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Search for Additional Replication Terminators in the *Bacillus subtilis* 168 Chromosome

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The *Bacillus subtilis* 168 chromosome is known to contain at least six DNA replication terminators in the terminus region of the chromosome. By using a degenerate DNA probe for the consensus terminator sequence and low-stringency hybridization conditions, several additional minor hybridizing bands were identified. DNA corresponding to the most intense of these bands was cloned and characterized. Although localized in the terminus region, it could not bind RTP and possibly represents a degenerate terminator. A search of the SubtiList database identified an additional terminator sequence in the terminus region, near *glnA*. It was shown to bind RTP and to function in blocking replication fork movement in a polar manner. Its orientation conformed to the replication fork trap arrangement of the other terminators. The low-stringency hybridization experiments failed to identify any terminus region-type terminators in the region of the chromosome where postinitiation control sequences (STer sites) are known to reside. The two most likely terminators in STer site regions, in terms of sequence similarity to terminus region terminators, were identified through sequence searching. They were synthesized and were found not to bind RTP under conditions that allowed binding to terminus region terminators. Neither did they elicit fork arrest, when present in a plasmid, under stringent conditions. It is concluded that the STer site terminators, at least the first two to the left of *oriC*, do not have the typical consensus A+B site makeup of terminus region terminators.

Escherichia coli and *Bacillus subtilis* both have a system for arresting a replication fork within the terminus region of their circular chromosomes (6). In each case, fork arrest requires interaction between a specific terminator protein (Tus in *E. coli*, RTP in *B. subtilis*) and a short DNA sequence known as a terminator. The terminator protein-DNA terminator complexes are polar in their action (i.e., they arrest a replication fork approaching from one particular direction only). Tus and RTP show no recognizable sequence similarity to each other, and neither do their cognate terminators. Tus functions as a monomer and interacts with a terminator sequence of 22 to 23 bp. The interaction that takes place between RTP and its terminator is more complex. A dimer of RTP binds to each of two adjacent and opposed sites (A and B) which make up a minimal terminator of 29 bp (Fig. 1, top section). This sequence encompasses the segment running from nucleotides (nt) −2 to 27 of the originally identified terminators (3). The 16-bp A and B sites each comprise a trinucleotide (common to both sites [boxed in Fig. 1]) plus a 13-bp sequence which is different between the two sites. Six terminators (*TerI* to *TerVI*) have so far been identified in *B. subtilis* 168, and two terminators have been identified in *B. subtilis* W23 (5) (the consensus is shown in Fig. 1). The consensus B site (nt 12 to 27) possesses a detectable level of symmetry about nt 21 (note arrows under G15, A17 and T25, C27 in Fig. 1) within its 13-nt outer portion. This symmetry presumably contributes to the high affinity of the B site (in contrast to the A site) for the symmetrical RTP dimer (17). (The B site alone, but not the A site alone, will bind RTP tightly.) Once a dimer has bound to the B site of the complete terminator, a second dimer binds cooperatively to the A site (nt −2 to 14). The A site shows little symmetry within itself, but the central nucleotide within its outer 13-nt portion is one of the few positions conserved (at nt 5) in all chromosomal terminators. *B. subtilis* terminators function only when the B site is proximal to an approaching replication fork, and it is likely that the sequence differences between the A and B binding sites are crucial to the polarity of action of such terminators (9, 17).

The arrangements of the multiple DNA terminators in *E. coli* and *B. subtilis* are similar in that they are dispersed over the terminus region as two opposed sets, each set orientated to arrest a replication fork moving from one direction only (4, 7). In *E. coli*, they are spread over 25% of the chromosome, while in *B. subtilis* they span a smaller region of 10% of the chromosome (Fig. 2). This arrangement generates a one-way passage to a replication fork entering the terminus region so that it can enter this region but cannot leave (8). Presumably the extra terminators provide backups to ensure that the approaching forks always meet within the region spanned by the terminators. All such terminators but one in *E. coli* and all in *B. subtilis* are located outside coding regions, suggesting that this may be an important feature in their functioning.

RTP is not only involved in fork arrest in the terminus region of the *B. subtilis* chromosome. Levine et al. (10) have shown that RTP has a role in the arrest of replication forks, at sites not far removed from the origin, under conditions that induce the stringent response. They have shown that a number (about five) of postinitiation control sequences (called STer sites) are positioned within 200 kb on either side of the origin. Under stringent conditions, these sequences function, in conjunction with RTP, to block replication fork movement. STer sites could be the same as terminus region terminators that are rendered inactive during normal growth (14), or they could just resemble such terminators in their makeup and require additional stringent response factors in order to bind RTP and become functional.

Originally, two DNA terminators were identified in each of *B. subtilis* strains 168 and W23 (3, 12). Subsequently, four additional terminators in the 168 strain were detected by probing chromosomal DNA with a degenerate oligonucleotide sim-

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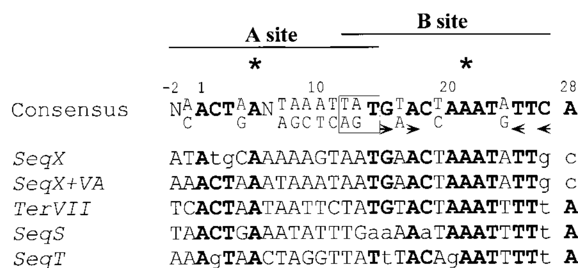

FIG. 1. Sequences of terminators and potential terminators. The minimal terminus region terminator consists of two overlapping (by 3 nt) RTP binding sites, A and B, as depicted at the top. Immediately beneath is the consensus for the six known chromosomal terminators of *B. subtilis* 168 plus the two terminators in the W23 strain; the consensus extends from nt −2 to 27. The positions in boldface are conserved in all terminators. Where the nucleotides at these positions are different, they are shown in lowercase. The asterisks are positioned over the centers of the 13-nt segments flanking the central trinucleotide, which is boxed. The arrows define the symmetry around the asterisked position in the B site. The nt 28 position is indicated for completeness, because it is conserved in all functional terminators and could be considered part of the terminator (17).

ilar in sequence to the consensus B site of those already identified (5). While a total of seven terminators have now been identified in *E. coli* (7), the earlier work of François et al. (4) has raised the possibility that there could be more than seven. This raises the question of are there more than six terminators in *B. subtilis* 168?

In the present work, we have addressed this question through an extension of the approach used previously (5), employing less-stringent hybridization conditions and a slight change in the sequence of the probe used. It will be shown that the most likely candidate terminator sequence detected in this way occurs within the terminus region but does not bind RTP, probably because of a defective A site. However, a search of the current *B. subtilis* database has uncovered a new functional, but relatively weak terminator in the terminus region. Its differences from the consensus shown in Fig. 1 raised the possibility that the earlier lack of detection of STer sites by hybridization (5) could have been due to additional sequence deviations from the terminus region terminators. A search of available sequences spanning the STer sites was carried out. The two most likely candidate STer sites, in terms of sequence similarity to the consensus terminus region terminator, could not bind RTP.

Detection of additional DNA terminator-like sequences on the chromosome of *B. subtilis* 168. The degenerate probe used in the present work was the 17-mer $^{T}/_{A}ATG^{T}/_{A}AC^{T}/_{C}AAA$ $T^{A}/_{G}TTCA$, which covers the nt 12 to 27 B site region of the consensus terminator, plus the additional nucleotide A, which is conserved at the nt 28 position in all chromosomal terminators (Fig. 1) but not in the *B. subtilis* plasmid pLS20 terminator (13). The probe differs from that used previously in that the first two positions have been altered to conform to the consensus of just the six *B. subtilis* 168 chromosomal terminators, the strain W23 sequences being ignored. Southern hybridizations to digests (*Eco*RI and *Bam*HI/*Sal*I) of *B. subtilis* 168 DNA were performed as described before (5), except the temperature of the TMAC (tetramethylammonium chloride) washes was lowered from 52 to 54°C to 46°C, which would permit the detection of sequences containing up to five mismatches to the 17-mer probe (21). The result of such an experiment is shown in Fig. 3B. Additional fragments (arrowed, with sizes shown) to those originally seen under the earlier conditions (Fig. 3A) are visible, suggesting that more B site-like sequences may be present in the chromosome. The most intensely hybridizing new band, a *Bam*HI/*Sal*I fragment of 9.1 kb, was cloned into pGEM3Zf(+) to give pAG6, and then an *Xba*I/*Sal*I 1.1-kb subfragment of the insert containing the hybridizing sequence was cloned into the same vector to give pAG7. The smaller fragment was sequenced (GenBank accession no. U68731), and a sequence that resembled a DNA terminator was identified (*SeqX* [Fig. 1]). Through hybridization to a YAC library (2, 5), this sequence was found to reside within the terminus region, close to *TerV* (data not shown). Like the previously identified terminators, *SeqX* did not reside in a coding region. Comparison with the consensus (Fig. 1) shows three of the fully conserved nucleotides to be different, two of the differences being in the A site portion. A *SeqX*-containing fragment (double-stranded synthetic oligonucleotide) was tested for RTP binding. The *SeqX* sequence did not bind even one dimer of RTP (Fig. 4A) despite its being almost identical to the consensus terminator over the nt 12 to 27 B site region (one mismatch). Note that, in contrast to *TerI* (center panel), neither of the more slowly moving bands (I or II) appears when more RTP is added. It was previously shown that deletion of the nt 25 to 27 segment from the minimal *TerI* terminator of *B. subtilis* 168 still afforded binding of two dimers of RTP and a significant level of in vivo fork arrest activity (16). Possibly *SeqX* could not bind RTP because of a defective A site. The nt −2 to 11 segment of *SeqX* was therefore replaced by the equivalent region of the *TerV* terminator (because it most closely resembles *SeqX* over the B site) to give *SeqX+VA* (Fig. 1), and this was cloned into pWS64-1 (18) to give pAG13. Figure 4A shows that a fragment containing this new sequence could bind two dimers of RTP as evidenced by the appearance

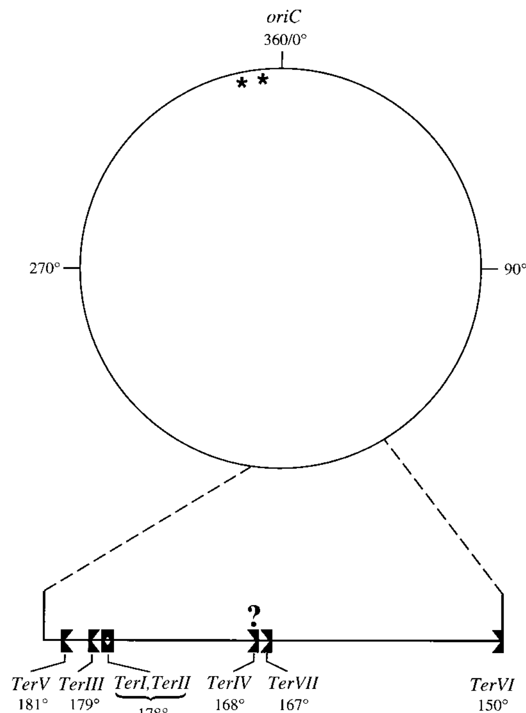

FIG. 2. The chromosome of *B. subtilis* 168 showing the positions of the six identified terminators (*TerI* to *TerVI*) and the additional terminator (*TerVII*) detected in the present work. Positions (in degrees) are in accordance with the map in reference (1). *TerI*, *TerIII*, and *TerV* block a fork progressing leftwards; the other terminators block a fork progressing rightwards. The orientation of *TerIV* (?) is not known. The asterisks to the left of *oriC* represent STer sites which reside in the sequence of the SubtiList database contig 348-1 (15).

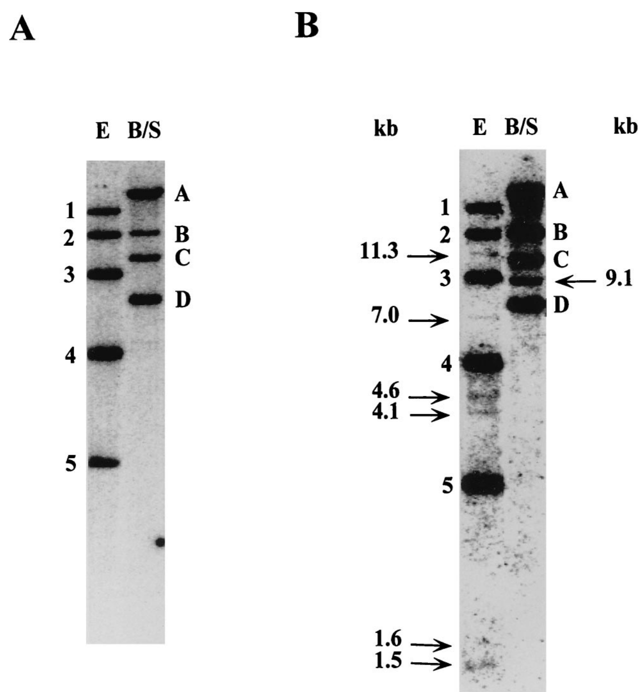

FIG. 3. Phosphorimages of Southern hybridizations of digests of *B. subtilis* 168 (SB19) DNA with a ^{32}P-labelled 17-mer B site probe (see text for probe sequence). The experiment was performed as described previously (5). In panel A, the tetramethylammonium chloride posthybridization washes (20) were at 52°C; in panel B, the wash temperature was reduced to 46°C. In each panel, the left lane represents an *Eco*RI digest and the right panel represents a *Bam*HI/*Sal*I digest. The phosphorimages are presented with a grey scale adjustment to show minor bands more clearly and appear overexposed. The additional bands in panel B (compared with panel A) are arrowed, with sizes shown in kilobases.

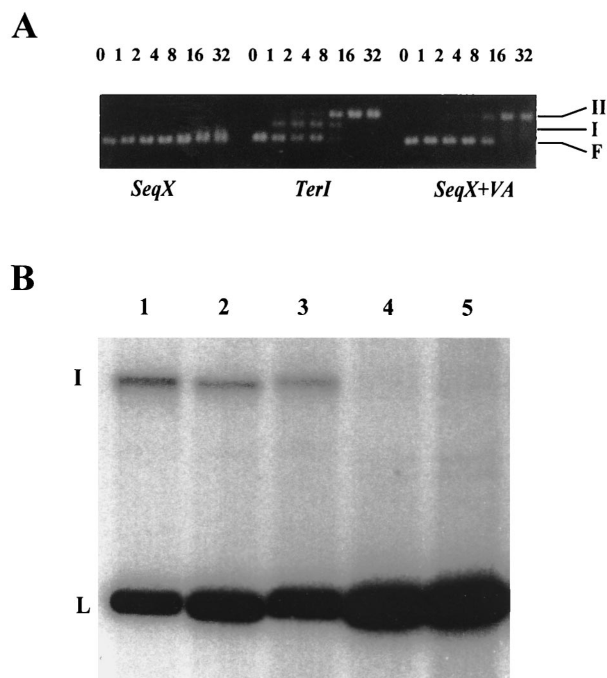

FIG. 4. RTP binding and fork arrest assays for various terminus-related sequences. (A) Band retardation assays for the binding of RTP to the sequences shown, performed as described previously (11). See the legend to Fig. 1 for a description of the sequences. The numbers at the top indicate the RTP dimer/DNA molar ratios in the respective lanes. F represents the free DNA fragment (~400 bp); I and II correspond to complexes containing one and two dimers of RTP, respectively. (B) Results of in vivo fork arrest assays (18). Lane 1 is a positive control with *TerI* (in pWS66-1); lanes 2 and 3 are for two independent transformants in which *TerVII* is in the functional orientation in the pWS64-1-derived pAG12; lanes 4 and 5 correspond to *TerVII* in the nonfunctional orientation in pAG11. L, linear DNA; I, forked DNA.

of the band II species. While the *SeqX+VA* sequence has not been tested for fork arrest activity, previous data on truncated terminators (16) strongly suggest that it would have at least partial activity compared with the major terminator *TerI*. While the chromosomal *SeqX* is unlikely to be a functional terminator and its orientation is not known, its terminus region location raises the possibility that it represents a previously functional terminator that has degenerated largely through changes in its A site.

Identification of an additional functional terminator through searching the database. Because *SeqX* does not bind RTP, it was thought unlikely that any of the minor hybridizing bands in the *Eco*RI digest of Fig. 3B would represent bona fide terminators. However, another likely functional terminator, which has been named *TerVII*, was identified through a search of the SubtiList database (15) with the consensus shown in Fig. 1. *TerVII* is located within the terminus region at 167°, close to *TerIV* and in the *glnA* region, which has been extensively studied by Strauch et al. (19). Typically, *TerVII* lies outside a coding region and is orientated to block the anticlockwise fork as might be expected (Fig. 2). It was synthesized and found to bind two dimers of RTP (data not shown). With the standard plasmid assay (18), *TerVII* was shown to arrest a replication fork in a polar manner with an efficiency of ~25% of that of *TerI* (Fig. 4B). Note the presence of the slowing-moving forked DNA (I) in lanes 2 and 3 and its absence from lanes 4 and 5 when *TerVII* was in the opposite orientation relative to replication fork approach. *TerVII* could be assigned to the 1.6-kb *Eco*RI fragment barely visible in Fig. 3B. The two mismatches with the B site probe would explain its low efficiency of hybridization. The presence of several minor bands in the *Eco*RI digest of Fig. 3B of an intensity greater than that of the band corresponding to *TerVII* raises the possibility that they do reflect other terminator sequences. However, like *TerVII*, they could be relatively weak compared with the major *TerI* terminator because of likely mismatches with the consensus B site (Fig. 1). Alternatively, the minor bands could represent just single B site-like sequences.

Searching for STer site sequences. Some of the minor bands in the *Eco*RI digest of Fig. 3B could represent STer sites. However, identifying such sequences among a collection of cloned DNAs would be difficult, and no attempt was made to isolate and test them for RTP binding. On the assumption that a STer site would resemble the terminus region consensus to some extent at least, a more intensive search of the available sequences in the SubtiList database corresponding to the regions in which the STer sites on each side of the origin are known to reside was performed. Contig 348-1 covers the 348 to 11° segment of the chromosome and contains at least two STer sites (at ~349 and 352 to 355° [asterisked in Fig. 2]). The 12 to 22° region, which spans at least one STer site (at 17 to 22°) and possibly yet another, has not been completely sequenced. It is represented by seven contigs. By using the Search Pattern program within SubtiList, a search of the sequences in the 348 to 22° region was performed for the A+B consensus (nt −1 to 27 of the seven known strain 168 terminators in Fig. 2 plus the two terminators of W23) with various levels of mismatch. A

total of five sequences with up to five mismatches from the consensus and in the active orientation were identified. None contained perfect B sites. The two best sequences are shown in Fig. 1 as *SeqS* and *SeqT*; they are located at ~21 and ~354°, respectively, and were therefore possible STer candidates. Both occur outside coding regions. In *SeqS*, three of the fully conserved positions (boldface in Fig. 1) were different (all in the B site); in *SeqT*, three were again different (one in the A site and two in the B site). Both *SeqS* and *SeqT* were synthesized and were found to show no detectable binding to RTP under standard assay conditions (reference 11 and data not shown). They were also inserted into plasmid pWS64-1, in the appropriate orientation, to test for fork arrest activity under stringent conditions. After induction of the stringent response with arginine hydroxamate hydrochloride (10), no forked molecule consistent with fork arrest at *SeqS* or *SeqT* could be detected (data not shown). Additional database searches were performed for consensus A and B sites (with various levels of mismatch) separately, and these were examined for opposed adjoining B-like and A- or B-like regions, respectively. Nothing of potential significance was uncovered despite the finding of >80 A-like sequences with up to one mismatch (38 in the active orientation) and >20 B-like sequences with up to two mismatches (15 in the active orientation) from the consensus. The possibility of two adjoining and opposed B-like sites was considered because of the recent discovery of a *B. subtilis* plasmid bidirectional terminator in which the A site was replaced by a B-like site (13).

Conclusions. The present work establishes that the *B. subtilis* 168 chromosome contains a complement of at least seven terminus region terminators. The seventh, *TerVII*, was uncovered by sequence searching. It gives a barely detectable hybridization signal with the probe and conditions employed here, and this raises the possibility that some of the other minor bands in Fig. 3B reflect the presence of even more terminators. However, it is likely that, as with *TerVII*, they would be relatively weak. While it has not been established directly that all terminus region terminators are related to the consensus shown in Fig. 1, reflecting the A+B site makeup, it is likely that this is the case. The equivalents of the *TerI* and *TerII* terminators in *Bacillus atrophaeus* conform completely to the *B. subtilis* 168 consensus despite extensive changes in the nucleotide sequence of the *rtp* gene (unpublished data).

It appears that the STer sites, at least the first two to the left of *oriC*, do not have the typical A+B site consensus makeup. The likelihood that STer sites differ appreciably from the established terminus region terminators is very relevant to the mechanism by which an RTP-terminator complex blocks movement of a replication fork. Identification and characterization of the STer sites, which might bind RTP in a somewhat different manner from those in the terminus region, would undoubtedly help to clarify the current uncertainty concerning the molecular mechanism of fork arrest (20). It is also possible that a STer site comprises just a single B-like site, which is rendered functional under stringent conditions.

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