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Expression of *divIB* of *Bacillus subtilis* during Vegetative Growth

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Expression of the division initiation gene, *divIB*, of *Bacillus subtilis* during vegetative growth was examined. *lacZ* fusion studies and transcription start point mapping have established that a σ^A promoter proximal to *divIB* is utilized in vivo. The −10 region of this promoter, which is located 93 bp upstream of the start codon, has been defined precisely by site-directed mutagenesis that destroys the promoter. Examination of transcripts by Northern (RNA) blotting has shown that there are at least two transcripts for *divIB*. The established proximal promoter was found to give rise to a very minor transcript which could not be convincingly demonstrated in wild-type cells but which became apparent upon insertion of a plasmid into the chromosome just upstream of this promoter. The major transcript for *divIB* originated from a site several kb upstream of the gene and is probably the same as the long polycistronic message also traversing the *murD-spoVE-murG* genes that was identified previously by others (A. D. Henriques, H. de Lencastre, and P. J. Piggot, Biochimie 74:735–748, 1992). Transcription from the proximal promoter alone, in an upstream-deletion mutant strain, provided sufficient DivIB for normal growth and division as well as sporulation.

Studies on cell division in *Bacillus subtilis* have identified at least three genes involved in septum formation: *ftsZ*, *ftsA* (3), and *divIB* (also called *dds* [4, 16]). The *ftsA* and *ftsZ* genes are homologous to the equivalent genes in *Escherichia coli* (3). No protein homologous to the DivIB protein has been identified and, as with most other cell division proteins of *B. subtilis* and *E. coli*, its precise function remains unknown. Mutational analyses have established that *divIB* has a role in the initiation stage of septum formation (6). Recently, it has been shown that DivIB is associated exclusively with the *B. subtilis* cell envelope (15). It has been proposed that the long C terminus of DivIB, which lies exterior to the membrane, is involved in initiating a change in the direction of peptidoglycan growth through interaction with one or more other proteins (15).

Knowledge of the regulation of expression of cell division genes in both *E. coli* and *B. subtilis* is far from complete. In *E. coli*, the *ftsQAZ* cluster in the *mra* region at 2 min on the chromosome has been the most intensively studied. It constitutes an atypical operon (21). There are numerous promoters in this region, and their transcriptional and translational regulation is complex (27). The *divIB* gene of *B. subtilis* is located, along with *ftsA* and *ftsZ*, at ~133° on the chromosome and within the equivalent of the *mra* region of *E. coli* (4, 9) (Fig. 1). All genes in this region are transcribed in the direction of chromosome replication. The *divIB* gene is separated from the downstream *ftsA* and *ftsZ* genes of *B. subtilis* by three unidentified open reading frames (3, 4). Recent genetic and RNA transcript studies of *ftsA* and *ftsZ* have suggested that the two genes are cotranscribed from promoters just upstream of *ftsA* (5, 13, 14). This makes it unlikely that *divIB* is cotranscribed with *ftsA* and *ftsZ*.

Recently, transcripts in the region upstream of *divIB*, in which *murD*, *spoVE*, and *murG* reside, have been investigated (17). Northern (RNA) blotting experiments indicated that, during vegetative growth and early stationary phase, this region of the chromosome is transcribed to produce three long polycistronic messages of 8.3, 6.0, and 4.9 kb. All three transcripts passed through the *murD* and *spoVE* genes, but only the 8.3-kb transcript traversed the *murG* gene. A putative σ^A promoter for *divIB* has been identified from the nucleotide sequence (4, 16) and is located in the intergenic region between *divIB* and *orf2*. It is not known whether this promoter functions in vivo or whether transcription of the *divIB* gene occurs from a site further upstream (or both). However, it was found that insertion of a plasmid just upstream of this putative promoter had no observable effects indicative of poor DivIB production, and it was concluded that this promoter probably allowed sufficient expression of *divIB* for normal growth (4). It has been suggested that the 8.3-kb transcript is unlikely to contain *divIB* due to the presence of an inverted repeat or terminator-like sequence just upstream of the gene (17; see Fig. 3). This sequence had been noted earlier (16). It lies just downstream of the putative σ^A promoter for *divIB* and would be in an unusual position to act as a terminator, unless it was involved in modulating expression of *divIB*.

In this paper, it is shown that the putative σ^A promoter just upstream of *divIB* is utilized in vivo and can provide sufficient expression of *divIB* during vegetative growth. It is also shown that there are at least two transcripts from which DivIB is produced; one (minor) originates from this σ^A promoter proximal to *divIB*, and the other (major) originates from a promoter upstream of *murG*.

MATERIALS AND METHODS

Bacterial strains. *B. subtilis* strains are listed in Table 1. *E. coli* strains XL-1 Blue (Stratagene) and DH5α (Gibco BRL) were used as hosts for various plasmids.

Plasmids. pLH5 was described previously (16). pMW26 (Fig. 2A) was constructed by inserting the 0.5-kb *Sma*I fragment from pLH5 into pCPP-3 (2) which had been digested

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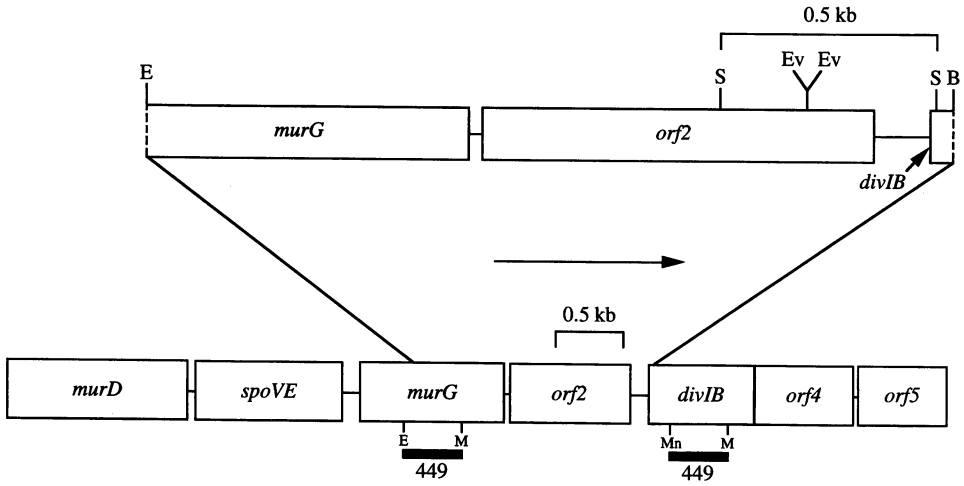

FIG. 1. Arrangement of genes in the *divIB* region of the *B. subtilis* chromosome. The lower section, covering *murD-orf5*, is based on Figure 8 of Errington's review (9); the *orf*s are numbered according to reference 4. All genes are transcribed left to right (arrow). The shaded regions define the *murG* and *divIB* probes used for analysis of transcripts (sizes are in base pairs). The upper section shows the 1.9-kb *Eco*RI-*Bam*HI insert cloned in pLH5. B, *Bam*HI; E, *Eco*RI; Ev, *Eco*RV; M, *Msc*I; Mn, *Mnl*I; S, *Sma*I.

with *Eco*RI and treated with Klenow enzyme to create blunt ends. pMW33, pMW50, pMW62, and pMW63 are derivatives of the *E. coli-B. subtilis* shuttle promoter-detection vector pMW30 (Fig. 2B), which is a derivative of pRL126, an *E. coli* promoter-detection vector (24). pMW30 was constructed by inserting a 1.7-kb *Cla*I fragment, containing the origin of replication and the *cat* gene of pC194 (18), between the multiple cloning site and ampicillin resistance gene (*bla*) of pRL126 and replacing the 2.0-kb *Eco*RI-*Sac*I fragment, containing the 5′ end of the *galK-lacZ* region, with the 2.0-kb *Eco*RI-*Sac*I fragment of pDH32 (see below) containing the 5′ end of the *spoVG-lacZ* region.

pMW33 comprises the 0.5-kb *Sma*I fragment of pLH5 (Fig. 1) cloned into pMW30 between the *Sma*I sites. pMW50 is a deletion derivative of pMW33. To construct pMW50, pMW33 was first digested with *Sph*I and *Xba*I. Unidirectional deletions from the *Sma*I site within *orf2* (Fig. 1) were then performed by using exonuclease III (Stratagene) and mung bean nuclease (MBN) (Pharmacia) sequentially. pMW50 was shown by DNA sequencing to be deleted from the *Sma*I site in *orf2* (Fig. 1) to nucleotide (nt) 128 (Fig. 3A). pMW62 and pMW63 are site-directed mutagenesis products of pMW33 in which the σ^A promoter −10 region, TATACT, has been altered (see below).

TABLE 1. Strains of *B. subtilis*

Strain	Genotype and markers	Source
SU5 (168)	*trpC2*	E. Nester
SU8	*trpC2 thyA thyB*	A. T. Ganesan
SU146	*arg(GH)15 leuA8* r(−) m(−) *recE4 stp thrA*	BGSC[a]
SU237	SU5 Ω pLH20	This work
SU241	SU8 *amyE*::pDH32[b]	This work
SU242	SU8 *amyE*::pLH33[b]	This work
SU243	SU8 *amyE*::pLH62[b]	This work
SU244	SU8 *amyE*::pLH63[b]	This work
SU265	SU8 *amyE*::pBS33[b]	This work

[a] BGSC, *Bacillus* Genetic Stock Center.
[b] In each case, the *Pst*I-linearized plasmid was inserted into the *amyE* locus of SU8.

pLH20 comprises the insert in pMW50 cloned in pJH101 (12) and was constructed by ligating the 0.25-kb *Bam*HI-*Cla*I fragment from pMW50, containing the partially deleted *Sma*I fragment, to the 5.0-kb *Bam*HI-*Cla*I fragment of pJH101. The plasmid pMW33* is a site-directed mutagenesis product of pMW33 in which the inverted-repeat region following the *divIB* promoter has been altered (Fig. 3B). pLH33, pLH62, pLH63, and pBS33 comprise the inserts in pMW33, pMW62, pMW63, and pMW33*, respectively, cloned in the *B. subtilis* integration vector pDH32 (29).

***B. subtilis* RNA preparation.** Total RNA, used in MBN transcript mapping, was isolated from *B. subtilis* SU146/pMW26 growing rapidly in Penassay broth (containing 10 μg of chloramphenicol per ml) (A_{590} = 1.0), using a modification of a method described previously (1). Total RNA, used for primer extension analysis, was extracted from a culture of *B. subtilis* 168 (SU5) growing rapidly in Penassay broth (A_{590} = 0.5) by using a procedure described previously (32) with slight modifications. For Northern (RNA) blotting experiments, RNA was extracted from cultures of *B. subtilis* 168 (SU5) and SU237 growing rapidly in Penassay broth (A_{590} = 0.5) by using the method of Wu et al. (31). DNase digestions were performed with RNase-free DNase I (Boehringer-Mannheim).

MBN mapping of transcripts. MBN transcript mapping was performed using a modification of the method described by Malo (23). A single-stranded template for synthesis of the DNA probe was first prepared by treating pLH5 sequentially with *Eco*RV and T4 DNA polymerase to create a gapped duplex. This template was annealed with an excess of primer 90 (see below and Fig. 3). The annealed primer was then extended using deoxynucleoside triphosphates plus [α-^{32}P]dCTP, and the product (labelled probe) was purified for use in the transcript mapping.

Primer extension analysis of transcripts. A modification of the method described by Wu et al. (31) was used. For the analysis, primers 90 and 97 were used and were labeled at their 5′ end as described previously (28). The sequences of these oligonucleotides were as follows: primer 90, 5′GGGTTCAT TGCCTGTTCACC3′; primer 97, 5′GCGATTTCCGCCGAT

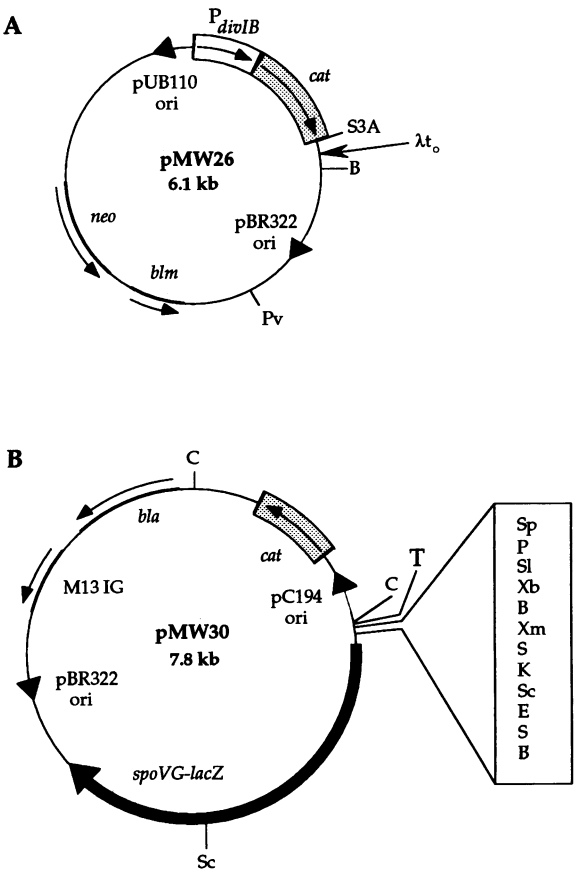

FIG. 2. Structures of plasmids. (A) Plasmid pMW26, used in MBN mapping experiments, comprises the *B. subtilis* promoter-detection vector, pCPP-3, containing the 0.5-kb *Sma*I segment from pLH5 cloned upstream of the promoterless chloramphenicol resistance gene (*cat*). The orientations of the *blm*, *cat*, and *neo* genes and the *divIB* promoter (P_{divIB}) within the *Sma*I insert are shown. (B) The *E. coli-B. subtilis* shuttle promoter-detection vector, pMW30, was used as a vector for various cloning experiments, for site-directed mutagenesis experiments, and for deletion analysis of the *divIB* promoter region. An *E. coli trp a* transcription terminator (T) is located just upstream of the multiple cloning site to prevent transcription readthrough into this region. B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; Pv, *Pvu*II; S, *Sma*I; Sc, *Sac*I; Sl, *Sal*I; Sp, *Sph*I; S3A, *Sau*3AI, Xb, *Xba*I; Xm, *Xma*I.

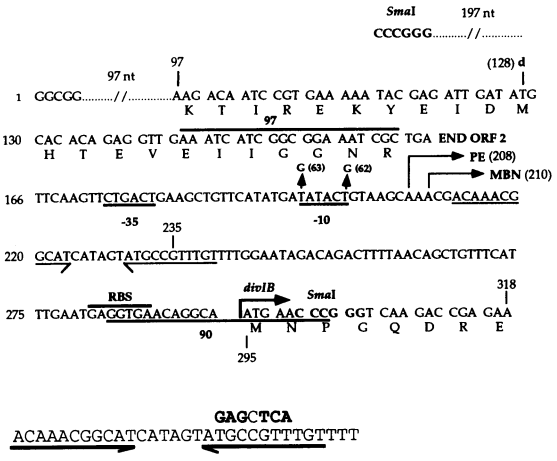

FIG. 3. Nucleotide sequence of the region upstream of *divIB*. The upper section (A) shows the sequence of approximately 200 nt upstream of *divIB* and the first eight codons of the gene. The two *Sma*I sites, separated by 0.5 kb, are indicated. The regions which are the complement of primers 90 (underlined) and 97 (overlined) are shown. The ribosome-binding site (RBS) is overlined, and the deletion limit (rightwards) of the insert in pMW50 is denoted by the **d** at nt 128. The 11-bp inverted repeats are underlined by arrows. The −35 and −10 regions of the putative promoter are shown. The transcription start points predicted from MBN mapping and primer extension (PE) analysis (this work) are identified. The two point mutations in the −10 region cloned in pMW62 and pMW63 are indicated by vertical arrows (62 and 63, respectively). The nucleotide numbering is the same as that published previously (18). The lower section (B) shows the base changes (in boldface type) made in the inverted repeat segment of strain SU242 (nt 213 to 243) to give SU265. The GenBank accession number for this sequence is M31800.

GATTT3′. They are complementary to the nucleotides underlined in Fig. 3.

Site-directed mutagenesis. Mutagenesis of the −10 region of the *divIB* promoter was performed as described previously (22), using single-stranded pMW33 as a template. The oligonucleotides used to create these changes were 5′CGTT TGCTTACCGTATATCATATGAACAGCTTC3′ to produce pMW62 and 5′CGTTTGCTTACAGTATCTCATATGAA CAGCTTC3′ to produce pMW63. The underlined nucleotide in each sequence represents the point mutation. Both oligonucleotides are complementary to nucleotides 180 to 212 in Fig. 3A.

Mutagenesis of the terminator-like sequence in the untranslated *divIB* DNA was performed by using an oligonucleotide-directed in vitro mutagenesis system (Amersham) and single-stranded pMW33 as the template. The oligonucleotide used was 5′GTCTATTCCAAAACATGAGCTCTACTATGATGC CGTTTG3′, the complement of the nt 214 to 252 segment of Fig. 3A. The underlined nucleotides represent the altered bases and are also part of the novel *Sac*I site (GAGCTC) introduced into the sequence, which was used to screen for the mutant plasmid.

β-Galactosidase assays. Strains were grown in Spizizen's minimal medium containing thymine (20 μg/ml), L-tryptophan (20 μg/ml), and 0.05% (wt/vol) Bacto-Casamino Acids. Samples were collected during exponential growth (A_{590} = 0.2 to 0.4) by centrifugation. Cell pellets were rapidly frozen in dry ice-ethanol and stored at −80°C. β-Galactosidase activity was determined by using MUG (4-methylumbelliferone-β-D-galactopyranoside; Sigma) with a modified version (10) of the procedure described previously (11).

Northern transfer and hybridization. Electrophoresis of RNA was performed in 10- to 15-mm-thick 1.2% (wt/vol) agarose gels containing 200 mM MOPS 3-[-*N*-morpholino]propanesulfonic acid), 500 mM sodium acetate, pH 7.0, 10 mM Na_2EDTA (MOPS buffer), and 8% (wt/vol) HCHO, using recommended precautions (28). Usually, 25 μg of denatured RNA was loaded into each lane of a preelectrophoresed gel. RNA transfer was accomplished by capillary blotting, as recommended (28), onto Biotrace NT nitrocellulose membranes. After being dried and fixed, membranes were hybridized with DNA probes as previously described (17). Probes (purified fragments from suitable plasmids; *Msc*I-*Eco*RI segment for *murG*, and *Mnl*I-*Msc*I segment for *divIB* [see Fig. 1]) were labeled with ^{32}P by using the Amersham Multiprime Labelling System. Radioactive bands were visualized by using a Molecular Dynamics PhosphorImager system.

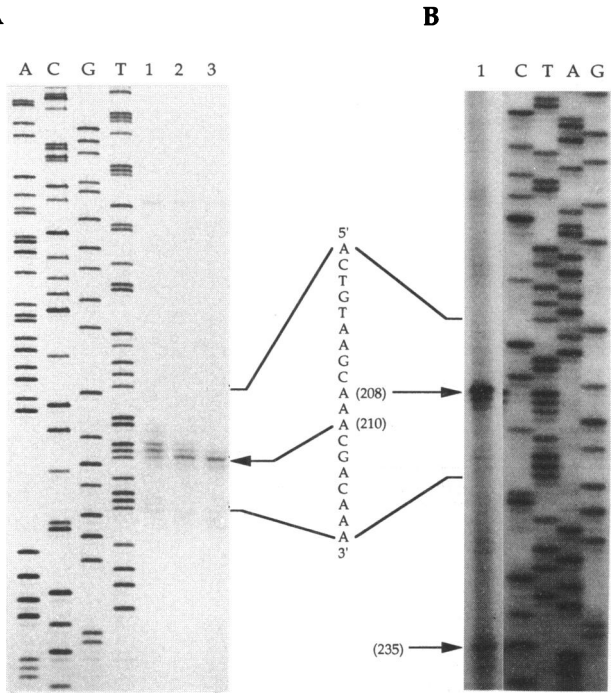

FIG. 4. Determination of the transcription start point for *divIB* by MBN mapping (A) and by primer extension (B). MBN mapping was performed by using RNA extracted from SU146/pMW26. Lanes 1, 2, and 3 in panel A are digestion products obtained by using 500, 650, and 800 U of MBN, respectively. Primer extension analysis of transcripts (B) was performed with RNA extracted from cultures of *B. subtilis* 168 (SU5). Lane 1 shows the result of this analysis. For both panels, sequencing ladders, obtained with the same primer used for making the probe in MBN mapping and primer extension experiments with pLH5 as a template, are shown. The numbers in parentheses are nucleotide positions of the DNA sequence shown in Fig. 3A, corresponding to the 3′ end of the extended product. The sequence indicated is for the nontranscribed strand and is the complement of the sequence that can be read from the autoradiographs. The arrows indicate the positions of the extension products.

RESULTS

A transcription start point just upstream of *divIB*. The 0.5-kb *Sma*I fragment of pLH5 (Fig. 1), encompassing the putative σ^A promoter, was inserted into the *B. subtilis* promoter-detection vector, pCPP-3 (2), just upstream of the *cat* gene. The putative promoter was in the same orientation as the *cat* gene. The resulting plasmid, pMW26 (Fig. 2A), was able to confer chloramphenicol resistance on the *recE* mutant *B. subtilis* strain SU146, indicating that this 0.5-kb *Sma*I fragment contained promoter activity in vivo. The 5′ end of mRNA starting from within this fragment was determined by MBN mapping using RNA extracted from SU146/pMW26 and a probe synthesized from primer 90, which is complementary to a region approximately 80 nt downstream of the putative −10 region (Fig. 3A). The 5′ end of the transcript was shown to correspond to an A residue at nt position 210, 9 nt downstream of the putative −10 region (Fig. 4A).

To determine whether *divIB* mRNA derived from the chromosome has the same 5′ end, primer extension experiments were performed using RNA extracted from exponentially growing *B. subtilis* 168 (SU5) and primer 90. A typical result is shown in Fig. 4B. The darkest band corresponds to an A, at position 208, 7 nt downstream of the putative −10 region (Fig. 3A). The position and intensity of this band suggest that it corresponds to a transcription start point for chromosomal *divIB*. This start point agrees well, within 2 nt, with that established by using RNA generated by plasmid pMW26. Three additional minor bands (or groups of bands), one at position 235 (Fig. 4B) and the other two further upstream of the identified transcription start point (not shown), were observed in the primer extension experiments. Upon further investigation, it was concluded that the latter two were unlikely to be due to extension of *divIB* mRNA (not shown). The band that migrated the furthest (nt 235) was probably the result of premature pausing of the reverse transcriptase at the base of a stable stem-loop structure (Fig. 3A). No strong promoters for σ^A or any of the minor forms of RNA polymerase (25) could be identified by sequence analysis in the region of the *B. subtilis* chromosome corresponding to all three minor bands. On the basis of these observations, the only transcription start point for *divIB* within the 0.5-kb region is suggested to be between nt 208 and 210, as shown in Fig. 3A. It will be shown below, by mutagenesis studies, that the only vegetative promoter in the 502-bp *Sma*I segment in Fig. 3A is indeed the one identified as such.

TABLE 2. β-Galactosidase activities of various strains carrying *divIB-lacZ* transcriptional fusions integrated at the *amyE* locus

Strain	Description[a]	β-Galactosidase activity[b]
SU241	pDH32 integrated	<1, <1
SU242	Wild type −10 region (TATACT)	714 ± 24.8, 592 ± 11.9
SU243	Mutant −10 region (TATACG)	<1, <1
SU244	Mutant −10 region (GATACT)	<1, <1

[a] Construction of these strains is described in Materials and Methods.
[b] Enzyme activity is expressed as picomoles of MUG hydrolyzed per minute per milliliter of culture (A_{590} = 1). Mean values (±SEM) for each separate experiment with each strain are shown.

Mutagenesis of the putative *divIB* promoter. The −10 region of the putative σ^A promoter, TATAcT (nt 196 to 201), matches the consensus sequence well. The 0.5-kb *Sma*I insert in pMW33 (see Materials and Methods) was mutagenized to produce pMW62 and pMW63. In pMW62, the last T is changed to a G (to give TATAcg), and in pMW63, the first T is substituted by a G (to give gATAcT) (Fig. 3A). If the proposed −10 region is correct, then both substitutions should result in a significant decrease in promoter activity in vivo. *divIB-lacZ* transcriptional fusions were constructed by inserting the 0.5-kb *Sma*I fragments of pMW33, pMW62, and pMW63, in the appropriate orientation, adjacent to the *spoVG-lacZ* region of pDH32, to produce pLH33, pLH62, and pLH63, respectively. These constructs (linearized) were introduced into the *B. subtilis* SU8 chromosome at the *amyE* locus. The desired integrations were confirmed by Southern hybridization experiments (data not shown). Of all the Cm^r transformants obtained, only the construct containing the wild-type *divIB* sequence produced blue colonies in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). This strongly suggests that both point mutations in the proposed *divIB* −10 region cause a significant decrease in promoter activity in vivo. β-Galactosidase assays were performed, and the results are shown in Table 2. The β-galactosidase activities of SU243 and SU244, containing the two mutant −10 sequences, are at least 500-fold lower than that for SU242, which contains the wild-type sequence. There is also no significant difference in enzyme activity between the mutant strains and SU241, which contains only pDH32 integrated into the chromosome. This establishes that there are no additional

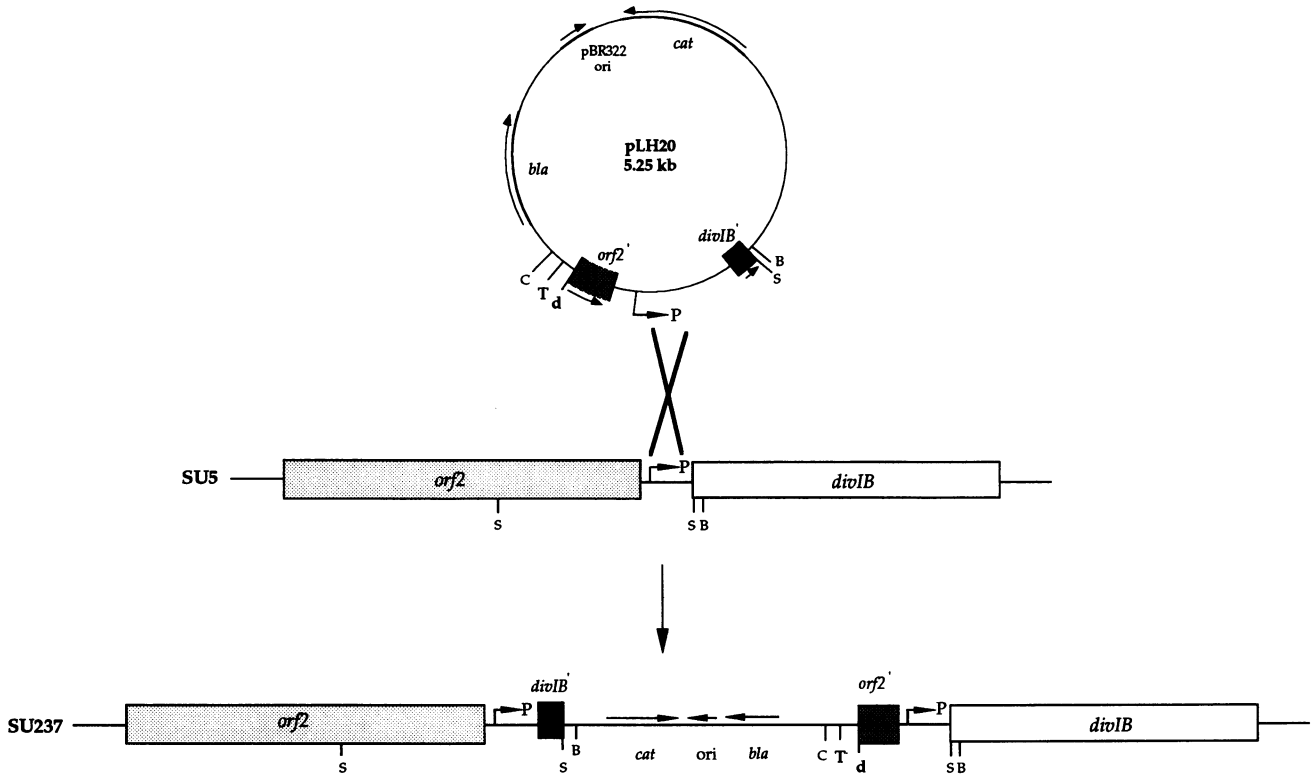

FIG. 5. Integration of pLH20 into the *B. subtilis* 168 (SU5) chromosome to give SU237. A single Campbell-type homologous recombination event occurs between the upstream region of *divIB* in the *B. subtilis* chromosome and pLH20 to give the structure shown in the lower section. The deletion site (**d**) is shown, as is the position of the *E. coli trpA* transcription terminator (T) and the position and orientation of the *divIB* promoter (P). Boxes represent the coding regions of the genes, and incomplete coding regions are designated *orf2'* and *divIB'*. Only part of the Tet^r gene is present in pLH20, and for simplicity it has been omitted. Note that the *Bam*HI-*Cla*I region of pLH20 (174 bp) and the region between *divIB'* and *orf2'* in SU237 are not drawn to scale. B, *Bam*HI; C, *Cla*I; S, *Sma*I.

promoter sequences in the 0.5-kb *Sma*I fragment that are active in vivo during exponential growth. These results show definitively that the predicted −10 region for the *divIB* promoter, TATAcT, is correct. We suggest that the −35 region is cTGACt, which is separated from the −10 region by 16 nt.

Deletion analysis of the *divIB* promoter. The transcriptional analyses described above have been confined to the 0.5-kb *Sma*I fragment upstream of *divIB*. To determine whether there are any additional promoters which are necessary for sufficient *divIB* expression during vegetative growth, a strain of *B. subtilis* was constructed to contain a deletion upstream of this promoter. Unidirectional deletions were performed on the 0.5-kb *Sma*I insert in pMW33, and pMW50 was one of the products. DNA sequencing of pMW50 showed that it was deleted to a T at position 128, 46 nt upstream of the −35 region (Fig. 3A). The insert in pMW50 was transferred to the *B. subtilis* integrable plasmid, pJH101, to produce pLH20 (Fig. 5). *B. subtilis* 168 (SU5) was transformed with pLH20, and two Cm^r colonies, named SU237A and SU237B, were chosen for further analysis. Southern hybridization experiments (not shown) confirmed that pLH20 had integrated as shown in Fig. 5. Strains SU237A and SU237B were examined for their ability to grow and divide. Since the strong *E. coli trp a* terminator is just upstream of the deletion site in pLH20, there should be no transcription readthrough from any promoters upstream of the established promoter. The *trp a* terminator should function efficiently in *B. subtilis*, as RNA polymerase from *B. subtilis* uses *E. coli* transcription terminators very well in vitro (26, 30). The transcription terminator for the *cat* gene in pLH20 might help to prevent transcription readthrough of the *divIB* gene from the *cat* promoter, but it is relatively weak (based on theoretical considerations; see reference 8). Growth curves (determined for Penassay broth, with chloramphenicol present for SU237A and -B) for both deletion strains and the SU5 wild-type strain were indistinguishable (not shown), and the generation times for all three strains were found to be essentially the same (SU5, 31 ± 2.3 min [standard error of the mean {SEM}]; SU237A, 28 ± 0.75 min; SU237B, 28 ± 1.5 min). The morphologies of both deletion strains were indistinguishable from that of the wild type, and they sporulated well. Cell lengths for SU5 and SU237A were identical (3.5 ± 0.08 μm). Thus, there was no filamentation in the deletion mutants.

The levels of DivIB in the deletion strains, relative to that in the wild type, were examined by Western blotting (immunoblotting) and immunodetection experiments using DivIB antiserum (see reference 15). The results (not shown) indicated that the levels of DivIB in the deletion strains, as a percentage of the levels in SU5, were somewhat lower (61% ± 4% [SEM] and 64% ± 8% for SU237A and SU237B, respectively). It is therefore concluded that at least 50% of DivIB produced in wild-type cells can be generated in the deletion strains from the established σ^A promoter located downstream of the deletion site at nt 128 (Fig. 3A).

Northern analysis of transcripts. The finding that the level of DivIB was reduced when the region upstream of the

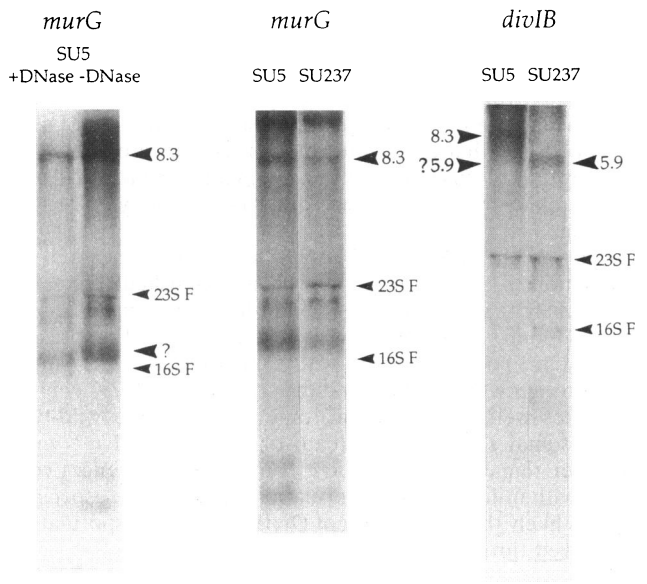

FIG. 6. Analysis of RNA transcripts in *B. subtilis* strains SU5 and SU237 by Northern transfer and hybridization with various probes. The probes used (see Materials and Methods) are shown above each set of lanes (also see Fig. 1). Bands are identified according to size (in kilobases) or as 16S F or 23S F (fronts of 16S and 23S rRNA, respectively).

identified *divIB* promoter was disrupted by plasmid insertion (in SU237) raised the possibility that transcription starting at sites even further upstream contributes to *divIB* expression. A long polycistronic message (8.3 kb) spanning the *murD-murG* region has been identified (17), but whether it extended as far as *divIB* was not investigated. The inverted repeat (or terminator-like) sequence that might function to block transcription into *divIB* (Fig. 3A) was assessed for such function by using the theoretical approach of d'Aubenton Carafa et al. (8). A calculated d value of -7.6 strongly suggested that such a sequence would be very inefficient as a terminator.

RNA was isolated from *B. subtilis* wild-type (SU5) cells harvested at mid-exponential phase in Penassay broth. The leftmost panel of Fig. 6 shows the result of electrophoresis, transfer, and hybridization with the *murG* probe (see Fig. 1 for probe definitions) after and before treatment of the RNA with DNase I. An 8.3-kb species could be clearly distinguished even in the presence of the contaminating DNA. Because DNase I treatment frequently lowered the hybridization signal from the RNA, it was not used routinely. The sharp bands, labeled 23SF and 16SF, represent the fronts of the 23S and 16S rRNA species, respectively. Two extra bands appeared between these two, one just below the 23S front with a size equivalent to 1.5 kb, and another that coincides exactly with the 16S rRNA (confirmed by hybridization with an *rrnO* probe). While the latter probably does reflect hybridization to 16S rRNA, it is of interest that of all the probes used in the present work, this band was detected only with the adjacent *murG* and *orf2* probes. For this reason, the band is marked with a question mark. The pair of faint bands at the bottom of the gel were 0.5 and 0.4 kb in size.

The central and rightmost panels of Fig. 6 compare RNAs isolated from SU5 and SU237 (pLH20 integrated just upstream of the identified *divIB* promoter; see Fig. 5). In one case, hybridization was with the *murG* probe, and in the other it was with the *divIB* probe. In SU5, the 8.3-kb species was detected by both probes. In other experiments with SU5 (not shown), the membrane was stripped after hybridization with one probe and then hybridized with the other to show that the SU5 8.3-kb species detected by the *murG* and *divIB* probes were coincident. The 8.3-kb species in SU5 was also detected with *orf2*, *orf4*, and *orf5* probes (not shown). It is concluded that the 8.3-kb species identified here reflects a transcript traversing the *murG-orf5* region. The 8.3-kb species was the major *divIB* transcript in SU5. There was no major transcript in SU5 which was detectable with the *divIB* probe and not by the *murG* and *orf2* probes.

In SU237, where pLH20, containing the *trp a* terminator, was integrated between *orf2* and *divIB*, it would be expected that transcription from a site upstream of *murG* would not proceed into *divIB*. The right-hand lanes of the central and rightmost panels in Fig. 6 tested this prediction. The *murG* (and *orf2*, not shown) probe again detected a species of 8.3 kb. It is surprising that the size of this transcript in SU237, compared with that in SU5, appears to be unaltered. However, a transcript proceeding through *murG* and *orf2* in SU237 could include >5 kb originating from the plasmid insert. This could have compensated precisely for the loss of RNA due to transcription not proceeding into *divIB* because of the *trp a* terminator. As expected, the 8.3-kb species was absent when the *divIB* probe was used. Instead, a well-defined though relatively weak signal at the 5.9-kb position was observed. This 5.9-kb transcript was also detected with *orf4* and *orf5* probes (not shown). It is highly likely that the 5.9-kb transcript originates from the identified proximal *divIB* promoter between *orf2* and *divIB*. That it does not come from transcription starting at the *cat* promoter in SU237 (Fig. 5) was established by use of a *bla* probe which gave no evidence of a 5.9-kb band (data not shown). But why wasn't the 5.9-kb *divIB* transcript identifiable in RNA from the wild type, SU5? Special efforts were made to detect it. In some experiments, including some with longer electrophoresis times, there were suggestions of its presence. Possibly its low abundance in SU5 RNA made it difficult to detect.

Effect of disruption of the upstream inverted-repeat region on transcription from the identified *divIB* promoter. The inverted-repeat region, or terminator-like sequence, between *orf2* and *divIB* did not appear to function as a transcription terminator. Its positioning just downstream of the identified *divIB* promoter raised the possibility that it was involved in regulating transcription from this promoter. The *B. subtilis* strain SU242 contained, at its *amyE* locus, the 0.5-kb *Sma*I segment of DNA shown in Fig. 3A transcriptionally fused to *lacZ*. SU265 is the same as SU242, except for disruption of the inverted-repeat region as shown in Fig. 3B. β-Galactosidase assays of extracts prepared from mid-exponential phase (Penassay) cultures of SU242 and SU265 gave levels of 651 ± 127 and 870 ± 158 MUG units, respectively (see Table 2 for unit definition). This makes it unlikely that the inverted-repeat region has a significant role in modulating transcription from the identified *divIB* promoter, at least during vegetative growth.

DISCUSSION

The present work relates to the expression of the cell division initiation gene, *divIB*, of *B. subtilis* during vegetative growth. It is clear that the putative σ^A promoter lying proximal to *divIB*, which was identified previously from sequence data (16), is functional in vivo. Transcripts starting at positions consistent with utilization of this promoter (labeled MBN and PE in Fig. 3A) have been shown to be present in RNA

generated by a *B. subtilis* plasmid containing the promoter, as well as in chromosomal DNA itself. The precise identification of the −10 region has been achieved through specific site mutations that destroy the functioning of the promoter. However, it is clear that the promoter is very weak. When fused to *lacZ* at *amyE*, it yields a β-galactosidase specific activity which is lower by a factor of 100 to 1,000 compared with that obtained with the *xylA* promoter analyzed in the same way (19). Its weakness is probably also reflected in the weak signal for its transcript, relative to other spurious bands, obtained in the primer extension experiments (Fig. 4B).

Previously, it was shown that insertion of a plasmid upstream of the −35 region of the proximal promoter, leaving only 160 bp in this segment uninterrupted, allowed normal growth and division (4), suggesting that this promoter provides sufficient expression of *divIB* for these purposes. However, it could not be ruled out that another promoter in the uninterrupted 160-bp segment was responsible for *divIB* expression in this situation or that expression from another (cryptic) promoter in the inserted plasmid was involved. The insertion of pLH20 containing the *trp a* terminator into the wild-type strain at a similar chromosomal site to give SU237 (Fig. 5) leaves only 46 bp upstream of the −35 region uninterrupted, and analysis of this sequence shows no consensus sequence corresponding to a promoter for σ^A or any of the minor forms of RNA polymerase (25). This makes it more likely that the identified σ^A promoter allows sufficient expression for normal growth and division, at least in SU237. Direct measurement of DivIB in SU237 shows that it contains at least 50% of the level present in the wild type.

Surprisingly, Northern blotting experiments have shown that the major transcript for *divIB* does not start from the proximal σ^A promoter. Rather, it starts from a promoter upstream of *murG* (Fig. 1) which yields a transcript of 8.3 kb. It is possible that this transcript is the same as the 8.3-kb species identified previously (17) and starts from a site upstream of *murD*. It appears that the putative transcription terminator between *orf2* and *divIB* does not function as such. The present work shows that *orf4* and *orf5*, both downstream of *divIB*, are also represented in the 8.3-kb transcript. Certainly this is feasible if transcription commences from a site upstream of *murD*; the distance covered by the *murD-orf5* segment in Fig. 1 is approximately 7 kb.

Insertion of pLH20, which contains the strong *trp a* transcription terminator, between *orf2* and *divIB* (Fig. 5) has been shown to block progression of the transcript, starting upstream of *murG*, into *divIB*. In this situation, a shorter *divIB* transcript of 5.9 kb becomes apparent. This shorter transcript almost certainly starts from the proximal *divIB* promoter. The 5.9-kb species was not detectable in the wild type, and this can be explained by the low activity of the proximal promoter. It is possible that the level of the 5.9-kb transcript is elevated in SU237 (containing the integrated pLH20) compared with that in the wild type in order to compensate for loss of transcription of *divIB* from the promoter upstream of *murG*.

A transcript of 5.9 kb starting at the proximal *divIB* promoter would be expected to pass through the downstream *ftsA* and *ftsZ* genes. A 5.9-kb transcript containing *ftsA* was not observed in wild-type cells (13), but it might not have been expected given the fact that it has not been possible to convincingly establish the presence of the 5.9-kb transcript in wild-type RNA in the present work.

The present data, of course, do not rule out the possibility that the 8.3-kb RNA detected in wild-type *B. subtilis* with the *murG* and *divIB* probes represents two separate transcripts. One of these transcripts could start significantly upstream of *murD*, as has already been suggested (7), and stop before *divIB*. The other could start closer to *murG* and proceed through *divIB*, *orf4*, and *orf5*. This might explain why the sizes of the *murG* transcripts in the wild-type and SU237 strains are essentially the same. However, such a situation would not alter the conclusion that there must be at least two promoters for transcription of *divIB*, one being the identified proximal promoter (relatively weak) and the other (stronger) lying further upstream.

It has been shown previously that DivIB is needed for sporulation (4). While it has not been established directly, it seems likely that DivIB is involved in formation of the sporulation septum, as are FtsA (20) and FtsZ (5). The present studies have been concerned with expression of *divIB* during vegetative growth, but it is significant that strain SU237 (Fig. 5) sporulates well. In this strain, only 46 bp of the wild-type chromosomal sequence upstream of the proximal σ^A *divIB* promoter remains. This segment contains no sequences consistent with utilization of a sporulation sigma factor, especially σ^H. It is likely that production of DivIB needed for sporulation is achieved through the action of σ^A promoters.

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