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Utilization of Subsidiary Chromosomal Replication Terminators in *Bacillus subtilis*

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The *Bacillus subtilis* merodiploid strain GSY1127 contains a large nontandem duplication of a portion of its chromosome within its left (anticlockwise) replication segment. This causes displacement of the replication terminus region to a noticeably asymmetric location relative to *oriC*. The utilization of the subsidiary replication terminators, *TerIII* and *TerV*, in the merodiploid strain has been compared with that in *B. subtilis* 168. It is shown that *TerIII* is utilized to a significant extent in GSY1127 and that *TerV* is used only marginally at the most. Neither of these terminators is used to a measurable extent in the 168 strain. It is concluded that *TerIII* and *TerV* do indeed function as backups to the major terminator *TerI*, as has been generally thought. It is further concluded that, in the 168 strain, the vast majority of clockwise forks are arrested at the highly efficient *TerI* terminator, with fork fusion between the approaching forks occurring frequently while the clockwise fork is stationary at *TerI*.

In the circular chromosomes of *Bacillus subtilis* and *Escherichia coli* the approach of replication forks towards the end of a round of replication is modulated by the presence of DNA replication terminators. Such terminators are short DNA sequences that bind a cognate terminator protein (RTP [for replication terminator protein] in *B. subtilis* and Tus [for terminus utilization substance] in *E. coli*). A terminator-terminator protein complex has the ability to arrest (or cause a severe pausing of) a replication fork in a polar manner. Both organisms contain multiple terminators (9 in *B. subtilis* and 10 in *E. coli*) in the terminus region, and they are organized as two opposed groups in each case (2, 6). Figure 1A shows the location and orientation of the terminators (*TerI* to *TerIX*) in the *B. subtilis* 168 chromosome. *Ter1*, *-III*, *-V*, and *-IX* are oriented to arrest the clockwise-moving fork, while *TerII*, *-VIII*, *-IV*, *-VII*, and *-VI* arrest the anticlockwise fork. The gene for RTP (*rtp*) lies between *TerII* and *TerVIII*, and its expression is autoregulated through a promoter that overlaps *TerI* (1). It has been generally agreed that the multiplicity of terminators reflects the use of the outer (subsidiary) ones as backups to those more centrally located within the terminus region. Thus, if the clockwise fork passed through *TerI*, its movement out of the terminus region would be subsequently impeded when it reached *TerIII*. Consistent with this concept was the observation that some of the outer terminators in *E. coli* operated only when the inner ones were deleted (7). The opposed arrangement of two groups of terminators in the chromosome has been referred to as constituting a "replication fork trap" (8).

In the present work, and with knowledge of the full complement of terminus region terminators in *B. subtilis*, we have used a new approach to demonstrate directly that the outer terminators do in fact function as backups in order to prevent passage of a fork out of the terminus region. We took advantage of the availability of a stable merodiploid strain of *B. subtilis*, GSY1127 (11). This strain contains a nontandem duplication of a 932-kb segment of the left replication half of the chromosome, which causes displacement of the terminus region to a markedly asymmetric location relative to *oriC* (Fig. 1B). The orientation of the duplication within the enlarged *oriC*-to-terminus segment is the same as in the primary segment. (It should be noted that the progenitor of GSY1127 and *B. subtilis* 168 were both derived from the *B. subtilis* Marburg strain.) *TerI* appears to be the most frequently used terminator in *B. subtilis* 168, and this probably reflects its asymmetric positioning at ~172° on the 360° map (9). Clearly, one would expect *TerIII* and *TerV*, if they were needed as backups, to be utilized more frequently in GSY1127 than in *B. subtilis* 168. It will be shown that this is indeed the case. Also, it can be firmly concluded that very few, if any, of the clockwise forks in *B. subtilis* 168 pass through *TerI*.

B. subtilis 168 *trpC2* and the GSY1127 strain (*hisH2 ilvC1*/*ilvC*$^+$) were obtained from E. W. Nester (Stanford collection) and C. Anagnostopoulos, respectively. DNA was extracted from mid-exponential-phase Penassay broth cultures at 37°C using procedures described previously (13). After being cut with the appropriate restriction enzyme, the DNA was examined for the presence of forked molecules arrested at the terminator being investigated by Southern transfer and hybridization using an appropriate ^{32}P-labeled probe (4). In all cases two completely independent experiments were performed, and they gave identical results.

Figure 2A shows the result of testing for fork arrest at *TerI*. This terminator lies within a 15.1-kb *Nco*I fragment. The forked molecule, indicative of arrest at *TerI*, migrates more slowly than the linear form; the faster-migrating species (6.7 kb) represents an arm released from the fork (13). It is clear that, as expected, the level of arrested fork in GSY1127 relative to the linear form is significantly higher (~5-fold) than in the 168 strain. In the case of the merodiploid, the ratio of the fork (after correcting for breakdown) to the linear form was quite high (~0.3). Obviously, *TerI* is a highly efficient terminator.

TerIII lies only 16.4 kb from *TerI* and within a 10.8-kb *Sal*I fragment. Figure 2B shows the results of Southern analysis of a *Sal*I digest of the DNA samples examined in Fig. 2A using an appropriate *TerIII* probe. Clearly there has been measurable fork arrest at *TerIII* in GSY1127, indicated by the presence of

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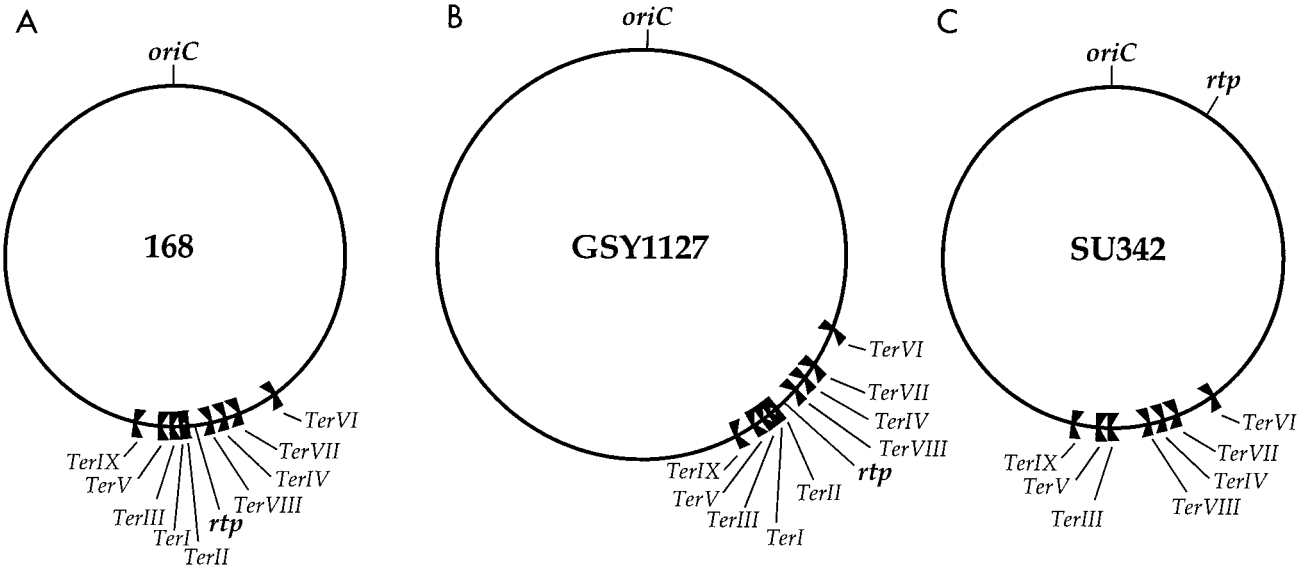

FIG. 1. Location of DNA replication terminators in the chromosomes of various *B. subtilis* strains. *TerI*, *-III*, *-V*, and *-IX* are oriented to block movement of the clockwise fork generated at *oriC*. The others are oriented to block the anticlockwise fork. The position of the *rtp* gene is also shown in each case.

forked DNA, a significant portion of which has broken down to yield the 4.4-kb arm. But the *TerIII*-related fork is completely absent from the 168 strain. That the band labeled "f" in the GSY1127 lane was indeed a forked molecule was established by two-dimensional (2-D) (neutral-alkaline) gel electrophoresis (Fig. 2C, right section). In the second (alkaline) dimension the forked molecule was resolved into its expected component single strands of 4.4 and 10.8 kb. The left section of Fig. 2C confirmed that the fork was undetectable in the 168 strain. The ratio of forked DNA at *TerIII* in GSY1127 (corrected for breakdown) relative to the linear (10.8-kb) species was ~0.05. Even in examining more intense phosphorimager images, no *TerIII*-derived forked molecules could be detected in DNA from the 168 strain. It is concluded that a small but significant fraction of the clockwise replication forks in GSY1127 pass through *TerI* to be arrested at *TerIII*, but this is not so for the 168 strain.

TerV lies 117 kb from *TerIII* and within a 20.3-kb *Nco*I fragment. Results of an examination of the DNA samples from the 168 and GSY1127 strains for forks arrested at *TerV* are shown in Fig. 3. The position expected for an arrested fork at *TerV* was established by examining DNA from the SU342

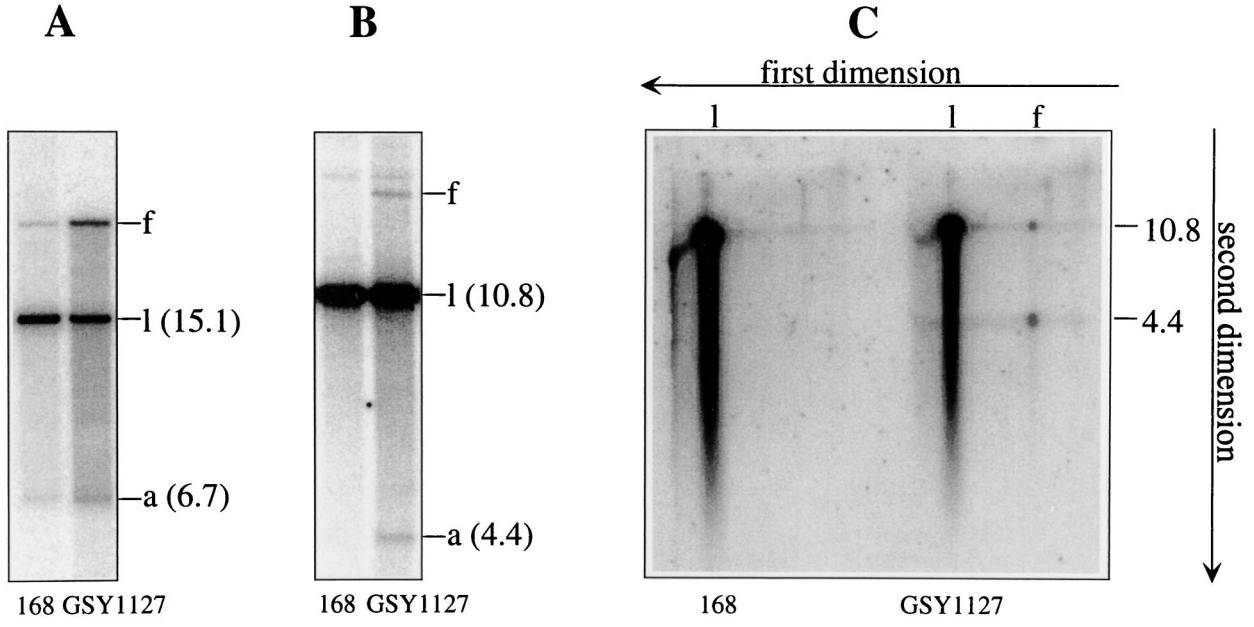

FIG. 2. Clockwise replication fork arrest at *TerI* and *TerIII* in *B. subtilis* 168 and GSY1127. (A and B) Results of neutral-gel assays (4, 13) for arrest at *TerI* and *TerIII*, respectively. (C) Results of analysis of the DNA samples analyzed in panel B by 2-D (neutral-alkaline) gel electrophoresis (14). *B. subtilis* 168 and GSY1127 were grown in Penassay broth at 37°C, and samples were collected at mid-exponential phase for DNA extraction and analysis as described previously (4, 13). For assays of fork arrest at *TerI* the DNA was cut with *Nco*I, and for *TerIII* assays *Sal*I was used. The ^{32}P-labeled probes used corresponded to a 0.53-kb segment of the 6.7-kb arm of the fork arrested at *TerI* and a 5.1-kb segment covering the complete arm (4.4 kb) plus part of the stem of the fork arrested at *TerIII*.

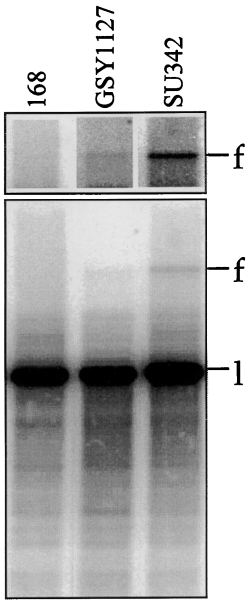

FIG. 3. Assays for fork arrest at *TerV* in various *B. subtilis* strains. The DNA samples were obtained and assayed, after being cut with *Nco*I, as described in the legend to Fig. 2. The ^{32}P-labeled probe corresponded to a 1.5-kb segment (labeling by PCR) from within the 12.0-kb arm of the fork arrested at *TerV*.

strain (Fig. 3), from which *TerI* and *TerII* had been deleted and *rtp* placed at *amyE* under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *spac-1* gene (3). The upper section of Fig. 3 shows the area of the forked molecule at a higher phosphorimager intensity. There was possibly a trace amount of forked DNA in GSY1127, but it was completely missing from the 168 strain. The ratio of forked to linear DNA in GSY1127 was <0.002. The band identified in SU342 as the *TerV*-related fork was too faint to establish its validity by 2-D electrophoresis. This was achieved by comparing DNA from SU342 and SU341 (parent of SU342 without *rtp*) and finding that, after cutting of the DNA individually with five different restriction enzymes, the putative forked molecule in SU342 (absent from SU341) in every case was of the expected apparent size (approximately the sum of the stem plus two arms).

The relative strength of individual terminators within their chromosomal context and at normal cellular levels of RTP is not known. However, we have previously shown that both *TerIII* and *TerV* function very efficiently and similarly to *TerI* in the presence of overproduced RTP in a plasmid assay system (4). The results described here show clearly that in the merodiploid strain GSY1127, *TerIII* is utilized to a small but significant extent and *TerV* is used only marginally at the most. (The potential usage of the next terminator, *TerVI*, was not investigated, but it would be expected to be negligible in both strains studied here.) On the other hand, neither *TerIII* nor *TerV* was utilized to a measurable extent in the 168 strain. Thus, in the 168 strain the vast majority of clockwise forks are effectively arrested at *TerI* and do not pass through the additional 16.4 kb to reach *TerIII*. This is completely consistent with *TerIII* and *TerV* functioning as backup terminators, being utilized only if some of the clockwise replication forks pass through *TerI*. This would be likely to occur more frequently in the merodiploid because of the clockwise fork entering the displaced terminus region well in advance of the anticlockwise fork.

TerI is a very efficient terminator, as evidenced by its presence as forked DNA to such a large extent in GSY1127. Under the conditions of growth used here, approximately 20% of all replicating chromosomes in GSY1127 would have contained a clockwise fork arrested at *TerI*, reflecting arrest of each individual fork for a significant portion of a round of replication. It is thus not surprising that no detectable clockwise forks pass through *TerI* in the 168 strain in the relatively short time available before arrival of the anticlockwise fork. Appropriate strains to test the behavior of the anticlockwise fork through the same type of approach are not available. While we have not tested directly for the level of fork arrest at *TerII* in the 168 chromosome, it is likely that on most occasions, the clockwise fork would encounter *TerI* (at 172°) in advance of the anticlockwise fork encountering *TerII*. Thus, in *B. subtilis* 168 it is highly likely that the majority of approaching forks fuse in the close vicinity of *TerI* and that in many instances fork fusion would take place while the clockwise fork was stationary.

Some years ago the approach of replication forks in the terminus region of GSY1127 was investigated by the use of outgrowing spores of this strain in conjunction with a density shift approach (10). The data showed severe blockage of the clockwise fork in the vicinity of *gltA*, but it could not be decided on what side of *gltA* the block was more likely to have taken place. *gltA* is now known to reside <4 kb from *TerI*, which is clearly the major site of arrest. One potentially interesting aspect of the behavior of GSY1127 should be mentioned. In this strain the clockwise fork appears to remain arrested at *TerI* for a significant portion of each round of replication. In an earlier study in which *TerI* was placed at *metD* (100°) in the 168 strain and well outside the normal terminus region (to yield SU227), the clockwise fork was estimated to be arrested at this site for the order of 15 min at 37°C (5). This caused the two forks to fuse, on the average, in the vicinity of 145°. Noticeable elongation of the cells was evident, with a small portion becoming filamentous. It was concluded that the cell elongation was most likely mediated through at least partial SOS induction as a result of the clockwise forks being arrested for such a long time. In spite of arrest of the clockwise fork for an extended period of time in GSY1127, this strain gave no indication of filamentation. In GSY1127 most of the approaching forks would still meet within the normal terminus region, in contrast to SU227, and this might be the causative factor in avoiding filamentation. Of course it is possible that GSY1127 carries a mutation suppressing any filamentation. The nonfilamentous behavior of GSY1127 deserves further investigation.

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