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±6 or 300±6 on the 360±6 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.

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). 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, 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, 1993; Løbner-Olesen 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). 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-‘ykcC, 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 BamHI 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); ‘ykcC, 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 BamHI 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 bggB–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::lacZara D139 Δ(ara, leu)7697 ΔlacX74, gagU, 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*, 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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Reference or construction</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRK6000</td>
<td>purA16 metB5 hisA3 gusA</td>
<td>Moriya et al. (1990)</td>
</tr>
<tr>
<td>NIS6451</td>
<td>CRK6000 argG::pAH3 (oriC cat) ΔoriC(dnaA-dnaN)::bgpB-ermC</td>
<td>Kadoya et al. (2002)</td>
</tr>
<tr>
<td>NIS6551</td>
<td>CRK6000 pnbA::pSM5022-′pnbA (oriC cat) ΔoriC(dnaA-dnaN)::bgpB-ermC</td>
<td>pSM5022-′pnbA→CRK6000 (Cp, pro−) (NIS6250)</td>
</tr>
<tr>
<td>NIS6552</td>
<td>CRK6000 purA::pSM5022-′purA (oriC cat) ΔoriC(dnaA-dnaN)::bgpB-ermC</td>
<td>Linear form of pRK4 (Kadoya et al., 2002)→NIS6250 (Em)</td>
</tr>
<tr>
<td>NIS6553</td>
<td>CRK6000 97l (ermC)::pSM5022-′ermC ΔoriC(dnaA-dnaN)::bgpB-ermC</td>
<td>pSM5022-′ermC and linear form of pRK4→CRK6000 (Cp, Em)</td>
</tr>
<tr>
<td>NIS6554</td>
<td>CRK6000 97l (ermC)::pSM5022-′erm′ ΔoriC(dnaA-dnaN)::bgpB-spc</td>
<td>Linear form of pEm::Spc (Lindow et al., 2002)→NIS6553 (Sp)</td>
</tr>
<tr>
<td>NIS6555</td>
<td>CRK6000 97l (ermC)::pSM5022-′ermC ΔoriC(dnaA-dnaN)::bgpB-spc</td>
<td>ydxAA-ermC-ydxB PCR products→CRK6000 (Em) (NIS6505)</td>
</tr>
<tr>
<td>SU6556</td>
<td>CRK6000 albF (ermC)::pSM5022-′ermC ΔoriC(dnaA-dnaN)::bgpB-spc</td>
<td>pSM5022-′ermC→CRK6000 (Cp, Em′) (NIS6515)</td>
</tr>
<tr>
<td>SU6557</td>
<td>CRK6000 ywoE (ermC)::pSM5022-′ermC ΔoriC(dnaA-dnaN)::bgpB-spc</td>
<td>NIS6554 chr. DNA→NIS6515 (Cp, Sp)</td>
</tr>
<tr>
<td>NIS6558</td>
<td>CRK6000 yyoD (ermC)::pSM5022-′ermC ΔoriC(dnaA-dnaN)::bgpB-spc</td>
<td>albEF-ermC-albG PCR products→CRK6000 (Em) (SU6506)</td>
</tr>
</tbody>
</table>

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 (Hasan 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 CCD 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 (Lobner-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 Brite 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...
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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.,

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, ΔoriC) 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.
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 cephalaxin 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Distribution of cell size (μm)</th>
<th>Average cell length (μm) (relative to wild-type)</th>
<th>Growth rate* (doublings per hour)</th>
<th>DNA:protein ratio† (relative to wild-type)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
<td>Experiment 2</td>
<td>Experiment 1</td>
<td>Experiment 2</td>
</tr>
<tr>
<td>Wild-type</td>
<td>2.36–7.26</td>
<td>2.00–6.03</td>
<td>4.27 (1.00)</td>
<td>3.41 (1.00)</td>
</tr>
<tr>
<td>oriC at −60 kb</td>
<td>2.33–6.97</td>
<td>–</td>
<td>4.29 (1.00)</td>
<td>–</td>
</tr>
<tr>
<td>oriC at −140 kb</td>
<td>2.72–7.79</td>
<td>–</td>
<td>4.49 (1.05)</td>
<td>–</td>
</tr>
<tr>
<td>oriC at −250 kb</td>
<td>2.00–6.03</td>
<td>–</td>
<td>–</td>
<td>3.91 (1.15)</td>
</tr>
<tr>
<td>oriC at −350 kb</td>
<td>2.76–10.67</td>
<td>–</td>
<td>5.37 (1.26)</td>
<td>–</td>
</tr>
<tr>
<td>oriC at −450 kb</td>
<td>2.03–15.59</td>
<td>–</td>
<td>5.71 (1.34)</td>
<td>–</td>
</tr>
<tr>
<td>oriC at −550 kb</td>
<td>–</td>
<td>2.49–11.06</td>
<td>–</td>
<td>4.75 (1.39)</td>
</tr>
<tr>
<td>oriC at pnbA</td>
<td>2.44–10.64</td>
<td>–</td>
<td>5.34 (1.25)</td>
<td>–</td>
</tr>
<tr>
<td>oriC at argG</td>
<td>1.94–11.26</td>
<td>–</td>
<td>5.43 (1.27)</td>
<td>–</td>
</tr>
</tbody>
</table>

*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 prematurely 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

\[\text{wild-type} \quad \text{oriC at pnbA}\]

\[\text{ Wild-type} \quad \text{oriC at pnbA}\]

\[\text{Wild-type} \quad \text{oriC at pnbA}\]

\[\text{Cell counts}\]

\[\text{Genome equivalents (origins per cell)}\]

\[\text{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 \(\sim 300°\) (~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 over-production 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. 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 −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 (Henkes 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 the nature of any DNA–membrane binding in B. subtilis may be different from the sequestration of newly replicated origins into the cell membrane.
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 macromdomain 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 oriCs (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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