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A Suppressor of Mutations in the Region Adjacent to Iterons of pSC101 *ori*

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Some single-base changes in a 14-bp region (the downstream region) adjacent to three repeated sequences (iterons) in pSC101 *ori* are very deleterious for replication. We isolated a host suppressor mutation for one of these mutations and found that the suppressor suppressed all the mutations tested in the downstream region. The nucleotide sequence of the suppressor revealed that the suppressor gene was identical to *dksA*, which encodes a multicopy suppressor of the heat shock gene *dnaK*.

Plasmid pSC101 has 21-bp repeats (iterons) in its replication origin (*ori*). We discovered that core *ori*, which is about 180 bp, extends from the DnaA binding sequence at the left end shown in Fig. 1 to the region (the downstream region) between the third iteron (DR-3) and an inverted repeat (IR-1) at the right end (13). IR-1 and the *par* region function as replication enhancers that increase the limited initiation frequency driven by core *ori* to the normal level (9, 10, 13). The plasmid-encoded initiator protein, Rep (or RepA), binds IR-1 in the form of a dimer in addition to IR-2, an operator site of the *rep* gene (8, 16), while *par* is a DNA gyrase binding site (17). The downstream region of the third iteron (DR-3) is required for the origin function in a sequence-specific fashion. Specific base changes at nucleotide positions 420 to 425, especially CAC at positions 420 to 422, in the downstream region (Fig. 1) have severe effects on the replication enhanced by either IR-1 or *par* (15).

To investigate the role of the downstream region in the replication of pSC101, we isolated host suppressors for these mutations. Plasmid pSS92 is a composite of pUC19 and the replication system of pSC101, which retained *ori* and *rep* but not *par* (nucleotide positions 223 to 1663 [20]), and was used as a wild-type control. Plasmids pSS420, pSS421, and pSS422 are single-base substitution mutants within the downstream region with mutations at positions C-420→G, A-421→G, and C-422→G, respectively (15). WA802*polA* (14) cells mutagenized with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (11) were transformed with pSS421, and ampicillin-resistant (Amp^r) colonies were isolated. Plasmid-free clones were selected, and their suppressor activities were again tested by transformation with pSS421. In this way, clone WA825 carrying a suppressor mutation was obtained.

Table 1 shows that WA825 was transformed with pSS421 at a higher frequency than was the parental strain, WA802*polA*. In addition to pSS421, mutations at position 420 (pSS420) or 422 (pSS422) were also suppressed by the suppressor. It is notable that WA825 permits the replication of wild-type plasmid pSS92 as well as pSS421.

To clone the suppressor gene, we first developed a new cosmid vector which is present at a low copy number to avoid isolating multicopy suppressors and to replicate in *polA* cells. A cosmid, pRCOS, was constructed by ligation of an *Eco*RI-*Rsa*I fragment containing *cos* and *aph*II from pHSG262 (1) with an *Mlu*I-*Eco*RI fragment containing the Rts1 basic replicon from pTW602 (5). We prepared a chromosomal DNA library of WA825 by use of pRCOS and transformed it with pSS421. A cosmid, pRCOS2.2, retained in one of colonies that was resistant to both kanamycin and ampicillin contained the shortest insert. The cells carrying pRCOS2.2 generated a large number of transformants with pSS421 (Fig. 2). This suppression suggests the dominance of the suppressor mutation over the wild type.

When Kohara's ordered DNA library was hybridized with pRCOS2.2 DNA (12), positive signals were detected on clones

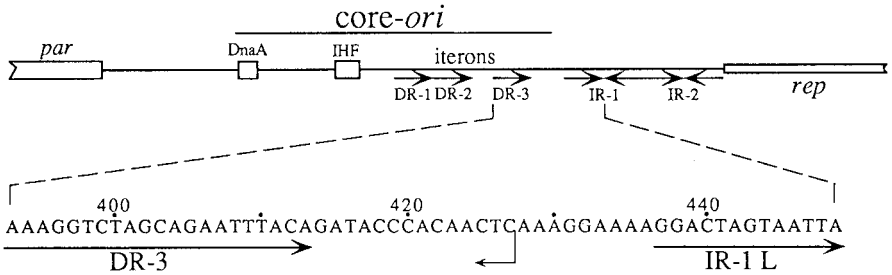

FIG. 1. Structural features of the replication origin and nucleotide sequence of the downstream region of DR-3. Boxes show the *par* region, the DnaA protein binding sequence, the integration host factor binding sequence (IHF), and the *rep* gene. Arrows indicate three direct repeats (DR-1, DR-2, and DR-3) and two inverted repeats (IR-1 and IR-2). Numerals above the sequence are the coordinates of the *ori* region (20). IR-1L means the left half of pseudosymmetric IR-1.

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TABLE 1. Transformation of host suppressor mutants with the downstream-region mutant plasmids

Strain	Relative transformation frequency[a] with:			
	pSSM2[b]	pSS420	pSS421	pSS422
WA802	1.0	1.0	1.0	1.0
WA802*polA*	<0.0012	0.0018	0.0057	0.017
WA825	<0.0004	0.45	0.41	0.62

[a] Competent cells were transformed with 20 ng of plasmid DNA. The plates were incubated at 37°C for 17 to 24 h. Transformants were selected with 75 μg of ampicillin per ml. Results are expressed as transformation frequencies of mutant cells relative to that of the wild-type WA802 cells. The transformation frequency of each strain was corrected by comparison with that of pSS92 (core *ori*, IR-1) in the same experiment.

[b] A pSS92 derivative missing the third iteron (nucleotide positions 382 to 413).

115 (11C5) to 120 (12D5) (data not shown), which cover the region from 3.2 to 4.3 min of the *Escherichia coli* chromosome. Subcloning of DNA segments from pRCOS2.2 showed that the suppressor is located in a 1.3-kb *Hin*cII-*Eco*RV fragment retaining the *dksA* (*dnaK* suppressor) operon (pDKS6). To further confirm the suppressor function of the *dksA* operon, we cloned the *dksA* operons of WA802*polA* and of WA825 by PCR. The *dksA* operon from WA825 (pDKM5) exhibited suppressor activity, while the *dksA* operon from the parental strain, WA802*polA* (pDKW8), showed little activity (Fig. 3). There are two nonoverlapping open reading frames on the *dksA* operon. To identify the open reading frame that actually encodes the suppressor protein, we introduced mutations into them and tested their suppressor activities. Figure 3 shows that open reading frame 2 (ORF2) (pDO21) encodes the suppressor. It has been reported that *dksA* is a multicopy suppressor for the temperature-sensitive growth of the *dnaK* deletion mutant (6). Since ORF2 has suppressor activity for the *dnaK* deletion and actually encodes an 18,000-M_r polypeptide (6), we concluded that the suppressor gene is identical to the *dksA* gene. This is the first indication that *dksA* is involved in DNA replication.

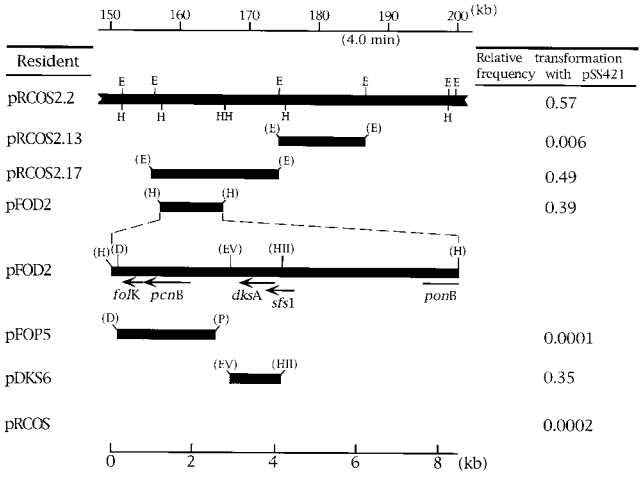

FIG. 2. Cloning of the suppressor (*dksA1*) gene. The upper line represents a part of the *E. coli* chromosome map described by Kohara et al. (7) and a scale for plasmids pRCOS2.2, pRCOS2.13, pRCOS2.17, and pFOD2. Plasmids pRCOS2.13 and pRCOS2.17 are derivatives of pRCOS2.2. Plasmids pFOD2, pFOP5, and pDKS6 are derived from pRCOS2.17. Plasmid pRCOS is a cosmid vector that has no host DNA. Each plasmid was first introduced into WA802*polA* cells, and then the cells were transformed with pSS421. The relative transformation frequencies, which were calculated as shown in footnote *a* of Table 1, are presented at the right. The coding frames and directions of transcription are indicated by arrows. The bottom line is a scale for plasmids pFOD2, pFOP5, and pDKS6.

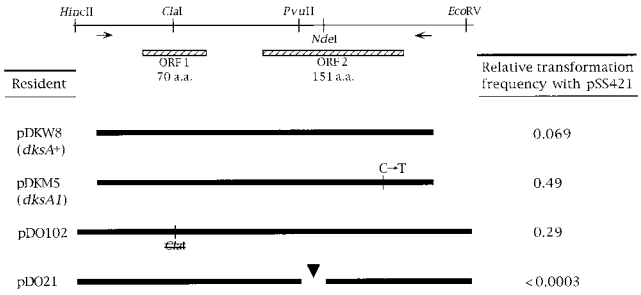

FIG. 3. Identification of the ORF2 suppressor gene in the *dksA* operon. The operon consists of ORF1 and ORF2, and its transcription proceeds to the right. The wild-type *dksA* operon (pDKW8) and the *dksA1* allele (pDKM5) were cloned by PCR amplification using Pfu DNA polymerase (Strategene). The PCR primers used as N- and C-terminal primers (shown by arrows) were 5′-CGTAT TGACTTTATGTTGCAGGCGG-3′ and 5′-GCGACAGGCCAATATACTGT GTGTC-3′, respectively. The PCR products were inserted into *Hin*cII-digested pRCOS. Plasmid pDKM5 retains the suppressor mutation in ORF2 (see text). Plasmids pDO102 and pDO21 are derivatives of pDKS6. The former has a +1 frameshift mutation in the *Cla*I site of ORF1, and the latter has an in-frame deletion in the *Pvu*II-*Nde*I region of ORF2. The relative transformation frequencies of WA802*polA* carrying an indicated plasmid are shown at the right.

We determined the nucleotide sequences of the *dksA* operons of both the wild type and WA825 (*dksA1*). In addition to four discrepancies in the published sequence (GenBank accession number M34945) (CC replaced the single C at position 217; GC replaced CG at positions 900 and 901, which changed Asn-94 and Val-95 of ORF2 to Lys and Leu, respectively; GGC and GC replaced CG at positions 1103 and 1104 and replaced CG at positions 1112 and 1113, respectively), a point mutation at position C-1010, to T, was found only in WA825, resulting in an amino acid substitution, Thr-131 to Ile of ORF2. Therefore, we regard the mutation at position C-1010 to T as the *dksA1* suppressor mutation.

To confirm the functional change of the DksA1 protein, we tested the ability of *dksA1* to suppress the temperature-sensitive growth of *dnaK204* mutant cells. Although *dnaK204* cells (KY1455 [4]) carrying either the *dksA*$^+$ plasmid (pDKW8) or the *dksA1* plasmid (pDKM5) had a higher plating efficiency at 37°C than the *dnaK204* strain carrying the vector plasmid, the efficiency of cells carrying the *dksA1* plasmid was about 100-fold higher at 40°C than was that of pDKW8-carrying cells. The amino acid change Thr-131 to Ile of the DksA protein seemed to increase the ability to suppress the temperature-sensitive growth of *dnaK204* cells.

Since *dksA* may involve the replication of pSC101, we examined the effect of disrupting *dksA* on the replication of pSC101. PK201 (*dksA*::*kan*) cells (6) were transformed with pSC101, as were their parental cells (MG1655). This shows that *dksA* is dispensable for the replication of pSC101 (data not shown). The plasmid copy number in PK201 was almost the same as in the wild-type cells.

One explanation of how *dksA1* suppresses the mutation at the downstream region of pSC101 *ori* is that DksA is a CAC motif binding protein. Thus, DksA1 can recognize the mutated CAC motif. Another explanation is that *dksA1* stimulates the initiation reaction by activating a protein (e.g., Rep) essential for the reaction, causing initiation without the downstream region. The initiator protein, RepA, of P1 is activated to bind to iterons by DnaJ/DnaK/GrpE proteins (2, 18, 19). Since the *dksA* gene not only rescues the *dnaK* mutation but also suppresses *grpE280* or a *dnaJ* deletion mutation, DksA may have

a function similar to that of DnaK, DnaJ, and GrpE (6). Although pSC101 can be maintained in either the *dksA* null mutant or a deletion mutant of both *dnaJ* and *dnaK* (unpublished data), the lack of a heat shock protein induces the synthesis of other heat shock proteins (3). Thus, it is likely that *dksA* and *dnaK*, *dnaJ*, and *grpE* are involved in the replication of pSC101 and complement each other for their functions. The present suppressor mutation, *dksA1*, was apparently dominant over $dksA^+$. Biochemical analysis of DksA and DksA1 is indicated in order for us to understand the function of *dksA*.

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