JOURNAL OF BACTERIOLOGY, Oct. 1995, p. 5711–5715 Vol. 177, No. 19
0021-9193/95/$04.00+0

Replication through the Terminus Region of the *Bacillus subtilis* Chromosome Is Not Essential for the Formation of a Division Septum That Partitions the DNA

L. J. WU, A. H. FRANKS, AND R. G. WAKE*

Department of Biochemistry, University of Sydney, Sydney, New South Wales 2006, Australia

Received 27 April 1995/Accepted 25 July 1995

Germinated and outgrowing spores of a temperature-sensitive DNA initiation mutant of *Bacillus subtilis* were allowed to initiate a single round of replication by being shifted from 34 to 47°C at the appropriate time. The DNA replication inhibitor 6-(parahydroxyphenylazo)-uracil was added to separate portions of the culture at various times during the round. Samples were collected from each around the time of the first division septation for measurements of the extent of the round completed, the level of division septation, the position of the septum within the outgrown cell, and the distribution of DNA (nucleoid) in relation to the septum. The extent of replication was measured directly through a hybridization approach. The results show clearly that a central division septum can close down onto a chromosome that is only partially replicated (to a minimum extent of about 60% of the round) such that DNA appears on both sides of the septum and frequently very close to it. It is concluded, as claimed previously on the basis of a less direct approach (T. McGinness and R. G. Wake, J. Mol. Biol. 134:251–264, 1979), that replication through the terminus region of the chromosome is not essential for the formation of a division septum that partitions the DNA.

During normal growth and multiplication of bacteria, the processes of chromosome replication and division are highly coordinated. In the case of *Escherichia coli*, a currently held view is that coordination is ensured because termination of replication is required for the movement of the daughter chromosomes away from one another, and this allows a potential division site at the cell center to be utilized (5). While blocking chromosome replication normally blocks division and causes filamentation, consistent with this view, it has been known that division can take place in the absence of chromosome replication in certain circumstances (15). But in these cases, division results in the formation of anucleate cells, i.e., the septum does not always form between segregating sister chromosomes. It is still generally accepted that division septation between sister chromosomes to give rise to two nucleated cells cannot occur until after termination of the round of replication that yields the sister chromosomes. An important question relating to this situation concerns whether the act of termination itself triggers some event or sequence of events that leads to or affects division. In 1991, this was listed as one of the remaining problems in the area of cell division in bacteria (4). While evidence to the contrary, i.e., that chromosome replication does not trigger cell division, has been described for *E. coli* (3), some consider that the system used in this work was perhaps not able to rule out a more direct biochemical link between replication and division (14).

Germinated and outgrowing spores of the rod-shaped bacterium *Bacillus subtilis* provide an attractive system for investigating aspects of the relationship between chromosome replication and division. Spores can be germinated to give reasonably good synchrony in initiation of the first round of replication and the first division septation that normally follows termination of this round. In 1979 such a system was used, in conjunction with the DNA polymerase III inhibitor 6-(parahydroxyphenylazo)-uracil (HPUra), to show that replication through the terminus region of the chromosome could be blocked while still allowing the central division septum to close down on the incompletely replicated chromosome (12). The conclusion drawn from this work was that replication through the terminus region of the chromosome was not a prerequisite for division septation. However, this particular finding appears to have had little impact, possibly because the DNA density labeling method used to measure the extent of replication was somewhat indirect and because DNA (nucleoid) positioning was observed through acridine orange staining, a less than optimal approach. Also, visual data describing the relationship between DNA positioning and division septation were not presented. In recent years, information on the location of the major site of fork fusion associated with the termination of replication process in *B. subtilis* (and *E. coli*) has become available (2, 7). In the present work, the relationship between termination of the first round of replication and the first division septation accompanying outgrowth of germinated spores of *B. subtilis* following initiation of just a single round was reinvestigated, taking advantage of this new information and improved experimental approaches. A DNA hydrization assay using probes for appropriate segments of the chromosome to monitor directly the extent of the first round of replication achieved after HPUra addition at various times was used. A new fixation procedure allowed better visualization of septa, and the location of DNA in the outgrowing cell, relative to the site of septation, was established by light microscopy after staining with the fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI). The results confirm the earlier conclusion and provide more convincing evidence that division septation (resulting in the presence of roughly equal amounts of DNA on both sides of the septum) is not absolutely dependent on completion of replication of the chromosome through its terminus region. The results are further discussed in the light of current views on the relationship between chromosome segregation (movement) and the division process.

Spores of the *B. subtilis* temperature-sensitive DNA initiation mutant *dna-1* [168 *trpC2 dnaB1*(Ts) *thyA thyB*] strain (19)

* Corresponding author. Phone: (612) 351-2504. Fax: (612) 351-4726.

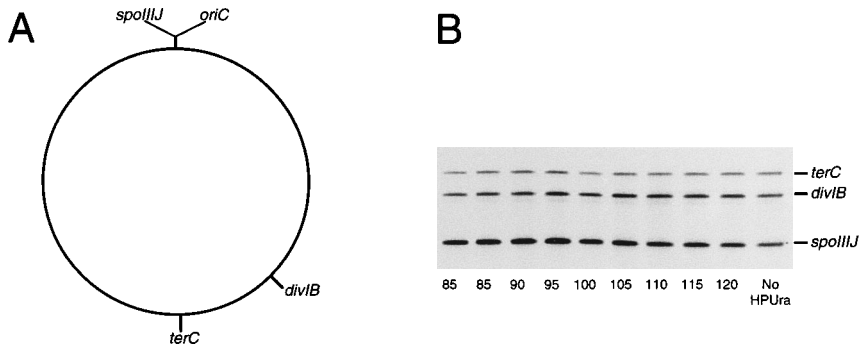

FIG. 1. Use of DNA hybridization probes to measure the extent of a round of chromosome replication. (A) Locations of the regions, *spoIIIJ*, *divIB*, and *terC*, from which restriction fragments of cloned DNA were isolated for ^{32}P labeling by standard procedures and use as hybridization probes. (B) Phosphor image results of Southern transfer and hybridization of *Eco*RI digests of DNA isolated from germinated and outgrowing spores of the *dna-1* strain. Numbers below the lanes indicate the times (in minutes) of addition of HPUra, following the shift from 34 to 47°C at 80 min. Samples were collected at 160 min for DNA extraction and analysis. The bands to which the *spoIIIJ*, *divIB*, and *terC* probes hybridized are indicated; their sizes are 6.9, 10.5, and 14 kb, respectively. Because the marker ratios for all samples were normalized to that for the 85-min sample, two lanes of the latter were routinely analyzed and averaged to obtain each set of data.

were prepared and germinated (10^8/ml) at 34°C in synthetic medium as previously described (12). The first round of replication initiates between 50 and 80 min (see below). At 80 min, the germination mixture was transferred into an equal volume of the same medium at 47°C to prevent initiation of a second round. Under these conditions, the round runs to completion by about 120 min, and most cells form a central division septum between two well-segregated nucleoids by 150 min (see also reference 8). At 5-min intervals (from 85 until 120 min), samples were removed quickly at 47°C and placed into flasks containing HPUra (final concentration, 50 μM) in order to block further progression of the initiated round (12). Portions were removed from each flask containing the HPUra at 140, 150, and 160 min for fixation and microscopic observation (see below). The bulk of the mixture from each flask was collected at 160 min, after addition of sodium azide (0.1%), for DNA extraction (6) and measurement of the extent of replication of the single round.

Progress of the single round of replication and the effect of HPUra addition. Three regions of the chromosome (*spoIIIJ*, *divIB*, and *terC*; Fig. 1A) were used as hybridization probes for monitoring replication. (The designation *terC* refers to the major site of fusion of the approaching forks in the terminus region.) The *spoIIIJ* probe (360° on the map of Anagnostopoulos et al. [1]), located close to *oriC*, was a 3.0-kb fragment of plasmid pSX3 (6a). The *divIB* probe (135°) was a 1.7-kb fragment of λLHI (9), and the *terC* region probe (179°) was a 1.5-kb fragment of pWS1 (17). The DNA samples were digested with *Eco*RI for Southern transfer and hybridization with a mixture of the ^{32}P-labeled probes (prepared by standard procedures), using a fivefold excess of each probe to hybridizable DNA on the membrane. Control experiments established that such conditions provided a linear response between probe bound and the amount of DNA on the membrane (data not shown). Figure 1B shows phosphor images (Molecular Dynamics PhosphorImager system) of the probed species in DNA samples prepared after HPUra addition at the various times. The amount of probe bound to each DNA species was quantitated by using Imagequant software (Molecular Dynamics). Results are expressed as ratios, *divIB*/*spoIIIJ* and *terC*/*spoIIIJ*, normalized to those of the 85-min sample, which were set at 1.0. Control experiments (data not shown) established that these ratios decreased by an average of 1.8 between 50 and 85 min, reflecting *spoIIIJ* replication soon after initiation of the round at 34°C. Figure 2A shows the change in the two ratios resulting from the addition of HPUra at 5-min intervals over the 85- to 120-min period. It should be noted, first, that in the absence of HPUra, the ratio in each case at 160 min was approximately double that of the 85-min sample (2.1 for *divIB*/*spoIIIJ* and 1.8 for *terC*/*spoIIIJ*), consistent with the initiation and completion of just one round of replication in the majority of germinated and outgrowing spores. *divIB* remained completely unreplicated at 160 min following addition of HPUra at 90 min. Addition of HPUra at 105 min allowed approximately 50% replication of *divIB*. Consistent with the expected later replication of *terC*, this region remained unreplicated upon addition of HPUra up until 100 min. Addition of HPUra at 115 min allowed approximately 50% replication of the *terC* region. It should be noted that in a previous study (12) in which density transfer experiments were used to monitor the extent of replication of the *leuB* and *metB* genes, the timing of the system was essentially the same as found here.

Septation and DNA (nucleoid) positioning after blocking the single round at various stages. For microscopic examination of samples taken at 140, 150, and 160 min, the cells were fixed with ethanol. The use of ethanol for detecting septa more readily than by other treatments has been discussed elsewhere (10). Samples were first mixed with sodium azide (0.1%), and the cells were pelleted and suspended in ice-cold 70% ethanol. They were stored at 4°C. The fixed cells were suspended in H_2O, processed for DAPI staining as previously described (20), and viewed by both phase-contrast and epifluorescence microscopy. Phase-contrast images showed the septa, while fluorescence images showed nucleoid distribution (Fig. 3). At 140 min, very few cells in any of the samples had septated. At 150 min, those cells that had septated contained mostly (80 to 95%; see below) one septum, while at 160 min, more cells had a second septum. Samples fixed at 150 min were therefore analyzed in detail (160 to 280 cells for each sample). Figure 2B shows the percentage septation at 150 min following addition of HPUra at various times. It increased gradually from <20% for HPUra addition at 90 min to >60% for addition at 120 min. In the absence of HPUra, a level of 73% was achieved. (In preliminary experiments in which ethanol and the previously used formaldehyde fixation were compared, similar levels of septation were measured with the two approaches, particularly after 95 min. However, the use of ethanol allowed easier detection of septa.) After additions of HPUra at 95 and 100 min, both of which completely blocked replication through the terminus region, approximately 30 and 45% of the cells, respec-

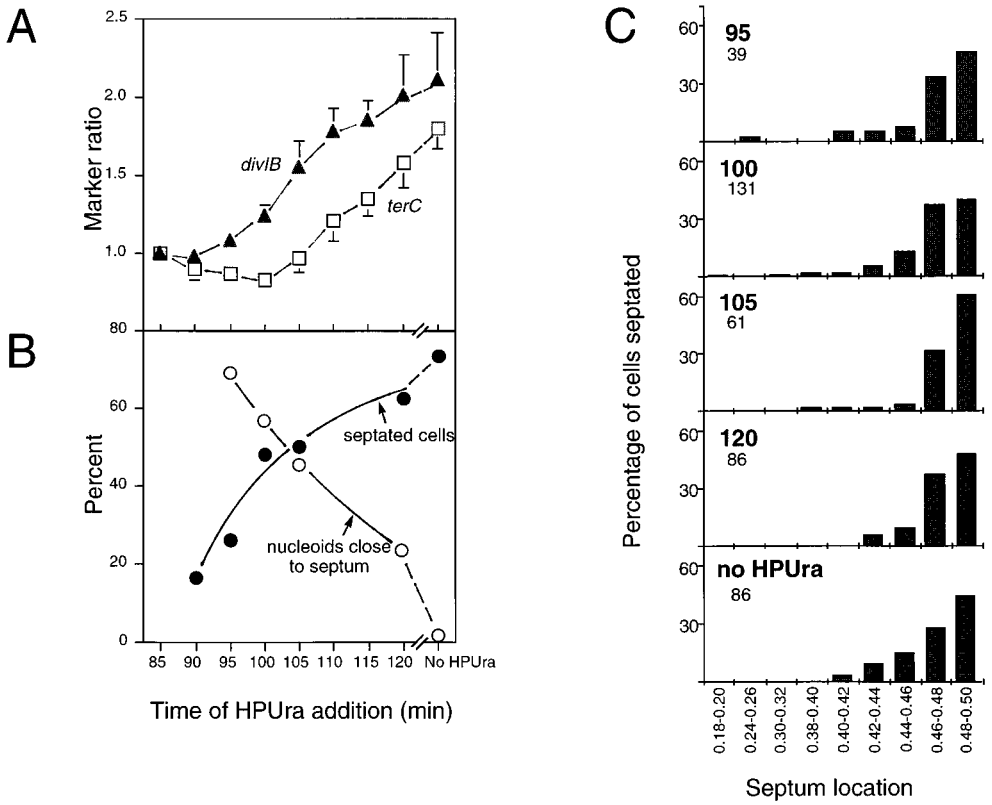

FIG. 2. Extent of the single round of replication and levels of septation and septum-apposed DNA (nucleoids) in germinated and outgrowing *dna-1* spores. The germination mixture was shifted from 34 to 47°C at 80 min, and HPUra was added to separate samples at 5-min intervals over the period from 85 to 120 min. Small samples of each of these mixtures were collected at 140, 150, and 160 min for fixation and microscopic examination, and the bulk of each was collected at 160 min for DNA extraction and analysis of the extent of the single round of replication completed. See text for other details. (A) *divIB*/*spoIIII* (▲) and *terC*/*spoIIII* (□) ratios (normalized to that for the 85-min sample) for the addition of HPUra at the times indicated. The ratios shown are the means from five separate membranes (± standard errors of the means) (see Fig. 1B). (B) Percentage of septated cells in the samples fixed at 150 min (●) and percentage of singly septated cells (majority of septated cells) at 150 min in which the DNA on each side of the septum had not moved a measurable distance away from it (○). (C) Histograms indicating the location of the septum in singly septated cells of the 150-min samples expressed as the fraction of the distance of the septum to the nearer pole of the total length. There were no cells falling into the septum location classes that are missing from the lower range of values (<0.38). The upper and lower numbers within each box indicate the times of addition of HPUra and the number of cells analyzed, respectively.

tively, had septated. These values corresponded to approximately 40 and 60% of the level of septation in the absence of HPUra. Clearly, the 50% septation level (relative to that in the absence of HPUra) was reached before 50% replication of *divIB* (at 105 min), and a high level of septation was achieved in the absence of any replication through the terminus region. Figure 2C shows that the septa that formed in the singly septated cells, when replication was inhibited by HPUra, were located centrally, as was the case without HPUra addition.

Of the septated cells at 150 min, following addition of HPUra at 95, 100, 105 and 120 min and with no addition, the singly septated cells made up 82, 87, 91, 95, and 92%, respectively. Examination of the nucleoid distribution in these cells by DAPI staining showed that in only 1% of the cases—for addition of HPUra at 95 and 100 min—did all of the DNA lie on one side of the septum to give an anucleate cell. In all other cases, and significantly for the 95-, 100-, and 105-min additions, DNA was located on both sides of the septum. (Of the small number of cells with more than one septum, all had DNA on both sides of one of the septa.) Typical examples from the 100-min addition of HPUra are shown in Fig. 3. The left panel comprises photographs taken under epifluorescence to show DNA (nucleoids), and the right panel shows the same under phase contrast, in which case septa were visible. (In some cases, an associated spore coat could be seen under phase contrast.) In a substantial proportion of the cells, the DNA on each side of the septum remained close to it (arrowheads). This is very different from the situation when DNA replication is not blocked and in which case the segregating nucleoids are positioned at approximately the midpoint of each new compartment (8). This finding obviously reflects noncompletion of the round of replication. Figure 2B shows that the percentage of singly septated cells in which the DNA did not move away from the septum to any measurable extent was relatively high for HPUra addition at 95 and 100 min, 69 and 56%, respectively, decreasing to 2% in the absence of HPUra. It is clear that blocking completion of the round of replication, while still allowing sufficient replication for division septation to proceed, had the added effect of impeding the movement of DNA away from the septum. This same conclusion was drawn on the basis of previous experiments in which formaldehyde fixation and acridine orange staining were used (12). However, septa and DNA were more difficult to detect under these earlier conditions.

Implications of the present findings. The results described here provide new and more convincing data to support the earlier conclusion that replication through the terminus region of the *B. subtilis* chromosome is not essential for the formation of a central division septum and that the septum can close down onto the partially replicated chromosome. While the

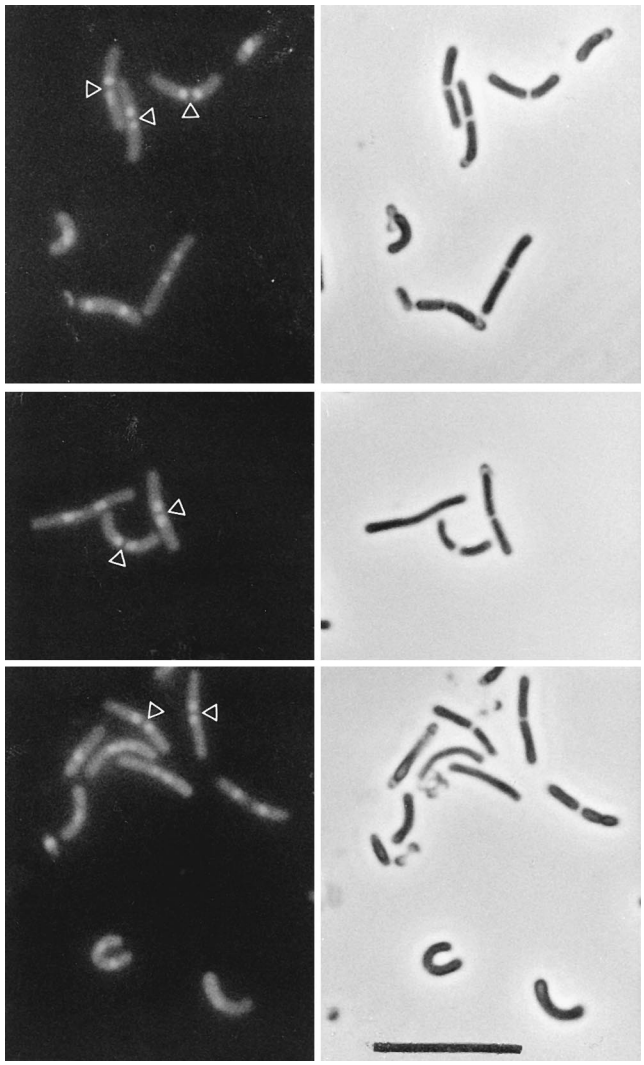

FIG. 3. Photographs of cells observed by epifluorescence and phase-contrast microscopy after staining with DAPI. The cells shown are from the sample in which HPUra was added to the outgrowing spores at 100 min and collected for fixation at 150 min. The left panel shows the appearance under epifluorescence microscopy using appropriate filters (11), in which case DNA (nucleoids) can be visualized. The right panel shows the same field under phase-contrast to allow detection of septa. The DNA (nucleoid) and septa could be visualized together by the fluophase technique (11) to confirm that the DNA was distributed on both sides of the septum when it was present. Cells in which the DNA remains very close to the septum and on both sides of it are indicated by arrowheads. The bar represents 10 μm.

results have been obtained by taking advantage of the favorable situation offered by the outgrowth of germinated spores and under conditions such that only a single round of replication was allowed to occur, there is no reason to believe that the finding is unique to this system (see below).

From the earlier series of experiments, it was concluded that replication through the single round needed to progress to approximately 70% in order for a central septum to form. An extension of this work (13), which also addressed the fact that the earliest additions of HPUra, up to 95 min under the conditions used here, can cause a reduction in the rate of cell length extension by up to 20%, suggested that the amount of replication needed to allow central septation was fixed at a minimum of about 60% of the round. The results described here show that of the outgrown cells that would have normally septated in the absence of HPUra, about 50% did so after its addition in the 95- to 100-min interval. This is 5 to 10 min earlier than the time of addition which allowed 50% replication of the *divIB* gene (corresponding to 75% progression into the round on the basis of its 135° position) and is consistent with the requirement for about 60% completion in order for a septum to form.

In a very recent study, Sharma and Hill (16) have presented convincing evidence to the effect that under certain conditions, septation can occur in *E. coli* when the chromosome is largely but not completely replicated. The septum severs the partially replicated chromosome. The conditions that allowed such septation were the absence of both the *sfi*-dependent and *sfi*-independent pathways for filamentation. It is interesting that *B. subtilis sfiA* or *sfiC* homologs have not been uncovered, and there is no evidence for the existence of either of these pathways in *B. subtilis*. It is possible that termination-independent division septation in *B. subtilis* reflects the absence of such pathways. Regardless of whether this is the case, the present and earlier findings (12) support the view that termination of chromosome replication does not directly trigger cell division. This is not to say that during normal growth and division, complete replication through the terminus region has not always occurred before septal growth proceeds, and that this sequence of events is essential for the production of two viable daughter cells, but it does raise the possibility that early events in the initiation of a division septum occur well before termination of replication.

The present results also establish that division septation can occur in the absence of the normal movement of sister nucleoids away from one another. How does this bear on the suggestion that nonsegregated nucleoids might act to prevent septation at the cell center through a steric hindrance effect (15)? It appears likely that segregation of the replicated segments of the partially completed chromosome, across which septal closure occurs, proceeds to at least a partial extent, and perhaps to an extent sufficient to release a steric hindrance effect, even in those cases in which the DNA does not move away measurably from the septum. This is because, in the majority of these cases, the amounts of DNA on each side of the septum are similar, suggesting that the septum specifically positions itself between the replicated segments of the nucleoid. It seems likely that the latter condense onto themselves into separate domains which gradually move apart as the round progresses. Experimental evidence for gradual movement of the replicated segments as the round progresses in *E. coli* has been presented (18). In the present experiments, the resolution of the replicated segments is much more pronounced than might be expected during normal growth and division. A key factor in achieving this situation could be cell length extension in the absence of replication. Length extension proceeds at 80 to 100% of the normal rate following HPUra addition (12). The replicated segments could continue to move apart after the addition of HPUra, along with cell length extension, until they are restrained by the unreplicated DNA connecting them. While this could explain why they do not move further apart, another possibility is that segregation is a multistep process which in its earliest stages is linked to continued progression into a round of replication. Perhaps the examples shown in Fig. 3, in which the DNA remains close to the septum in spite of considerable cell length extension, reflect a failure of the replicated domains to proceed to a subsequent replication-dependent stage in the segregation process rather than separation being simply restrained through their linkage by unreplicated DNA.

This work was supported by a grant from the Australian Research Council.

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