JOURNAL OF BACTERIOLOGY, July 1996, p. 4258–4265 Vol. 178, No. 14
0021-9193/96/$04.00+0

Replication Fork Arrest at Relocated Replication Terminators on the *Bacillus subtilis* Chromosome

A. H. FRANKS† AND R. G. WAKE*

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

Received 7 February 1996/Accepted 7 May 1996

The replication terminus region of the *Bacillus subtilis* chromosome, comprising *TerI* and *TerII* plus the *rtp* gene (referred to as the *terC* region) was relocated to *serC* (257°) and *cym* (10°) on the anticlockwise- and clockwise-replicating segments of the chromosome, respectively. In both cases, it was found that only the orientation of the *terC* region that placed *TerI* in opposition to the approaching replication fork was functional in fork arrest. When *TerII* was opposed to the approaching fork, it was nonfunctional. These findings confirm and extend earlier work which involved relocations to only the clockwise-replicating segment, at *metD* (100°) and *pyr* (139°). In the present work, it was further shown that in the strain in which *TerII* was opposed to an approaching fork at *metD*, overproduction of the replication terminator protein (RTP) enabled *TerII* to function as an arrest site. Thus, chromosomal *TerII* is nonfunctional in arrest in vivo because of a limiting level of RTP. Marker frequency analysis showed that *TerI* at both *cym* and *metD* caused only transient arrest of a replication fork. Arrest appeared to be more severe in the latter situation and caused the two forks to meet at ~145° (just outside or on the edge of the replication fork trap). The minimum pause time effected by *TerI* at *metD* was calculated to be ~40% of the time taken to complete a round of replication. This significant pause at *metD* caused the cells to become elongated, indicating that cell division was delayed. Further work is needed to establish the immediate cause of the delay in division.

Both *Bacillus subtilis* and *Escherichia coli* contain several DNA replication terminators on their chromosomes which, when complexed to their cognate terminator proteins, arrest replication fork movement in a polar manner (4, 7, 9). The terminators are located approximately opposite *oriC* within a relatively broad region and are organized as two opposed groups. This organization provides a replication fork trap which ensures that the approaching forks always meet within a restricted (terminus) region. The arrangement of terminators on the *B. subtilis* chromosome is shown in Fig. 1. *TerI*, *III*, and *V* are oriented to block movement of the clockwise fork generated at *oriC*, while *TerII*, *IV*, and *VI* block the anticlockwise fork (7). *TerI* and *II* (originally IRI and IRII) were the first chromosomal terminators to be identified (5). They lie just upstream of the gene (*rtp*) that encodes the replication terminator protein (RTP). The overall terminus region extends from *TerV* to *TerVI*. In the present paper, the chromosomal segment comprising *TerI*, *TerII* plus *rtp*, will be referred to as the *terC* region because it encompasses the major arrest site, *TerI*. When this *terC* region was relocated to the *pyr* and *metD* loci (at 139° and 100°, respectively) it was found that the clockwise fork was arrested at each of these loci only when the orientation of the inserted segment placed *TerI* in opposition to the approaching clockwise fork (6). *TerII* was inactive when opposed to the clockwise moving fork at these sites. Because *TerI* and *TerII* were the only known terminators at the time, two possible explanations for the nonfunctioning of *TerII* were offered: (i) the level of RTP was too low to saturate *TerII* in the presence of the higher-affinity *TerI*, and, less likely, (ii) *TerI* was designed to function on the clockwise half of the chromosome and *TerII* was designed to function on the anticlockwise half. It is shown here that the first suggestion is the correct one. Two additional questions have been asked in the present work. First, what is the effect of placing a functional terminator much closer to *oriC*; and second, how severe is fork arrest at a relocated *TerI*? It will be shown that *TerI*, in the situations examined, functions as a pause site. However, when located at *metD*, at least, the pausing achieved by *TerI* is of a severity which would provide an essentially complete block to clockwise fork movement at its normal location (shown in Fig. 1).

* Corresponding author. Phone: (61-2) 351-2504. Fax: (61-2) 351-4726.

† Present address: Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, CO 80309.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *B. subtilis* strains used or constructed in this work are listed in Table 1. The plasmids pWC5 and pWC6 have been described previously (6).

Spore preparation, vegetative cell growth, and DNA preparations. Spores of SU225 were prepared on potato medium supplemented with 50 μg of methionine per ml and 50 μg of thymine per ml as described previously (15). Bacterial DNA for transformation and for the detection of forked molecules was prepared from stationary- or exponential-phase cultures grown in Penassay broth as described previously (22). Sodium azide (100 mM) was added to exponential-phase cultures at the harvesting stage to inhibit further growth.

DNA hybridization probes, Southern transfer, and hybridization. The probes WC1 and WC2 for fork arrest assays were described previously (6). They were labelled with ^{32}P with an Amersham Megaprime DNA labelling system. Transfer of DNA to nylon membranes and hybridization were carried out under standard conditions (16).

MFA by Southern hybridization. The marker frequency analysis (MFA) approach used has been outlined previously (23). The hybridization probes used in the present analyses are listed in Table 2. Cells were grown in Spizizen medium plus methionine (50 μg/ml), thymine (50 μg/ml), tryptophan (40 μg/ml), and Casamino Acids (0.1%). DNA was extracted as described above and cut with *Bam*HI-*Eco*RI or *Eco*RI alone, and 200 ng of each digest per lane was electrophoresed on 0.7% agarose gels in Tris-phosphate-EDTA (14) at 45 V for 18 to 24 h. After being stained with 25 μg of ethidium bromide per ml, the DNA was transferred from the gels to nylon membranes with a Bio-Rad Vacuumblotter according to Bio-Rad's instructions. After transfer, the gels were restained to verify the complete transfer of DNA. The membrane was hybridized with a ^{32}P-labelled hybridization probe mix containing a fivefold molar excess of purified fragments from various chromosomal locations. The membranes were exposed to PhosphorImager screens, and the amount of bound radioactivity was quantitated with the ImageQuant program (Molecular Dynamics). On each gel,

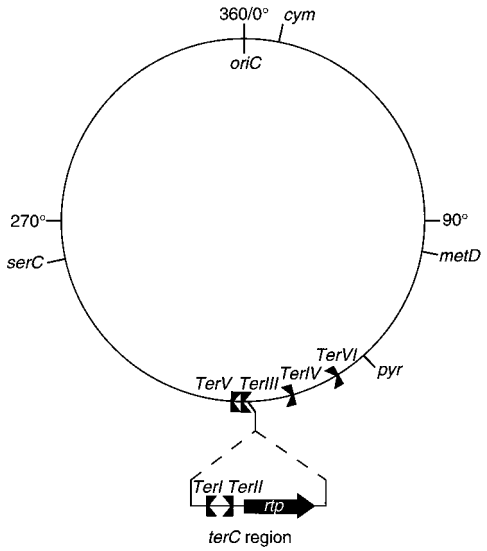

FIG. 1. Abbreviated map of the *B. subtilis* 168 chromosome showing the location and orientation of the DNA terminators, *TerI* to *TerVI*. (Note that the orientation of *TerIV* is assumed.) The six terminators form a replication fork trap in which two opposing groups of terminators allow forks to enter but not exit the terminus region of the chromosome. *TerI* and *TerII* plus the *rtp* gene make up the *terC* region, which has been relocated to the *cym* (10°) and *serC* (257°) loci in this study and was previously relocated at the *metD* (100°) and *pyr* (139°) loci (6).

samples of digested DNA from SU225 spores were included to normalize the hybridization to the different markers.

Microscopy. *B. subtilis* cells were fixed with ethanol by the method of Hauser and Errington (8) and stained with 4′,6-diamidino-2-phenylindole (DAPI) by the method of Setlow et al. (18) before being viewed by both phase-contrast and epifluorescence microscopy with a Zeiss photomicroscope.

All other materials and methods were as described previously (6).

RESULTS

Relocations of the *terC* region to *serC* and *cym*. *serC*, at 257°, was chosen as a site for relocating the *terC* region because of its presence in the left (anticlockwise) half of the chromosome. *cym*, at 10°, was chosen because it is close to *oriC*. Relocations of the *terC* region to these sites were achieved by the approach described previously (6). First, Tn*917* was inserted at *serC* and *cym*, respectively, by transformation of the *terC* region-deleted strain, SU187, with DNA from the appropriate Tn*917*-containing strains (SU281 and SU280). Linearized pWC5 and pWC6 (containing the *terC* region-*cat-amp* segment in alternate orientations within Tn*917*) were then used to transform these strains (SU283 and SU282) to chloramphenicol resistance. There was no marked difference in the number of transformants obtained with pWC5 and pWC6 in each case. The structures of the chromosomal segments spanning the inserted *terC* regions (Fig. 2) were established by Southern transfer experiments. In the new strains, SU290 and SU291, the insertions (alternate orientations) were at *serC*; in SU286 and SU287, they were at *cym*.

Anticlockwise fork arrest at the *terC* region relocated to *serC*. Figure 3 (left panels) shows the analyses of *Eco*RI digests of DNA from SU290 and SU291 growing exponentially in Penassay broth by Southern transfer and hybridization with different ^{32}P-labelled probes. The membrane was first hybridized with the WC1 probe, and after being stripped, it was hybridized with the WC2 probe. These probes hybridize to the *Eco*RI fragments in which the *terC* region is located (Fig. 2) and to each side of the *TerI-II* segment, with WC1 recognizing the *rtp* side and WC2 recognizing the other side (6). With the WC1 probe, SU290 showed the major species expected (4.9 kb) plus two extra bands (labelled I and II); SU291 showed the expected 6.8-kb band only, and there was no evidence of extra bands. This result suggests that SU290 gives rise to an arrested fork (species I) and that WC1 hybridizes to the arm of this fork; band II corresponds to the free arm (breakdown product). This interpretation was confirmed with the WC2 probing, where band II was absent from SU290. WC2 is clearly a stem probe in this case. No forked DNA was evident in SU291. Referring to the maps in Fig. 2, it is clear that the fork which is arrested in SU290 must enter the segment of the chromosome shown from the right in order to account for WC1 (which hybridizes to the *rtp* side of the terminators) as an arm probe and WC2 (which hybridizes to the other side) as a stem probe. *TerI* is in the functional orientation in such a situation. Thus, as found before for relocations of the *terC* region to the clockwise half of the chromosome, only the orientation that places *TerI* in opposition to the approaching anticlockwise fork is functional.

Clockwise fork arrest at the *terC* region relocated to *cym*. Figure 3 (right panels) show the analyses of *Eco*RI digests of DNA from exponentially growing SU286 and SU287 for fork arrest. In all cases, the expected major species was observed. Only in the case of SU286 was forked DNA evident. (The

TABLE 1. Characteristics of *B. subtilis* strains used in this study

Strain[a]	Genotype	Resistance	Source
SU187	Δ*rtp* Δ*TerI* Δ*TerII thyA1 thyB1*		Carrigan et al. (6)
SU225	*metD83*::Tn*917* Δ*rtp* Δ*TerI* Δ*TerII thyA1 thyB1*	Em^r	Carrigan et al. (6)
SU227	*metD83*::Tn*917*::*cat*(pWC5) *thyA1 thyB1*	Em^r Cm^r	Carrigan et al. (6)
SU228	*metD83*::Tn*917*::*cat*(pWC6) *thyA1 thyB1*	Em^r Cm^r	Carrigan et al. (6)
SU280 (1A600)	(SPβC2)*cym-84*::Tn*917 trpC2*	Em^r	Bacillus Genetic Stock Center
SU281 (1A621)	(SPβC2)*serC82*::Tn*917 trpC2*	Em^r	Bacillus Genetic Stock Center
SU282	*cym-84*::Tn*917* Δ*TerI* Δ*TerII thyA1 thyB1*	Em^r	This study
SU283	*serC82*::Tn*917* Δ*TerI* Δ*TerII thyA1 thyB1*	Em^r	This study
SU286	*cym-84*::Tn*917*::*cat*(pWC5) *thyA1 thyB1*	Em^r Cm^r	This study
SU287	*cym-84*::Tn*917*::*cat*(pWC6) *thyA1 thyB1*	Em^r Cm^r	This study
SU290	*serC82*::Tn*917*::*cat*(pWC5) *thyA1 thyB1*	Em^r Cm^r	This study
SU291	*serC82*::Tn*917*::*cat*(pWC6) *thyA1 thyB1*	Em^r Cm^r	This study
SU292 (OMG211)	168*trpC2 spac-1 rtp amy*::*cat lacI*	Nm^r Cm^r	S. Seror
SU296	*metD83*::Tn*917*::*cat*(pWC6) *spac-1 rtp amy*::*cat lacI*	Nm^r Cm^r Em^r	This study
SU327	Δ*rtp* Δ*TerI* Δ*TerII thyA1 thyB1*		This study

[a] Original strain name is in parentheses.

TABLE 2. Plasmids used as sources of DNA probes for marker frequency analysis

Plasmid	Gene	DNA probe fragment (kb)	Map position (°)[a]	Size of chromosomal fragment (kb)	Source
pUEC14′	*recM*	*Eco*RI-*Bam*HI (1.8)	5	2.2[b]	N. Ogasawara
pAmy10	*amyE*	*Xba*I-*Bfr*I (2.4)	25	15.0[b]	D. Henner
pUL720	*argA*	*Eco*RI-*Pvu*I (3.0)	102	12.0[b,c]	S. Baumberg
pLH1	*divIB*	*Bam*HI-*Hin*dIII (1.8)	135	8.6[b] 10.5[c]	E. Harry
p1747	*che*	*Eco*RI-*Eco*RI (4.0)	144	4.0[c]	G. Ordal
pOR101	*cotE*	*Hin*dIII-*Hin*dIII (2.0)	150	10.1[c]	O. Resnekov
pAG3	*TerIV/cotC*	*Eco*RI-*Bam*HI (1.8)	165	16.0[c]	A. Griffiths
pLS23-17	*gltA*	*Eco*RI-*Pvu*II (1.9)	174	10.1[b,c,d]	A. Sonenshein
pSX3	*spoIIIJ*	*Bam*HI-*Eco*RI (3.0)	360	6.9[b]	J. Errington

[a] According to Anagnostopoulos et al. (3).
[b] Size in *Bam*HI-*Eco*RI chromosomal digests.
[c] Size in *Eco*RI chromosomal digests.
[d] In the strains with a relocated *terC* region, the size of *gltA* is 10.1 kb, not 7.0 kb as in the wild type.

additional band, labelled P, was of a size [~16 kb] expected for a partial-digest fragment, and this was confirmed by two-dimensional neutral-alkali gel electrophoresis.) Again, only the WC1 probe detected the free arm of the fork (band II). Reference to Fig. 2 allowed the conclusion that the fork which was arrested entered the SU286 region from the right; i.e., *TerI* was the functional terminator. In SU287, where *TerII* would have opposed the fork, no arrest was effected, as expected.

cym is positioned very close to *oriC*, and it was therefore somewhat surprising that the relative amount of forked DNA in SU286 was not greater than observed. (Complete arrest so close to *oriC* would result in forked DNA being by far the major species.) Even after correction for the breakdown product, the amount of forked DNA appeared to be significantly less than that of the linear 5.6-kb species. This would suggest that fork arrest at the *terC* region relocated to *cym* was not complete.

Fork arrest by chromosomally located *TerII* in the presence of an increased level of RTP. Previous results (6), as well as those presented here so far, establish that *TerII*, contained within the *terC* region chromosomal segment, is unable to arrest either the clockwise- or anticlockwise-moving replication fork. To find out if this was due to a limiting amount of RTP in the cell, causing the lower-affinity *TerII* (compared with *TerI* [13]) to be incompletely complexed with RTP, an appropriate strain was constructed in which the *rtp* gene was inducible and was capable of yielding high levels of RTP. SU292 is a strain of *B. subtilis* that has the *rtp* gene under *spac-l* control and inducible because of the presence of *lacI* at *amyE* (17a). It was transformed to erythromycin resistance with DNA from strain SU228 which contains the *terC* region at *metD* in the nonfunctional orientation; i.e., *TerII* in SU228 is opposed to the approaching clockwise fork (6). The new strain was called SU296. The structure of the chromosomal segment spanning the *terC* region in SU296 (and SU228) is shown at the top of Fig. 4. This structure was verified by Southern hybridization, and SU296 was checked for the overproduction of RTP in the presence of IPTG (isopropyl-β-D-thiogalactopyranoside) by Western blotting (immunoblotting) with antiserum to RTP (data not shown). Figure 4 shows the analysis of *Eco*RI digests of chromosomal DNA extracted from exponentially growing SU228 and SU296 (with or without IPTG) for fork arrest obtained with the WC2 probe. In the case of SU228 (lane 1), only the linear species with a size of 5.5 kb was observed, confirming the previous conclusion of the lack of fork arrest by *TerII* (6). Lane 2 shows the behavior of DNA from SU296 grown in the absence of IPTG. Again, only the 5.5-kb species was present. However, when SU296 was grown in the presence of IPTG (0.5 mM), two additional bands (I and II) were observed (lane 3). They were at positions completely consistent with their representing forked DNA and the released arm (4.5 kb), respectively. It is concluded that chromosomally located *TerII* can be made to function as a fork arrest site in the presence of excess RTP.

Clockwise fork arrest at *cym* is relatively weak. The levels of forked DNA detected in exponentially grown SU286 (*TerI* in

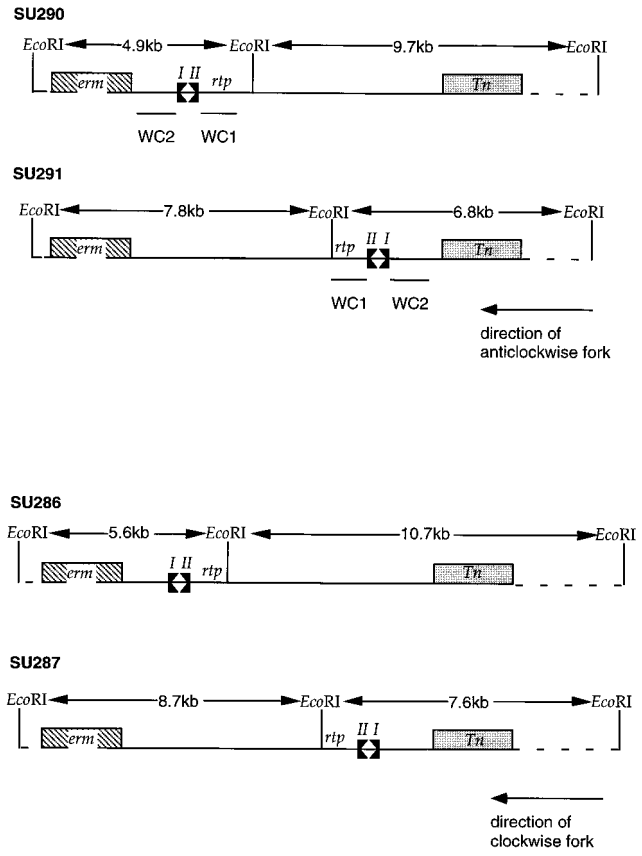

FIG. 2. Restriction maps of the chromosomal segments spanning the *terC* regions relocated to the *cym* and *serC* loci. SU290 and SU291 contain the relocations at *serC*; SU286 and SU287 contain the relocations at *cym*. For clarity, the *amp* and *cat* genes are not shown. The regions corresponding to the WC1 and WC2 probes are shown for SU290 and SU291. They are in equivalent positions for SU286 and SU287. Sizes are in kilobases.

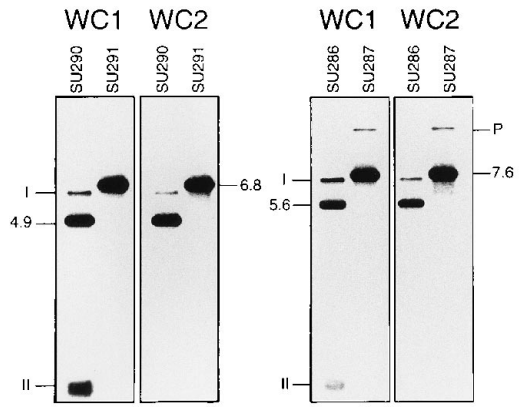

FIG. 3. Analysis of DNA from *B. subtilis* SU290, SU291, SU286, and SU287 for fork arrest at the relocated *terC* regions. *Eco*RI digests of DNA from cells growing exponentially in Penassay broth were fractionated on neutral gels, transferred to membranes, and hybridized with either ^{32}P-labelled WC1 or WC2 DNA as described previously (6). The two panels on the left show the results for SU290 and SU291, and those on the right show the results for SU286 and SU287. In each case, the membrane, after hybridization with WC1 DNA, was stripped and hybridized with WC2 DNA. I, forked DNA; II, released arm of fork; P, partial-digest fragment. Sizes are in kilobases.

the functional orientation at *cym*) suggested that the fork was only being stalled transiently at this position. Six regions of the chromosome were chosen for use as hybridization probes to establish where the two forks (clockwise and anticlockwise) met. The probes used lie mainly on the clockwise arm of the chromosome (*spoIIIJ*, *recM*, *amyE*, *argA*, *divIB*, and *gltA* [Fig. 5]). DNAs were extracted from spore DNA (SU225) and SU286 and SU287 growing exponentially in minimal medium. They were digested with *Bam*HI-*Eco*RI and MFA by Southern hybridization was performed (23). Control experiments established that such conditions provided a linear response between bound probe and the amount of DNA on the membrane. Figure 6A shows the relative frequency of the various chromosomal markers. The results are the average of data obtained from the separate probing of four membranes and are expressed as ratios of each marker to *gltA*, normalized to the spore DNA. *gltA* was set at 1.0. The upper panel represents SU286, while the lower one shows the results for SU287. It can be seen that in both instances, the forks are meeting in the vicinity of the normal terminus region, certainly beyond *divIB*. It is clear that although fork arrest was detected in SU286 by Southern analysis (Fig. 3, right panels), it is not apparent in this strain by MFA with the probes desribed. It cannot be ruled out, however, that in SU286 there is a minimum marker ratio between *divIB* and *gltA*. It is concluded that the block to replication fork movement effected by *TerI* at *cym* is only transient and is not sufficient to alter appreciably the region in which the two forks eventually meet.

Clockwise fork arrest at *TerI* relocated to *metD* is more severe but not complete. The MFA described in the previous section was applied to DNA from SU227 and SU228, the strains containing the *terC* region in alternate orientations at *metD*. Figure 6B shows the averaged data from 12 separate hybridizations. In the lower panel (SU228 [inactive orientation]), it can be seen that *divIB* (135°) is replicated before *gltA* (174°), as expected, and the forks fuse, presumably, around 180°. However, in the upper panel (SU227 [active orientation]), *divIB* is replicated after *gltA*, with the forks fusing around this region. While the impediment to fork movement effected by *TerI* at *metD* is obviously more severe than that at *cym*, it cannot be complete because *argA* (102°) is replicated before *divIB* (135°). The MFA described was extended with the use of additional probes, with the aim of establishing more precisely the region in which the forks meet. It was first established that the forks in SU227 were meeting, on the average, in the *divIB-che* region (Fig. 7A). Further experiments (Fig. 7B) showed that the forks in SU227 were meeting in a region past *divIB* and closer to the *che-cotE* segment of the chromosome, at around 145°. Thus the anticlockwise fork in SU227 must move through the 180° to 145° segment of the chromosome.

SU227 cells are elongated. SU227 and SU228, in which the replication forks meet at approximately 145° and 180°, respectively, showed no significant difference in generation times in Penassay broth or minimal medium, but in the case of SU227, the cells, on average, were longer. Some appeared as moderately long filaments. To determine if this was associated with fork arrest at the relocated *terC* region, the latter was removed from SU227 by transforming it to Met$^+$ with DNA from SU187 (a *terC* region-deleted strain) to give SU327. SU227, SU228, and SU327 were grown to the mid-exponential phase in Penassay broth, and the cells were fixed with ethanol (8). Each sample was stained with DAPI (17) and viewed by both phase-contrast and fluorescence microscopy. Samples from eight separate cultures were analyzed. The phenotypes of the three strains (phase-contrast microscopy) are compared in Fig. 8. Elongated SU227 cells (Fig. 8C) are clearly observable. Figure 9 plots the distribution of cell lengths in each strain; it is clear that a significant number of the SU227 cells (~20%) are greater than twice the average length of the SU228 and SU327 cells. The latter two strains had average cell lengths of 3.04 ± 0.07 and 2.93 ± 0.08 μm, respectively. The average cell length of SU227 was 6.29 ± 0.38 μm. The DAPI staining and fluorescence microscopy did not show any marked differences in nucleoid segregation between the three strains (data not shown).

DISCUSSION

In this work, the *terC* region of the *B. subtilis* chromosome has been relocated to two new positions on the chromosome,

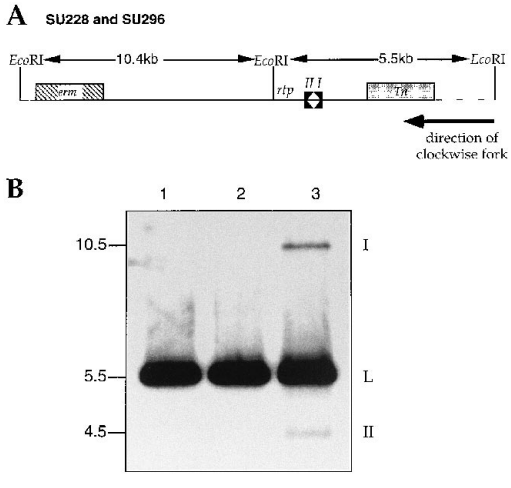

FIG. 4. Arrest of a chromosomal replication fork by *TerII* in the presence of overproduced RTP. (A) Restriction map of the chromosomal segment spanning the *terC* region at *metD* in SU228 and SU296. (B) *Eco*RI digests of DNA fractionated on neutral gels, transferred to membranes, and hybridized with WC2. Lanes: 1, SU228; lane 2, SU296; 3, SU296 plus IPTG. I, forked DNA; II, released arm of fork; L, linear DNA. Sizes are in kilobases.

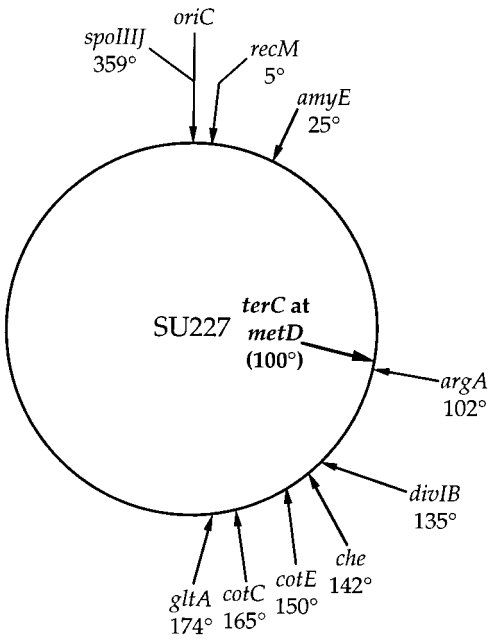

FIG. 5. Locations of the regions of the *B. subtilis* 168 chromosome used as hybridization probes for MFA. Restriction fragments of cloned DNA were isolated for ^{32}P labelling as outlined in Materials and Methods. The positions of these regions and the fragment sizes are given in Table 2.

serC (257°) and *cym* (10°), extending the work of Carrigan et al. (6). The relocations were performed to examine the effects of relocating the *terC* region to the anticlockwise-replicating arm of the chromosome and of placing it close to *oriC*.

The results of relocating the *terC* region to *serC* rule out the possibility that *TerII* can function on the anticlockwise-moving arm because of some recognition differential between the two replication forks. Fork arrest at *serC* occurs only when *TerI* is positioned to oppose the anticlockwise-moving fork (SU290 [Fig. 2 and 3]). When *TerII* is positioned to impede the anticlockwise-moving fork (SU291), it fails to do so. Given the sequence similarities between *TerI* and *TerII* and the fact that both can function when located in a plasmid in the presence of overproduced RTP (20), the simplest explanation for the nonfunctioning of *TerII* on the chromosome is that it is unoccupied at normal intracellular concentrations of RTP. It is known that the intracellular level of RTP is very low and is barely or not detectable by antiserum to RTP (1, 12). The functioning of *TerII* at *metD* (100°) in strain SU296 when RTP was overproduced (Fig. 4) shows that chromosomally located *TerII* can indeed function in the presence of elevated levels of RTP. Thus, it is reasonable to conclude that *TerII* in its normal position on the wild-type chromosome and under normal circumstances cannot function, although it could under conditions that cause an increase in RTP. Because expression of the *rtp* gene is normally repressed through the binding of RTP to the high-affinity *TerI*, thus occluding the closely adjacent promoter (2), damage to *TerI* would cause the intracellular level of RTP to rise. Whether or not this is a significant factor in the utilization of *TerII*, and possibly other terminators, is not known. The present finding also raises the possibility that a number of terminators, in *B. subtilis* and *E. coli*, are nonfunctional because of a limiting intracellular concentration of the terminator protein.

For the relocation close to *oriC*, the *cym* locus (10°) was chosen, because this was the position closest to *oriC* for which a Tn*917* insertion was available. The clockwise fork would

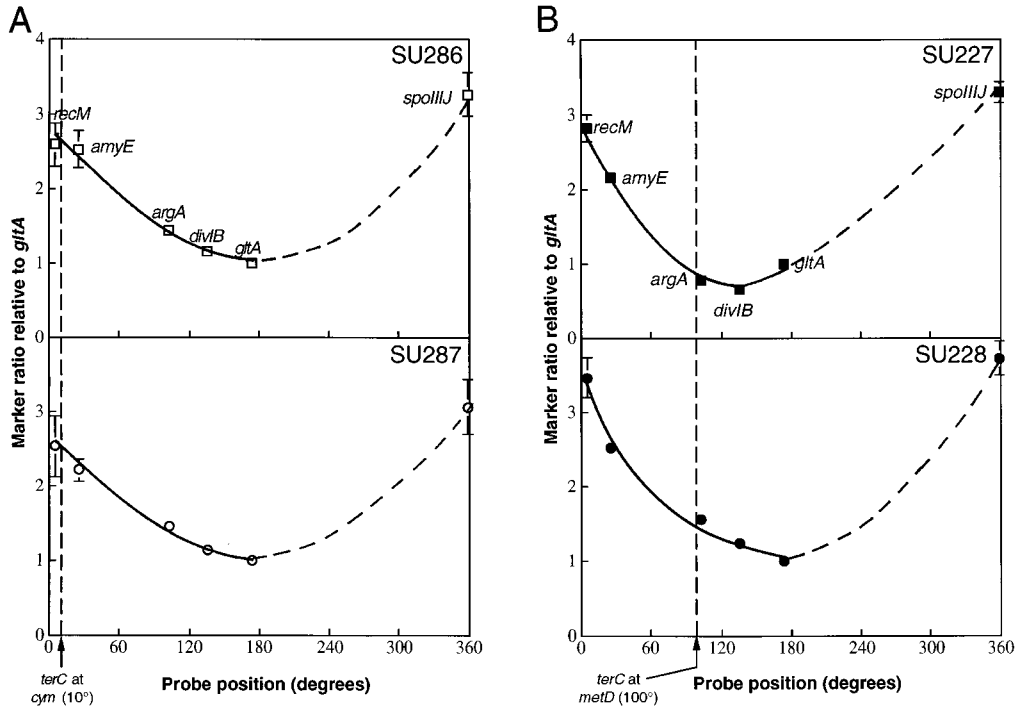

FIG. 6. MFA of DNA from exponentially growing cells of *B. subtilis* strains with relocated *terC* regions. Marker ratios are relative to *gltA* (178°) and have been normalized to SU225 spore DNA; *gltA* has been set at 1.0. (A) SU286 (□) and SU287 (○). (B) SU227 (■) and SU228 (●). The vertical bars represent the standard error of the mean of the datum points. Where no error bar is visible, the error is smaller than the height of the plotted point.

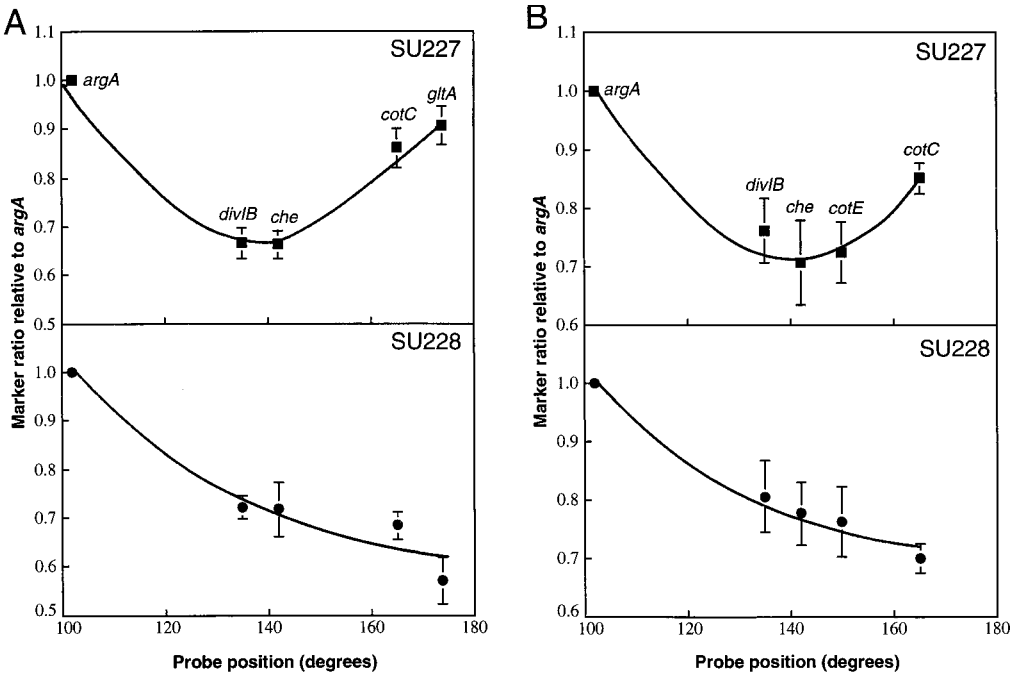

FIG. 7. More refined MFA of DNA from SU227 and SU228. Marker ratios are relative to *argA* (102°) and have been normalized to spore DNA; *argA* has been set at 1.0. ■, SU227; ●, SU228. In section A, analysis is for the chromosomal region 102° to 178°, and in section B, it is for the 102° to 165° region. The vertical bars represent the standard error (see Fig. 6).

encounter the relocated *terC* region soon after its generation at *oriC*, and it was thought that this might have severe effects on the growth and other features of the cell. Fork arrest at this position is again only effected by *TerI* (SU286 [Fig. 2 and 3]), confirming the earlier results (6). However, the relatively low amounts of forked DNA detected in this case and the fact that the strain in question did not have a significantly slower growth rate than that with the inactive *terC* region orientation (data not shown) suggest that although a block to fork movement is detectable, it is only transient. This is confirmed by MFA, which shows that the clockwise and anticlockwise forks meet in the general vicinity diametrically opposite *oriC*. MFA was also performed for the *terC* region relocations to *metD*. It is shown that, while fork arrest by *TerI* is again only transient, it is of a severity that causes the forks to meet at a position (145°) significantly displaced from diametrically opposite the origin (SU227 [Fig. 6B and 7]). Assuming that the clockwise and anticlockwise forks move at the same constant speed (when unimpeded by a terminator-RTP complex), this would mean that the clockwise fork pauses longer at the *metD* (100°)-located *TerI* than it does at the *cym* (10°)-located *TerI*. This finding is somewhat surprising, because in both cases, *TerI* is embedded within the same flanking sequence (several kilobases on each side), and it raises the possibility that other factors such as chromosome location and/or organization can influence the efficiency of a terminator. The possibility that the level of RTP in the *terC* region relocation at *cym* is abnormally low has not been investigated.

It is possible to estimate approximately the minimum time for which the clockwise fork stops at the *metD*-located *TerI*. For this calculation, it is assumed that the fork stops at the terminator for a fixed period, that it is then released to travel at its original speed, and that the anticlockwise fork travels unimpeded at the same constant speed until it fuses with the released clockwise fork. The presence of the additional terminators, *TerIV* and *TerVI*, which would impede the anticlockwise fork after it progresses past the 180° position, would only cause an underestimate of the pause time. For two forks generated at *oriC*, the anticlockwise fork would be at the 260° position when the clockwise fork reaches *TerI* at 100°. The clockwise fork must then stop for enough time to allow the anticlockwise fork during this interval to travel a further distance (before the clockwise fork starts to move again) to reach a position that would place the fusion site (145°) midway between this position and 100°. This means that the anticlockwise fork would be at 190° when the clockwise fork starts to move again. The time taken for the anticlockwise fork to travel from 260° to 190° is therefore equivalent to the pause time. Assuming that the rate of fork movement is 180°/40 min (40 min is the average time to replicate the chromosome at 37°C), the time taken to replicate 70° (or the average pause time) is ~15 min. In relatively early work leading up to the discovery of replication terminators in *B. subtilis*, it was concluded that in the wild-type situation, the clockwise replication fork was arrested at a site in the terminus region, now known to be *TerI*, for a period of <5 min before being met by the anticlockwise fork (11, 21). It was concluded that the block to fork movement at the arrest site was either complete or very severe (22). While a minimum pause time of ~15 min would not hold for arrest by *TerI* at all chromosomal sites (in view of the data for *TerI* at *cym*), it is clear that this terminator is certainly capable of stalling a fork for a period sufficient to provide an essentially complete block in the wild-type chromosome such that the anticlockwise fork can progress up to the same site, as was observed in the initial investigations. It is concluded that arrest of fork movement by *TerI* can be severe, on the order of 40% of the time taken to replicate the whole chromosome.

SU227 (*TerI* opposed to the clockwise fork at 100°) is the only *B. subtilis* strain for which it has so far been established that the forks fuse at a site significantly removed from the

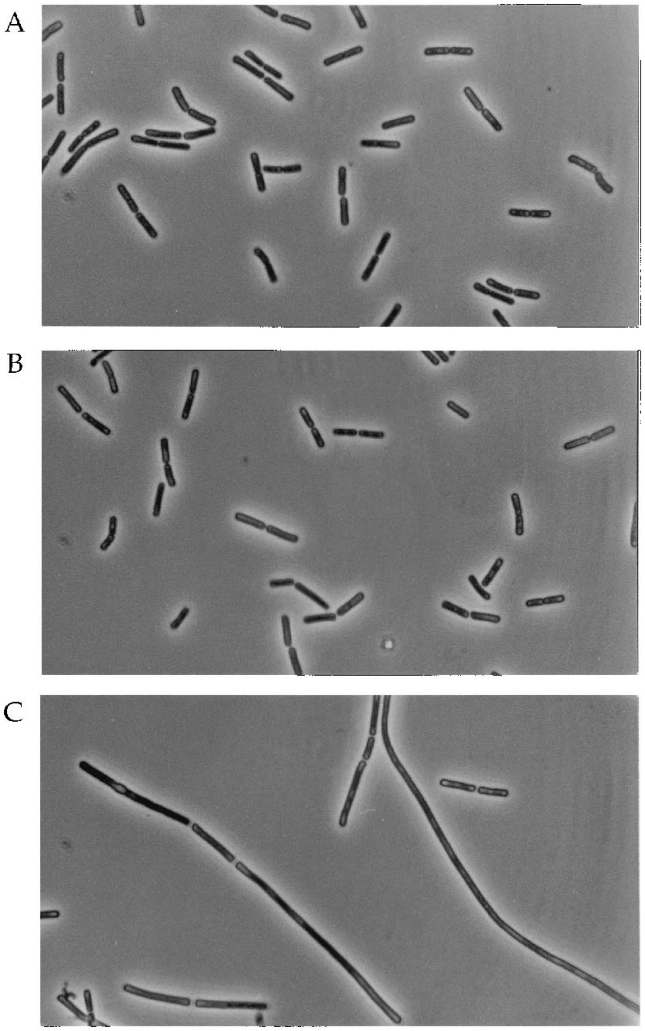

FIG. 8. Photographs of cells observed by phase-contrast microscopy. The cells are from exponentially growing cultures in Penassay broth at 37°C and fixed in ethanol. (A) SU228. (B) SU327. (C) SU227.

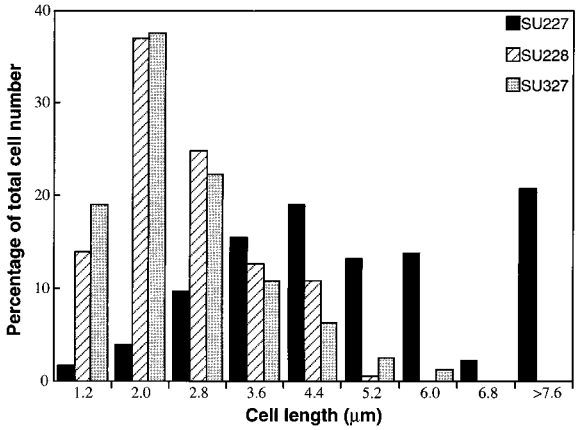

FIG. 9. Cell length distributions for exponentially growing SU227, SU228, and SU327. The cell lengths (distance between septa) were measured from photographs taken in the experiment described in the legend to Fig. 8. A total of 178 cells were measured for SU227, and 147 cells (each) were measured for SU228 and SU327.

"normal" terminus. The site of fork fusion at 145° is just short of *TerVI* (150°). This places it just outside or on the edge of the replication fork trap. SU227 cells are elongated, and this has been shown to result from the pausing of the clockwise fork at the 100°-positioned *TerI*. This was not accompanied by any marked alteration in nucleoid distribution. Clearly, cell division is delayed relative to DNA replication in SU227. While this could be caused by the forks meeting outside the replication fork trap, a more likely explanation is that it is a result of at least partial induction of the SOS system in SU227. Horiuchi and Fujimura (10) inserted a replication terminator within the *lacZ* gene of *E. coli*, at the equivalent of about the 80° position on a 360° map, with *oriC* at 0°. While the site of fork fusion when the terminator was functional was not established, the cells showed an elongated-cell phenotype similar to that of SU227. It was established that constitutive SOS induction occurred in such cells as a result of fork arrest at the additional terminator and that growth was dependent on $recA^+$ and $recB^+$ (C^+). It was concluded that the elongated-cell phenotype was mediated through at least partial SOS induction. Sharma and Hill (19) also observed SOS induction and filamentation in *E. coli* when inverted *Ter* sites were inserted into the terminus region in order to delay completion of DNA replication. SOS induction in *B. subtilis* also causes filamentation, but it is different from that in *E. coli* in not requiring a functional RecA product (24). Attempts are currently being made to establish the immediate cause of cell elongation in SU227. In the merodiploid strain of *B. subtilis*, GSY1127, the "normal" terminus region is positioned significantly asymmetrically relative to *oriC* (at 142° on a 360° map [17]). It could be particularly informative to know the effect of deleting the *rtp* gene from this strain on both the region of fork fusion and cell morphology.

ACKNOWLEDGMENTS

We thank the numerous people listed in Tables 1 and 2 for providing bacterial strains and plasmids.

This work has been supported by the Australian Research Council.

REFERENCES

1. **Ahn, K. S.** 1994. Expression of the *rtp* gene of *Bacillus subtilis*. PhD. thesis. University of Sydney, Sydney, Australia.
2. **Ahn, K. S., M. S. Malo, and R. G. Wake.** 1993. Autoregulation of the gene encoding the replication terminator protein of *Bacillus subtilis*. Gene **132:**7–13.
3. **Anagnostopoulos, C., P. J. Piggot, and J. A. Hoch.** 1993. The genetic map of *Bacillus subtilis*, p. 425–461. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington, D.C.
4. **Baker, T. A.** 1995. Replication arrest. Cell **80:**521–524.
5. **Carrigan, C. M., J. A. Haarsma, M. T. Smith, and R. G. Wake.** 1987. Sequence features of the replication terminus of the *Bacillus subtilis* chromosome. Nucleic Acids Res. **15:**8501–8509.
6. **Carrigan, C. M., R. A. Pack, M. T. Smith, and R. G. Wake.** 1991. Normal *terC*-region of the *Bacillus subtilis* chromosome acts in a polar manner to arrest the clockwise replication fork. J. Mol. Biol. **222:**197–207.
7. **Franks, A. H., A. A. Griffiths, and R. G. Wake.** 1995. Identification and characterization of new DNA terminators in *Bacillus subtilis*. Mol. Microbiol. **17:**13–23.
8. **Hauser, P. M., and J. Errington.** 1995. Characterization of cell cycle events during the onset of sporulation in *Bacillus subtilis*. J. Bacteriol. **177:**3923–3931.
9. **Hill, T. M.** 1992. Arrest of bacterial DNA replication. Annu. Rev. Microbiol. **46:**603–633.
10. **Horiuchi, T., and Y. Fujimura.** 1995. Recombinational rescue of the stalled DNA replication fork: a model based on analysis of an *Escherichia coli* strain with a chromosome region difficult to replicate. J. Bacteriol. **177:**783–791.
11. **Iismaa, T. P., M. T. Smith, and R. G. Wake.** 1984. Physical map of the *Bacillus subtilis* replication terminus region: its confirmation, extension and genetic orientation. Gene **32:**171–180.
12. **Levine, A., S. Autret, and S. J. Seror.** 1995. A checkpoint involving RTP, the

replication terminator protein, arrests replication downstream of the origin during the stringent response in *Bacillus subtilis*. Mol. Microbiol. **15:**287–295.
13. **Lewis, P. J., G. B. Ralston, R. I. Christopherson, and R. G. Wake.** 1990. Identification of the replication terminator protein binding sites in the terminus region of the *Bacillus subtilis* chromosome and stoichiometry of the binding. J. Mol. Biol. **214:**73–84.
14. **Lewis, P. J., M. T. Smith, and R. G. Wake.** 1989. A protein involved in termination of chromosome replication in *Bacillus subtilis* binds specifically to the *terC* site. J. Bacteriol. **171:**3564–3567.
15. **McGinness, T., and R. G. Wake.** 1979. Division septation in the absence of chromosome termination in *Bacillus subtilis*. J. Mol. Biol. **134:**251–264.
16. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
17. **Schneider, A.-M., M. Gaisne, and C. Anagnostopoulos.** 1982. Genetic structure and internal rearrangements of stable merodiploids from *Bacillus subtilis* strains carrying the *trpE26* mutation. Genetics **101:**189–210.
17a. **Seror, S. J.** Personal communication.
18. **Setlow, B., N. Magill, P. Febbroriello, L. Nakhimovsky, D. E. Koppel, and P. Setlow.** 1991. Condensation of the forespore nucleoid early in sporulation of *Bacillus* species. J. Bacteriol. **173:**6270–6278.
19. **Sharma, B., and T. M. Hill.** 1995. Insertion of inverted *Ter* sites into the terminus region of the *Escherichia coli* chromosome delays completion of DNA replication and disrupts the cell cycle. Mol. Microbiol. **18:**45–61.
20. **Smith, M. T., and R. G. Wake.** 1992. Definition and polarity of action of DNA replication terminators in *Bacillus subtilis*. J. Mol. Biol. **227:**648–657.
21. **Weiss, A. S., and R. G. Wake.** 1983. Restriction map of DNA spanning the replication terminus of the *Bacillus subtilis* chromosome. J. Mol. Biol. **171:** 119–137.
22. **Weiss, A. S., and R. G. Wake.** 1984. A unique DNA intermediate associated with termination of chromosome replication in *Bacillus subtilis*. Cell **39:**683–689.
23. **Wu, L. J., A. H. Franks, and R. G. Wake.** 1995. Replication through the terminus region of the *Bacillus subtilis* chromosome is not essential for the formation of a division septum that partitions the DNA. J. Bacteriol. **177:** 5711–5715.
24. **Yasbin, R. E., D. Cheo, and D. Bol.** 1993. DNA repair systems, p. 529–537. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington, D.C.