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Regulation of initiation of the chromosomal replication by DnaA-boxes in the origin region of the *Bacillus subtilis* chromosome

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A gene homologous to the *Escherichia coli dnaA* gene and two flanking 'regulatory' regions which contain nine and four DnaA-boxes respectively, are located in the replication origin region of the *Bacillus subtilis* chromosome. Attempts to isolate an autonomously replicating fragment from these 'regulatory' regions in order to identify *oriC* have been unsuccessful because the DnaA-box-containing regions strongly inhibited plasmid transformation particularly when inserted into a high-copy number plasmid pUB110. Using two plasmids differing in copy number, the two regions were subdivided into three regions, A, B and C, each containing five, four and four DnaA-boxes respectively, which differed in level of inhibition of transformation. Region C is downstream of the '*dnaA*' gene and inhibits transformation in high-copy but not in low-copy number plasmids. When a part of the DnaA-boxes was deleted from the incompatible plasmids, they became transformable and produced slow-growing transformants in which the initiation frequency of chromosomal replication was selectively reduced. Fast-growing revertants were found containing the same number of plasmids as the parent but with single base changes in the DnaA-boxes. These mutations were in the most highly conserved bases of the DnaA-box sequence. This indicates that a sequence-specific interaction of the DnaA-box, probably with the *B.subtilis* DnaA protein is responsible for the observed incompatibility and thus appears to be involved in control of initiation frequency of the chromosomal replication.

Key words: DnaA-box/*dnaA* gene/incompatibility/initiation frequency/*oriC*/replication

Introduction

In *Escherichia coli*, the *dnaA* gene is essential for initiation of chromosomal replication (see von Meyenburg and Hansen, 1987). The DnaA protein activates *oriC* through binding to a repeating sequence, DnaA-box, present in the *oriC* (Fuller *et al.*, 1984; Sekimizu *et al.*, 1987). We have reported that a gene highly similar to the *E.coli dnaA* gene is located in the replication origin region of the *Bacillus subtilis* chromosome; it is flanked by two 'regulatory' regions containing respectively nine and four repeated DnaA-box sequences identical to the DnaA-box (Ogasawara *et al.*, 1985b). Recently we found that *Pseudomonas putida* also has a *dnaA* gene homologue accompanied by an upstream 'regulatory' region containing eight DnaA-boxes (manuscript in preparation). The region was found to exert *oriC* function in *P.putida* (D.Smith, personal communication). These results may be taken to suggest that the DnaA protein–DnaA-box interaction is a common feature of chromosomal replication of bacteria.

There is no direct evidence for involvement of either the *dnaA* gene or the DnaA-boxes in initiation of chromosomal replication in *B.subtilis* due to the lack of *dnaA* mutations and the failure to isolate autonomously replicating *oriC* minichromosomes as in *E.coli*. On the other hand we observed that fragments containing the 'regulatory' regions flanking the '*dnaA*' gene of *B.subtilis* were highly inhibitory for transformation of *B.subtilis* cells when cloned into plasmids (Ogasawara *et al.*, 1986a). We assumed that growth inhibition of such plasmids is due to incompatibility with the chromosome due to competition for the limited amount of DnaA protein by its binding to the DnaA-boxes. Such incompatibility is commonly observed in plasmid replication (Scott, 1984). This paper describes a genetic analysis of this incompatibility by the DnaA-box. By subcloning subsegments of the DnaA-box containing flanking regions of the '*dnaA*' gene, the growth inhibition was shown to be due to frequency of initiation of chromosomal replication. Subsequently, single base change mutations in DnaA-boxes could be isolated which suppressed the growth inhibition. This demonstrates that at increased copy number the DnaA-boxes influence the initiation of chromosomal replication presumably through competition for the DnaA protein, the presumptive initiator as in *E.coli*.

Results

Inhibition of transformation by plasmid containing the regulatory regions with DnaA-boxes flanking the 'dnaA' gene

Figure 1 shows the nucleotide sequence of the two 'regulatory' regions flanking the '*dnaA*' gene in the origin region of the *B.subtilis* chromosome (Ogasawara *et al.*, 1985a). When fragments derived from these two 'regulatory' regions, regions I and II, were inserted into the high-copy plasmid pUB110, the resulting plasmids could not transform the competent cells (Ogasawara *et al.*, 1986a). Deletion analysis suggested direct involvement of DnaA-boxes in this inhibition of transformation (Ogasawara *et al.*, 1986a). We assumed that the plasmid containing DnaA-boxes became incompatible with the chromosome, probably through interaction with DnaA protein.

To examine this possibility various fragments from regions I and II were inserted into two plasmids differing in copy number. One is a chimera plasmid between pBR322 and pUB110 with a high copy number (40–50 copies/chromosome). The other is a low-copy number chimera between pBR322 and pE194 (5–6 copies/chromosome; Scheer-Abramowitz *et al.*, 1981). Testing the resulting plasmids, we found that transformation was inhibited by

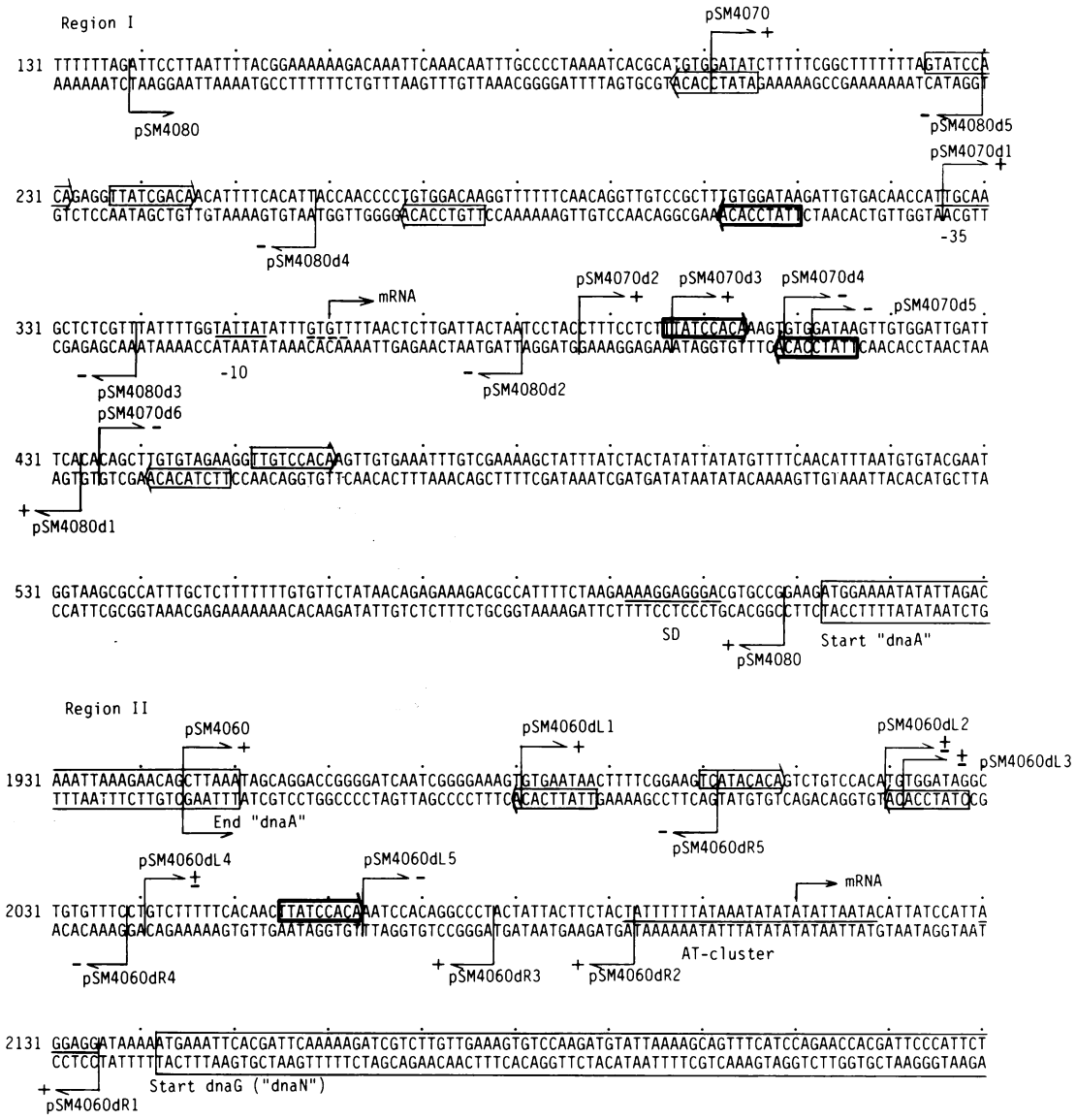


Fig. 1. Nucleotide sequence of the 'regulatory' regions flanking the '*dnaA*' gene and end points of deletion plasmids in these regions. Nucleotide sequence and identification of ORFs for the 'DnaA' and 'DnaN' proteins, transcriptional start sites and DnaA-boxes, consensus (thick arrow box) and one base change (thin arrow box), are as reported previously (Ogasawara *et al.*, 1985b). Arrows dividing the sequence indicate end points of deletion in each plasmid named above the arrow. Arrows pointing right indicate deletions from left and arrows pointing left are deletions from the right. − or + attached to arrows indicates whether each plasmid is capable or incapable of transforming *B.subtilis* respectively at high-copy number. See the text and Figure 4 for ±.

those fragments containing DnaA-boxes (Figure 2), confirming the previous results (Ogasawara *et al.*, 1986a). It should be noted that promoters for '*dnaA*' gene in region I and for '*dnaN*' [equivalent to *dnaG* in *B.subtilis* (Ogasawara *et al.*, 1987b)] in region II have no effect on transformation (plasmids pSM4080d2 and pSM4060dL5 respectively).The 'regulatory' regions containing DnaA-boxes were divided into three regions, A, B and C, each exhibiting a distinctly different level of transformation inhibition (Figure 2). Region A, consisting of five DnaA-boxes, did not result in any inhibition nor any effect on growth even in the high-copy plasmid. Region B, with four DnaA-boxes, showed strong inhibition of transformation even in the low-copy number plasmid. In some cases, a small number of transformant colonies appeared when using the low-copy plasmid with region B or part of it (Figure 2). The plasmids in these transformants showed drastic changes in the structure of the inserted B region (Figure 3A). Region C with four boxes showed inhibition of transformation at high copy number only (Figure 2). Approximately six copies of plasmids could be maintained in the cell without apparent structural changes (Figure 3B). The differences in the levels of inhibition by the three regions essentially similar in number and relative position of DnaA-boxes suggest that the exact positioning of DnaA-boxes is important for the extent of transformation inhibition. It is interesting to note that *oriC* from *E.coli* containing four DnaA-boxes had no effect on *B.subtilis* either at high or low copy number. The deletion anlaysis also demonstrates that in each of the two regions, B and C, one of the DnaA-boxes is particularly critical for the inhibition effect; in region B it is the left-most, in region C the right-most box (Figures 1 and 2).

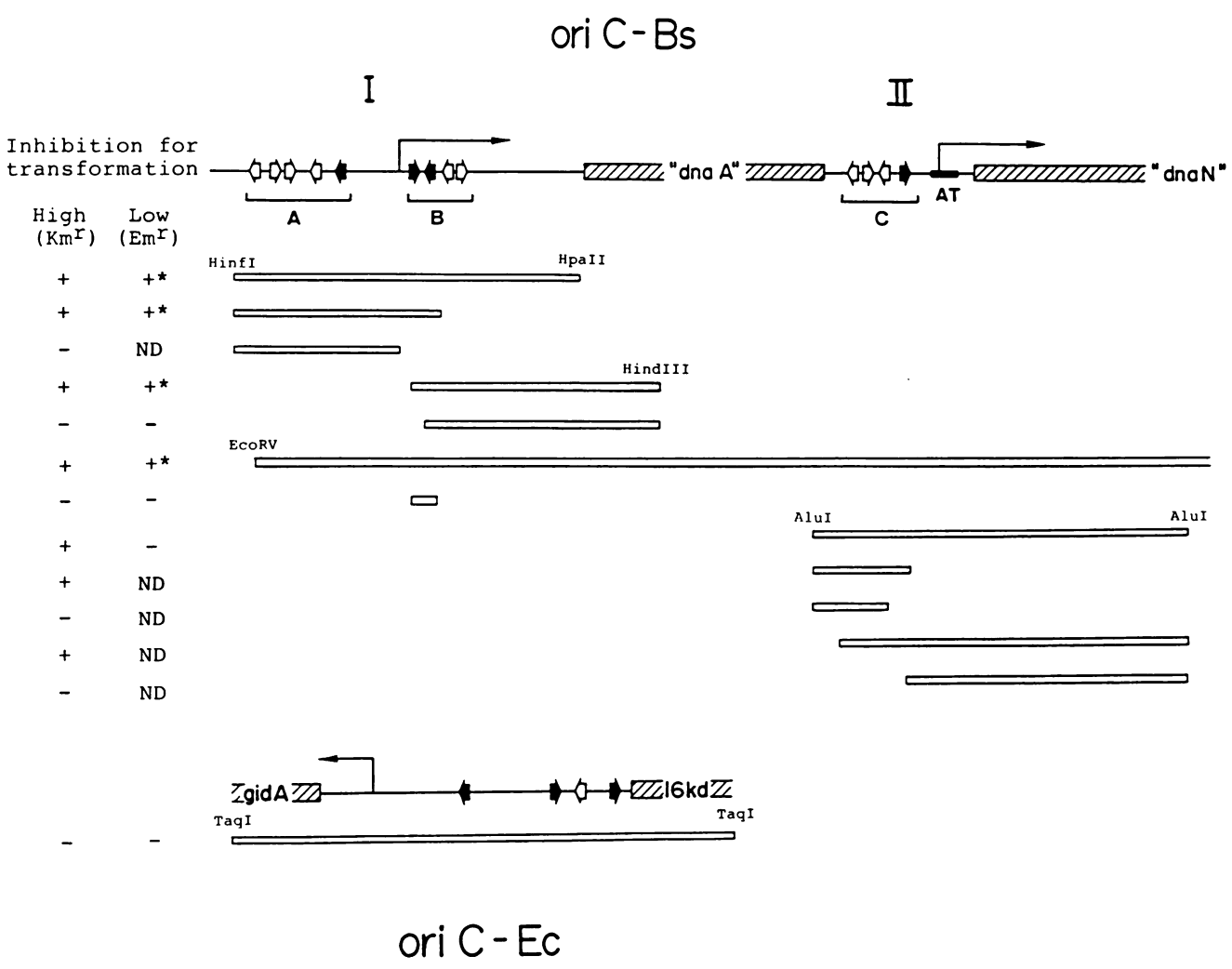


Fig. 2. Transformation of *B.subtilis* by two types of plasmids (high- and low-copy number) containing various fragments in the origin region. At the top and bottom, DNA structures of the origin region of *B.subtilis* and *E.coli* are schematically drawn respectively. Shaded bars are open reading frames. Arrows are DnaA-box (TTATC/ACACA) sequences either consensus (black) or with one base difference (open). Transcription initiation sites (broken arrow) are indicated. Open thin bars under the maps show locations of fragments inserted into plasmids. + or − in inhibition for transformation indicates that frequency of transformation was $<10^{-7}$ or $\sim 5 \times 10^{-5}$ respectively. +* shows that a small number of heterogeneous transformants appeared after prolonged incubation. 'High' and 'Low' indicate that a given fragment was inserted into plasmids of high (pN04) and low-copy number (pTF1000) respectively, as described in Materials and methods. N.D. signifies not done.

Region C inhibits initiation of chromosomal replication

In the analysis of transformation by the high-copy plasmid containing various fragments from region C, we observed with some plasmids the appearance of slowly growing transformants with doubling times about twice as long as those of the parental cells in rich media (Figures 4 and 5A). Plasmids giving this phenotype contained the most rightward DnaA-box either alone or together with one additional box. A plasmid with the three leftward boxes (pSM4060dR4) did not inhibit growth. In all slow growers, normal numbers of the plasmid, some 50 copies/chromosome, were present without detectable changes in the size of the insert or the vector (data not shown).

To examine the nature of the slow growth, we measured the marker frequency of a genetic marker (*purA*) near the replication origin region and a marker (*metB*) near the terminus of the chromosome in DNA prepared from exponentially growing cells of these slow growers and then compared these with the same markers in parental cells. The *purA/metB* ratios when normalized with the same ratio of the DNA from stationary phase cells correspond approximately to the number of replication origins per chromosome (Yoshikawa and Sueoka, 1963). The data in Figure 4 show that the ratios in slow growers are markedly lower than those of control cells. Values of 2.3 origins/chromosome in control cells, which contain the parental plasmid (pN04), or of 1.9−2.1 in cells with compatible plasmids (pSM4060dL5, pSM4060dR4) indicate that most cells in these rapidly growing cultures are in a replicating state throughout the division cycle. On the other hand, the ratios in slow growers were on average 1.4 origins/chromosome, indicating that only ~40% of these cells are replicating at a given time and that the remaining cells are in a resting state of DNA replication. In the slowly growing cells, DNA:protein ratios were decreased on average by 43% compared to control cells (Figure 4). This indicates that chromosomal replication is selectively inhibited by the plasmid. From the decrease in marker frequency ratio (*purA/metB*), the decreased DNA: protein ratio and the increased doubling time (Figure 5A),

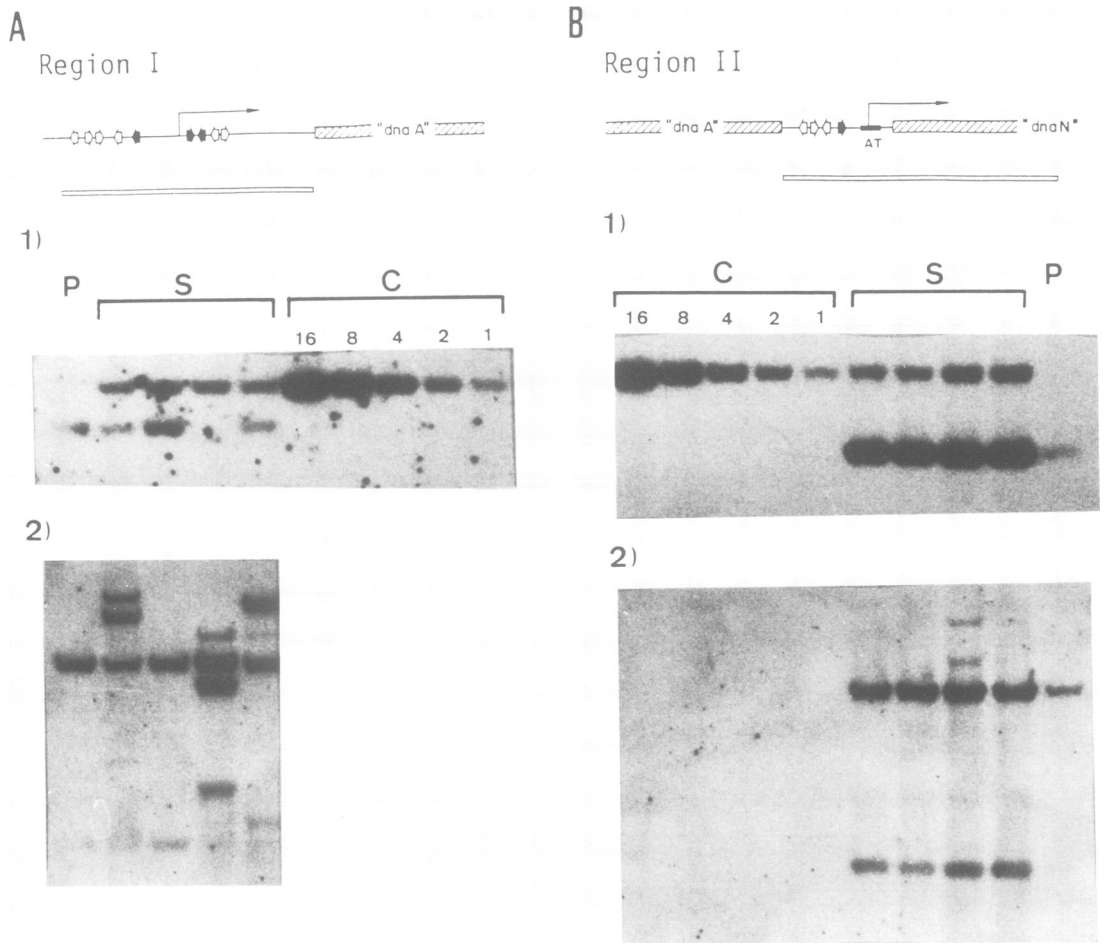
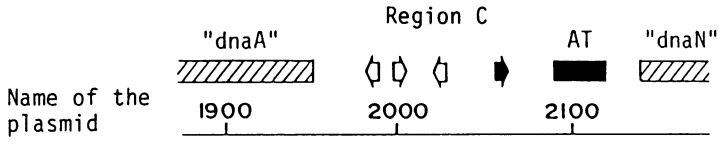


Fig. 3. Structure and copy number of plasmids in the cell transformed by low-copy number plasmids containing DnaA-boxes. Experimental procedures as described in Materials and methods. DNA structures of region I (**A**) and region II (**B**) are schematically drawn with flanking regions. Open thin bars show fragments originally inserted into the low-copy plasmid. Fragments identical to those inserts were used as probes in experiment 1, while vector portions of the plasmids were used in experiment 2. Purified DNAs were charged after digestion with a mixture of *Pst*I and *Hin*dIII, separated by electrophoresis in agarose gel and then blotted to nitrocellulose filter. The filter was first probed by the inserts (experiment 1). The same paper was used for the second vector probe (experiment 2) after removing the first probe by washing. In lanes marked **C**, chromosomal DNA alone was charged in 1-, 2-, 4-, 8- and 16-fold for density standard. Samples prepared from four independent transformants were charged in lanes marked **S**. **Lane P** is the parental plasmid used for the transformation.



Name of the plasmid	Inc	Doubling time (min.)	purA/metB (normalized) exp.1	purA/metB (normalized) exp.2	DNA/protein exp.1	DNA/protein exp.2
pSM4060	+					
pSM4060dL1	+					
pSM4060dL2	±	68	1.41	1.39	0.023	0.025
pSM4060dL3	±	75	1.48	1.39	-	0.031
pSM4060dL4	±	74	1.26	1.48	0.029	-
pSM4060dL5	−	35	1.95	2.21	0.050	0.044
pSM4060dR1	+					
pSM4060dR2	+					
pSM4060dR3	+					
pSM4060dR4	−	35	1.89	-	0.046	0.043
pN04	−	33	2.37	2.29	0.050	0.048

Fig. 4. Frequency of initiation of chromosomal replication in cells transformed by plasmids containing DnaA-boxes. In the left half, structure of the 'regulatory' region II (= region C) and locations of fragments are schematically drawn. Names of plasmids containing these fragments are the same as those in Figure 1. Inc, incompatibility. Inc^+ signifies that the plasmid with the indicated fragment as an insert could not transform *B.subtilis*, while plasmids containing Inc^- fragments produced several hundred transformants per plate containing $\sim 10^7$ cells. $Inc^\pm$ produced similar numbers but much smaller transformed colonies as compared to Inc^- fragments. pN04 is the parental plasmid without chromosomal insert. Doubling time was measured in a rich (1.9% brain heart infusion) medium in the presence of 10 μg/ml kanamycin. The *purA/metB* and DNA:protein ratios were determined as described in Materials and methods.

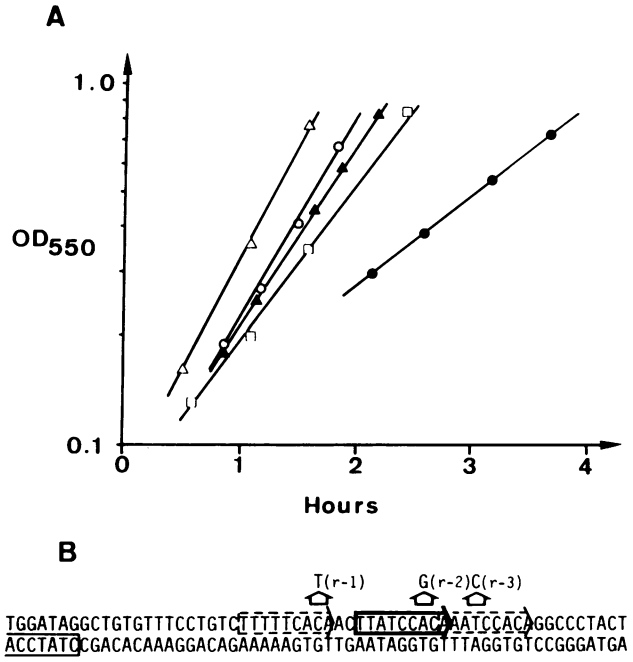


Fig. 5. Growth of the slowly growing cell and its revertants and mutation sites found in the plasmid DNA in the revertants. (**A**) Growth curves of the parental cell containing pN04 (no DnaA-box insert) [○——○, doubling time (dt) 33 min], the slowly growing transformant wiht pSM4060dL3 (●——●, dt 72 min) and three revertants r-1 (△——△, dt 29 min), r-2 (▲——▲, dt 35 min) and r-3 (□——□, dt 42 min) are shown. Cells were grown as described in the legend to Figure 4. (**B**) Sequence of the left terminal portion of the insert of pSM4060dL3 containing one DnaA-box is shown. Single base changes found in plasmid in the three revertants are indicated. Consensus DnaA-box sequence is indicated by an arrow-shaped box, and sequences differing two bases from the consensus sequence are shown by dotted lined arrows.

we can infer that it is not elongation of chromosomal replication but rather initiation which is inhibited by the 'partially incompatible' plasmids (pSM4060dL2, pSM4060dL3 and pSM4060dL4). Therefore one can also suggest that the stronger incompatibility of the plasmids with the complete C region is due to an even more extensive inhibition of initiation of chromosomal replication.

Mutations in DnaA-boxes in region C suppress growth inhibition

To determine genetic factors responsible for the decrease in the initiation frequency of chromosomal replication, normally growing revertants were isolated from the slowly growing cells (Figure 5A). Three such fast growers were isolated from a pSM4060dL3 transformant. Since they were found to carry plasmids in about the same number of copies as before, without detectable changes in size, the nucleotide sequence of the chromosomal insert of the plasmid was determined. In each case a single base substitution was detected in the right-most DnaA-box of region C or very close to it (Figure 5B). Close examination of the sequence near the mutated sites revealed that there are two sequences flanking that DnaA-box which are identical to the consensus sequence of the DnaA-box except for two base changes. In order to examine the significance of these base changes the sequences of DnaA-boxes were collected from replication origin regions of bacterial chromosomes so far identified including those with up to two base changes from the consensus sequence TTATCCACA (Figure 6). Within the

oriC-Pp	oriC-BsI	oriC-BsII	oriC-Ec
TGATCAACA	ATATCCACA	TTATTCACA	TTATCCACA
TTATCCACT	GTATCCACA	TCATACACA	TTATCCAAA
TTATCCACA	TTATCGACA	CTGTCCACA	TTATACACA
TCATCCACA	TTGTCCACA	CTATCCACA	TCATTCACA
TTATCCACA	TTTTCAACA	TTTTTCACA	TTATCCACA
TTATCAACA	TTATCCACA	TTATCCACA	
CTATGCACA	TTATCCACA	AAATCCACA	
TTATCCACA	TTATCCACA		
TTATTCACA	CAATCCACA		
TTATCGACC	TTCTACACA		
GTGTCCACA	TTGTCCACA		
TTATCCACA	TTTTCGACA		
	TTATCTACT		
	TTTTCAACA		

A_2	A_2	A_{29}	A_3	A_4	A_{38}	A_1	A_{35}
C_4	C_3	C_1	C_{30}	C_{30}		C_{37}	C_1
G_2	G_1	G_4	G_1	G_3			
T_{30}	T_{32}	T_4 T_{38}	T_4	T_1			T_2

Fig. 6. List of DnaA-box sequences and their cumulative sequence. DnaA-boxes including those differing by one or two bases from the consensus TTATCCACA found in the replication origin region of the bacterial chromosomes: Pp, *Pseudomonas putida* (manuscript in preparation); Bs, *B.subtilis* (Moriya *et al.*, 1985); Ec, *E.coli* (Hirota *et al.*, 1981) are listed. All the sequences in the list are cumulated at the bottom to deduce a consensus sequence shown in bold letters.

nine nucleotides, the fourth (T), seventh (A) and eighth (C) bases appear to be the most highly conserved. The three mutations found in the plasmids from the rapid growing revertants coincide with these most conserved bases (Figure 5B).

This surprising result also reveals that the slow growth phenotype is not only due to the right-most *bona fide* DnaA-box in region C but is dependent on the neighboring *quasi* DnaA-boxes as well. The contributions of the three boxes, however, may not be equal; one of the mutations (r-3) only relieves growth inhibition partially (Figure 5A). Although we have not demonstrated to what extent marker frequency and DNA contents were normalized by these single base change mutations, we can interpret these results as demonstrating that it is the presence of an increased number of the DnaA-boxes which leads to inhibition of initiation by pSM4060dL3.

Discussion

Co-existence of two types of plasmids which share common factors for replication in the same cell is mutually restricted in many plasmids (see Novick and Hoppensteadt, 1978; Nördstrom, 1983). This is known as incompatibility between replicons. In *E.coli* multiple copies of *oriC* plasmids can co-exist with the chromosome (von Meyenburg *et al.*, 1978; Yamaguchi *et al.*, 1982; Stuitje and Meijer, 1983). Lack of incompatibility between the *oriC* plasmid and the *E.coli* chromosome is rather mysterious considering the fact that initiation frequency of the chromosomal replication is strictly regulated. In some *dnaA*(ts) mutants of *E.coli*, however,

plasmids containing DnaA-boxes have been shown to exhibit 'incompatibility' which is interpreted to be due to lack of active DnaA protein (F.G.Hansen, personal communication). In *dnaA*$^+$ strains the competition for DnaA protein by minichromosomes or DnaA-box-containing plasmids might not be apparent since derepression of the *dnaA* gene expression seemed to lead to a compensatory increase in total DnaA protein level (Hansen *et al.*, 1987). Failure to isolate *oriC* plasmids in *B.subtilis* suggested that the *oriC* containing plasmids are strictly incompatible with the chromosome in this bacterium. Thus, insertion of DNA segments from the '*dnaA*' gene region containing several copies of a DnaA-box sequence into two types of plasmids of low and high copy numbers appeared to make them incompatible with the chromosome, yet to different extents.

One of the three clusters of DnaA-boxes, the two closest to the '*dnaA*' gene, regions B and C, both resulted in strong incompatibility, i.e. lack of transformability at high copy number; the latter did not exhibit incompatibility at low copy number, whereas region B did. In this latter case the presence of one of the DnaA-boxes appeared to be critical for the effect; its elimination relieved the effects almost completely.

For the C region, it could be demonstrated that incompatibility was correlated with inhibition of growth and of initiation of chromosomal replication. The isolation of suppressing point mutations in DnaA-boxes rules out the possibility that this incompatibility is due to inhibition of plasmid replication by the secondary structure of the unit (Hagan and Warren, 1983) or by strong promoters contained in it (Seiki *et al.*, 1981). The direct involvement of the primary structure of the DnaA-boxes—in particular the most highly conserved bases—in yielding inhibition of initiation suggests that the effect is due to competition between the DnaA-boxes on the plasmid and the chromosome for proteins essential for initiation of chromosomal replication. We suggest that it is the DnaA protein which is being titrated out because of its binding—in analogy to the *E.coli* case—to DnaA-box sequences. Although we could not test the incompatibility exerted by region B, because it appeared to be complete even at the low copy number, we suspect that it will turn out to be due to inhibition of chromosomal initiation as well. If it is due to lack of free active DnaA protein as initiator, as in *E.coli* (von Meyenburg and Hansen, 1987), controlled overexpression of the *B.subtilis* '*dnaA*' gene on a plasmid should suppress this incompatibility.

Materials and methods

Bacterial strains and plasmids

Bacillus subtilis PSL1 (*leuA8, arg15, thrA, hsrM, hsmM, recE4, stp*) (Ostroff and Pène, 1983) was a gift from J.Pène, University of Delaware, and used for plasmid transformation. *Bacillus subtilis* strain CRK3000 (*purA16, leuA8, hisA3, metB*) (Yamaguchi and Yoshikawa, 1975) was used to determine *purA/metB* ratios by transformation. *Escherichia coli* HB101, TC1963 [*dnaA46, int*(mini-R1)] (Andrup *et al.*, 1988) and C600r$^-$m$^-$ were used as hosts for DNA cloning using derivatives of pBR322 as cloning vector. Plasmid pN04 (Ogasawara *et al.*, 1984), a chimera between pBR322 and pUB110, and pTF1000, a chimera between pBR322 and pE194, were representatives of high- (40–50 copies/cell) and low-copy number (5–6 copies/cell) plasmids; they were used to construct plasmids containing various DnaA-box fragments. A fragment from the upstream non-translation region (region I) of *B.subtilis* '*dnaA*' gene, a *Hin*fI–*Hpa*II fragment, nt 139–608 (Moriya *et al.*, 1985) (see Figures 1 and 2), was inserted into the *Cla*I site of pN04 to construct pSM4080. A second fragment from the same region, an *Eco*RV–*Hin*dIII fragment, nt 201–1037 (Moriya *et al.*, 1985) (see Figure 2), was inserted by replacing a *Hin*dIII–*Eco*RV fragment of pN04 to construct pSM4070. The two inserts were oriented in opposite directions relative to the *Bam*HI site of the vector from which site a series of deletions was produced (see below).

An *Eco*RI–*Bam*HI fragment derived from pSM2060 (Ogasawara *et al.*, 1986b) including the downstream non-translation region (region II), nt 1945–2472 (see Figure 2), was inserted by replacing the smaller *Eco*RI–*Bam*HI fragment of pN04 to obtain pSM4060. pSM4080, pSM4070 and pSM4060 were used to make fragments containing a series of deletions in regions I and II (see Figure 1). They were then cut out from the plasmids, repaired by Klenow enzyme and transferred to the *Eco*RI site of pTF1000 to obtain a series of low-copy plasmids containing various portions of regions I and II (see Figure 2). pKY507 (Yamaguchi *et al.*, 1982), a gift from K.Yamaguchi, University of Kanazawa, contains *oriC* of *E.coli*. A *Taq*I fragment of pKY507, an *E.coli oriC* fragment, was transferred to pN04 at the *Cla*I and to pTF1000 at the *Eco*RI site respectively (see Figure 2).

Chemicals

A calf intestine alkaline phosphatase was purchased from Boehringer Mannheim GmbH (Mannheim, FRG). Restriction enzymes, T4 DNA ligase, Klenow enzyme, T4 polynucleotide kinase, low-melting-point agarose, M13 sequencing kit and 5′-phosphorylated *Sal*I and *Eco*RI linkers were from Takara Shuzo Co. Ltd (Kyoto, Japan). A multiprime DNA labelling system, [γ-^{32}P]ATP (PB10168, 3000 Ci/mmol) and [α-^{32}P]dCTP (PB10205, 3000 Ci/mmol), were from Amersham International Ltd (Amersham, UK). A nick-translation kit and M13mp18 and mp19 RF DNA were from Nippon Gene Co. Ltd (Toyama, Japan).

Preparation of series of deletions in regions I and II

Plasmids pSM4080 and pSM4070 containing two portions of region I in opposite orientations were linearized by *Bam*HI and then digested with appropriate units of *Bal*31 at 37°C for various times (see Figure 1). 5′-Phosphorylated *Sal*I linkers were ligated to the digests by T4 DNA ligase after it was treated with both Klenow enzyme and calf intestine alkaline phosphatase. After digestion with *Sal*I and circularization, *E.coli* cells were transformed. Plasmid DNA from each transformant was analyzed to estimate the extent of deletions by agarose gel electrophoresis after digestion with appropriate enzymes. pSM4060 was used to construct a series of deletions in region II from two directions, either from *Bam*HI site as above ligated with *Sal*I linker, or from *Eco*RI site and ligated with *Eco*RI linker.

Determination of the end points of the deletion

Each deletion plasmid was cleaved at either *Sal*I or *Eco*RI site nearest the end point of the deletion. They were treated with calf intestine alkaline phosphatase, digested with the second restriction enzyme and fractionated in low-melting-point agarose. Fragments were recovered from the gel and 5′-end-labelled by [γ-^{32}P]ATP and T4 polynucleotide kinase. The sequence around the end point was determined by the method of Maxam and Gilbert (1980).

Plasmid transformation of B.subtilis

Plasmid DNA (2–4 μg) containing various portions of the *B.subtilis* replication origin region were introduced into PSL1 cells by the plasmid transformation method (Contente and Dubnau, 1979). Transformants by derivatives of the plasmids of high and low copy were selected on a rich (1.9% brain heart infusion with 10 μg/ml kanamycin) and a synthetic Cg medium (Yoshikawa, 1966) (Cg–0.5% glucose-requirements with 5 μg/ml erythromycin) respectively.

Estimation of copy number of plasmids in B.subtilis

From transformants with high-copy number plasmids, plasmid DNA was extracted (Birnboim and Doly, 1979) and the amount of DNA was estimated by measuring the density of the stained band in agarose gel after electrophoresis of the samples digested with appropriate restriction enzymes. Plasmid DNA in transformants with low-copy number plasmids was determined by Southern blot hybridization using cloned origin fragments as probes, as indicated in Figure 3. The probe was labelled with [α-^{32}P]dCTP with a multiprime DNA labelling system according to the supplier's manual. Whole DNA was extracted from the cell, purified and separated in agarose gel after digestion with restriction enzyme appropriate for separating two origins, one from chromosome and the other from plasmid insert, by size. The 'origin DNA' derived from the chromosome should serve as an internal control to estimate copy number of the chromosomal origin insert in plasmids. The vector portion of the plasmids was detected using the parental plasmid pTF1000 as a probe labelled by nick translation.

Determination of purA/metB ratios

Whole DNA was extracted from cells either in the exponentially growing state or in stationary phase and purified by treatment with phenol/chloroform/

isoamylalcohol. The purified DNA of unsaturated concentration was used to transform *B.subtilis* CRK3000 by the conventional method (Haas and Yoshikawa, 1969). Transformants for *purA* and *metB* were scored and the *purA/metB* ratios were determined. The *purA/metB* ratios obtained for DNA from exponentially growing cells were normalized by the same ratio obtained for DNA from the stationary phase cells of *B.subtilis* strain PSL1.

Determination of DNA:protein ratios

Nucleic acid fraction was extracted from cell pellets by a modified Schneider's method (Herbert *et al.*, 1971) and the residuals were dissolved in 1 N NaOH to solubilize protein fraction. DNA or protein content was determined by the method of Burton (1956) or Lowry (1951) respectively.

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