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Transcription Regulation of *ezrA* and Its Effect on Cell Division of *Bacillus subtilis*

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The EzrA protein of *Bacillus subtilis* is a negative regulator for FtsZ (Z)-ring formation. It is able to modulate the frequency and position of Z-ring formation during cell division. The loss of this protein results in cells with multiple Z rings located at polar as well as medial sites; it also lowers the critical concentration of FtsZ required for ring formation (P. A. Levin, I. G. Kurster, and A. D. Grossman, Proc. Natl. Acad. Sci. USA 96:9642-9647, 1999). We have studied the regulation of *ezrA* expression during the growth of *B. subtilis* and its effects on the intracellular level of EzrA as well as the cell length of *B. subtilis*. With the aid of promoter probing, primer extension, in vitro transcription, and Western blotting analyses, two overlapping σ^A-type promoters, P1 and P2, located about 100 bp upstream of the initiation codon of *ezrA*, have been identified. P1, supposed to be an extended −10 promoter, was responsible for most of the *ezrA* expression during the growth of *B. subtilis*. Disruption of this promoter reduced the intracellular level of EzrA very significantly compared with disruption of P2. Moreover, deletion of both promoters completely abolished EzrA in *B. subtilis*. More importantly, the cell length and percentage of filamentous cells of *B. subtilis* were significantly increased by disruption of the promoter(s). Thus, EzrA is required for efficient cell division during the growth of *B. subtilis*, despite serving as a negative regulator for Z-ring formation.

Binary fission in rod-shaped bacteria entails the formation of a transverse septum that divides a progenitor cell into two daughter cells of equal size. In the initiation of cell division, the tubulin-like cell division protein FtsZ polymerizes at the mid-cell into a ring structure that is required for subsequent recruitment of other cell division proteins and assembly of the cell division machinery (7, 10, 29, 36, 40). Temporally, the division process is tightly coupled to chromosome replication, chromosome segregation, and cell growth to ensure that both daughter cells inherit complete genomes and are of appropriate size and shape (12, 16, 17, 35).

Two mechanisms, nucleoid occlusion and the Min system, are involved in selection of the correct mid-cell site for cell division (1, 12, 17, 30). Nucleoid occlusion, although poorly defined, was revealed by the observation that cell division is largely inhibited in the vicinity of the nucleoid of cells in which DNA replication and/or segregation is perturbed (33). In other words, Z-ring assembly and cell wall synthesis is inhibited in the immediate vicinity of the actively replicating nucleoid. Since the Z ring appears to form at a position where the DNA concentration is low compared to the wild-type situations (9, 15, 35, 38), it is assumed that it is not the presence of DNA per se but the concentration of DNA which determines the position of Z-ring formation. The Min system, which inhibits FtsZ polymerization and also division at cell poles, has been exten-

sively characterized for both *Escherichia coli* and *Bacillus subtilis*. For *B. subtilis*, the MinCD complex is recruited to the pole by a cell pole-associated protein, DivIVA, probably through a direct interaction with MinD (5, 11, 23, 31). Moreover, the DivIVA-MinCD complex remains associated with the newly formed pole after division, thereby preventing future division at these polar sites (11, 31).

The *B. subtilis* EzrA protein is a negative regulator for Z-ring formation. It is able to modulate the frequency and position of Z-ring formation during cell division. The lack of this protein causes cells with multiple Z rings located at polar as well as medial sites and lowers the critical concentration of FtsZ required for ring formation (26). The EzrA protein is homogeneously distributed in the cell membrane and localized to the cell division site once the Z ring is assembled, presumably via an interaction with FtsZ (26). A null mutation of *ezrA* has been found to suppress the defects in FtsZ polymer stability associated with *minCD* overexpression (27). Moreover, the effect of the loss of EzrA on cell division is enhanced by ZapA (a Z-ring-associated protein). The absence of ZapA and EzrA, but not ZapA itself, causes a severe block in cytokinesis of *B. subtilis* (14), suggesting that EzrA may play a positive role during cell division. Furthermore, EzrA may also participate in asymmetric division, since it is detectable in the spiral-like structure in sporulating cells (3). Recently, it was reported that EzrA can be degraded by an ATP-dependent CodWX protease in vitro (22). However, it remains elusive whether the intracellular level of EzrA is regulated during the growth of *B. subtilis*. This work was aimed at studying the transcription regulation of *ezrA* and its effect on *B. subtilis* cell division.

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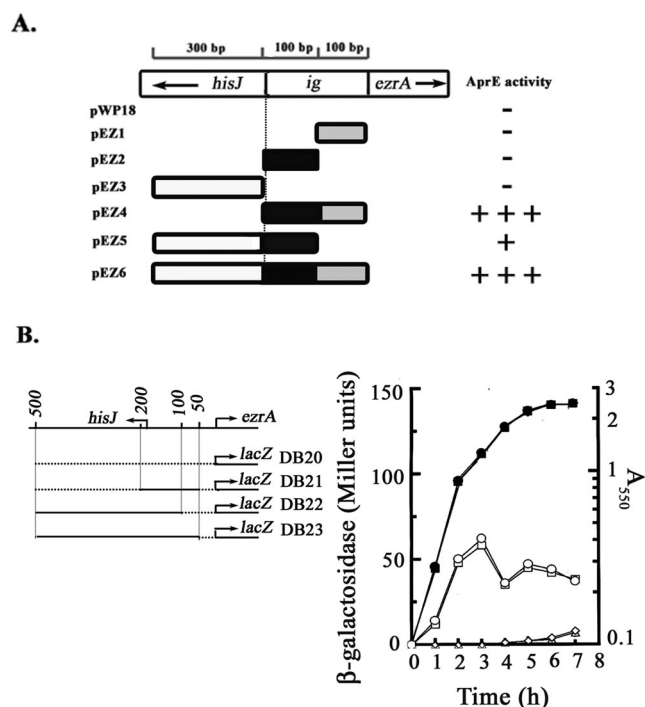
FIG. 1. Localization of putative promoter region(s) upstream of *ezrA* and expression of putative promoter-*lacZ* fusions during *B. subtilis* growth. (A) Localization of the putative promoter region(s) upstream of *ezrA*. The DNA fragment containing the intergenic region (*ig*), *hisJ* coding region, or both regions was cloned into the promoter probing vector, pWP18, to generate pEZ1 through pEZ6. *B. subtilis* DB430 harboring each of the plasmids was then patched on the skim-milk plate (1% skim milk in 2× SG medium) and analyzed for the halo size. + to +++ indicates the relative size of the halo. − indicates no significant halo observed. (B) Expression of the putative promoter-*lacZ* fusions in *B. subtilis*. The solid lines indicate the DNA fragments inserted upstream of *lacZ*. Each of the *B. subtilis* strains was grown in 2× SG medium and analyzed for *lacZ* expression throughout the growth. The procedure used for β-galactosidase assay was derived from the work of Miller (32). The symbol ▲, ■, ♦, or ● indicates the growth curve of *B. subtilis* DB20, DB21, DB22, or DB23, respectively. The symbol, △, □, ◇, or ○ indicates the expression of *lacZ* in the corresponding strain of *B. subtilis*.

The putative promoter region(s) upstream of *ezrA*. On the basis of the data of sequence analysis of the DNA encompassing *ezrA* and its upstream region, it was believed that *ezrA* constitutes a single-gene operon with the transcription direction opposite to that of *hisJ* (Fig. 1A). To search for the promoter(s) controlling *ezrA* expression, six different DNA fragments of up to 500 bp in length and upstream of the initiation codon of *ezrA* (Fig. 1A) were first synthesized by PCR using specific sets of primers (Table 1), digested with designed restriction enzymes, inserted into the promoter-probing vector, pWP18 (34), transformed into *B. subtilis* DB430, and analyzed for AprE activity on a skim-milk plate (Fig. 1A). Our data showed that both the DNA fragments containing the whole intergenic region (*ig*) between *ezrA* and *hisJ*, as shown for pEZ4 and pEZ6, were relatively high in producing the AprE activity (Fig. 1A). Dissection of this intergenic region into the two 100-bp halves, as shown for pEZ1 and pEZ2, completely eliminated the activity of each half, indicating that

a putative promoter region is located in the junction of these two halves of DNA. However, only relatively weak AprE activity was observed for the DNA inserted into pEZ5. This weak activity was eliminated as the inserted DNA was further separated into two shorter halves, as shown for pEZ2 and pEZ3, suggesting that a second putative promoter region is located about 200 bp upstream of *ezrA*.

The promoter region 100 bp upstream of *ezrA* is responsible for most of the expression of *ezrA*. To analyze whether both of the putative promoter regions were critical for *ezrA* expression, transcriptional fusions of promoter-*lacZ*, as shown in the left part of Fig. 1B, were constructed. To make these constructs, each of the promoter regions was amplified by PCR, using the chromosomal DNA of *B. subtilis* DB2 as the template. The forward, reverse, and overlapping primers are shown in Table 1. The synthesized putative promoter DNA fragments were digested with PstI and BamHI and cloned into the integration vector, pCoiZA (34). The resultant plasmids, pEZ10, pEZ8, and pEZ11 (Table 2), as well as the vector, pCoiZA, were integrated, respectively, into the *B. subtilis* DB2 chromosome through homologous recombination at the *aprE* locus to generate *B. subtilis* DB21, DB22, DB23, and DB20. The expression of *lacZ* in each of the strains was then measured throughout the growth in 2× SG medium (25). Similar to that observed for the negative control strain, *B. subtilis* DB20, no significant expression of *lacZ* was detected for *B. subtilis* DB22 in which the *lacZ* gene was under the control of the putative promoter region located at about 200 bp upstream of *ezrA* (Fig. 1B). These results suggested that this putative promoter region is of no significance to the expression of *ezrA*. In contrast, a significant two-peak expression pattern of *lacZ* was observed when DNA fragments containing the putative promoter region located at about 100 bp upstream of *ezrA* were cloned in front of *lacZ* as shown for *B. subtilis* DB21 and DB23 (Fig. 1B), indicating that this putative promoter region is responsible for most of the expression of *ezrA*.

Identification and characterization of promoters critical for ezrA expression. To locate exactly the position of the promoter(s) about 100 bp upstream of *ezrA*, primer extension analysis of total RNA extracted from *B. subtilis* DB430 harboring pEZ6 was performed. The protocol used for primer extension was similar to that reported previously (28). The primer used is 22 bases long, starting from the base immediately upstream of the initiation codon (Table 1). As shown in Fig. 2A, two major transcription initiation sites, A-71 and A-96, upstream of the initiation codon (ATG) of *ezrA* were found. The promoter identified by A-96 was designated as P1; it had nice fits (four of six and five of six, respectively) to the −35 (TTGACA) and −10 (TATAAT) consensus of the *B. subtilis* σ^A-type promoter besides the possession of a TG motif (TGnTATAAT) immediate upstream of the −10 consensus, indicative of an extended −10 promoter (Fig. 2B) (2, 4, 24). Initiation at A-71 identified another promoter, designated P2. This promoter had poor fits (three of six and two of six, respectively) to the −35 and −10 consensus of the σ^A-type promoters. No consensus for other σ-type promoters was found at this region. Thus, the expression of *ezrA* is controlled by two overlapping σ^A-type promoters, with P1 probably playing the major role (Fig. 2B).

To confirm that P1 and P2 promoters are of the σ^A type, in vitro transcription assays were performed. The procedure used

TABLE 1. Primers used in this study

Description of primer	Direction and sequence[a]
Overexpression of EzrA	Forward, 5′-AGACTA**CCATGG**AGTTTGGATTA-3′
	Reverse, 5′-CGTTAC<u><u>CTCGAG</u></u>AGCGGATATGTCAGCTTTG-3′
Construction of pWP18 derivatives	
pEZ1	Forward, 5′-AGACTA**GAATT**CTAACAATGAAAACGACAGTTTTTC-3′
	Reverse, 5′-CGTTAC*GGATCC*AATGAGCCCCCTTGCTGTT-3′
pEZ2	Forward, 5′-AGACTA**GAATT**CGCATTGATGTCACCCCATG-3′
	Reverse, 5′-CGTTAC*GGATCC*TTATGATACCATGTAACAAAC-3′
pEZ3	Forward, 5′-AGACTA**GAATT**CATGTAATTTCGTCTTCAAATTC-3′
	Reverse, 5′-CGTTAC*GGATCC*AAAAGCGAGACGGACATATTC-3′
pEZ4	Forward, 5′-AGACTA**GAATT**CGCATTGATGTCACCCCATG-3′
	Reverse, 5′-CGTTAC*GGATCC*AATGAGCCCCCTTGCTGTT-3′
pEZ5	Forward, 5′-AGACTA**GAATT**CATGTAATTTCGTCTTCAAATTC-3′
	Reverse, 5′-CGTTAC*GGATCC*TTATGATACCATGTAACAAAC-3′
pEZ6	Forward, 5′-AGACTA**GAATT**CATGTAATTTCGTCTTCAAATTC-3′
	Reverse, 5′-CGTTAC*GGATCC*AATGAGCCCCCTTGCTGTT-3′
Construction of pCoiZA derivatives	
pEZ8	Forward, 5′-AGACTA**CTGCAG**ATGTAATTTCGTCTTCAAATTC-3′
	Reverse, 5′-CGTTAC*GGATCC*TTATGATACCATGTAACAAAC-3′
pEZ10	Forward, 5′-AGACTA**CTGCAG**ATGTAATTTCGTCTTCAAATTC-3′
	Reverse, 5′-CGTTAC*GGATCC*AATGAGCCCCCTTGCTGTT-3′
pEZ11	Forward, 5′-AGACTA**CTGCAG**ATGTAATTTCGTCTTCAAATTC-3′
	Reverse, 5′-CGTTAC*GGATCC*TATTCAGAAAGATTTCAGG-3′
pEZ12	Forward, 5′-AGACTA**CTGCAG**GTTTGTTACATGGTATCATAATAAC-3′
	Reverse, 5′-CGTTAC*GGATCC*TATTCAGAAAGATTTCAGG-3′
pEZ13	Forward a, 5′-AGACTA**CTGCAG**GCATTGATGTCACCCCATG-3′
	Reverse b, 5′-CAGAAAGATTTCATTGTTATTATGATACCATGTAAC-3′
	Forward c, 5′-ATAACAATGAAATCTTTCTGAATAAATAAAAC-3′
	Reverse d, 5′-CGTTAC*GGATCC*TATTCAGAAAGATTTCAGG-3′
Deletion of *ezrA* promoters	
P1 deletion strain (DB2001)	Forward, 5′-AGACTA**GAATT**CGTTTGTTACATGGTATCATAATAAC-3′
	Reverse, 5′-CGTTAC**CTGCAG**TCCGCTCGTTTCAGCTGG-3′
P2 deletion strain (DB2002)	Forward a, 5′-AGACTA**GAATT**CGCATTGATGTCACCCCATG-3′
	Reverse b, 5′-CAGAAAGATTTCATTGTTATTATGATACCATGTAAC-3′
	Forward c, 5′-ATAACAATGAAATCTTTCTGAATAAATAAAAC-3′
	Reverse d, 5′-CGTTAC**CTGCAG**TCCGCTCGTTTCAGCTGG-3′
P1P2 deletion strain (DB2003)	Forward, 5′-AGACTA**GAATT**CCGTGGAAGATTGAAATTCTG-3′
	Reverse, 5′-CGTTAC**CTGCAG**TCCGCTCGTTTCAGCTGG-3′
Primer extension	5′-AATGAGCCCCCTTGCYGTTTAC-3′

[a] The underlined, doubly underlined, bold, italic, and bold italic bases indicate the NcoI, XhoI, EcoRI, BamHI, and PstI restriction sites, respectively. Forward a, Reverse b, Forward c, and Reverse d primers were used for construction and amplification of P2-disrupted promoter DNA fragments by overlapping extension PCR. Reverse b and Forward c are primers containing overlapping sequences and mutation sites.

for the in vitro transcription assay was similar to that reported previously (28). Four different *B. subtilis* σ factors, including σ^A, σ^B, σ^D, and σ^H, were overexpressed, purified from *E. coli* (6, 8, 28, 39), and reconstituted with core RNA polymerase (20). Subsequently, each RNA polymerase holoenzyme or a mixture of two different RNA polymerase holoenzymes was examined for activity on transcribing the pKM3 plasmid containing the DNA sequence encompassing both P1 and P2 (Table 2). As shown in Fig. 3, only in the presence of σ^A-RNA polymerase holoenzyme was the promoter activity observed. Two mRNA transcripts that were supposed to be initiated from P1 and P2, respectively, and terminated at the T1 site of the T1T2 terminators (21) were produced. Thus, the two promoters controlling *ezrA* expression are both of the σ^A type.

P1 plays the major role for *ezrA* expression during the growth of *B. subtilis*. To examine the relative importance of each of the two promoters for *ezrA* expression, two more strains of *B. subtilis*, DB24 and DB25, with a mutant *ezrA* promoter (P1 or P2 disruption) transcriptionally fused to *lacZ* (Fig. 4A, upper part) were constructed using the same strategy as described for *B. subtilis* DB21, DB22, and DB23. Then, *lacZ*

expression was measured for these strains throughout the growth in 2× SG medium. As shown in the lower part of Fig. 4A, *B. subtilis* DB25 with P2 being disrupted by removing the −10 region had a high expression pattern for *lacZ* similar to that of the wild-type counterpart, *B. subtilis* DB21, indicating that P2 plays only a minor role in transcription of *ezrA*. In contrast, disruption of P1 by removing its −35 region drastically reduced the expression of *lacZ* in *B. subtilis* DB24, albeit with a gradual increase in *lacZ* expression during growth. Thus, P1 is the major promoter for transcriptional control of *ezrA* in *B. subtilis*, while P2 is a supplemental promoter for the control, especially during the later stage of *B. subtilis* growth.

The difference in the importance of P1 and P2 to *ezrA* expression indicated that the intracellular level of EzrA might be also differentially affected by disruption of each of the promoters. To check this idea, *B. subtilis* DB2001, DB2002, and DB2003, with P1, P2, or both P1 and P2 being disrupted, respectively, were constructed (Fig. 4B, upper part) and analyzed for relative intracellular levels of EzrA by Western blotting (Fig. 4B, lower part). As expected, P1 disruption led to a substantial reduction in the content of EzrA in *B. subtilis*

TABLE 2. *B. subtilis* strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
B. subtilis strains		
DB2	*trpC2 ezrA* (wild type)	*B. subtilis* 168
DB430	*trpC2 npr apr epr bfp isp1*	18
DB2001	*trpC2 ezrA* (ΔP1) Cm^r	This study
DB2002	*trpC2 ezrA* (ΔP2) Cm^r	This study
DB2003	*trpC2 ezrA* (ΔP1P2) Cm^r	This study
Plasmids		
pET21d	*E. coli* protein overexpression vector, Amp^r	Novagen Inc.
pKM1	Derivative of pET21d, containing an expressible *ezrA* from *B. subtilis* DB2	This study
pCT20	pCT18-derived plasmid which contains P1P2, P7, and P8 promoters of *sigA* operon, followed by the T1T2 terminators of *E. coli rrnB* operon	28
pKM3	pCT20-derived plasmid containing DNA fragment encompassing the 500-bp of DNA upstream of *ezrA* followed by T1T2 terminators of *E. coli rrnB* operon	This study
pWP18	Promoter probing vector with *aprE* reporter	34
pEZ1 to -6	pWP18 derivative containing putative promoter DNA fragments with different length	This study
pCioZA	Integration vector with *lacZ* as reporter; Amp^r Erm^r	34
pEZ8, -10 to -13	pCoiZA derivative containing different putative promoter DNA fragments and used for construction of *B. subtilis* DB22, DB21, DB23, DB24, DB25, respectively, by integration into the *aprE* locus on *B. subtilis* DB2 chromosome	This study
pDP1	Integration vector used for construction of *B. subtilis* DB2001	This study
pDP2	Integration vector used for construction of *B. subtilis* DB2002	This study
pDP12	Integration vector used for construction of *B. subtilis* DB2003	This study

DB2001; P2 disruption caused only a minor reduction in EzrA (see *B. subtilis* DB2002). Deletion of both P1 and P2 completely abolished EzrA in *B. subtilis* DB2003. These results were consistent with those obtained for *lacZ* expression (Fig. 4A), strongly supporting that the expression of *ezrA* is exclu-
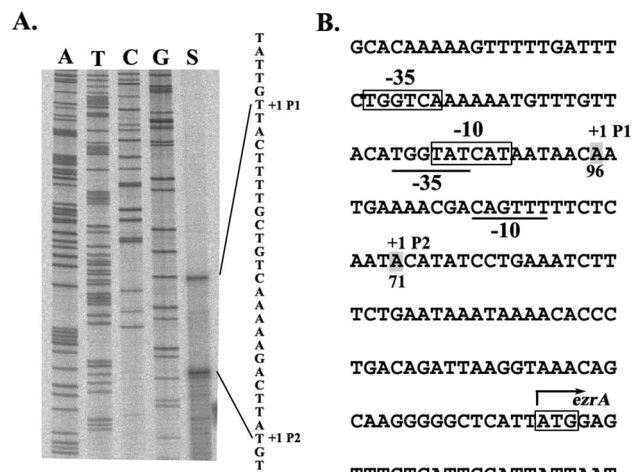

FIG. 2. Determination of the transcription initiation sites of *B. subtilis ezrA*. (A) Primer extension analysis of the transcription initiation sites of *ezrA*. The letter A, T, C, or G above each lane indicates the dideoxynucleotide used to terminate the sequencing reaction. The transcription initiation sites are indicated as +1. The DNA sequence shown in the right margin is read directly from the gel and represents the sequence of noncoding strand DNA. (B) Coding-strand DNA sequence of the two overlapping promoters of *B. subtilis ezrA*. The nucleotide sequence is given in the 5′-to-3′ direction. The −10 and −35 regions of P1 and P2 are boxed and underlined, respectively. The transcription +1 sites of the two overlapping promoters are indicated by the shadowed letter, A. The translation initiation codon (ATG) for *ezrA* is boxed and indicated by the rightward arrow.
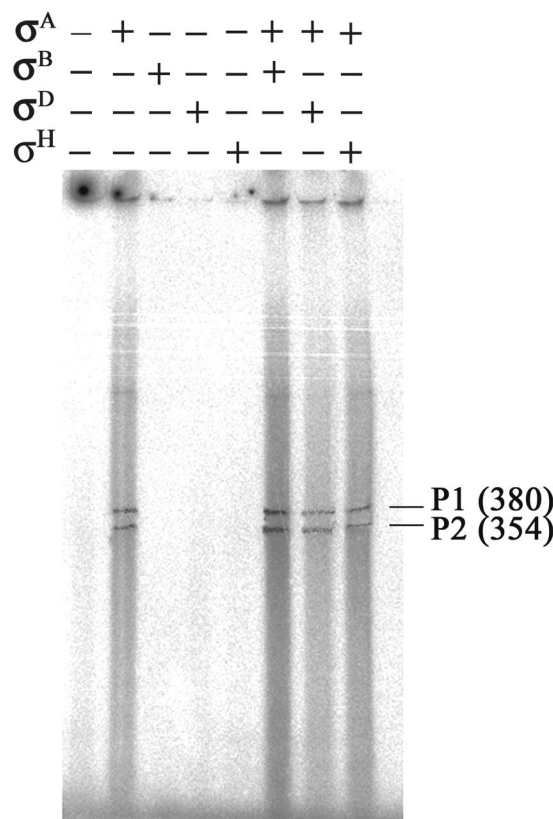

FIG. 3. Determination of the σ specificity of *ezrA* promoters by in vitro transcription assays. The pKM3 plasmid bearing the DNA sequence encompassing both P1 and P2 promoters upstream of *ezrA* was used as a template for in vitro transcription. A single RNA polymerase holoenzyme or a mixture of two RNA polymerase holoenzymes, as indicated by + and − signs, was used to identify the σ type of the promoters. The transcripts initiated from P1 and P2 and terminated at the first termination site (T1) of the T1T2 terminators are indicated by P1 and P2 with transcript length (in bases) shown in the right margin.
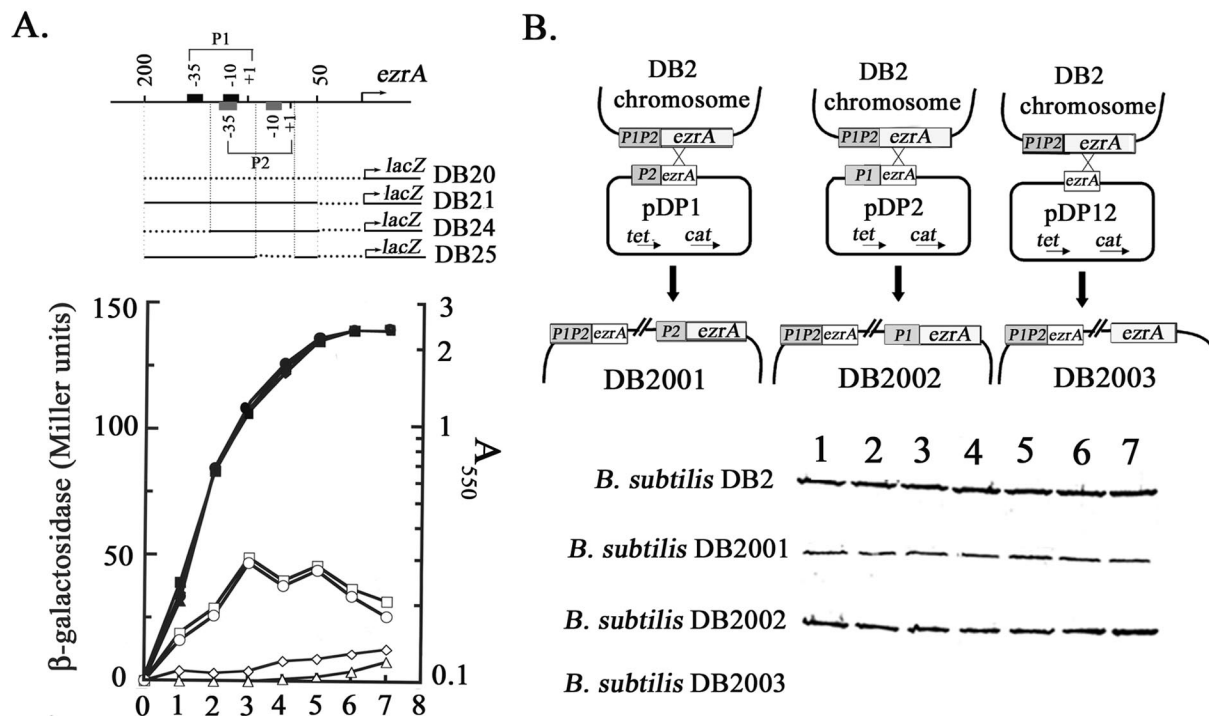
FIG. 4. Effects of promoter mutations on transcription and intracellular level of EzrA. (A) The effect of promoter mutation on transcription of *ezrA* in *B. subtilis*. The transcriptional promoter-*lacZ* fusions are shown in the upper part of the figure. The horizontal dotted lines indicate the DNA sequences removed in the mutant promoters. Each of them was synthesized directly by PCR or indirectly by overlapping extension PCR (19). Each of the *B. subtilis* strains was grown in 2× SG medium and measured for *lacZ* expression. ▲, ■, ♦, or ● indicates the growth curve of *B. subtilis* DB20, DB21, DB24 or DB25, respectively, while △, □, ◇, or ○ indicates the expression of *lacZ* throughout growth. (B) Effect of promoter mutation on intracellular level of EzrA during the growth of *B. subtilis*. The designs for constructing *ezrA* promoter mutants by homologous recombination are shown in the upper part of the figure. The boxed *ezrA* in pDPx (where x = 1, 2, or 12) indicate a 3′-truncated *ezrA* gene which is about 700 bp in length. *B. subtilis* DB2001, DB2002, and DB2003 are strains in which the P1, P2, and P1P2 promoters of *ezrA*, respectively, are disrupted. The promoter designs are the same as those shown at the upper part of panel A. The lower part of the figure shows the intracellular levels of EzrA during growth of *B. subtilis*. The cell samples analyzed were collected every hour throughout growth in 2× SG medium with an initial cell density (A_{550}) of 0.1. Equal amounts of total proteins were loaded for Western blot analysis of EzrA. To prepare anti-EzrA, His-tagged EzrA was overexpressed in *E. coli* BL21(DE3)/pKM1, purified with TALON resin (CLONTECH), concentrated, mixed with adjuvant, and injected into rabbit.

sively controlled by the two promoters. Furthermore, a rather constant level of EzrA was observed for each of the *B. subtilis* strains throughout growth, indicating that EzrA is constitutively expressed and that the synthesis and degradation of EzrA are maintained at equal rates during the growth. The constitutive expression of EzrA is consistent with the participation of EzrA in Z-ring formation in both vegetative and sporulation phases of *B. subtilis*.

The intracellular level of EzrA affects the cell length and percentage of filamentous cells of *B. subtilis* during growth. It has been reported that the *ezrA* null mutation appears to cause a delay in cell division, resulting in cells 20% longer on average than the wild-type counterpart grown in minimal medium (26); however, it remains unclear whether the difference in the intracellular level of EzrA would have differential effects on cell division of *B. subtilis* during growth. To answer this question, the overnight culture of *B. subtilis* DB2, DB2001, DB2002, or DB2003 was inoculated into 2× SG medium to an initial cell density (A_{550}) of 0.1. The cell length and filament size of each strain of *B. subtilis* were then measured at mid-log ($A_{550} = 0.9$) and stationary ($A_{550} = 2.4$) phases after fixation with 70% ethanol. The phase-contrast micrographs for each strain of *B.*

subtilis are shown in Fig. 5A. During mid-log phase, the cells of *B. subtilis* DB2001, DB2002, and DB2003 were 21, 5, and 25%, respectively, longer on average than the wild-type counterpart (DB2). During stationary phase, both *B. subtilis* DB2001 and DB2003, in which the intracellular level of EzrA was either drastically reduced or completely abolished, remained 17% longer on average than DB2. However, *B. subtilis* DB2002, with only a minor reduction in the intracellular level of EzrA, was of about the same size as DB2. Moreover, higher percentages of septate filaments were observed for all of the three mutant strains of *B. subtilis* than with the wild-type at both growth stages (Fig. 5), and nonseptate filaments were clearly observed only for *B. subtilis* DB2001 and DB2003. The increase in the percentage of filamentous cells of *B. subtilis* correlated with the decrease in the intracellular level of EzrA, regardless of the size distribution of filaments (Fig. 5B). The increase in both cell length and percentage of filamentous cells for *B. subtilis* containing reduced levels of EzrA indicates that EzrA is required for efficient cell division.

The possible roles of EzrA during cell division. It has been reported that EzrA recruited to the division site, partially dependent on FtsA, is a negative regulator for Z-ring formation
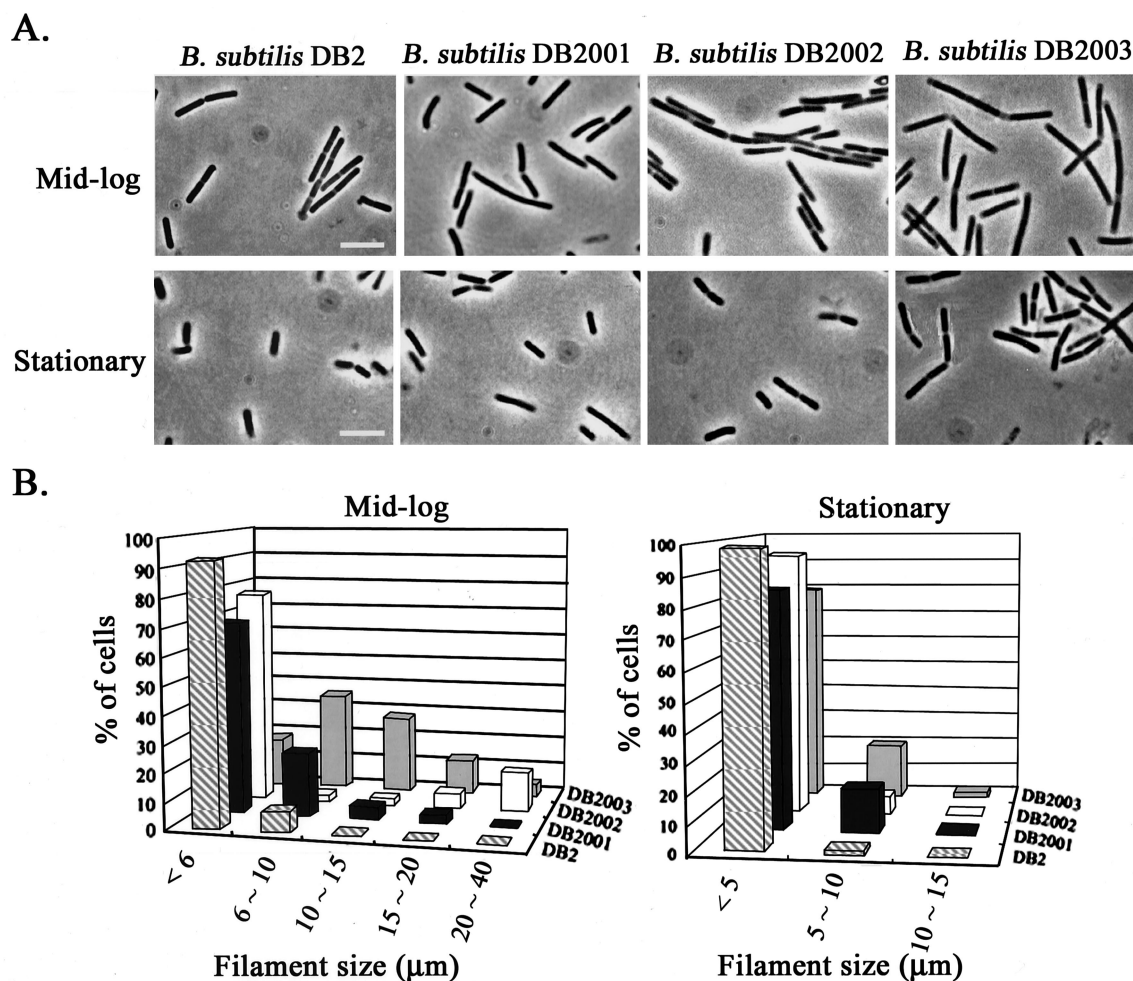
A.

B.

FIG. 5. The effects of *ezrA* promoter disruption on cell length and filament size of *B. subtilis*. (A) Phase-contrast microscopy of *B. subtilis* at ×1,000 magnification. Bar, 10 μm. (B) The filament sizes for each strain of *B. subtilis*. The overnight culture of *B. subtilis* was diluted with 2× SG medium to an initial cell density ($A_{550} = 0.1$), grown at 37°C, harvested at mid-log ($A_{550} = 0.9$) or stationary ($A_{550} = 2.4$) phases, fixed in 70% ethanol for 2 h, and resuspended with PBS buffer (5.4 mM Na_2HPO_4, 1.7 mM NaH_2PO_4, 137 mM NaCl, and 3 mM KCl) before measuring the cell length (×1,000) and filament size (×400) with a phase-contrast microscope. The measurement was repeated at least five times, and the data are reproducible.

(13, 26). However, EzrA was shown in this study to be required for efficient cell division. How does EzrA work to fulfill the two seemingly contradictory functions? The paradox can be partly solved if the effect of EzrA on Z-ring formation is not restricted to a single round of cell division: more precisely, if the negative effects of EzrA on a round of Z-ring formation become positive for the next. It was proposed that EzrA contributes to Z-ring remodeling by accelerating the disassembly of Z ring (14, 37). Thus, in the absence or deficiency of EzrA, the slow disassembly of Z ring in a round of cell division may reduce the concentration of free FtsZ monomers or protofilaments in the cytoplasmic pool and therefore the efficiency of the next round of Z-ring formation. However, Z-ring remodeling seems unable to explain why the *ezrA* null mutation is capable of suppressing the inhibition of cell division caused by overexpression of MinCD (27). Probably, EzrA is also able to interact either directly or indirectly with MinC or MinCD complex to affect their functions before being recruited to the division site. Actually, EzrA may also serve as a membrane

anchor for coordinate membrane invagination at the later stage of cell division, since it shares similar membrane topologies with the homologous *E. coli* ZipA (12). Thus, EzrA may be multifunctional during cell division and present in *B. subtilis* all the time. The presence of a constant level of EzrA in *B. subtilis* during growth (Fig. 4B) and the requirement of EzrA for efficient cell division are consistent with this idea.

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