Effects of *oriC* relocation on control of replication initiation in *Bacillus subtilis*

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In bacteria, DNA replication initiation is tightly regulated in order to coordinate chromosome replication with cell growth. In *Escherichia coli*, positive factors and negative regulatory mechanisms playing important roles in the strict control of DNA replication initiation have been reported. However, it remains unclear how bacterial cells recognize the right time for replication initiation during the cell cycle. In the Gram-positive bacterium *Bacillus subtilis*, much less is known about the regulation of replication initiation, specifically, regarding negative control mechanisms which ensure replication initiation only once per cell cycle. Here we report that replication initiation was greatly enhanced in strains that had the origin of replication (*oriC*) relocated to various loci on the chromosome. When *oriC* was relocated to new loci further than 250 kb counterclockwise from the native locus, replication initiation became asynchronous and earlier than in the wild-type cells. In two *oriC*-relocated strains (*oriC* at *argG* or *pnbA*, 257° or 300° on the 360° chromosome map, respectively), DnaA levels were higher than in the wild-type but not enough to cause earlier initiation of replication. Our results suggest that the initiation capacity of replication is accumulated well before the actual time of initiation, and its release may be suppressed by a unique DNA structure formed near the native *oriC* locus.

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INTRODUCTION

Replication of genomic DNA is indispensable for cell growth. In bacteria, replication begins at a fixed site (*oriC*) within the genome and proceeds bidirectionally. In order to coordinate replication with cell growth, the most important control is carried out at the initiation stage (von Meyenburg & Hansen, 1987). In fast-growing cells (generation time <60 min), the time required for replication and cell division is longer than the generation time. To compensate for this potentially serious problem, cells start a new round of replication before previous rounds terminate in a process known as dichotomous replication (Yoshikawa & Wake, 1993). It is unclear how cells precisely recognize the right stage in the cell cycle to initiate replication.

In *Escherichia coli*, several positive and negative factors appear to participate in the regulation of replication initiation. DnaA protein level is a positive factor, as increased levels of DnaA cause overinitiation and advance the initiation event to an earlier time in the cell cycle (Atlung & Hansen, 1993; Løbner-Olesen *et al.*, 1989). The level of the ATP-bound form of DnaA, which is the active form for replication initiation, correlates with this property (Kurokawa *et al.*, 1999). A recent finding has indicated that DiaA tetramers stimulate assembly of active ATP–DnaA–*oriC* initiation complex by directly binding to multiple DnaA molecules (Keyamura *et al.*, 2007). Three negative mechanisms are known to inhibit extra initiations of DNA replication. The first one is sequestration of newly duplicated hemimethylated origins into the cell membrane by SeqA protein (Campbell & Kleckner, 1990; Lu *et al.*, 1994; Ogden *et al.*, 1988). Two others are inactivation of DnaA initiation protein by Hda and DnaN (β subunit of DNA polymerase III) (Katayama *et al.*, 1998; Kato & Katayama, 2001) and sequestration of DnaA at the *datA*

Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; PLR, polar localization region.

A supplementary table, listing nucleotide sequences of primers used in this study, is available with the online version of this paper.

region on the chromosome (Kitagawa *et al.*, 1998; Ogawa *et al.*, 2002). Accordingly, in *E. coli*, initiation of replication is tightly regulated by these factors and mechanisms so that the chromosome is replicated only once at the correct stage in every cell cycle.

DnaA is also essential for initiation of replication in *Bacillus subtilis* (Moriya *et al.*, 1990) and high levels of DnaA protein advance the stage of replication initiation to earlier in the cell cycle (Ogura *et al.*, 2001). It has not yet been examined whether both ATP- and ADP-bound forms of DnaA exist in *B. subtilis* cells (only ATP-DnaA is active for replication initiation). As to negative factors, intriguingly, there are no homologues of SeqA, Dam methylase and Hda (Kunst *et al.*, 1997). It is therefore unlikely that these two negative regulation mechanisms, sequestration of new origins into the cell membrane and inactivation of DnaA by Hda, are acting in *B. subtilis* as they do in *E. coli*. However, a novel negative regulator of replication initiation, YabA, has been recently found in *B. subtilis* (Hayashi *et al.*, 2005; Noirot-Gros *et al.*, 2002). When the YabA level is reduced, replication begins at a decreased cell mass, whereas when the YabA level is increased, replication initiation is delayed. This protein does not have a significant homology with *E. coli* Hda, but like this protein, it appears to interact with both DnaA and DnaN (sliding clamp of DNA polymerase III) (Noirot-Gros *et al.*, 2002, 2006). Very recently, it has been proposed that YabA spatially sequesters DnaA initiator molecules from *oriC* by binding with the replisome during most of the cell cycle to prevent inappropriate reinitiation of replication. DnaA colocalizes with replication origin regions only at early and late stages of the cell cycle (Soufo *et al.*, 2008). Intriguingly, it has also been very recently reported that Soj, which is a *B. subtilis* orthologue of ParA engaged in plasmid partitioning, interacts with DnaA directly and controls its activity (Murray & Errington, 2008). Furthermore, although the mechanism has not yet been determined, DNA replication initiates at a lower cell mass in *pgcA* disruption mutants (Weart *et al.*, 2007).

Previously, we constructed an *oriC*-relocated strain of *B. subtilis* by transferring *oriC* from the native locus to *argG*, which is very far (~1200 kb) from the native *oriC* on the chromosome (Kadoya *et al.*, 2002). Here we show by flow cytometry that in this *oriC*-relocated strain, synchrony of replication initiation is disrupted and initiation frequency is enhanced. In order to examine the correlation between relocation of *oriC* and the control of replication initiation, new *oriC*-relocated strains were constructed by transferring *oriC* to various loci on the chromosome. When *oriC* was relocated to loci further than 250 kb counterclockwise from the native locus, the strict control of replication initiation was disrupted, resulting in asynchronous initiation and early initiation during the cell cycle. Although DnaA levels were measurably higher in the *oriC*-relocated strains compared with the wild-type, this increase was not enough to cause the early initiation. Our results suggest that the potential for replication initiation is accumulated

well before the actual time of initiation, and that release of this potential is suppressed by specific DNA structures in the *oriC* region of the *B. subtilis* chromosome such as so-called 'Spo0J domains' (Breier & Grossman, 2007; Murray *et al.*, 2006), the polar localization region (PLR) (Wu & Errington, 2002) and other unknown higher-order DNA structures.

METHODS

Plasmids. Nucleotide sequences of primers used in this study are shown in Supplementary Table S1.

For pSM5022-*proA*', pSM5022-*ykxC*, pSM5022-*pnbA* and pSM5022-*purA*', an internal DNA fragment of each gene was amplified by PCR using the appropriate F and R primers shown in Table S1 and cloned into the *Bam*HI site of an *oriC* plasmid, pSM5022 (Moriya *et al.*, 1992). The lengths of each internal DNA fragment amplified were as follows: *proA*', 1.07 kb (Hassan *et al.*, 1997); *ykxC*, 621 bp; *pnbA*, 595 bp; *purA*', 660 bp. For pSM5022-*ermC*', an internal DNA fragment (1188 bp) of the *ermC* gene was amplified from pMutinT3 (Moriya *et al.*, 1998) with two primers, *erm*-iF and *erm*-iR, and cloned into the *Bam*HI site of pSM5022.

Strains. *B. subtilis* strains used in this study are listed in Table 1. Strains in this table were constructed as follows.

For NIS6553, 5' and 3' halves of the *iolJ* gene were amplified by PCR from *B. subtilis* wild-type chromosomal DNA with two primer sets, -140kAF-AR and -140kBF-BR (Table S1), respectively. The *ermC* gene was amplified from pMutinT3 plasmid DNA using two primers, *ermF* and *ermR* (Table S1). Because -140kAR and -140kBF contain sequences complementary to *ermF* and *ermR*, respectively, the two *iolJ* DNA fragments amplified as above can be joined to the *ermC* gene by PCR using the -140kAF-*ermR* and *ermF*-140kBR primer sets, respectively. These two PCR fragments, *iolJA-ermC* and *ermC-iolJB*, can be further joined to *iolJB* and *iolJA* PCR fragments, respectively, by PCR with two primers, -140kAF and -140kBR. The resultant PCR product containing *iolJA-ermC-iolJB* was used as donor DNA to transform CRK6000. Erythromycin-resistant transformants were selected and the insertion of the resistant gene into *iolJ* gene by double crossover was confirmed by PCR. Subsequently, this strain, NIS6503 (Table 1), was again transformed with DNA of plasmid pSM5022-*ermC*'. Chloramphenicol-resistant transformants were selected and checked for erythromycin-sensitivity. The plasmid DNA is integrated at the *iolJ* locus by a single crossover using the homologous *ermC* genes for recombination. Proper integration was confirmed by PCR. Finally, this strain, NIS6513, was again transformed with chromosomal DNA of NIS6551 to introduce a deletion of *oriC* at the native locus. Transformants were selected on a plate containing erythromycin. The replacement of *dnaA-dnaN* (*oriC*) with the *bgaB-ermC* construct was confirmed as indicated in Fig. 1(d).

For NIS6555, SU6556, SU6557 and NIS6558, the strains were constructed as indicated above for NIS6553, except that chromosomal DNA of NIS6554 was used as donor followed by selection on a plate containing spectinomycin to introduce the deletion of the native *oriC* (Table 1). The *ermR2* primer was used instead of *ermR* to construct SU6556 and SU6557.

Escherichia coli, TC1963 [*dnaA46 int*(mini-R1) RB1-*dnaA::lacZ,araD139 Δ(ara, leu)7697 ΔlacX74, galU, galK, strA*] (Andrup *et al.*, 1988) was used as a cloning host throughout this study.

Table 1. *B. subtilis* strains used in this study

Arrows indicate transformation. Donor DNA and the recipient strain are shown at the left and right of arrows, respectively, with antibiotics and markers for selection. Cp, chloramphenicol; Em, erythromycin; Sp, spectinomycin; Em^s, erythromycin-sensitive; *pro*⁻, proline requirement; *purA*⁻, adenine requirement. Names of strains temporarily made during construction are shown in parentheses after selection methods. Transformation of *B. subtilis* cells was carried out as described elsewhere (Kunst *et al.*, 1994). chr., chromosomal.

Strain	Relevant genotype	Reference or construction
CRK6000	<i>purA16 metB5 hisA3 guaB</i>	Moriya <i>et al.</i> (1990)
NIS6451	CRK6000 <i>argG</i> ::pAH3 (<i>oriC cat</i>) <i>ΔoriC(dnaA-dnaN)::bgaB-ermC</i>	Kadoya <i>et al.</i> (2002)
NIS6251	CRK6000 <i>proA</i> ::pSM5022-' <i>proA</i> (<i>oriC cat</i>) <i>ΔoriC(dnaA-dnaN)::bgaB-ermC</i>	pSM5022-' <i>proA</i> →CRK6000 (Cp, <i>pro</i> ⁻) (NIS6250) Linear form of pRK4 (Kadoya <i>et al.</i> , 2002)→NIS6250 (Em)
NIS6261	CRK6000 <i>ykxC</i> ::pSM5022-' <i>ykxC</i> (<i>oriC cat</i>) <i>ΔoriC(dnaA-dnaN)::bgaB-ermC</i>	pSM5022-' <i>ykxC</i> and linear form of pRK4→CRK6000 (Cp, Em)
NIS6551	CRK6000 <i>pnbA</i> ::pSM5022-' <i>pnbA</i> (<i>oriC cat</i>) <i>ΔoriC(dnaA-dnaN)::bgaB-ermC</i>	pSM5022-' <i>pnbA</i> and linear form of pRK4→CRK6000 (Cp, Em)
NIS6552	CRK6000 <i>purA</i> ::pSM5022-' <i>purA</i> (<i>oriC cat</i>) <i>ΔoriC(dnaA-dnaN)::bgaB-ermC</i>	168 chr.→CRK6000 (<i>purA</i> ⁺) (NIS6502) pSM5022-' <i>purA</i> and linear form of pRK4→NIS6502 (Cp, <i>purA</i> ⁻)
NIS6553	CRK6000 <i>iolJ</i> (<i>ermC</i>)::pSM5022-' <i>ermC</i> ' <i>ΔoriC(dnaA-dnaN)::bgaB-ermC</i>	<i>iolJA-ermC-iolJB</i> PCR products→CRK6000 (Em) (NIS6503) pSM5022-' <i>ermC</i> '→NIS6503 (Cp, Em ^s) (NIS6513) NIS6551 chr. DNA→NIS6513 (Em)
NIS6554	CRK6000 <i>iolJ</i> (<i>ermC</i>)::pSM5022-' <i>ermC</i> ' <i>ΔoriC(dnaA-dnaN)::bgaB-spc</i>	Linear form of pEm::Spc (Lindow <i>et al.</i> , 2002)→NIS6553 (Sp)
NIS6555	CRK6000 <i>yxIA</i> (<i>ermC</i>)::pSM5022-' <i>ermC</i> ' <i>ΔoriC(dnaA-dnaN)::bgaB-spc</i>	<i>yxIAA-ermC-yxIAB</i> PCR products→CRK6000 (Em) (NIS6505) pSM5022-' <i>ermC</i> '→NIS6505 (Cp, Em ^s) (NIS6515) NIS6554 chr. DNA→NIS6515 (Cp, Sp)
SU6556	CRK6000 <i>albF</i> (<i>ermC</i>)::pSM5022-' <i>ermC</i> ' <i>ΔoriC(dnaA-dnaN)::bgaB-spc</i>	<i>albEF-ermC-albG</i> PCR products→CRK6000 (Em) (SU6506) pSM5022-' <i>ermC</i> '→SU6506 (Cp, Em ^s) (SU6516) NIS6554 chr. DNA→SU6516 (Cp, Sp)
SU6557	CRK6000 <i>ywoE</i> (<i>ermC</i>)::pSM5022-' <i>ermC</i> ' <i>ΔoriC(dnaA-dnaN)::bgaB-spc</i>	<i>ywoE-ermC-ywoF</i> PCR products→CRK6000 (Em) (SU6507) pSM5022-' <i>ermC</i> '→NIS6507 (Cp, Em ^s) (SU6517) NIS6554 chr. DNA→SU6517 (Cp, Sp)
NIS6558	CRK6000 <i>yvyD</i> (<i>ermC</i>)::pSM5022-' <i>ermC</i> ' <i>ΔoriC(dnaA-dnaN)::bgaB-spc</i>	<i>yvyDA-ermC-yvyDB</i> PCR products→CRK6000 (Em) (NIS6508) pSM5022-' <i>ermC</i> '→NIS6508 (Cp, Em ^s) (NIS6518) NIS6554 chr. DNA→NIS6518 (Cp, Sp)

Growth conditions. *B. subtilis* cells were grown in a rich medium, Antibiotic Medium 3 (Penassay medium; Becton Dickinson), supplemented with adenine (20 µg ml⁻¹) and guanosine (20 µg ml⁻¹) at 30 or 37 °C throughout this study. When necessary, various concentrations of antibiotics were added: 7.5 µg chloramphenicol ml⁻¹, 1.0 µg erythromycin ml⁻¹, 40 µg spectinomycin ml⁻¹. As addition of antibiotics reduced the growth rate slightly, cells were grown without antibiotics for flow cytometry.

4',6-Diamidino-2-phenylindole (DAPI) staining of cells. Cells were collected at mid-exponential phase (OD₆₀₀ 0.2) and stained with DAPI, as previously described (Hassan *et al.*, 1997). Briefly, after collection by centrifugation, cells were fixed in 70 % (v/v) ethanol at 4 °C overnight and spread on a glass slide coated with poly-L-lysine. After drying, cells were stained with DAPI solution [1 µg ml⁻¹ in 50 % (v/v) glycerol] followed by observation under a BX50 fluorescence microscope (Olympus). DAPI-staining images of cells were captured by a colour chilled 3CCD camera (C5810, Hamamatsu) or a high-sensitivity digital black and white cooled CCD camera (ORCA II-ER-1394, Hamamatsu). Cell length was measured from 250–500 cells of each strain using MacScope or V-Windows+ programs.

Flow cytometry. When the OD₆₀₀ of the *B. subtilis* cell culture reached 0.1–0.3, chloramphenicol was added to the culture at a concentration of 200 µg ml⁻¹ in order to inhibit initiation of new rounds of replication, and the cell culture was further incubated for 5 h to finish ongoing replication. Cells were collected and fixed as described elsewhere (Løbner-Olesen *et al.*, 1989). Genomic DNA within cells was stained with a mixture of ethidium bromide and mithramycin, and the number of genome equivalents in individual cells, namely the number of replication origins which had existed in individual exponential-phase cells, was estimated with a Bryte HS flow cytometer (Bio-Rad Laboratories). Alternatively, the same analysis was carried out using another flow cytometer, an LSR II (BD Biosciences), after fixed cells had been stained with Hoechst 33258 and FITC, as described by Torheim *et al.* (2000).

Measurement of DNA and protein concentrations by colorimetry. Measurement was carried out exactly as described previously (Kadoya *et al.*, 2002). Briefly, cells were collected by centrifugation from 30 ml exponentially growing cell culture, and both nucleic acids and proteins were extracted by a slightly modified Schneider method (Kadoya *et al.*, 2002). DNA and protein concentrations in the fractions were quantitatively determined by the Burton (1956) and Lowry

Fig. 1. Flow cytometry and DAPI-staining images of *oriC*-relocated strains. (a) *B. subtilis* circular genome schematically represented as a map of 360°. Genes to which *oriC* has been relocated are indicated. (b) DAPI-staining images of wild-type (CRK6000) and three *oriC*-relocated strains, NIS6451 (*oriC* at *argG*), NIS6251 (*oriC* at *proA*) and NIS6261 (*oriC* at *ykcC*), which were exponentially grown in Penassay medium at 30 °C. Bar, 4 μm. (c) Flow cytometry of wild-type and NIS6451 *oriC*-relocated cells exponentially grown in the rich medium. (d) Schematic structures of the integration site (*ykcC* gene) of an *oriC* plasmid and the native *oriC* site in the novel *oriC*-relocated strain NIS6261. (i) Structure of the integration site. Thin and thick lines indicate genomic and plasmid DNA, respectively. Thick and thin arrows represent genes and primers, respectively. Shaded arrows show complete and partial *ykcC* genes at the recombination site between genomic and plasmid DNA. (ii) Structures of the *oriC* region in the *oriC*-relocated (NIS6261, $\Delta oriC$) and the wild-type strains. The thick line represents part of the pRK4 sequence (Kadoya *et al.*, 2002). ERV, restriction site for *EcoRV*. (iii) PCR products digested with *EcoRV*. The *oriC* region was amplified by long-range PCR using Ex *Taq* DNA polymerase (Takara Bio) with p5 and p6 primers [shown in (ii)]. After digestion with *EcoRV*, DNA fragments were separated in a 1% agarose gel by electrophoresis and visualized by staining with ethidium bromide. Lanes: M, standard markers; 1–2, independent clones of *oriC*-relocated strain NIS6261; w, wild-type strain.

methods (Lowry *et al.*, 1951), respectively. The DNA:protein ratio was calculated from the total amounts of DNA and protein in the fractions.

Western blotting. Cells were collected from 3 ml cell culture at mid-exponential phase (OD₆₀₀ 0.2) and further treated for Western

blotting as described previously (Moriya *et al.*, 1990). After proteins in samples had been separated in an SDS-polyacrylamide gradient (10–20%) gel by electrophoresis, they were blotted onto a Hybond-P PVDF membrane (GE Healthcare Life Sciences). DnaA protein signals were detected with the ECL-Plus enhanced chemifluorescence system (GE Healthcare Life Sciences) as described previously (Ogura *et al.*,

2001). The DnaA bands detected were scanned and the signal intensity was quantitatively estimated using NIH image.

RESULTS

Control of replication initiation is defective in an *oriC*-relocated strain

We previously constructed an *oriC*-relocated strain in which the replication origin (*oriC*) was transferred from its native position (0°) to the *argG* locus at 257° of the *B. subtilis* chromosome (a circular map of 360°) (Kadoya *et al.*, 2002). To examine the effects of relocating *oriC* on control of replication initiation, the number of replication origins in individual cells was analysed by flow cytometry. *B. subtilis* cells generally tend to form chains, which can make flow cytometry difficult to interpret. However, our parent strain (CRK6000) does not form chains under usual cultivation conditions, and thus is suitable for flow cytometry (Ogura *et al.*, 2001).

In the wild-type cells, synchrony of replication initiation is strictly maintained. Under our cultivation conditions, either four or eight replication origins were detected in individual wild-type cell particles (Fig. 1c). No peak was detected for cells with 16 genome equivalents (data not shown), which was consistent with no chain formation of this strain, as described above. Treating *B. subtilis* cells with cephalixin to inhibit cell division caused cell lysis, unlike in *E. coli*. This drug, therefore, was not added to *B. subtilis* cells to prepare cell samples for flow cytometry. Furthermore, as two different techniques, flow cytometry

and fluorescence *in situ* hybridization, appeared to show a similar result for the number of replication origins per cell in exponentially grown cells (Kadoya *et al.*, 2002), the procedure used for flow cytometry in this study seemed to inhibit cell division in *B. subtilis*.

Flow cytometry revealed that many cells appeared to contain six origins in cells in which *oriC* was relocated to *argG* on the *B. subtilis* chromosome (Fig. 1a, c). The DNA replication rate may be significantly slowed down in the *oriC*-relocated strain because of collision of the replication forks with head-on transcription on most of the chromosome (Wang *et al.*, 2007a). Therefore, incubation of cells for 5 h after addition of chloramphenicol may not be long enough to complete ongoing replication in this mutant for flow cytometry. However, in the DNA histogram of the mutant, cells with genome equivalents between four and eight would distribute randomly, because replication forks do not completely stop at particular positions on the chromosome. Thus, detection of a peak of cells with six genome equivalents in the DNA histogram of this *oriC*-relocated strain suggests that asynchronous initiation of replication was actually occurring in this mutant.

In addition, many cells in this *oriC*-relocated strain seemed to contain more than eight origins, suggesting that replication initiation was also enhanced. When individual cells from this strain were observed using phase-contrast microscopy they seemed to be longer than wild-type cells (Fig. 1b). Indeed, when the cell length was measured from 500 cells, the average cell length of NIS6451 was approximately 1.2 µm longer than that of the wild-type cells (Table 2) (note that, as described above, our parent

Table 2. Distribution of cells in size, average cell length, growth rate and DNA:protein ratio of *oriC*-relocated strains used in this study

Cells were grown in a rich medium (Penassay medium) at 30 °C. At mid-exponential phase, cells were fixed with 70 % ethanol and the cell length was measured in 250–500 cells. Experiments 1 and 2 were carried out in different laboratories, and thus the distribution of cell length of the wild-type strain probably reflects the variation of aeration from the use of different shaker incubators. –, Not measured/done.

Strain	Distribution of cell size (µm)		Average cell length (µm) (relative to wild-type)		Growth rate* (doublings per hour)	DNA:protein ratio† (relative to wild-type)
	Experiment 1	Experiment 2	Experiment 1	Experiment 2		
Wild-type	2.36–7.26	2.00–6.03	4.27 (1.00)	3.41 (1.00)	2.00	0.041 (1.00)
<i>oriC</i> at –60 kb	2.33–6.97	–	4.29 (1.00)	–	2.00	0.042 (1.02)
<i>oriC</i> at –140 kb	2.72–7.79	–	4.49 (1.05)	–	2.00	0.043 (1.05)
<i>oriC</i> at –250 kb	–	2.14–6.94	–	3.91 (1.15)	1.88	0.046 (1.12)
<i>oriC</i> at –350 kb	2.76–10.67	–	5.37 (1.26)	–	2.00	0.046 (1.12)
<i>oriC</i> at –450 kb	2.03–15.59	–	5.71 (1.34)	–	1.76	0.049 (1.20)
<i>oriC</i> at –550 kb	–	2.49–11.06	–	4.75 (1.39)	1.69	0.050 (1.22)
<i>oriC</i> at <i>pnbA</i>	2.44–10.64	–	5.34 (1.25)	–	1.69	0.052 (1.27)
<i>oriC</i> at <i>argG</i>	1.94–11.26	–	5.43 (1.27)	–	–	0.048 (1.17)

*Doubling time was determined from the growth curve of each strain in Penassay medium at 37 °C by measuring OD₆₀₀. Growth rate was calculated by dividing one hour by the doubling time.

†These values are averages from two or three independent experiments. For every strain, SD was less than 0.001.

strain does not form chains at least under these cultivation conditions, and thus cell length was determined by measuring the distance between the two ends of individual cells). The *oriC*-relocated cells with more than eight replication origins might be produced simply due to the increase in cell length. If this were the case, only cell division would be delayed and the balance between DNA replication and mass increase would be still maintained in the *oriC*-relocated strain as in the wild-type strain. To check this possibility, the ratio of DNA to protein was measured quantitatively using a chemical method. The results indicated that the ratio was significantly higher (0.048 ± 0.001) in the *oriC*-relocated strain (NIS6451) than in the wild-type (0.041 ± 0.001). Thus, DNA replication was actually enhanced in this *oriC*-relocated strain.

Cells divide abnormally at a high frequency when *oriC* is relocated near 117° of the genetic map

In order to estimate whether replication initiation is always enhanced by relocation of *oriC*, we constructed another *oriC*-relocated strain, NIS6261, in which *oriC* was transferred to the *ykcC* locus at 116° of the *B. subtilis* circular genome map (Fig. 1a).

Integration of an *oriC* plasmid into the *ykcC* locus and replacement of the native *oriC* with an unrelated sequence containing an antibiotic-resistance marker were simultaneously performed through a single transformation using two different donor DNA sources (Table 1). Integration of the plasmid into the *ykcC* locus in both chloramphenicol- and erythromycin-resistant transformants was confirmed by PCR using plasmid- and *ykcC*-specific primer sets, as shown in Fig. 1d(i) (p1–p2 and p3–p4 primer sets). PCR products with the expected lengths were detected in several transformants (data not shown). Subsequently, the replacement at the native *oriC* in the transformants was confirmed by PCR followed by digestion with *EcoRV*. If the *oriC* locus was correctly replaced by the *bgaB* and *ermC* genes, two *EcoRV* DNA fragments (2394 and 2640 bp) would be detected instead of four fragments [Fig. 1d(ii)]. As shown in Fig. 1d(iii), two candidates displayed the expected pattern. The results indicate that these transformants contain the *oriC* locus relocated into the *ykcC* locus.

Exponentially growing *oriC*-relocated cells were stained with DAPI to localize the DNA and observed using a fluorescence microscope. Surprisingly, they were found to be very small compared with the wild-type cells, probably due to increased cell division (Fig. 1b). This was not due to transfer of *oriC* into the *ykcC* locus, because the same small-cell phenotype, including anucleate cells produced by polar division, was also observed when *oriC* was transferred into another locus close to *ykcC*, *proA*, at 118° (Fig. 1b), and when *oriN*, the replication origin of a plasmid pLS32, was integrated into the *proA* locus as a new origin of chromosome replication (Moriya *et al.*, 1997). These results indicate that when DNA replication begins near 117° on the *B. subtilis* chromosome, cells divide prema-

turely for unknown reasons (see Discussion). Because of the abnormal cell division events, these two *oriC*-relocated strains are unsuitable for analysis by flow cytometry.

Replication initiation is enhanced in another *oriC*-relocated strain

Another *oriC*-relocated strain was constructed to determine whether replication initiation is always enhanced by relocation of *oriC*. In the new *oriC*-relocated strain, *oriC* was integrated downstream of the *pnbA* gene at 300° on the *B. subtilis* genome map (Fig. 1a). Both integration of *oriC* at the *pnbA* locus and replacement of *oriC* with the unrelated plasmid sequence were confirmed as described above.

Unlike the previous *oriC*-relocated strains NIS6251 and NIS6261 (*oriC* at *proA* and *ykcC*, respectively), cell division appeared to be normal in this new *oriC*-relocated strain, NIS6551 (Fig. 2a). Therefore, replication initiation in this strain was examined by flow cytometry. The majority of the *oriC*-relocated cells contained eight replication origins per cell, compared with four origins in the wild-type cells (Fig. 2b) and six origins in NIS6451 *oriC*-relocated cells (*oriC* at *argG*; Fig. 1c). This result suggests that the timing of replication initiation in the cell division cycle is advanced in this *oriC*-relocated strain compared with that in the wild-type strain. However, the *oriC*-relocated cells

Fig. 2. DAPI-staining images (a) and flow cytometry (b) of a novel *oriC*-relocated strain. Wild-type, CRK6000; *oriC* at *pnbA*, NIS6551 in which *oriC* is relocated to the *pnbA* locus at ~300° (~700 kb counterclockwise from the *oriC*) on the *B. subtilis* genome map, as shown in Fig. 1(a).

were slightly longer than the wild-type cells (Table 2), and this elongation could lead to an apparent increase in cells with eight replication origins. To determine whether DNA replication was enhanced in this *oriC*-relocated strain, the ratio of DNA to protein was quantitatively measured. The DNA:protein ratio was significantly higher (0.052 ± 0.001) in this *oriC*-relocated strain, NIS6551, compared with the wild-type (0.041 ± 0.001). These results indicate that normal control of replication initiation is disturbed and replication initiation is upregulated in this new *oriC*-relocated strain.

DnaA protein levels in *oriC*-relocated strains were similar to those in the wild-type strain

Relocation of *oriC* alters the gene organization around *oriC* and could affect gene expression of the *oriC*-linked *dnaA*–*dnaN* operon. An increase in the level of DnaA caused by relocation of *oriC* would advance timing of replication initiation during the cell division cycle, because overproduction of DnaA protein advances the timing of replication initiation (Ogura *et al.*, 2001). To test this possibility, DnaA protein levels were compared between *oriC*-relocated and wild-type strains.

A series of dilutions was prepared for each sample and DnaA protein in the dilutions was detected by Western blotting (Fig. 3b). As shown in Fig. 3(a), amounts of total protein were approximately equal between samples prepared from *oriC*-relocated strains and the wild-type. Densitometry of the DnaA bands revealed that DnaA levels in NIS6451 (*oriC* at *argG*) and NIS6551 (*oriC* at *pnbA*) were 1.45 ± 0.20 and 1.12 ± 0.22 times the wild-type level, respectively. Thus, the DnaA level in NIS6451 was significantly higher than that in the wild-type but the DnaA level in another *oriC*-relocated strain, NIS6551, was similar to that in the wild-type strain. It has previously been found that when the DnaA level is increased 1.5-fold,

only a slight advance is observed in replication initiation (Ogura *et al.*, 2001). Therefore, the present results indicate that the asynchronous and advanced initiation of replication observed in *oriC*-relocated strains is not attributable to an increased cellular level of DnaA.

Flow cytometry of new *oriC*-relocated strains in which *oriC* was transferred to loci between the native *oriC* and *pnbA* at ~100 kb intervals

When *oriC* was relocated to two different loci (*argG* and *pnbA*) on the genome, both of which were very far from the native *oriC*, strict control of replication initiation was lost. To maintain this strict control, some unique conformation of the chromosome may be formed near the native *oriC*, or perhaps special genes exist near the native *oriC*. In order to test this possibility, several new *oriC*-relocated strains were constructed by transferring *oriC* to new loci between the native *oriC* and *pnbA* at ~100 kb intervals (Fig. 4a), and control of replication initiation was analysed by flow cytometry.

As shown in Fig. 4b(ii), a DNA histogram obtained by flow cytometry from a new *oriC*-relocated strain in which *oriC* was transferred to *purA*, ~60 kb away from the native locus, was nearly the same as that of wild-type cells, having mainly cells with four origins. Furthermore, a DNA histogram from another *oriC*-relocated strain in which *oriC* was transferred to *iolJ*, ~140 kb away from the native one, was also very similar to that of the wild-type (data not shown). These results indicate that replication initiation is normally controlled as long as *oriC* is relocated within a 140 kb region between *iolJ* and *oriC*.

In contrast, when *oriC* was relocated to three loci, 450, 550 and 700 kb away from the native locus, the majority of cells contained eight replication origins per cell, compared with four origins in wild-type cells [Fig. 4b(ii)]. Moreover,

Fig. 3. DnaA protein levels in *oriC*-relocated strains. (a) Total protein levels in samples prepared for Western blotting from wild-type (w) and two *oriC*-relocated strains, NIS6451 (*oriC* at *argG*) (lane 1) and NIS6551 (*oriC* at *pnbA*) (lane 2). Total proteins were extracted from exponentially growing cells as described in Methods and visualized by staining with Coomassie brilliant blue. (b) Western blotting with rabbit anti-DnaA antibody. Various amounts of total protein were loaded in the lanes of an SDS-polyacrylamide gradient gel. Total protein at a similar concentration to that in each lane of (a) was loaded in lane 10, and total protein corresponding to 2/5, 3/5 and 4/5 of that in lane 10 was loaded in lanes 4, 6 and 8, respectively.

Fig. 4. Flow cytometry of *oriC*-relocated strains. (a) Genetic map of the left region near *oriC* on the *B. subtilis* chromosome, from the native *oriC* to *pnbA*, which is ~700 kb away from *oriC*. The map is schematically depicted with loci where *oriC* has been relocated in this study, and with the distance from *oriC*. The numbering of the *B. subtilis* circular chromosome is clockwise, and thus the distance between several loci shown here and the native *oriC* is indicated with a minus sign. (b) DNA histograms obtained by flow cytometry of various *oriC*-relocated strains. (i) and (ii) are two independent experiments. Penassay broth is a complex medium, and thus the composition of the components slightly varies between lots. The subtle differences slightly affect growth rate, and thus change the DNA histogram of the wild-type cells.

asynchronous initiation of replication was commonly observed in these *oriC*-relocated strains.

When *oriC* was relocated to a locus ~350 kb away from the native one, the *oriC*-relocated cells showed an intermediate DNA histogram by flow cytometry [Fig. 4b(ii)]. The majority of the cells contained four replication origins per cell like wild-type cells, but asynchronous initiation was detected in this *oriC*-relocated strain [Fig. 4b(ii)]. A similar pattern was also observed in a DNA histogram obtained from another *oriC*-relocated strain in which *oriC* was relocated to a locus 250 kb away from the native *oriC* (Fig. 4b).

The number of cells having eight origins was slightly higher in three *oriC*-relocated strains (*oriC* at -60, -250 and -350 kb) than in the wild-type strain (Fig. 4b). Since cells were slightly elongated by relocation of *oriC* (Table 2), the small increase in the number of cells that had initiated replication might simply be due to cell elongation. If this were the case, then the DNA:protein ratio would remain constant in both *oriC*-relocated and wild-type strains. Indeed, the DNA:protein ratios in two *oriC*-relocated strains (*oriC* at -60 and -140 kb) were nearly the same as in the wild-type strain (Table 2). In contrast, the DNA:protein ratio was significantly increased in two other *oriC*-relocated strains (*oriC* at -250 and -350 kb) (Table 2). When *oriC* was relocated to further away from its native locus (to -450, -550 kb and in *pnbA*), the DNA:protein ratio was further increased by about 20% (Table 2). These results clearly indicated that replication initiation was actually upregulated in these *oriC*-relocated strains. In other words, the increase in the number of cells with eight replication origins detected by flow cytometry in the -250 and -350 kb *oriC*-relocated strains is not simply due to cell elongation.

Replication initiation is partially inhibited in double *oriC* strains

During the course of constructing *oriC*-relocated strains, double *oriC* strains can be temporarily created. We were interested to know how replication initiation is controlled in these double *oriC* strains. Using two double *oriC* strains in which the extra *oriC* was located at -250 or -550 kb, the number of replication origins in the cells was examined by flow cytometry.

The analysis revealed that the number of cells having eight origins was remarkably reduced in the double *oriC* strains compared with that in the wild-type strain (Fig. 5). This change was not due to reduction in cell size because the average cell lengths of the double *oriC* strains were almost the same as that of the wild-type (0.99 and 0.88 relative to the wild-type 1.00). These results indicate that replication initiation is slightly delayed in double *oriC* strains. Furthermore, a significant number of cells were found between the peaks of representing four and eight origins in a DNA histogram from a double *oriC* strain (extra *oriC* at

Fig. 5. Flow cytometry of double *oriC* strains in which additional *oriCs* are located at sites 250 and 550 kb left of the native *oriC*.

-550 kb) (Fig. 5), suggesting that synchrony of replication initiation was also disturbed in the double *oriC* strain.

DISCUSSION

In this report we show that when *oriC* is relocated to *argG*, which is very far from the native *oriC* (257° on the 360° map), normal control of replication initiation is lost. Initiation became asynchronous and enhanced as the DNA:protein ratio increased in the *oriC*-relocated strain. Very recently, more frequent replication initiation has been independently observed in the same *oriC*-relocated strain using genome-wide hybridization on a DNA microarray (Wang *et al.*, 2007a). When *oriC* was relocated to other loci, *ykcC* and *proA*, which are located very close to each other on the chromosome (116 and 118°, respectively) and also very far from the native *oriC*, cells were much shorter than the wild-type and appeared to divide more frequently. This extra cell division may be caused by an increase in the FtsZ protein level in the *oriC*-relocated strains. The *ftsZ* gene is located at ~200 kb downstream of *proA* (Kunst *et al.*, 1997) and thus replication starts near *ftsZ* in these strains. This situation leads to an increase in *ftsZ* gene dosage in these *oriC*-relocated cells compared with wild-type cells and may result in elevated levels of FtsZ in the *oriC*-relocated strains. Alternatively, rRNA synthesis may be greatly reduced in the two *oriC*-relocated mutants because transcription of rRNA operons clustered near 0° of the chromosome (Kunst *et al.*, 1997) is inhibited by collision with replication forks in these mutants. The reduction of ribosomal synthesis decreases the rate of mass increase, resulting in production of small cells by cell division at normal frequency. To examine this possibility, transcription of the rRNA cluster should be compared between the mutants (*oriC* at *proA* and *ykcC*) and another *oriC*-relocated strain in which *oriC* is located on the left arm of the chromosome (*oriC* at *argG*) and does not produce small cells. Due to the abnormal small cell phenotype, replication initiation was not analysed by flow

cytometry in these *oriC*-relocated strains. However, in another *oriC*-relocated strain (*oriC* at *pnbA*, 300°), both asynchronous and enhanced initiation of DNA replication were again observed. These results indicate that loss of normal regulation and enhanced initiation of replication do not only occur in a special strain, but are perhaps common in many *oriC*-relocated strains and are not unique to *oriC* relocation at *argG*.

The abnormal regulation of replication initiation was not simply due to an increase in the DnaA initiator protein level within the cell. Although the DnaA levels were slightly elevated (by about 1.1- and 1.5-fold in the two *oriC*-relocated strains), the increase was not enough to disrupt the tight regulation of replication initiation in *B. subtilis* (Ogura *et al.*, 2001). However, the increase in replication initiation was not observed when the *dnaA*-independent heterologous origin *oriN* was used as the sole origin for chromosome replication at *argG* and near the native *oriC* sites (Wang *et al.*, 2007a). Thus, it still may be that overinitiation of replication in the *oriC*-relocated strain is caused by the increased DnaA level. Nevertheless, it appears that some characteristic of the region surrounding the native *oriC* locus (within 140 kb) contributes to the tight regulation of replication initiation, since control of replication initiation was disturbed when *oriC* was relocated to loci further than 250 kb counterclockwise from the native locus. When *oriC* was relocated to loci at either –250 or –350 kb (counterclockwise), control of replication initiation was slightly disturbed and DNA:mass ratios increased by 10 % compared with that of the wild-type. When relocated to loci further than 450 kb from the native locus, replication initiation was more enhanced and DNA:protein ratios increased further by about 20 %. Thus, two regions seem to participate in the tight control of replication initiation. One lies between 140 and 250 kb and another between 350 and 450 kb counterclockwise from the native *oriC*.

What mechanism could exist in this origin region to regulate replication initiation? A candidate may be membrane binding of the origin region, similar to that reported in *E. coli*. It has been demonstrated in *B. subtilis* that the *purA* gene near the origin is enriched in the membrane fraction (Winston & Sueoka, 1980; Yamaguchi & Yoshikawa, 1977). A membrane-binding DNA sequence near *purA* has been isolated from the membrane fraction and identified by membrane association abilities *in vivo* and *in vitro* (Sargent & Bennett, 1986; Sato *et al.*, 1991). However, this sequence is located ~45 kb from *oriC* (Itaya *et al.*, 1992). Moreover, counterparts of SeqA and Dam methyltransferase, which play essential roles in sequestering newly replicated origins to the cell membrane in *E. coli*, do not exist in *B. subtilis* (Kunst *et al.*, 1997). Therefore, the nature of any DNA–membrane binding in *B. subtilis* may be different from the sequestration of newly replicated origins into the cell membrane.

In *B. subtilis*, DNA replication is arrested at ~200 kb downstream on either side of *oriC* during the stringent

response and premature initiation of replication after temperature shift-down of *dnaB*-ts initiation mutants (Henckes *et al.*, 1989; Levine *et al.*, 1991). The replication terminator protein (RTP) has been proposed to be involved in the arrest of replication during the stringent response and the arrest sites have been mapped in detail (Autret *et al.*, 1999; Levine *et al.*, 1995), although the replication arrest by the stringent response has been found very recently to occur throughout the chromosome independently of Rtp (Wang *et al.*, 2007b). According to the previous reports, approximately half of the replication forks are arrested at the LSTer1 site between –94 and –104 kb and the remaining half at the LSTer2 site between –127 and –135 kb. When *oriC* was relocated to the –140 kb locus, which is outside these arrest regions, significant effects on the control of replication initiation were not observed. Thus, it is unlikely that the mechanism that arrests replication forks during the stringent response and premature initiation of replication is related to the regulation of replication initiation observed in this study.

A recent paper has shown that replication elongation is slowed down by head-on transcription in an extended chromosomal region of an *oriC*-relocated strain (Wang *et al.*, 2007a). In this case, *oriC* was relocated to *argG*, the same as NIS6451 used in our study. The authors observed, by DNA microarray analysis, that replication initiation was more frequent in this mutant. This result appears to be consistent with our result obtained by flow cytometry. However, DNA microarray analysis only shows the relative ratio of amounts of origin- and terminus-proximal DNA, thus the relative increase of origin-proximal DNA may be explained by, for example, selective degradation of the terminus-proximal region of the chromosome, without affecting control of replication initiation. Such degradation could occur in cells by treatment with DNA-damaging agents or by collision of replication with transcription. Indeed, the same group detected a relative increase in the amount of origin-proximal DNA after treatment with mitomycin C (Goranov *et al.*, 2006). If such degradation is occurring, the DNA:protein ratio would be reduced compared with that under normal conditions. Our results indicated that this was not the case for the *oriC*-relocated strains in this study, because the DNA:protein ratio was actually higher in NIS6451 than in the wild-type. Therefore, our results confirm that replication initiation is actually enhanced in the *oriC*-relocated strain. Our study also showed that a small difference (~100 kb) in relocation of *oriC*, for instance between –350 and –450 kb, caused a drastic change in control of replication initiation. Thus, a mechanism other than the slowing of replication elongation may be an appropriate interpretation of the asynchronous and enhanced initiation of replication in *oriC*-relocated strains.

A special higher-order DNA structure may be formed on the *oriC* region of the chromosome and such a structure may be important for the regulation of replication initiation in *B. subtilis*. It has been suggested in *E. coli*

that two large regions (~900 kb) near *oriC* and *terC* are organized to form domains (Niki *et al.*, 2000). Indeed, very recently, it has been shown that the terminus region (800 kb) of the *E. coli* chromosome is organized into a macrodomain and plays a role in chromosome segregation (Mercier *et al.*, 2008). In *B. subtilis*, eight Spo0J-binding sites are located in the origin-proximal region (~20 %) of the chromosome (Lin & Grossman, 1998), and indeed, Spo0J binds to these sites *in vivo* (Breier & Grossman, 2007; Murray *et al.*, 2006). This binding is not just on the short recognition sequence but is spread over 10–20 kb (Breier & Grossman, 2007; Murray *et al.*, 2006). It has been suggested that this large nucleoprotein structure (called 'Spo0J domains') is involved in organizing the *oriC* region of the chromosome (Murray *et al.*, 2006). In *B. subtilis*, the DnaA protein level in the wild-type cells appears to have a potential which is enough to initiate replication once (Moriya *et al.*, 1990). However, the potential is usually suppressed until it becomes the right time for firing origins. The Spo0J domains in the *oriC* region may be involved in this suppression by inhibiting contact of free DnaA molecules with replication origins bound to DnaA. Indeed, replication initiation is greatly enhanced in *spo0J* null mutants (Lee *et al.*, 2003; Ogura *et al.*, 2003), and a recent paper suggests that the stimulation of replication initiation in the mutant is perhaps caused by alteration of DNA topology near *oriC* (Murray & Errington, 2008). In *oriC*-relocated strains in which *oriC* is moved to new loci outside the Spo0J domains, namely outside the suppression region, free DnaA molecules can attack the new *oriC*, and thus extra initiation of replication may occur. Six Spo0J binding sites are located on the left half of the *B. subtilis* chromosome (Breier & Grossman, 2007). Four out of the six binding sites are clustered within ~60–70 kb counterclockwise from *oriC*, one ~300 kb apart from *oriC* and the last one close to *terC* (Breier & Grossman, 2007). Control of replication initiation was almost normal when *oriC* was relocated to –60 or –140 kb and obviously disrupted when it was relocated to loci further than 250 kb. Thus, our results would be consistent with the model that the Spo0J binding site located at 334° (Breier & Grossman, 2007) is important to form a higher-order structure between dispersed Spo0J domains.

The PLR which is essential to bring the *oriC* region close to the cell pole in sporulating cells lies ~150–300 kb to the left of *oriC* (Wu & Errington, 2002). This long sequence may also play a role in the control of replication initiation. However, when *oriC* was relocated to loci further than 450 kb counterclockwise from the native locus, the frequency of initiation of replication increased further. Therefore, our results also suggest that some DNA structure outside the two regions (Spo0J domains and PLR) is involved in suppression of replication initiation. When *oriC* is moved over those DNA regions, all suppression mediated by the regions would be lost completely and free DnaA molecules would make contact with *oriC* without any hindrance, resulting in early and

asynchronous initiation of replication. If this is the case, the same abnormal control of replication initiation would also be observed in double *oriC* mutants. Although asynchronous initiation was observed in these mutants, early initiation was not detected even when an extra *oriC* was inserted at –550 kb (Fig. 5). Rather, replication initiation was delayed compared with the wild-type. This is probably due to the strong autorepression activity of DnaA (Ogura *et al.*, 2001). The two *dnaA* promoters present in these double *oriC* mutants would be completely repressed at the wild-type DnaA level. Since free DnaA molecules would be titrated by DnaA boxes in two *oriC*s (Moriya *et al.*, 1988), there may be much fewer residual free DnaA molecules in double *oriC* cells than in wild-type cells. This low level of free DnaA molecules may delay replication initiation in double *oriC* strains. Another possibility is that *oriC* at the native position is dominant over translocated *oriC* sequences and that the moderate asynchrony observed in double *oriC* strains is due to leaky and uncontrolled initiation of replication at the translocated origin.

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