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Full Length Research Paper

RepC as a negative copy number regulator is involved in the maintenance of pJB01 homeostasis

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The plasmid pJB01 contains a single operon consisting of three orfs, *copA, repB* and *repC* cistrons. The operon, also called *repABC* operon, starts transcription at T695 or A696 on the pJB01 genetic map. CopA (called RepA in pMV158 family) or ctRNA (counter-transcript RNA) of this plasmid play roles as a repressor of RepB, a replication initiator, on the transcriptional and translational level, respectively. RepC did not bind 73 bp PCR product including three tandem repeats (5′-CAACAAA-3′), the binding sites for RepB and any other regions on pJB01. However, when RepB and RepC were added simultaneously in the reaction mixture for gel mobility shift assay, unexpectedly, three kinds of retarded bands were observed. It suggests that RepC can interact with RepB by protein-protein interaction. In addition, the copy numbers of RepC-deleted pJB01 *ermC* (erythromycin-resistant methylase C) plasmids are increased 1.37-1.45 folds when compared with that of parent pJB01 *ermC*. From these, it could be proposed that RepC plays a role as a negative regulator to modify RepB function in the initiation of pJB01 replication, and therefore, the copy number of pJB01 is maintained via mutual global regulation of various replication factors, such as CopA, ctRNA, RepB and RepC.

Key words: pJB01, *repABC* operon, replication initiator, RepC, global regulation.

INTRODUCTION

Genetic analysis of replication control mechanisms had first been attempted for plasmid R1 through isolation of mutants to increase copy number (Nordstrom et al., 1972). The determinants of copy number control had been discovered in plasmid itself via characterization of these mutants, in which negative regulators (inhibitors) acting at the initiation step were involved in this control. A model of replication control by negative effectors was first proposed and described in quantitative terms by Pritchard et al. (1969). When a plasmid colonizes a new host, these negative regulators will be negligible owing to a little accumulated concentration of the inhibitors at initial stage. This seems desirable for successful establishment, since uninterrupted plasmid replication permit the normal copy number in a short time. Once the characteristic copy number is reached, maintenance of the average copy number in the population will then require adjustments to fluctuations in this value in individual cells. The control systems constantly maintain copy number by either

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Abbreviations: ctRNA, Counter-transcript ribonucleic acid; ***ermC***, erythromycin-resistant methylase C; **RT-PCR,** reverse transcription polymerase chain reaction; **RNA,** ribonucleic acid; **EDTA,** ethylenediaminetetraacetic acid; **DNA,** deoxyribonucleic acid; **TBE,** Tris/Borate/ethylenediaminetetraacetic acid; **NCBI,** national center for biotechnology information; ***sso,*** single strand origin; ***dso,*** double strand origin; **RC,** rolling-circle.

increasing or decreasing the rate of replication per plasmid copy and cell cycle. Although mechanisms to counter-select newly replicated-plasmid molecules exist, for example, hemimethylation and supercoiling (Abeles et al., 1993; Nordstrom et al., 1984), individual plasmid copies are selected for replication at random from a pool including replicated and non replicated copies.

The inhibition of plasmid replication associated with an increase in the gene dosage of copy number control genes has been used to identify these genes (Pritchard, 1978; Nordstrom, 1985; Novick, 1987; Austin and Nordstrom, 1990; Kittell and Helinski, 1992; Chattoraj and Schneider, 1997). Control of replication by inhibitors requires measurement of the concentration of plasmid copies within the cell. This is probably achieved by an unstable inhibitor expressed constitutively or by a stable inhibitor synthesized shortly after each initiation event (Pritchard et al., 1969). The regulators via these alternatives modulate the initiation frequency after each initiation event and lead to increase or decrease in the rate of initiation of replication when the average copy number is, res-pectively, lower or higher than required. Otherwise, when the frequency of initiation is determined by the level of an initiator protein, one mechanism for controlled plasmid replication (unlike phage replication) includes inactivation of the initiator protein after each replication event (Rasooly and Novick, 1993; Wojtkowiak et al., 1993). In this study, in order to examine RepC function of pJB01, we performed RT-PCR (reverse transcription-PCR), gel mobility shift assay and constructs of *repC*-deleted mutants to demonstrate a single operon consisting of *copA, repB* and *repC* genes, protein-protein interaction between RepB and RepC, and copy number control of pJB01 by RepC, respectively.

MATERIALS AND METHODS

Bacterial strains and plasmids

A pathogenic *Enterococcus faecium* JS2 (Amp^S, Erm^S, Tet^S, and Van^I), a strain non-harboring pJB01 plasmid, was isolated from patients in Samsung Biomedical Research Institute in Korea. In order to clone pJB01, *Escherichia coli* TG1 strain was employed. Cloned plasmids were transformed to *E. faecium* JS2 by the electroporation method (Dunny et al., 1991; Bensing and Dunny, 1993). Transformed plasmids were isolated from *E. faeium* JS2 by a modified alkaline lysis method (Sambrook et al., 1989; O'Sullivan and Klaenhammer, 1993). For selection of a host harboring target plasmids from *E. coli* TG1 and *E. faecium* JS2, an *ermC* (Khan et al., 2002) gene of plasmid pGKV21, after amplification by PCR using the portion of pE194 from this plasmid (van der Vossen et al., 1985**)**, was transferred into pJB01 and named pJB01 *ermC*.

RT-PCR (reverse transcription polymerase chain reaction) - RT-PCR

Total RNAs were prepared from exponentially growing *E. faecium* JS2 cultures harboring pJB01 *ermC* (RNeasy Mini Kit, Qiagen). For the 1st strand of three cDNAs containing *copA* and a part of *repB* genes, *copA*, *repB* and a part of *repC* genes, and *copA*, *RepB* and *RepC* genes, each of oligonucleotides such as the 3′-end of RepB as 5′-AATTTCATAAAAGCTTCCCC-3′, the 3′-end of E104Y as 5′-GTCTTTTGAATAATGCGTTAAATAC-3′ and the 3′-end of RepC as 5′-AAGCTTTAGTTATCCGCCCTTTCAAC-3′ was annealed to total Ribonucleic acid (RNA) (15 ∝g) at 65℃ for 5 min, and continuously, the reactions were done as described by a Superscript™ RNaseH⁻ RTase catalogue of Invitrogen. The next PCR was performed by the above three kinds of PCR products used as templates, and the 5′-end of CopA [5′-GGATCCATGGCTAGAGAAAAATCAGA-3′] as the 5′-end primer and the same oligonuclotides used for cDNA syntheses as the 3′-end primers.

Purification of repC and repB and gel mobility shift assay

Each of *repB and repC* genes was amplified by PCR and subcloned to the pQE30 vector for over expression. Subcloned *repB* and *repC* genes were over expressed by T7 promoter in *E. coli* JM109, and then, purified according to manufacturer's directions (Invitrogen, Korea). A 73-nucleotide stretch from nucleotides 583 to 655, the three tandem repeats-containing region of plasmid pJB01, was amplified by PCR. One pmol of each PCR product was incubated with various concentrations of RepB and RepC in 20 ∝l of reaction buffer [NB buffer (20 mM Tris HCl, pH 8.0; 1 mM Ethylenediaminetetraacetic acid (EDTA), pH 8.0; 100 mM KCl; 5 mM dithiothreitol), 50 ∝g/ml calf thymus Deoxyribonucleic acid (DNA) and 50 ∝g/ml bovine serum albumin] for 30 min at room temperature. Reactions were stopped by adding non-denaturing loading buffer [20% (v/v) glycerol, 0.01% (w/v) Bromophenol Blue in Tris/Borate/ Ethylenediaminetetraacetic acid (TBE) buffer]. Reaction products were separated on 5% native polyacrylamide gels. The resolved bands were stained with Ethidium Bromide (0.5 ∝g/ml) in TBE buffer for 30 min, and destained with distilled water for 30 to 120 min.

Site-directed mutagenesis of the pJB01 repC region and copy-number calculation

All mutants were obtained by inverse PCR (Mcpherson et al., 1993) using pJB01 *ermC* as a template. To remove *repC* gene from the parent plasmid pJB01 *ermC*, the 3′-end of RepB as 5′-AATTTCATAAAAGCTTCCCC-3′ and the 5′-end of Em 5′-GCTAGCATCGATTCACAA AAAATAGG-3′ oligonucleotides were used as primers. The desired mutant plasmids were harvested by using *E. coli* TG1, and then, transformed to *E. faecium* JS2. To calculate their copy numbers, cultural broths of *E. faecium* JS2 harboring plasmids were grown to mid-exponential phase, and adjusted to 0.5 optical densities at 600 nm in wavelength. Equal aliquots of each culture were withdrawn. Cells were collected by centrifugation and plasmids were prepared by an Accuprep™ plasmid extraction kit (Bioneer, Korea). Isolated plasmids were separated on agarose gels and stained by Ethidium Bromide (0.5 ∝g/ml). Monomer covalently closed circular plasmids were quantified by Gel doc 2000 (Bio-Rad, USA) and covalently closed monomers of mutant plasmids were compared with that of parent pJB01 *ermC*.

RESULTS AND DISCUSSION

Demonstration of a single repABC operon and its surrounding structure

The plasmid pJB01 (GenBank accession number

AY425961) was isolated originally from *E. faecium* JC2 (Kim et al., 2006), but it was not harbored by *E. faecium* JS2. It was assumed that this plasmid should have a single operon which consists of three orfs as *copA, repB* and *repC* by the sequencing analysis using a Translation of ExPASy tool. This operon begins transcription at T695 or A696 on the pJB01 genetic map (Kim et al., 2008). CopA (as RepA in pMV158 and pE194)), the product of *copA* gene, binds to the promoter (or operator) region of the operon and regulates it at transcriptional level, as in case of pMV158 (del Solar et al., 1995, 1997) and pE194 (Kwak and Weisblum, 1994). RepB, as a product of *repB* gene and a replication initiator, performs nicking and nick/closing reactions on the nick site in the *dso* region (Kim et al., 2006). However, the corresponding function of RepC, the product of *repC* gene, has not been identified yet, through primary sequence analysis using the BLAST search of NCBI (National Center for Biotechnology Information). The transcription of *copA* (171 nts), *repB* (666 nts), and *repC* (462 nts) genes begin at the 721st, 964th and 1661st nts and terminate at the 891st, 1629th and 2122nd nts, respectively. Since consensus sequences of the promoter region were not existed in the intergenic regions of 72 nts (*copA*-*repB*) and/or 31 nts (*repB*-*repC*), it was thought that these genes are a single operon. As shown in Figure 1, RT- PCR results using each oligonucleotide set also revealed that these genes consist of a single operon.

Binding on three tandem repeats by protein-protein interaction of RepB and RepC

Following termination of the leading strand replication of pT181 plasmid, repC as an initiator protein is released with a short oligonucleotide attached to one subunit of homodimer repC, which prevents it from being recycled, as an essential feature of the plasmid's replication control system. It was reported that the oligonucleotide probably results from the passage of the replication fork past the RepC nick site after one round of replication, the subsequent cleavage and re-ligation of the DNA by repC (Khan, 1997; Zhao and Khan, 1997). Although repC can bind to the recognition sequence in the leading strand origin, repC*, as a modified one with a short oligonucleotide, cannot induce cruciform extrusion, which is an essential structure for replication initiation. RepC* is defective in its ability to oligomerize on the DNA for the next round replication. DNA binding and replication activities of RepC* is greatly decreased, implying that it may play only a minor regulatory role in pT181 replication *in vivo* (Zhao et al., 1998).

On the contrary, Rep proteins of pMV158 family do not lead to a covalent binding with nicked DNA in itself (Moscoso et al., 1997). Therefore, it should be existing in other mechanism for deactivation of generated Reps in its family including pJB01. Since in this work the pJB01 *repC-RepC* gene was co-expressed as a component of the polycistronic mRNA of *copA* and *repB* gene, as shown in Figure 1, it could not be excluded from the possibility that RepC might play a role as a regulator of pJB01 replication through modification of RepB. In Figure 2, RepC did not show binding on the DNA segment containing three tandem repeats, known as DNA binding sites for RepB (as an initiator). However, simultaneous addition of RepB and RepC, particularly in higher concentrations of RepC, showed three extra retarded bands compared to the case of RepB only. It suggests that RepC interacts with RepB by protein-protein interaction. Interestingly, plasmid pLC2 (Vogel et al., 1991) which belongs to pMV158 family seems to be very similar to plasmid pJB01, in the aspect of the organization of *sso* (single strand origin), *dso* (double strand origin) and genes encoding for replication initiation factors. Particularly, unidentified pLC2 orf 3, which composed of 153 amino acid residues and a weakly acidic protein as a theoretical pI of 5.11, is homologous up to 96% to corresponding pJB01 RepC (Figure 3). Based on the similar organization and amino acid sequence homology, it could not be excluded from the possibility that pLC2 orf 3 also regulates the initiation of replication via protein-protein interaction with orf 2.

Increase of the copy numbers of the repC-deleted mutants

In order to examine the role of RepC in the regulation of pJB01 copy number, three mutants of RepC-deleted pJB01 *ermC* were created by inverse PCR (Figure 4a). All RepC-deleted pJB01 *ermC* mutants increased the copy numbers of 1.37-1.45 times when compared with that of parent plasmid pJB01 *ermC* (Figure 4b). In our previous work (Kim et al., 2008), the copy numbers of ctRNA mutants showed 1.78-5.43 times higher than that of parent pJB01 *ermC*. Based on these, it is assumed that RepC inhibits the initiation of replication weakly by modification of RepB, on the contrary to ctRNA does as a main repressor on the translation level.

A hypothetical model for the copy number control of pJB01

Based on all the data obtained in this work, RepC seems to be involved in the regulatory system of pJB01 DNA replication, in addition to various factors such as CopA, ctRNA and RepB. In some detail, according to our study it was demonstrated that CopA represses transcription of a *repABC* operon by interaction on partial palindromic sequence formed near the -35 box consensus sequence in the promoter region (unpublished data). In addition, pJB01 ctRNA, a 54 nucleotide transcript encoded on the opposite strand from the *copA/repB* intergenic region and

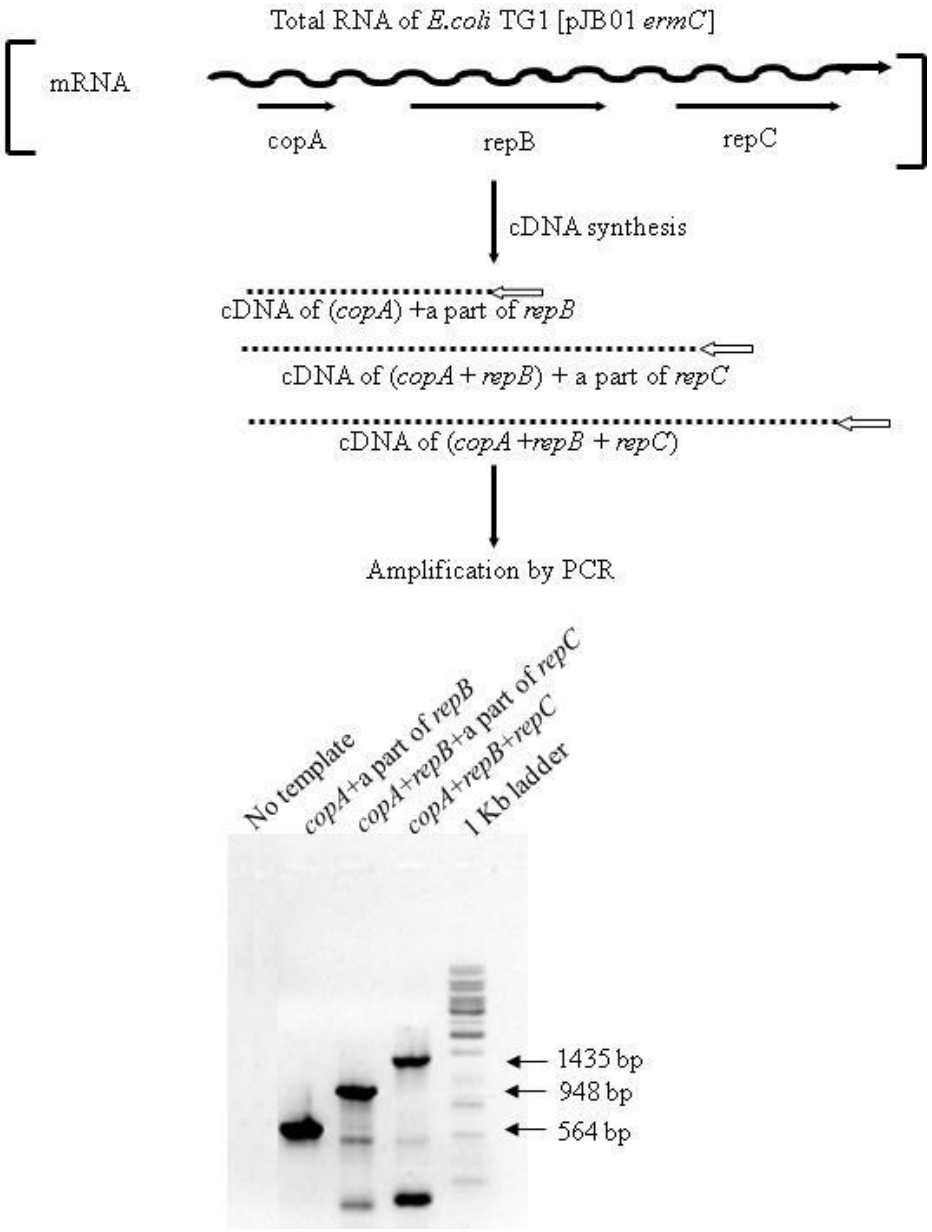


Figure 1. Demonstration of a single operon system consists of *copA*, *repB* and repC cistrons. Each primers marked by open arrows at the experimental strategy in the upper panel was annealed to total RNA isolated from *E. coli* TG1 culture harboring the pJB01 *ermC*, and extended by reverse transcriptase. The RT-PCR products consist of 564, 948 and 1435 bps as indicated on the right side of the gel, respectively. A full-length mRNA transcript of the *repABC* operon was represented by a thick-waved arrow. Each cistrons of a putative operon was marked below the *repABC* mRNA, primers for the first cDNA synthesis were represented in open arrows, and their reverse transcripts were shown in dotted lines. PCR products were separated on a 1% agarose gel and those products corresponding to three parts of a putative operon were marked by arrows on the right of the gel image.

also negatively regulates RepB expression on the translational level by partially overlapping an ARBS (Kim et al., 2008). Interestingly, RepB promotes replication activity on appropriate concentrations, but under overexpressed conditions, it not only decreases the replication activity of plasmid pJB01 but threatens viability of its host (unpublished data). Since the initiators of many rolling-circle (RC) plasmids do rate-limiting for replication, these proteins seems to be usually inactivated after accomplishment of one round of replication. In the pT181 plasmid, inactivation of the initiator RepC protein occurs by the attachment of an oligonucleotide to its active

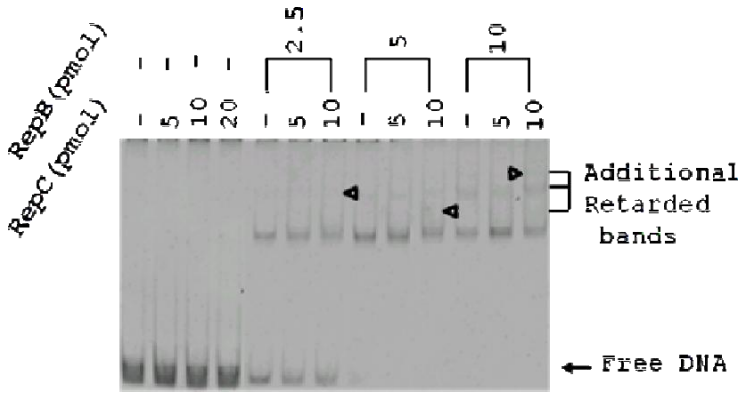
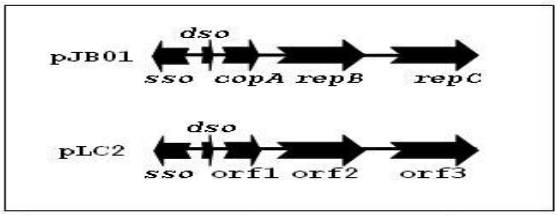


Figure 2. Confirmation of the protein-protein interaction between RepB and RepC by gel-shift assay. Free DNA indicates 73 bps PCR product containing three tandem repeats. Three additional retarded bands were produced by simultaneous addition of RepB and RepC to the reaction mixture for gel-shift assay. Concentrations of RepB and RepC added in pmol are indicated on the top of the gel image.

A



B

Name	pJB01	pLC2	Description
Origin	*Enterococcus faecium* JC1	*Lactococcus curvatus*	
Family Of RCR	pMV158	pMV158	
Full length	2235 bp	2489 bp	
Direct/tandem Repeats	CAACAAA	ACAAACCC (or CAACAAA)	pJB01, 3 tandem repeats; pLC2,2 direct (or 3 tandem) repeats
CopA	56 aa	56 aa	Homology up to 64%
RepB	221 aa	221 aa	Homology up to 86%
RepC	146 aa	153 aa	Homology up to 96%

C

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                10        20        30        40        50        60
pJB01     MKFEETLLPEKSDVMTLQNMIRKYNKQNFETANQTDFAIYIKDDSENVMGGISGEIFGNW
pLC2      MKFEETLLPEKSDVMTLQNMIRKYNKQNFETANQTDFAIYIKDDSENVMGGISGKIFGNW
Consensus MKFEETLLPEKSDVMTLQNMIRKYNKQNFETANQTDFAIYIKDDSENVMGGISGeIFGNW

                70        80        90       100       110       120
pJB01     MDIEYLVIHESVRRNGLGRDLLKKAEELAIRSNCKNIFLYTFDFQGKDFYPKFGFKEVYV
pLC2      MDIDYLVIHESLRKNGLGRDLLKKAEDLAIRSNCKNIFLYTFDFQGKDFYPKFGFKEVYV
Consensus MDI#YLVIHES1RrNGLGRDLLKKAE#LAIRSNCKNIFLYTFDFQGKDFYPKFGFKEVYV

                130       140       150
pJB01     KRQYPLTGTEHFFVKKLSNEIDKEAGEILEETE
pLC2      KRQYPLTGTEHFFVKKLSNEIDKETE--------
Consensus KRQYPLTGTEHFFVKKLSNEIDKEae
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Figure 3. Comparison of replication factors between pJB01 and pLC2. (A) A schematic model of their gene organizations. Plasmid pJB01 is the same as pLC2 in view of gene organization, for example, orientations and arrangements of *dso*, *sso*, and three orfs. (B) Some features of their replication factors. Various features of replication factors in two plasmids were compared such as direct/tandem repeats, and orfs including origin of plasmids and prototype of RCR. (C) Alignment of the amino acid sequence of RepC in pJB01 with that in pLC2. Two RepCs show about 96% homology. Upper-case letters are residues conserved more than 90%, Lower-case letters are residues conserved 50 to 90%, and # indicates the NDQE conserved positions.

tyrosine residue (Rasooly and Rasooly, 1996; Jin et al., 1996; Zhao et al., 1998). However, since Rep proteins as a replication initiator of pMV158 family do not bind covalently with an oligonucleotide after one round replication, its inactive forms are not observed (Moscoso et al., 1997), on the contrary to pT181 RepC.

Therefore, pMV158 family may need a new system for preventing accumulation of active Rep proteins, in other

A

Mutant name	Deleted region	Description
23-2	pJB01Δ1667-2167/GCTAGC(Em5′)	PCR primer : RepB 3′ end-Em 5′, total synthesized size : 2746 bp
23-3	pJB01Δ1669-2167/CTAGC(Em5′)	Deletion of G residue in *Nhe*I site (5′ -GCTAGC-3′), total synthesized size : 2747 bp
23-5	pJB01Δ1669-2167/GCTAGC(Em5′)	total synthesized size : 2748 bp

B

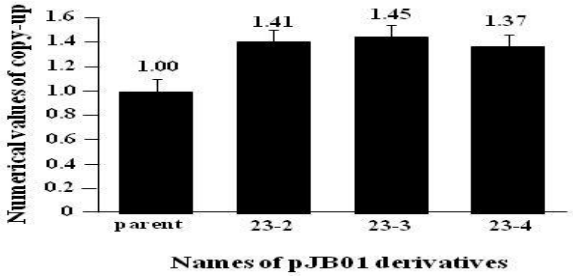

Figure 4. Changes of copy numbers of a few pJB01 *ermC repC* mutants. (A) Constructions of three pJB01 *ermC repC* mutants. The mutants were constructed by deletion of most parts of the *repC* gene, remaining some amino acid residues encoded at start and end portions. (B) Copy number ratios of three *repC* mutants. X-axis represents names of pJB01 *ermC* and its *repC* mutants as 23-2, -3 and - 4, and Y-axis indicates the copy-up ratios of three mutants against parent plasmid pJB01 *ermC*.

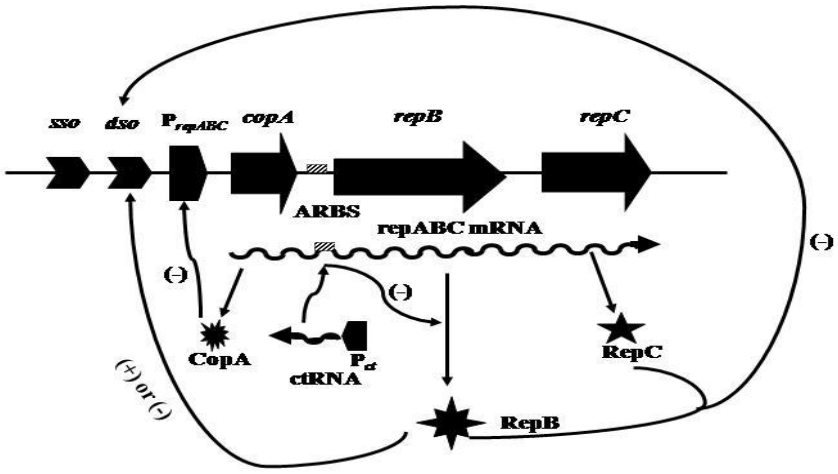

Figure 5. A hypothetical model for copy number control of pJB01. RepB is a positive regulator in the initiation of pJB01 RC replication at a normal level *in vivo*, whereas it functions as a negative regulator at an overexpressed level. The *copA*, *repB* and *repC* genes consist of a single operon, repABC and its transcription is autoregulated through CopA's binding on the putative operator region (P_{repABC}). Furthermore, the translation of *repB* is regulated by binding of ctRNA to the ARBS positioned between *copA* and *repB*. On these points of view, including co-expression of *copA* and *repB* with *repC* as a polycistronic mRNA, it could be assumed that RepC plays negative roles in the initiation of plasmid pJB01 replication through RepB-RepC interaction.

words, for rate-limiting of replication. Based on our data in this work, it could be hypothesized that RepC modifies RepB by protein-protein interaction and this leads to lessen the optimal initiation activity of RepB replication. It is supported in that the *repC*-delteted mutants show a few copy-ups by 1.37 -1.45 times against parent pJB01 *ermC* plasmid (Figure 4). The copy number control of pJB01 might be hypothesized as shown in Figure 5. The *dso* is

controlled positively or negatively depending on the expressed amounts of RepB. CopA binds a putative operator and then, auto-regulates transcription of the *repABC* operon. The ctRNA inhibits translation of RepB by binding on ARBS. RepC interacts with RepB and may play a role as a putative negative regulator at the *dso*. In this study, it was demonstrated that *copA, repB and repC* genes consist of a single operon, and that RepC interacts with RepB by protein-protein interaction. Moreover, a few copy-ups of its *repC*-deleted mutants against parent pJB01 *ermC* plasmid were observed. Taken all, it was suggested that the maintenance of the coy number of pJB01 is accomplished by mutual interactions of various replication factors, such as CopA, ctRNA, RepB and RepC.

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