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DNA Sequence Requirements for Replication Fork Arrest at *terC* in *Bacillus subtilis*

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The replication terminus, *terC*, of *Bacillus subtilis* is the chromosomal site at which movement of the clockwise replication fork is blocked. The effect of deletion or modification of DNA sequences on either side of *terC* (defined by the sequence location of the arrested clockwise fork junction) has been investigated. Deletion of sequences ahead of *terC* to within 250 base pairs (bp) had no effect on fork arrest, whereas removal of a further 130 bp abolished it. The 250-bp segment immediately ahead of *terC* encompassed the previously identified inverted repeat region as well as potential promoters for the transcription of an adjoining open reading frame (ORF). Deletion of DNA from the other side of *terC* up to 80 bp from it also abolished fork arrest. This deletion removed the bulk of the ORF. Disruption of this ORF by the insertion of 4 bp also abolished fork arrest. A model for clockwise fork arrest at *terC*, implicating both the inverted repeat region and the protein product of the ORF, is proposed.

The replication terminus, *terC*, of the *Bacillus subtilis* chromosome is located approximately opposite the origin, *oriC* (23). *terC* is unique (13, 14, 18), and at this site movement of the clockwise replication fork, which is the first of the two forks generated at the origin to reach *terC*, is blocked (8, 14, 20, 21). Clockwise fork arrest appears to represent the first stage in the overall process of termination of replication in *B. subtilis*. The anticlockwise fork arrives at *terC* a few minutes later, presumably to fuse with the arrested fork and so complete the process. In *Escherichia coli* the situation is different. The chromosome contains two regions, T1 and T2, which function as terminators of replication. They are separate by a distance of about 5% of the chromosome and each terminator is polar in its action, causing arrest of just one of the forks, clockwise or anticlockwise (3, 6). T1 has been mapped to within 20 kilobases (kb) and T2 to within 4 kb (7); neither region has been sequenced.

terC of *B. subtilis* has been cloned (16), and recently, a 1.3-kb segment of DNA spanning *terC* has been sequenced (2). The farthest that a clockwise fork moves within this sequence is to within about 20 nucleotides (at the most) of a region containing two imperfect inverted repeats, each of 47 to 48 nucleotides and separated by 59 nucleotides. (Note that each inverted repeat referred to here could be considered as one-half of a palindrome.) The inverted repeat region is located just upstream of an open reading frame (ORF) which has the potential to code for a protein of 122 amino acids. It is not known whether this ORF is expressed. A diagrammatic representation of these features is shown in the upper portion of Fig. 3 (see below); the clockwise fork enters the region shown from the right.

The block to movement of the clockwise fork at *terC* in *B. subtilis* is very severe, if not complete (5), and the site of the junction between the daughter arms and unreplicated DNA in this arrested fork has been used to define the location of *terC*. Clearly, the nucleotide sequence in the vicinity of *terC*, especially but not exclusively that of the unreplicated DNA just ahead of the arrested clockwise fork, must hold at least part of the key to the molecular basis of fork arrest. The work reported here explores the sequence requirements for clockwise fork arrest at *terC* in *B. subtilis*.

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MATERIALS AND METHODS

Bacterial strains. The *E. coli* strains used were DH5α, HB101, and RR1. The *B. subtilis* strains used were GSY1127, a class II stable merodiploid (*hisH2*, *ilvC1/ilvC*$^+$) from C. Anagnostopoulos; and SB19, a strain 168 prototroph, from E. Nester. Strains SU158, -161, -162, -163, -164, -165, -171, -185, and -186 were constructed in this work and are derivatives of GSY1127 transformed with linearized plasmids pWS24, pWS26, pWS27, pWS28, pWS29, pWS32, pWS35, pWS37, and pWS37, respectively.

Plasmids and plasmid constructions. The plasmids used have been described previously: pJH101 (4), pLS23-17 (1), pWS8 (21), pWS10 (16), and pWH47 (5). New plasmids were constructed as follows.

For pWS24, the 4.6-kb *Pst*I-*Eco*RI fragment of pJH101 was ligated to the 1.95-kb *Pst*I-*Eco*RI fragment of pWS10, resulting in pWS23. pWS23 was cleaved with *Eco*RI and *Bam*HI, and the large fragment was ligated to the 3.0-kb *Eco*RI-*Bam*HI insert of pWS8.

For pWS25, pWS24 was cleaved with *Bam*HI and digested with 2 U of exonuclease III per μg of DNA in 50 mM Tris hydrochloride (pH 8.1)–5 mM $MgCl_2$–10 mM 2-mercaptoethanol at 37°C for 45 min. The DNA was deproteinized, ethanol precipitated, dissolved in 30 mM sodium acetate (pH 4.6)–50 mM NaCl–1 mM $ZnCl_2$–5% glycerol, and digested with mung bean nuclease (0.5 U/μg of DNA) at 37°C for 10 min. The DNA was deproteinized and recircularized with T4 DNA ligase. Approximately 1 kb of DNA was deleted; this removed single *Bam*HI, *Hin*dIII, and *Pst*I sites.

For pWS26, -27 and -29 through -32, pWS25 was cleaved with *Pst*I and *Bgl*II and treated with exonuclease III for either 7 min (pWS27) or 10 min (pWS26 and pWS29 through -32) at 23°C, followed by digestion with mung bean nuclease, all according to the Stratagene exo/mung deletion protocol. DNAs were then recircularized with T4 DNA ligase. Approximate deletion sizes were determined after cleavage of

the plasmids with *Bgl*I and *Hin*dIII, which span the area of deletion.

For pWS28, pWS25 was cleaved with *Pst*I and *Hin*dIII, treated with mung bean nuclease, and recircularized as described above for pWS26.

For pWS35, pJH101 was cleaved with *Cla*I and *Hin*dIII and ligated to the 827-base-pair (bp) *Cla*I-*Hin*dIII fragment of pWS10, resulting in pWS33. pWS33 was cleaved with *Cla*I and *Eco*RI and ligated to an approximately 0.8-kb fragment of pLS23-17.

For pWS37, pWS27 was linearized with *Hin*dIII, the ends were filled in with the Klenow fragment of DNA polymerase I (12), and it was recircularized with T4 DNA ligase. All new plasmids were checked to establish that their structures were as expected.

Construction of bacterial strains. Competent cells of GSY1127 were prepared by the method of Wilson and Bott (22) and mixed with the relevant linearized plasmid DNA at a concentration of 2 to 10 μg/ml. After incubation at 37°C for 30 to 60 min, the mixture was diluted 10-fold in PenAssay broth and incubated at 37°C with aeration for 3 to 4 h before plating onto tryptose blood agar base (TBAB) with 5 μg of chloramphenicol per ml.

Bacterial and plasmid DNA extractions. Bacterial DNA was prepared as described previously (21) from resting-phase cultures grown in GM11 medium (11) lacking isoleucine and valine and supplemented with histidine (100 μg/ml), tryptophan (50 μg/ml), and thymine (20 μg/ml). *Bam*HI osmolysate DNA was prepared from mid-exponential-phase growing cells as described previously (see the legend to Fig. 2 in reference 5).

Plasmid DNA was prepared by the alkaline lysis method (10), followed by CsCl-ethidium bromide density gradient purification.

Southern transfer and probing. Gel electrophoresis, electrotransfer of DNA to nylon membranes, and hybridization with ^{32}P nick-translated DNA were done as described previously (19) by the dot blot hybridization method, except that the posthybridization washes were carried out at 65°C with the three final stringency washes in 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate.

S1 nuclease treatment of *Bam*HI osmolysate DNA. A 400-μl amount of *Bam*HI osmolysate DNA was precipitated with ethanol and dissolved in the same volume of TE (10 mM Tris hydrochloride [pH 8.0], 1 mM EDTA). The DNA was deproteinized, reprecipitated, and dissolved in 200 μl of 50 mM sodium acetate (pH 4.5)–200 mM NaCl–1 mM $ZnCl_2$–0.5% glycerol. To 20-μl samples was added 0.1 to 2.0 U of S1 nuclease, and digestion occurred at 37°C for 15 min.

Segregation analysis for merodiploid duplication in GSY1127 and derivatives. GSY1127 has a spontaneous low-level loss of the merodiploid duplication under nonselective conditions, resulting in an Ilv^- phenotype. To affirm that SU164 and SU171 were merodiploid derivatives of GSY1127, the percentage of Ilv^- segregants after growth under nonselective conditions was compared with that of GSY1127 as follows. All three strains were grown overnight in selective medium (GM11 lacking isoleucine and valine) at 37°C. Cultures were diluted 50-fold in PenAssay broth and incubated at 37°C with aeration for 4 h, when a further 50-fold dilution was made in the same medium. After further incubation under the same conditions for 2.5 h, glycerol was added to 15% (vol/vol), and the culture was frozen and stored at −80°C. Cells were thawed and plated on TBAB. One thousand colonies of each strain were patched onto TBAB and then replica plated onto minimal medium either containing isoleucine and valine (both at 50 μg/ml) or lacking them. The percentages of Ilv^- segregants were: GSY1127, 0.7%; SU164, 4.4%; and SU171, 1.4%.

DNA sequencing. Dideoxy sequencing was performed on double-stranded plasmid DNA according to the Stratagene protocol. Two sequencing primers were used to obtain the DNA sequence in the region of the deletion endpoints in pWS27 and pWS29. For pWS27 sequencing, a 25-mer complementary to positions 721 to 745 in the *B. subtilis terC* sequence (2) was used. For pWS29 sequencing, the pBR322 *Pst*I site clockwise sequencing primer (17) was used.

RESULTS

Deletion from the left towards *terC*. The upper half of Fig. 1 includes a 30-kb portion of the terminus region restriction map of *B. subtilis* 168. *terC*, identified with a solid arrowhead, was located within a 2.0-kb *Pst*I-*Eco*RI segment (hatched) and about 100 bp to the left of the internal *Hin*dIII site. The clockwise replication fork enters this region of the chromosome from the right and is arrested at *terC*. After agarose gel electrophoresis of *Bam*HI-cleaved DNA from exponentially growing cells, the forked termination intermediate migrates more slowly than its 24.8-kb linear counterpart (21). This species, called band I, is more readily detected in DNA from the merodiploid strain GSY1127, which has the same terminus region structure as the 168 strain and carries a large nontandem duplication of the chromosome, so that *terC* is grossly asymmetrically located in relation to *oriC*. In the present approach to establishing the sequence requirements for fork arrest, the effect of deleting portions of the *terC* region sequence from GSY1127 on the appearance of band I was investigated. The absence of band I has been interpreted as loss of the arrest function of *terC*. This overall approach is possible because it has been established that *terC* can be deleted from the *B. subtilis* chromosome without any obvious effect on cell growth and division (9). While such *terC*-deleted strains might use a secondary terminus (under investigation), the clockwise fork would approach the altered region in the newly constructed derivatives of GSY1127 in the normal manner.

Deletion of portions of the sequence to the left or right of *terC* was achieved by transformation of GSY1127 with linearized plasmid DNA containing a Cm^r gene flanked by appropriate *B. subtilis* sequences. The deleted portion of the chromosome was replaced by vector DNA. In the overall experimental approach followed, a number (two to four) of separate colonies from among the Cm^r transformants of GSY1127 in a single experiment were used for subsequent growth of larger cultures and DNA extraction by the *Bam*HI osmolysate procedure (5). It is possible that, in some experiments which yielded low numbers of transformants, some colonies chosen were segregants derived from a common transformant, but in every case the DNA preparations would have originated from at least two independent transformant colonies, and they are referred to here by the same strain number. One reason for using this approach was the possibility of loss of the chromosomal duplication in GSY1127, which can occur at a significant frequency (15). Such a loss would make detection of band I difficult. It will be seen that in the DNA preparations (*Bam*HI osmolysates) from a number of colonies in a single transformation experiment, band I was either present in all cases or absent in all cases. The variability in band I level, when band I was detectable, was not investigated further. It could reflect loss of the

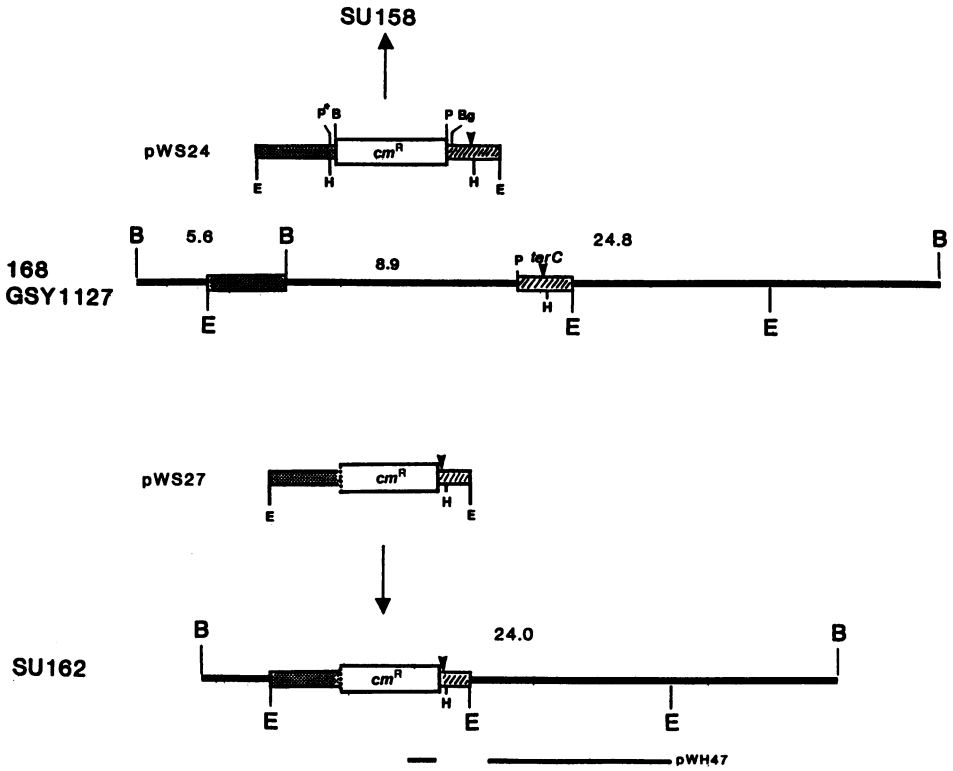

FIG. 1. Strategy for deleting portions of the *B. subtilis* chromosome from the left towards *terC*. The upper portion of the diagram shows a restriction map of the 30-kb region of the wild-type strain 168 (and merodiploid GSY1127) chromosome spanning *terC* (solid arrowhead). Plasmids were constructed to contain cloned portions (stippled and hatched boxes) which would, in the linearized form of the plasmids, facilitate recombination with the chromosome, exchanging the intervening chromosomal sequences for vector sequences (open box containing Cm^r gene). Insertion of pWS24 into the GSY1127 chromosome yielded SU158. Insertion of pWS27 into the GSY1127 chromosome yielded SU162. pWS27 is shown with a dotted line at the left end of the vector moiety to indicate that this vector-insert junction was not precisely defined (see Materials and Methods). pWH47 was used as a hybridization probe for the detection of fork arrest at *terC*, and the homology with the SU162 chromosome is indicated. All restriction sites of a particular enzyme are shown for the plasmid DNAs. All *Bam*HI and *Eco*RI sites are shown for the chromosomal DNA maps; other enzyme sites (smaller letters) define some particular sites only. B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; P, *Pst*I. Sizes are in kilobases; 5.6, 24.0, and 24.8 refer to *Bam*HI fragments; the 8.9-kb fragment is bounded by *Bam*HI and *Pst*I sites.

GSY1127 chromosomal duplication from a transformant or differences in growth rate of the cultures, stage of growth, efficiency of extraction of DNA in the osmolysate procedure used, or extent of breakdown of band I. When band I was not detectable in a series of DNA preparations, one of the transformant colonies used, in crucial cases only, was examined by segregation analysis to establish that the chromosomal duplication had not been lost.

The first deletion from the left towards *terC* was achieved with linearized pWS24 (top section of Fig. 1). Integration into the GSY1127 chromosome yielded SU158, in which the 8.9-kb chromosomal segment immediately to the left of the chromosomal *Pst*I site was replaced by vector DNA so that *terC* was now contained within a *Bam*HI segment of 20.2 kb. In this case, as well as in all others to be presented below, the expected chromosomal structure was established by direct analysis of all DNA preparations (data not shown). *Bam*HI-cleaved DNA from exponentially growing cultures prepared from two separate transformant colonies of SU158 showed a band migrating more slowly than the linear 20.2-kb *terC*-containing fragment (Fig. 2A). That this band represented a forked molecule of the expected dimensions of a band I equivalent, reflecting the arrest of the clockwise fork at *terC*, was confirmed by testing one of the DNA preparations for its sensitivity to S1 nuclease. S1 nuclease caused rapid destruction of the putative band I species, with release of one of the expected 15.4-kb arms (5). (In all cases of deletion at the left of *terC*, where a putative band I species was observed, its sensitivity to S1 nuclease was established for at least one of the DNA preparations, but only the data for the more significant SU162 strain are shown; see below.) It is clear that in SU158 arrest of the clockwise fork at *terC* is still effected, and this establishes that sequences to the left of the proximal *Pst*I site, which is 0.8 kb away, are not required for the arrest function of *terC*.

To gain more precise information on the amount of sequence to the left of *terC* needed for fork arrest, derivatives of pWS24 which carried increasingly larger deletions from this region were used for integration into GSY1127. To construct the new plasmids, the second *Pst*I site (asterisk) in pWS24 and its adjacent *Bam*HI site (Fig. 1) were first removed to yield pWS25. Unidirectional nuclease digestion was then applied to produce a series of plasmids in which the deletion of *B. subtilis* DNA extending from the remaining *Pst*I site in the direction of *terC* ranged from 430 to 780 bp (scale shown in Fig. 3). Another plasmid, pWS28, was deleted for the 895-bp *Pst*I-*Hin*dIII region in a separate construction. Integration of these plasmids (linearized) into GSY1127 yielded the strains SU161 to SU165 (see bottom section of Fig. 1 for integration resulting in SU162). For each

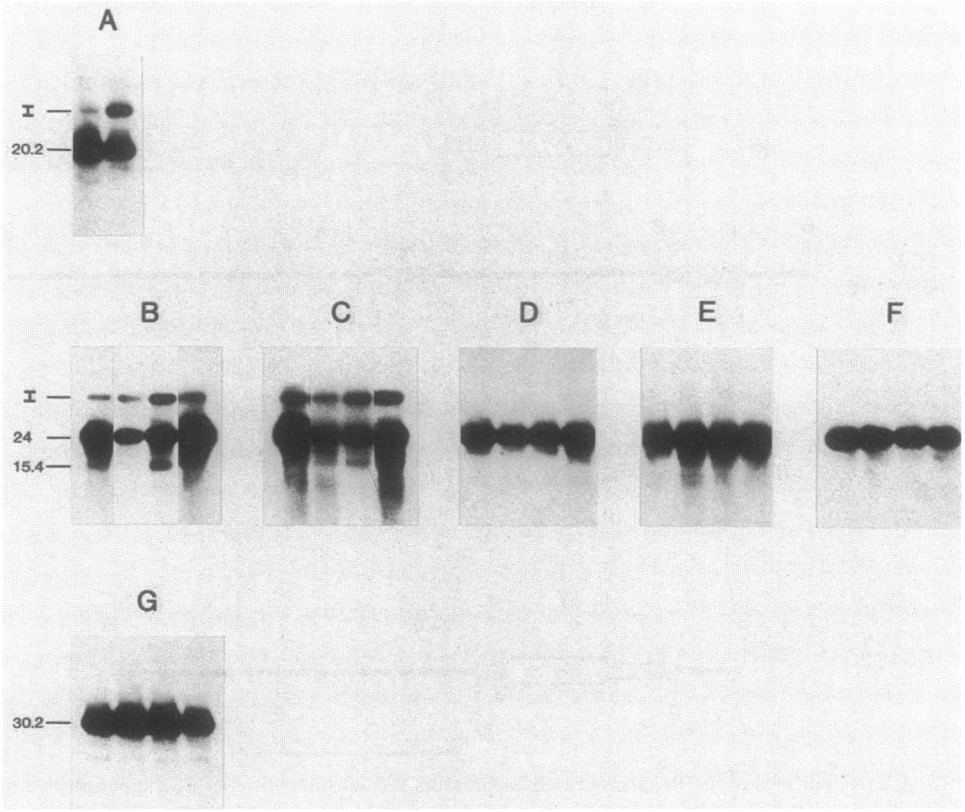

FIG. 2. Detection of the presence or absence of a forked termination intermediate (band I) in DNA from exponentially growing cultures of various GSY1127 derivatives. In each case, two to four colonies from the given transformation experiment were grown to mid-exponential phase, and *Bam*HI osmolysates were prepared. These DNAs were electrophoresed, transferred to nylon membranes, and probed with ^{32}P-labeled pWH47 (see Fig. 1 for pWH47 homology). (A) SU158; (B) SU161; (C) SU162; (D) SU164; (E) SU165; (F) SU163; (G) SU171. Sizes are in kilobases and refer to the linear species probed; the size of the 24-kb species in panels B to F varied slightly.

integration, four Cm^r transformants colonies were used for DNA extractions and analysis. The structures of the chromosomal regions in the vicinity of *terC* of the various strains are shown in the lower portion of Fig. 3. The precise endpoints of the rightward deletions in SU162 and SU164 (up to positions 582 and 712 bp, respectively, of the sequenced region) were established by sequencing the relevant regions of the plasmids used in their construction. The rightward deletion endpoints shown for SU161 and SU165 are only approximate and are based on the sizes of restriction fragments derived from the relevant plasmids. The rightward deletion of SU163, obtained with pWS28, was shown to extend past *terC* to the *Hin*dIII site. The precise location of the endpoint of the leftward deletion in SU161 to SU165 is unknown, but it is the same for all.

Figure 2B through F shows the results of analysis of DNA from exponentially growing cultures of SU161, -162, -164, -165, and -163 for the band I species. All lanes (DNA preparations) in panels B and C (SU161 and SU162) showed band I. The band that sometimes appeared below the 24-kb species at the 15.4-kb position represents the band I breakdown product, band II. (Note that the size of the 24-kb species varied slightly between the individual constructs). Figure 4 shows directly that the slowly migrating species in SU162 identified as band I was preferentially sensitive to S1 nuclease, as expected for a forked molecule. It broke down to yield the faster-migrating 15.4-kb species. All lanes in panels D, E, and F (SU164, -165, and -163) were missing band I (even after much longer exposures; not shown). One of the SU164 transformants was examined by segregation analysis to establish that it had not lost the merodiploid duplication.

These results established the loss of fork arrest ability when the chromosomal deletion towards *terC* extended beyond that in SU162, i.e., to that in SU164. The extra 130 bp in SU162 (Fig. 3) must contain a sequence essential for clockwise fork arrest. This segment encompasses the first inverted repeat, IR1. It also contains a potential promoter which could be used for transcription of the ORF (see below).

Deletion from the right towards *terC*. Clockwise replication fork arrest occurs to the left of the *Hin*dIII site shown in the map at the top of Fig. 3; the newly replicated strands in the arms of the fork extend at most to the region designated *terC*, although it has not been ruled out that some forks, while passing through the *Hin*dIII site, might stop short of this region. It was of significance to examine the effect of deleting DNA in the direction approaching *terC* from the right and up to this *Hin*dIII site. This would eliminate most of the ORF, which is read rightwards, and leave the whole inverted repeat region intact. For this purpose, plasmid pWS35 was constructed. It is shown in linearized form in Fig. 5, and integration into GSY1127 yielded SU171, whose *terC* region structure is shown. Figure 2G shows the result of examining DNA from exponentially growing cultures of four SU171 transformant colonies for a band I species. There was

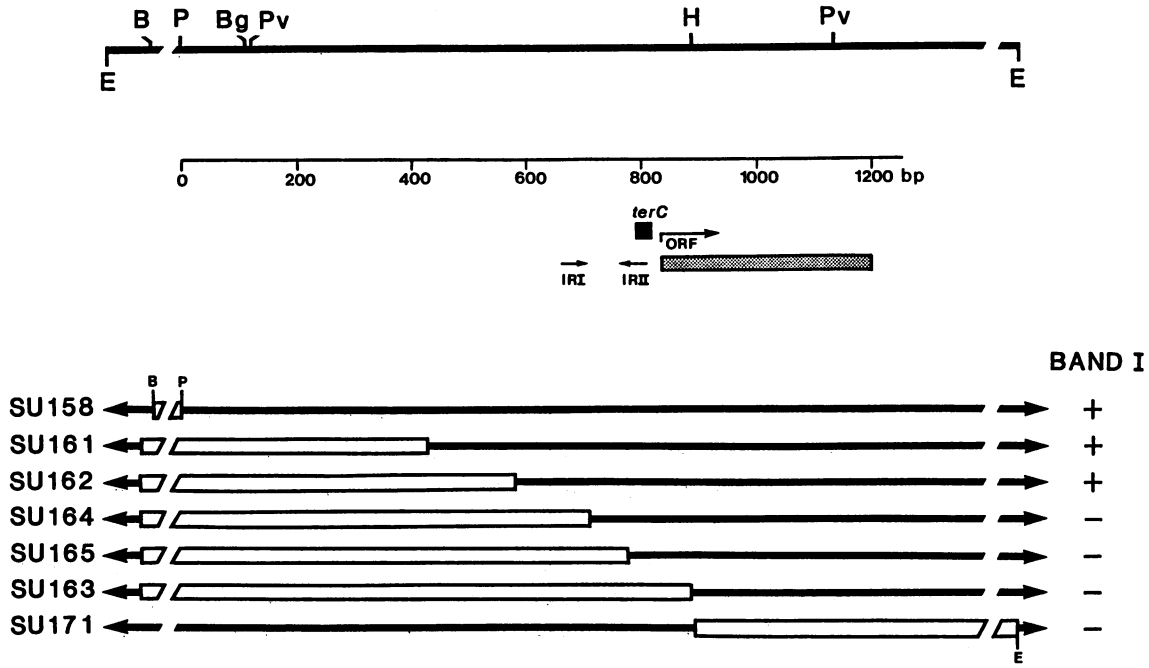

FIG. 3. Summary of chromosomal deletions approaching *terC* from either direction and their effect on arrest of the clockwise fork. The unbroken portion of the restriction map at the top covers the *terC*-containing 1,267-bp sequenced segment (2) extending rightward from the *Pst*I site adjacent to the hatched box in the wild-type 168 (and GSY1127) chromosomal segment of Fig. 1. Sequence features of this segment are shown immediately below this map. The clockwise fork, which enters from the right, is arrested at (or just before) it reaches the region defined by the solid square (labeled *terC*). IRI and IRII represent 47 to 48-bp imperfect inverted repeats which lie just upstream of an ORF, identified as a stippled box. The lower half of the figure shows the extent of the chromosomal deletions (open boxes) in various *B. subtilis* strains and the effect of these on arrest of the clockwise fork (band I present or absent). The right-hand endpoints of the deletions which approach *terC* from the left (SU158, -161, -162, -164, -165, and -163) range from the *Pst*I to the *Hin*dIII site; the endpoints in all except SU161 and -165 are known at the single-nucleotide level; those in SU161 and -165 are shown to within ±15 bp. The left-hand endpoint of the deletions in SU161, -162, -164, -165, and -163 is the same in all cases and lies approximately 0.5 kb to the left of the *Bam*HI site shown in SU158. The deletion approaching *terC* from the right (SU171) extends to the *Hin*dIII site from the *Eco*RI site 1 kb away. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II.

no evidence of a band migrating more slowly than the linear 30.2-kb fragment. It should be noted that had fork arrest occurred at *terC* in SU171, the arms of the *Bam*HI-derived molecule would have been approximately 20 kb and a forked structure of the dimensions expected would have been well resolved from its linear counterpart. One of the transformant colonies used for DNA analysis in panel G was examined to establish that it had not lost the chromosomal duplication characteristic of GSY1127.

The absence of band I from SU171 DNA raises the possibility that the putative protein product of the ORF is needed for clockwise fork arrest at *terC*.

Disruption of the ORF. The plasmid pWS27 (Fig. 1) has already been shown to contain sufficient sequence to the left of *terC* to effect clockwise fork arrest when incorporated into the GSY1127 chromosome. It also contains the *Hin*dIII site of the ORF as a unique site. This site was cut, the ends were filled by use of the Klenow enzyme, and the DNA was recircularized to yield pWS37. The presence of the expected additional four nucleotides at the altered *Hin*dIII site was established by sequencing this region. pWS37 provided the opportunity to disrupt the ORF in GSY1127 and to establish the effect of this on clockwise fork arrest.

Insertion of *Eco*RI-linearized pWS37 into the GSY1127 chromosome and selection for Cm^r colonies should yield two alternate chromosomal structures in the vicinity of *terC*. The difference in the structures will reflect on whether the altered *Hin*dIII site, H^0, is incorporated into the chromosome fol-

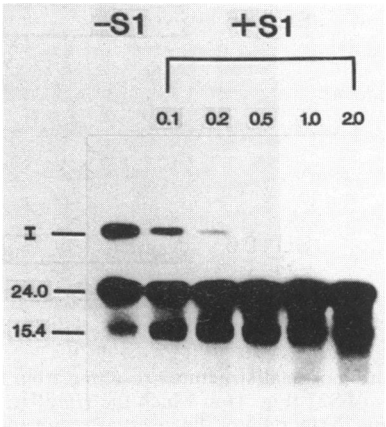

FIG. 4. S1 nuclease sensitivity of the putative band I species in DNA from strain SU162. For this experiment, the preparation corresponding to the third lane in panel C of Fig. 2 was used. The left lane shows untreated DNA. The other lanes show the effect of increasing levels of S1 (0.1 to 2.0 U; see Materials and Methods) in removing the slowly migrating band I and producing the expected 15.4-kb (band II) species.

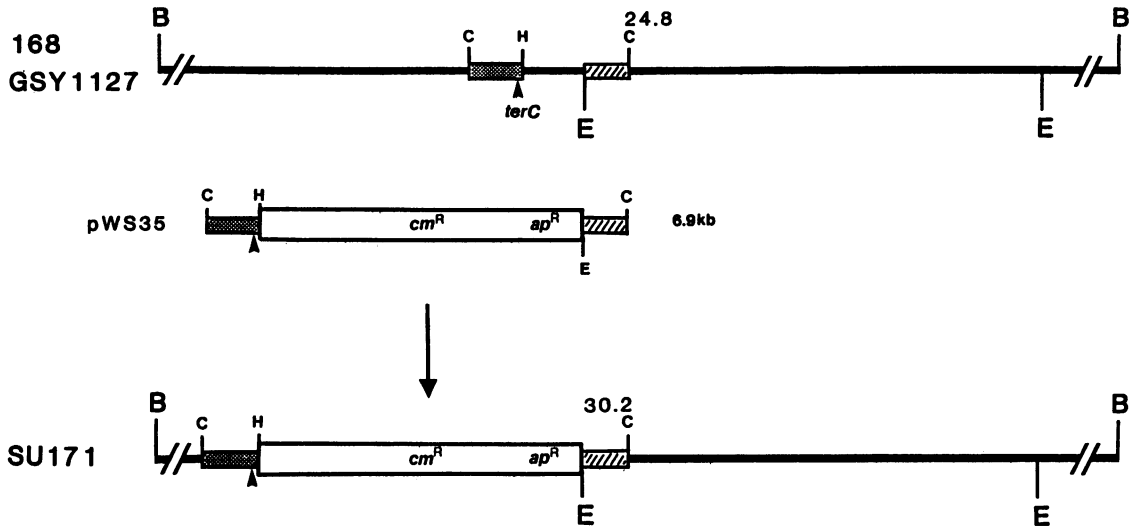

FIG. 5. Strategy for deleting a portion of the *B. subtilis* chromosome from the right towards *terC*. The restriction map for the 24.8-kb *terC*-containing segment of the wild-type 168 (and GSY1127) chromosome is shown and highlights the regions (stippled and hatched boxes) which are homologous to the ends of linearized pWS35. Insertion of pWS35 into GSY1127 yields SU171. All *Bam*HI and *Eco*RI sites in the chromosomal map are shown; the smaller letters in these maps define just some sites for a particular enzyme. All sites of a particular enzyme in the plasmid are shown. B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII. Sizes are in kilobases.

lowing crossover within the segment of DNA to the right of the vector in the linearized plasmid (Fig. 6). In SU185, crossover in this region occurred to the left of H^0; SU185 is thus identical to SU162. (The presence or absence of the *Hin*dIII site was established by hybridization analysis of appropriate digests.) In SU186, crossover was to the right of H^0 so that it was incorporated into the bacterial chromosome. The alteration in amino acid sequence of the protein encoded by the ORF resulting from this incorporation is shown (Fig. 6). The new sequence changed markedly after residue 21 (asterisk) of the original 122-amino-acid sequence. Furthermore, a stop codon was encountered after only 39 amino acids in the new sequence. DNA from two independent transformant colonies each of SU185 and SU186 was examined for the presence of band I (Fig. 7). As expected, SU185 DNA contained the band I species, but it was absent

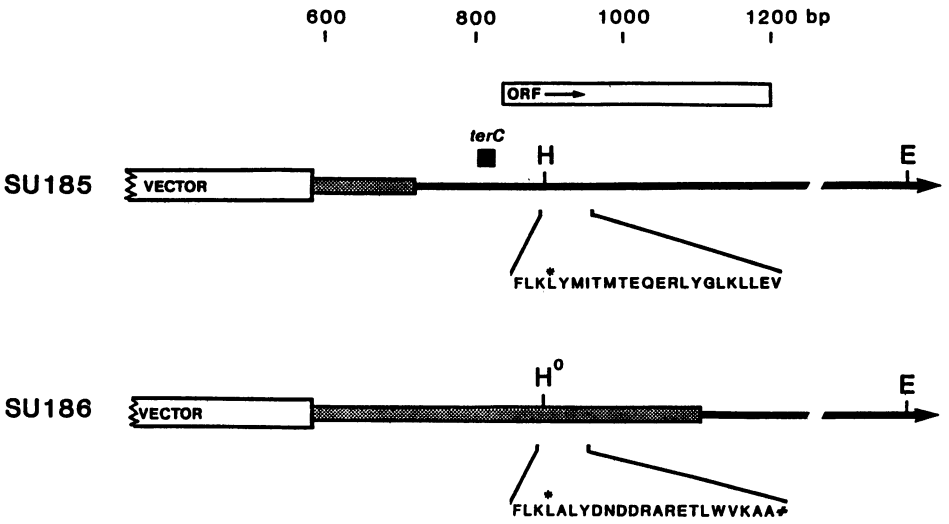

FIG. 6. Chromosomal structures resulting from the insertion of *Eco*RI-linearized pWS37 into the GSY1127 chromosome. pWS37 is a derivative of pWS27 (Fig. 1) in which the *Hin*dIII site has been altered (see text). For the new strains, only portions of the chromosomal structures lying to the right of the inserted vector (open box) and spanning the *terC* site and its associated ORF are shown. The scale at the top is the same as in Fig. 5. SU185 resulted from recombination between the linearized plasmid and the bacterial chromosome to the left of the altered *Hin*dIII site (H^0) in the plasmid, so that it was not incorporated into the chromosome. In SU186 the region of recombination was to the right of the H^0 site in the linearized plasmid, resulting in incorporation of the H^0 site into the chromosome. The stippled boxes represent integrated *B. subtilis* DNA from the plasmid, but the precise endpoints are not known. Incorporation of the H^0 site causes an altered reading frame within the ORF to produce the new amino acid sequence shown. Only portions of the amino acid sequences for the two strains are shown; the sequences start at residue 18 and begin to differ after residue 21, which is marked with an asterisk. H, *Hin*dIII; H^0, altered *Hin*dIII site; E, *Eco*RI; #, stop codon.

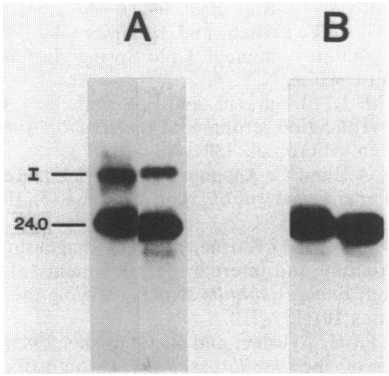

FIG. 7. Analysis of *Bam*HI osmolysate DNA from exponentially growing SU185 and SU186 for the presence or absence of the forked termination intermediate, band I. The DNA preparations in panels A (SU185) and B (SU186) were from independent transformants obtained in the experiment performed to construct the strains. See legend to Fig. 2 for details.

from SU186 DNA. Thus, disruption of the ORF abolished clockwise fork arrest.

DISCUSSION

The experiments described here, done to obtain information on the sequence requirements for *terC*, used the bacterial chromosome itself, rather than a *terC*-containing plasmid, in the assay for fork arrest. This eliminates any ambiguities that could arise from the use of plasmids for this purpose. Within the DNA sequence of the chromosomal region spanning *terC* in *B. subtilis*, the junction of the daughter arms and unreplicated DNA in the arrested clockwise fork occurs very close to one of two imperfect inverted repeats (2). These inverted repeats, IRI and IRII (Fig. 3), were the most notable feature of the *terC* region sequence. That they lay at the leading edge of the arrested fork suggested a possible role for them in the arrest mechanism. This could involve secondary structure formation within the separated template strands. Alternatively, the binding of a protein, or other factor, to the inverted repeats or some other sequence in the region might be responsible for blocking fork movement. It is unlikely that the sequence through which the clockwise fork had already passed would provide a template structural feature for arrest. The deletion experiments aimed at establishing the sequence requirement ahead of *terC* (summarized in Fig. 3) show that it is, at the most, about 250 bp. This is the distance between the endpoint of the rightward deletion in SU162 and *terC*. Deletion of a further 130 bp (in SU164), which encompasses IRI, abolished arrest. This finding could suggest a direct role for IRI in blocking movement of the clockwise fork.

If IRI, either alone or in conjunction with IRII or some other sequence in the 250-bp region, were sufficient to cause arrest, deletion of sequences from the right (Fig. 3) which left the IRI-IRII region intact would have had no effect on arrest. The properties of SU171, in which the deletion (up to the *Hin*dIII site) extended to within only 80 bp of IRII, showed that this was not the case. Thus, the 250-bp sequence ahead of the arrested fork is not sufficient for arrest, and it cannot be explained solely by a sequence-dependent secondary structure that forms in the separated template strands in the IRI-IRII region; something else is required. The *Hin*dIII site resides within the sequence defining the adjoining ORF and <20% of the overall distance from its initiation codon. The absence of clockwise fork arrest in SU171 raised the possibility that the putative protein product of the ORF had a direct role in blocking the fork. The insertion, in SU186, of an additional 4 bp at the *Hin*dIII site, causing a minimal change to the overall sequence but severely disrupting the ORF, abolished fork arrest. While there is no available experimental evidence for expression of the ORF, its sequence features (potential promoters, ribosome-binding site, and transcription terminator) indicate that it is expressed (2). The present findings also point to its expression and strongly suggest that the protein product is the other factor that has a role in fork arrest at *terC*.

The overall organization of the ORF, potential promoter sites for its transcription, and the inverted repeat region in relation to *terC* raise an interesting possibility which would point to a relatively sophisticated mechanism of fork arrest. Two potential promoter sites for transcription through the ORF are located just upstream of or overlapping IRI (−10 regions centered at positions 653 and 717). The protein product of the ORF might bind to the inverted repeat region. In doing so, it could function to block movement of the clockwise fork as well as its own synthesis. Such an arrangement would enable replication-dependent control of expression of the ORF. Transcription through the ORF would only proceed when the approaching anticlockwise fork fused with the stationary fork to complete termination and remove the bound protein (if this had not occurred earlier). Synthesis of the protein and its binding to the inverted repeat region would then shut down further synthesis. This would be a unique mechanism for cell cycle control of gene expression. Very recently, convincing evidence for the existence of a *trans*-acting factor, possibly a protein, in the termination of replication in *E. coli* has been described (7). It was shown that deletion of the T2 termination site inactivated T1. Supplying the T2 region, as a 4-kb segment in a plasmid, restored T1. The cloned 4-kb segment was considered to encode both a *cis*-acting (T2) and a *trans*-acting (*tus*) function. Perhaps the ORF implicated in fork arrest in *B. subtilis* is analogous to *tus*.

More detailed analysis and manipulation of the *terC* region of *B. subtilis* are needed to clarify the roles of the inverted repeat region and the adjoining ORF in fork arrest and the overall termination process. It is possible, for example, that deletion of IRI abolished fork arrest solely because it also destroyed the promoter for transcription of the ORF. Further work will be facilitated by the recent demonstration that the 1.75-kb *Eco*RI-*Bgl*II *terC*-containing segment of DNA (Fig. 1) functions as a terminus when relocated at another site and between new flanking sequences in the chromosome (7a). This 1.75-kb segment of DNA encompasses both the inverted repeats and the ORF. It is of particular significance to know whether just the 600-bp region encompassing these features is also functional.

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