in a *dnaA* temperature-sensitive mutant. DNA of pYTA1 was transformed into WM301 (*dnaA*$^+$) and *dnaA* temperature-sensitive mutant WM448 (isogenic with WM301 except *dnaA46*), harboring pMMB2 or pMMB2Δ67 as a helper plasmid. The ampicillin (20 μg/ml)-resistant transformants were selected at a permissive temperature (30°C) and the numbers of the transformants were scored (Table 2). pYTA1 could replicate in the temperature-sensitive mutants WM448 as well as in the wild type strain, when RepA, RepB', and RepC were supplied by the helper plasmid pMMB2. The result is consistent with the observation that the initiation processes in *ori$V_{RSF1010}$* are independent of host factors required for the initiation of replication of *E. coli* chromosome. However, when pYTA1 was transformed into WM448 (*dnaA46*) (pMMB2Δ67), no ampicillin-resistant transformant was obtained, indicating that pYTA1 could not replicate in WM448 (*dnaA46*) strain even at the permissive temperature when RepA and RepC, but not RepB', were supplied. However, pYTA1 could replicate in WM448 harboring pMMB2Δ67 and pBXDnaA, which can supply DnaA$^+$ exogenously. This indicates that the inability of replication in WM448 (*dnaA46*) even in the presence of RepA and RepC was rescued by the supplement of a *dnaA*$^+$ gene encoded by a helper plasmid, pBXDnaA.

4. Discussion

We showed that the *ori$V_{RSF1010}$*-dnaA box in pYTA1, isolated in this study, could replicate without RepB' primase, while it required RepA and RepC. This shows that the single dnaA box in this origin can substitute for the RSF1010-specific priming signals *ssiA* (RSF1010) and *ssiB* (RSF1010), which require RepB' primase, and that the dnaA box can direct a priming pathway different from the RSF1010-specific one. Furthermore, this *ori$V_{RSF1010}$*-dnaA box in pYTA1 could not replicate in the strain carrying the *dnaA46* allele even when RepA and RepC were supplied. This inability of replication in the *dnaA46* mutant was rescued by the supplement of a *dnaA*$^+$ gene encoded by a helper plasmid, pBXDnaA. These results suggest that DnaA protein can participate in the initiation event of the *ori$V_{RSF1010}$*-dnaA box in pYTA1, especially in the priming reaction which may be directed by the inserted dnaA box. It has been reported that plasmid pSC101, the replication origin of which has a dnaA box, reduces its copy number when it is in a *dnaA* temperature-sensitive mutant even at the permissive temperature [25]. This inability of replication of the pYTA1 in the *dnaA46* mutant seems to be similar to the case of pSC101. It has been shown in vitro that DnaA protein functions only in loading the primosome, binding to the dnaA box at an A-site isolated from plasmid R6K or near the pBR322 replication origin [17,18]. In this *ori$V_{RSF1010}$*-dnaA box in pYTA1, DnaA protein would direct priming by loading the primosome onto the unwound region as in the above two cases. However, duplex opening and unwinding at the*ori$V_{RSF1010}$*-dnaA box may be directed by RepA and RepC which associate with *ori$V_{RSF1010}$*, not by DnaA itself. If so, this *ori$V_{RSF1010}$*-dnaA box in pYTA1 is an interesting example that shows in vivo that DnaA protein functions only in directing the primosome onto the unwound region.

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