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Forespore-Specific Transcription of the *lonB* Gene during Sporulation in *Bacillus subtilis*

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The *Bacillus subtilis* genome encodes two members of the Lon family of prokaryotic ATP-dependent proteases. One, LonA, is produced in response to temperature, osmotic, and oxidative stress and has also been implicated in preventing σ^G activity under nonsporulation conditions. The second is encoded by the *lonB* gene, which resides immediately upstream from *lonA*. Here we report that transcription of *lonB* occurs during sporulation under σ^F control and thus is restricted to the prespore compartment of sporulating cells. First, expression of a *lonB-lacZ* transcriptional fusion was abolished in strains unable to produce σ^F but remained unaffected upon disruption of the genes encoding the early and late mother cell regulators σ^E and σ^K or the late forespore regulator σ^G. Second, the fluorescence of strains harboring a *lonB-gfp* fusion was confined to the prespore compartment and depended on σ^F production. Last, primer extension analysis of the *lonB* transcript revealed −10 and −35 sequences resembling the consensus sequence recognized by σ^F-containing RNA polymerase. We further show that the *lonB* message accumulated as a single monocistronic transcript during sporulation, synthesis of which required σ^F activity. Disruption of the *lonB* gene did not confer any discernible sporulation phenotype to otherwise wild-type cells, nor did expression of *lonB* from a multicopy plasmid. In contrast, expression of a fusion of the *lonB* promoter to the *lonA* gene severely reduced expression of the σ^G-dependent *sspE* gene and the frequency of sporulation. In confirmation of earlier observations, we found elevated levels of σ^F-dependent activity in a *spoIIIE47* mutant, in which the *lonB* region of the chromosome is not translocated into the prespore. Expression of either *lonB* or the P_{lonB}-*lonA* fusion from a plasmid in the *spoIIIE47* mutant reduced σ^F -dependent activity to wild-type levels. The results suggest that both LonA and LonB can prevent abnormally high σ^F activity but that only LonA can negatively regulate σ^G.

Sporulation in the rod-shaped bacterium *Bacillus subtilis* is initiated by an asymmetric division that produces a smaller prespore and a larger mother cell (11, 36, 49). Progress through the morphological stages of sporulation is governed by a cascade of four compartment-specific RNA polymerase sigma factors that appear in the order σ^F, σ^E, σ^G, and σ^K (11, 28, 49). The first compartment-specific sigma factor, σ^F, initiates the prespore-specific program of gene expression and is replaced by σ^G in this sporangial chamber at later stages of development (16, 19, 24, 29, 34, 49). Conversely, the mother cell-specific line of gene expression is initiated by the activation of σ^E, which is later replaced by σ^K (3, 4, 6, 60). σ^F is synthesized prior to the formation of the sporulation septum, together with three other proteins, SpoIIAA, SpoIIAB, and SpoIIE, required for its prespore-specific activation (13, 14, 57). SpoIIAB is an anti-sigma factor that binds to σ^F and holds it inactive in the predivisional cell and in the mother cell compartment of the sporulating cell (9, 31). SpoIIAA is an anti-anti-sigma factor, which can bind to and counteract SpoIIAB, releasing active σ^F (1, 5, 8, 31). SpoIIAB is also a serine protein kinase that can phosphorylate SpoIIAA, and phosphorylated SpoIIAA is unable to bind to SpoIIAB (1, 5, 9, 31). The third protein, SpoIIE, is a membrane-bound serine phosphatase that can dephosphorylate SpoIIAA (7, 12). Dephosphorylation of SpoIIAA by the SpoIIE phosphatase occurs preferentially in the prespore chamber, promoting the binding of SpoIIAA to SpoIIAB and the prespore-specific activation of σ^F (20, 25), which in turn leads to the synthesis of σ^G in the prespore. However, σ^G is kept in an inactive form until the engulfment stage of sporulation (stage III), presumably as the result of direct binding by the SpoIIAB anti-sigma factor (19, 21). Activation of σ^G seems to require the proteolysis of SpoIIAB (19, 21). Once active, σ^G transcribes its own gene, allowing a rapid increase in the cellular concentration of σ^G. Because of its positive autoregulatory nature, σ^G synthesis and activity are subject to multiple levels of control that prevent the expression of genes unnecessary or even deleterious for nonsporulating cells as well as the premature expression of the σ^G regulon during development (19, 30, 38, 42, 43). For example, mutations in either the *lonA* gene, encoding a member of the Lon family of prokaryotic ATP-dependent serine proteases, or in *spoIIAB* permit inappropriate expression of σ^G -dependent genes under conditions that do not promote sporulation (38, 42). The *lonA* gene is induced in response to several stresses, such as salt, ethanol, and oxidative stress or heat shock, but its precise role in stress management has not been determined (39). *B. subtilis* also possesses a second Lon-like

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TABLE 1. *B. subtilis* strains used

Strain	Genotype and phenotype	Origin
MB24	*trpC2 metC3*	Laboratory stock
AH38	*leuA8 spoIIGB55* Spo$^-$	Laboratory stock
AH45	*trpC2 metC3 spoIIGΔ1* Spo$^-$	Laboratory stock
AH77	*trpC2 metC3* Δ*sigK*::*erm* Spo$^-$	Laboratory stock
AH392	*trpC2 metC3* Δ*amyE*::*spoIID-lacZ* Cm^r	Laboratory stock
AH524	*trpC2 metC3* SPβ*sspE -lacZ* Cm^r	Laboratory stock
AH969	*trpC2 metC3* Δ*amyE*::*spoIIIG-lacZ* Cm^r	Laboratory stock
AH2350	*trpC2 metC3* pMK3 Nm^r	This work
AH2351	*trpC2 metC* pMS56 Nm^r	This work
AH2356	*trpC2 metC3* Δ*amyE*::*lonB-lacZ* Cm^r	This work
AH2357	*trpC2 metC3 spoIIAC*::*erm* Erm^r	Laboratory stock
AH2358	AH2357 Δ*amyE*::*lonB-lacZ* Cm^r Erm^r	This work
AH2359	AH38 Δ*amyE*::*lonB-lacZ* Cm^r	This work
AH2360	AH45 Δ*amyE*::*lonB-lacZ* Cm^r	This work
AH2361	AH77 Δ*amyE*::*lonB-lacZ* Cm^r	This work
AH2368	*trpC2 metC3* pMS72 Nm^r	This work
AH2369	AH2368 Δ*amyE*::*spoIID-lacZ* Nm^r Cm^r	This work
AH2370	AH2368 SPβ*sspE-lacZ* Nm^r Cm^r MLS^r	This work
AH2372	AH2350 Δ*amyE*::*spoIID-lacZ* Nm^r Cm^r	This work
AH2373	AH2350 SPβ*sspE-lacZ* Nm^r Cm^r MLS^r	This work
AH2382	*spoIIIE47* Δ*spoIIIG*::*spec* Δ*amyE*::*lonB-lacZ* Spc^r Cm^r	This work
AH2383	*spoIIIE47* Δ*spoIIIG*::*spec* Δ*amyE*::*spoIIIG-lacZ* Spc^r Cm^r	This work
AH2385	AH2382 pMK3 Spc^r Cm^r Nm^r	This work
AH2386	AH2382 pMS56 Spc Cm^r Nm^r	This work
AH2387	AH2383 pMK3 Spc^r Cm^r Nm^r	This work
AH2388	AH2383 pMS56 Spc^r Cm^r Nm^r	This work
AH2389	AH2383 pMS72 Spc^r Cm^r Nm^r	This work
AH2421	AH2356 pMK3 Cm^r Nm^r	This work
AH2422	AH2356 pMS72 Cm^r Nm^r	This work
AH2423	AH969 pMK3 Cm^r Nm^r	This work
AH2424	AH969 pMS72 Cm^r Nm^r	This work
AH2425	AH2382 pMS72 Spc^r Cm^r Nm^r	This work
AH2426	AH2383 pMS76/ Spc^r Cm^r Nm^r	This work
AH2427	*trpC2 metC3 lonB*::*spc* pMS72 Spc^r Nm^r	This work
AH2433	*trpC2 metC3* pMS76 Nm^r	This work
AH2434	*trpC2 metC3* pMS94 Nm^r	This work
AH2435	*trpC2 metC3 lonB*::*lonB-lacZ* Cm^r	This work
AH2436	*trpC2 metC3 spoIIAC*::*erm lonB*::*lonB-lacZ* Erm^r Cm^r	This work
BSM105	*trpC pheA lonB*::*spec* Spc^r	This work
BSM110	*trpC pheA lonB*::pSH4 Cm^r	This work
BSM111	*trpC pheA spoIIAC*::*erm lonB*::pSH4 Cm^r Erm^r	This work
JH642	*trpC pheA*	Laboratory stock
MO512	*trpC pheA spoIIGB*::*erm* Erm^r	P. Stragier
MO1073	*trpC pheA spoIIAC*::*erm* Erm^r	P. Stragier

protease that has been implicated in posttranslational regulation of σ^H.

Since Lon proteases have already been shown to play a role in differentiation processes in other microorganisms (47, 52, 56), we decided to investigate their possible role in the regulation of compartment-specific gene expression during endospore development. We found *lonB* transcription itself to be compartmentalized during sporulation, dependent on σ^F, and hence restricted to the forespore compartment. *lonB* did not seem to interfere with the activities of either σ^F or σ^G in a wild-type strain. In contrast, in confirmation and extension of earlier results, we show that *lonA* can act specifically to reduce σ^G activity (but not that of σ^F) when expressed in the forespore in an otherwise wild-type strain.

MATERIALS AND METHODS

Bacterial strains, media, and general methods. *Escherichia coli* DH5α was used for routine cloning experiments. The *B. subtilis* strains used in this work are listed in Table 1. The wild-type strain MB24 (*trpC2 metC3*) and congenic derivatives bearing different *spo* alleles (Table 1) were used for the analysis of β-galactosidase production driven by various *lacZ* fusions. The efficiency of sporulation was determined 18 h after the onset of sporulation as described previously (18). Sporulation of *B. subtilis* was induced by growth and exhaustion in Difco sporulation medium (DSM) or by resuspension (2, 33). Antibiotics and 5-bromo-4-chloro-3-indolyl-β-galactosidase-D-galactopyranoside (X-Gal) were used as previously described (17, 18).

Construction of a *lonB* insertional mutation. A 2,090-bp DNA fragment containing the entire *lonB* coding sequence as well as 336 bp upstream of its start codon was generated by high-fidelity PCR with oligonucleotides *lonB*-61D (5′-CGCAAGACTGCAGCACGCGGACTCCG-3′) and *lonB*-2151R (5′-TAAAA CAGTCTCCTGCAGTAGTATACCC-3′). The amplified product was purified, doubly digested with *Xho*I and *Bgl*II, and cloned between the *Sal*I and *Bam*HI sites of pLITMUS 38 (New England Biolabs), yielding plasmid pMS58 (Fig. 1). Next, a spectinomycin resistance (Spc^r) determinant was obtained by PCR with pAH256 (17) as the template and the primers pAH256-sf2 (5′-CGAATCCAT GGCGCGCACCGTACGTC-3′) and pAH256-sr2 (5′-GAGACGTCACCATG GGAAGC-3′). After digestion with *Nco*I, the Spc^r cassette was inserted at the unique *Nco*I site within the *lonB* gene of pMS58, a step that produced pSH5. Competent cells of strain JH642 were transformed with *Sca*I-linearized pSH5, with selection for Spc^r cells. This cross-generated the *lonB* insertional mutant BSM105, which was shown by Southern blot hybridization to result from the integration of the plasmid into the chromosomal *lonB* region by a double-crossover (marker replacement) event.

Construction of transcriptional fusions of *lonB* to *lacZ* and *gfp*. The 2,090-bp DNA fragment generated by high-fidelity PCR with oligonucleotides *lonB*-61D and *lonB*-2151R (see above) was digested with *Eco*RI and *Sau*3AI, and a 353-bp fragment was isolated. This fragment was inserted into the *amyE* integrational plasmid pSN32 that had been cut with *Eco*RI and *Bam*HI (32), producing pMS64, which carries a *lonB-lacZ* transcriptional fusion (Fig. 1). Integration of *Sca*I-linearized pMS64 into the *amyE* locus of strain MB24 produced the chloramphenicol-resistant (Cm^r) AmyE$^-$ strain AH2356 (Table 1). Chromosomal DNA was prepared from this strain and used to transfer the *lonB-lacZ* fusion into various Spo$^-$ recipients by DNA-mediated transformation with selection for chloramphenicol resistance (Table 1). Plasmid pMS64 was then digested with *Eco*RI and *Sac*I to release a 2,356-bp fragment that was purified and inserted into *Eco*RI- and *Sac*I-digested pJM783 (35), creating pMS92. Plasmid pMS92 was used to transfer a *lonB-lacZ* transcriptional fusion to the *lonB* locus of a wild-type and a *sigF* host by a single reciprocal crossover (Campbell-type recombination) that created strains AH2435 and AH2436, respectively (Table 1). For construction of a transcriptional fusion of *lonB* to the *gfp* gene, a 423-bp PCR fragment containing the regulatory region of *lonB* was generated with the primers *lonB*-SH3 (5′-GAGAGCGGCCGCAACGGATTCTTTATTGATTTCG-3′) and *lonB*-SH2 (5′-GAGACCCGGGCAAGACTGGAGCACGC-3′). After digestion with *Not*I and *Sma*I, this PCR fragment was inserted into pFSB79, an *amyE* integrational plasmid with a promoterless *gfp* gene (44; F. Spiegelhalter and E. Bremer, unpublished data) that had been cut with the same enzymes. The resulting plasmid, pSH4, was introduced into the wild-type strain JH642 and its corresponding *sigF* mutant MO1073 by transformation followed by selection for chloramphenicol resistance (Table 1). Cm^r colonies of this transformation that had retained amylase activity were screened by PCR. Derivatives of the wild-type strain JH642 and the *sigF* mutant MO1073 that had pSH4 integrated into the *lonB* region by a Campbell-type integration were named BSM110 and BSM111, respectively.

Multicopy plasmids bearing different fragments from the *lonB* region. To produce a version of the *lonB* gene in a multicopy plasmid, the same PCR fragment used in the construction of pMS58 was cloned between the *Bam*HI and *Sal*I sites of pMK3 (50) to create pMS56 (Fig. 1A). The Quickchange protocol (Stratagene) was used to convert the lysine-encoding 27th codon of *orf61* (Fig. 1B) to the nonsense codon TAA, creating the pMS56 derivative pMS94. The whole *Bam*HI-*Sal*I insert in pMS94 was sequenced to ensure that no other mutations were fortuitously introduced by the mutagenesis protocol. A plasmid containing just the *lonB* regulatory region in pMK3 (pMS76 [Fig. 1A]) was constructed by inserting a 354-bp *Xho*I-digested PCR product obtained with the oligonucleotide primers *lonB*-61D (see above) and *lonB*-415R (5′-CCCTGTCC AACCATGGTGGTCCC-3), between the *Sma*I and *Sal*I sites of pMK3. A version of *lonA* fused to the *lonB* promoter was constructed as follows. The *lonA* gene was PCR amplified with primers *lonA*-286D (5′-GGAGGTGTCAGTCCA TGGCAGAAG-3′) and *lonA*-2798R (5′-GGCCAATGCGAATTCCGGAAGC CC-3′). The PCR fragment was digested with *Eco*RI and inserted into pUC18 that had been cut with *Sma*I and *Eco*RI, creating pMS65, in which an *Nco*I site overlaps the *lonA* initiation codon. The *lonB* promoter fragment was generated by PCR with primers *lonB*-61D and *lonB*-415R (see above), digested with *Nco*I

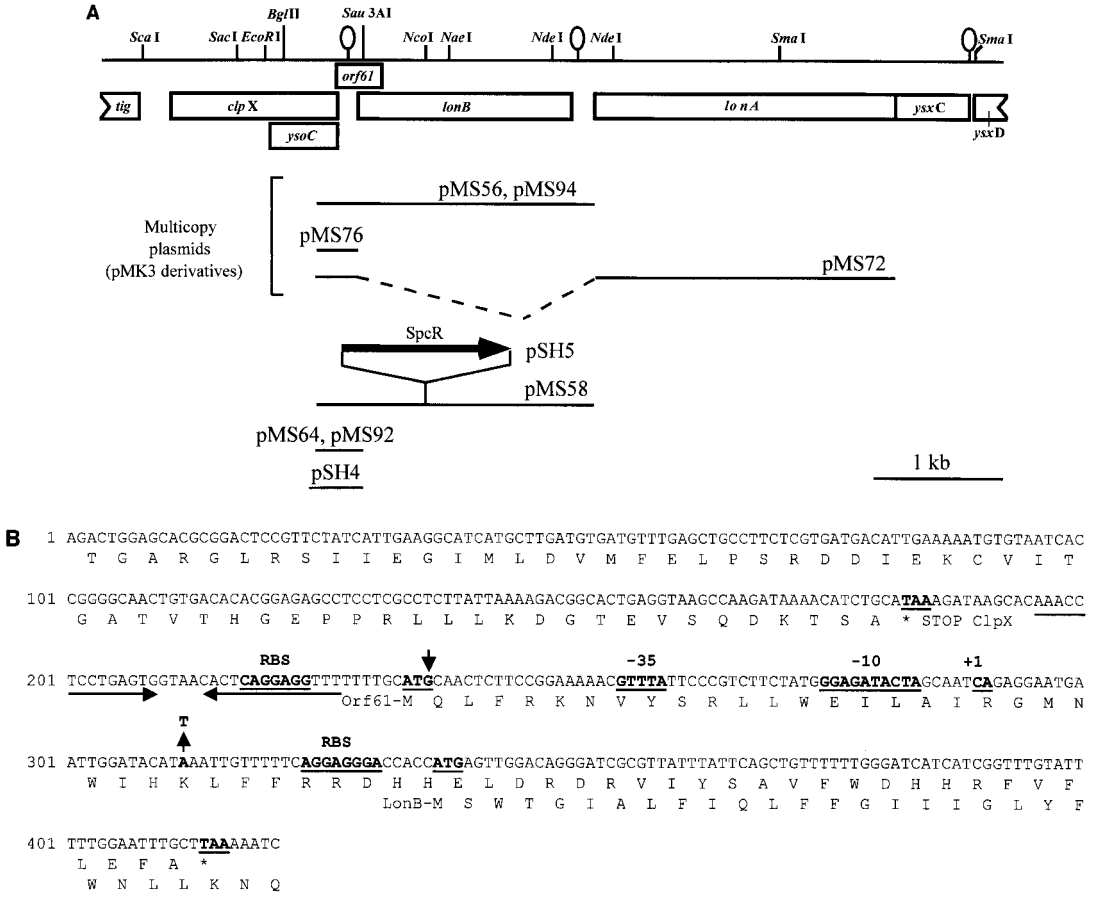

FIG. 1. *lonB* region of the *B. subtilis* chromosome and sequence of the *lonB* promoter region. (A) Partial restriction map and genetic organization of the *lonB* locus. The boxes below the restriction map indicate coding regions of the different genes in the region, as deduced from the analysis of the *B. subtilis* genome sequence (22). Note that *orf61* was not considered in the annotation of the *B. subtilis* genome sequence but has previously been suggested by Liu et al. (26). The stem-loop structures downstream of *clpX* and downstream of *lonB* and of *lonA* indicate the positions of possible transcription terminators. Lines below the restriction map depict DNA fragments cloned into the indicated plasmids. In pMS72, the dashed region was deleted to fuse the *lonB* promoter to the start codon of the *lonA* gene. Plasmid pMS94 carries the same insert as pMS56 but has a nonsense mutation at codon 27 of *orf61*. Plasmid pMS64 was used to transfer a *lonB-lacZ* fusion to the *amyE* locus, whereas pMS92 (which carries an identical insert) was used to transfer the fusion to the *lonB* locus by a single reciprocal crossover. pSH5 contains the same insert as pMS58, but the *lon* gene is disrupted by the insertion of an spc^r cassette. In plasmid pSH4, the regulatory region of *lonB* from −342 to +68 with respect to the ATG start codon was fused to a *gfp* gene lacking its own promoter.(B) Sequence of the *lonB* promoter region as well as the first 29 codons of the gene and the complete *orf61*. The sequence is the same as in plasmid pMS76, a multicopy plasmid that carries the *lonB* promoter region. Potential ribosome binding sites (RBS) as well as start and stop codons (bold and underlined) are indicated. The region of dyad symmetry downstream of *clpX* that may act as a transcriptional terminator is indicated by horizontal arrows (see above). The *lonB* transcriptional start site determined by primer extension analysis is underlined and labeled "+1" just downstream of −10 and −35 sequences that may be utilized by E σ^F (see text). The second weak but σ^F-independent signal observed in the primer extension analysis is indicated by the arrowhead above the G in the starting codon of *orf61*. The site of an A-to-T transversion that creates a nonsense mutation at codon 27 of *orf61* is also indicated.

(an *Nco*I site was introduced that overlapped the *lonB* start codon), and inserted between the *Nco*I and *Sal*I sites of pMS65. This ligation created pMS70. A fragment carrying the *lonB* promoter fused to the *lonA* gene was obtained from pMS70 by digestion with *Pst*I and *Eco*RI and inserted between the same sites of pMK3 (50), yielding pMS72.

RNA primer extension and Northern blot analysis. RNA was prepared by a modified acid phenol method (54) from cultures of a wild-type strain (JH642), a *sigF* mutant (MO1073), and a *sigE* mutant (MO512) at different times after resuspension in sporulation inducing medium as well as from a wild-type strain, JH642, which expressed either *sigB* or *sigF* under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter P_{spac} from plasmids pDG1481 and pSDA4, respectively (27, 48). The RNA (10 μg) was subject to Northern blot or primer extension analysis essentially as described before (41, 55). The probe for the Northern blot analysis was a 1,605-bp-long digoxygenin-labeled antisense RNA produced in vitro with T7 RNA polymerase from a PCR fragment internal to the *lonB* gene, generated with oligonucleotides *lonB*-SH1 (5′-ACAACGTTG AGCTTGAGTTTG-3′) and *lonB*-SH5 (5′-GAGATAATACGACTCACTATA GGGAGGTTCTTCAGCTATTCCT GTG-3′, which incorporates a T7 promoter at its 5′-end). For primer extension experiments, the oligonucleotide *lonB*-SH6 (5′-ATACAAACCGATGATGATCCCA-3′) was labeled at its 5′ end with [γ-^{32}P]ATP (3,000 mCi/mmol). A sequencing ladder was generated with the same oligonucleotide using plasmid pSH4 as the template.

Enzyme assays. β-Galactosidase activity was measured using the substrate *o*-nitrophenyl-β-D-galactoside as previously described (18, 45). The data reported in figures 2, 6, and 7 are derived from representative experiments that were repeated at least two times.

Fluorescence microscopy. Samples of cultures of a wild-type strain (BSM110) and its isogenic *sigF* mutant (BSM111) bearing a transcriptional *lonB-gfp* fusion integrated into the *lonB* region were collected about 2.5 h after the onset of sporulation in DSM. The samples were observed in a Zeiss fluorescence microscope using a 450-490/FT510/LP520 filter set. Images were recorded and processed for publication using Adobe Photoshop.

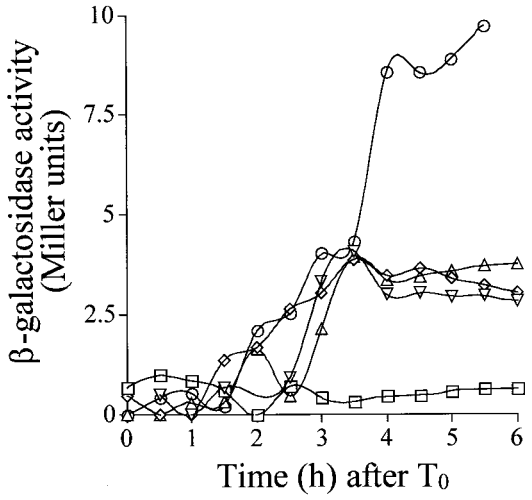

FIG. 2. Dependency of *lonB-lacZ* expression, illustrated by time courses of *lonB-lacZ*-driven β-galactosidase production in a wild-type strain (AH2356; inverted triangles) and in strains bearing deletion mutations of the following loci: *sigF* (AH2358; squares), *sigE* (AH2359; circles), *sigG* (AH2360; diamonds), and *sigK* (AH2361; triangles). Sporulation was induced by the resuspension method. Samples were collected every 30 min after the resuspension and onset of sporulation (T_0) and assayed for β-galactosidase activity. Enzyme activity is indicated in Miller units (see Materials and Methods). Background levels of enzyme activity in the wild-type strain MB24 were subtracted in all cases.

RESULTS

Transcription of *lonB* is under the control of σ^F. A wild-type strain (AH2356) and congenic derivatives with deletions of the genes encoding the four sporulation-specific sigma factors (Table 1) were induced to sporulate by growth and resuspension in a minimal sporulation medium (2, 33), and the formation of β-galactosidase from a *lonB-lacZ* transcriptional fusion inserted into the *amyE* locus was monitored during the course of sporulation. In a wild-type strain, *lonB-lacZ*-driven β-galactosidase production showed only background levels at the onset of sporulation. However, the enzyme levels increased sharply around 120 min after the initiation of sporulation, reaching a maximum level around hour 3 of sporulation (Fig. 2). Induction of *lonB-lacZ* transcription was prevented by deletion of the *sigF* gene but not by mutation of the gene coding for σ^E, σ^G, or σ^K (Fig. 2). A similar *lonB-lacZ* induction pattern was observed when the strains were induced to sporulate by growth and exhaustion in DSM (data not shown). Thus, during sporulation the σ^F form of RNA polymerase controls transcription of *lonB*. *lonB* did not seem to be transcribed by RNA polymerase carrying the other forespore-specific sigma factor, σ^G (Fig. 2), nor did its transcription require a functional copy of *spoIIGB*, which encodes the mother cell regulator σ^E. In both DSM and resuspension medium, *lonB-lacZ* transcription not only appeared to be independent of σ^E production but also showed a twofold increase in a *sigE* mutant (Fig. 2). This increase is likely to reflect the disporic phenotype of *sigE* mutants, in which σ^F is active in both prespore compartments of the sporangium (24).

***lonB* is transcribed during sporulation from a σ^F -dependent promoter.** The results described above suggested the location of a σ^F-dependent promoter, within the 336 bp preceding the *lonB* translational start site (Fig. 1A and B). A primer extension analysis with RNA prepared from wild-type cells at different times after the onset of sporulation revealed two major extension products, which corresponded to the adjacent nucleotides CA, 45 bp upstream of the *lonB* start codon (Fig. 1B and 3). Upstream from this position, we found sequences that strongly resembled the −10 and −35 regions recognized by σ^F in several other promoters (15). σ^F specificity of this promoter gained additional support when RNA isolated from *sigF* and *sigE* mutants at 90 min after resuspension was subject to primer extension analysis. Whereas no signal was observed when RNA from a *sigF* mutant was used, the utilization of RNA from the *sigE* mutant produced the same two major extension products observed in the wild type (Fig. 3). These extension products were also observed when σ^F was artificially produced during exponential growth in rich medium from the IPTG-inducible promoter P_{spac} in JH642 harboring the plasmid pSDA4 (Fig. 3). Thus, the CA dinucleotide at positions 333 and 334 (Fig. 1B and 3) is likely to represent the transcriptional start site for the σ^F-recognized promoter of *lonB*. σ^F -dependent expression of *lonB* during sporulation has independently been discovered by Piggot and coworkers (O. Amaya and P. Piggot, personal communication).

Liu et al. (26) reported the existence of a short open reading frame (*orf61*) which starts 103 bp upstream from the *lonB* start codon and overlaps by 25 codons the 5′ end of the *lonB* coding region (Fig. 1). However, the primer extension analysis presented above indicates that σ^F-dependent transcription during

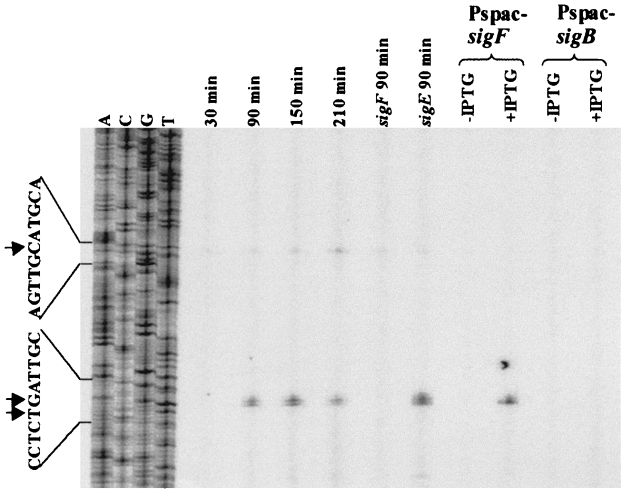

FIG. 3. Mapping of the 5′ end of the *lonB* transcript. Total RNA was isolated from wild-type strain JH642 (lanes 1 to 4) as well as from mutants lacking SigF (MO1073; lane 5) and SigE (MO512; lane 6) at different times after initiation of sporulation in resuspension medium. Additional RNA samples were prepared during exponential growth in rich medium (2×YT) from strain JH642 carrying plasmid pSDA4 or pDG1481, which allow production of active SigF (lanes 7 and 8) or SigB (lanes 9 and 10), respectively. Samples for lanes 7 and 9 were collected from cultures grown in the absence of the inducer IPTG, and samples were analyzed in lanes 8 and 10 were collected 30 min after IPTG addition to 1 mM. Primer extension was performed as described in Materials and Methods. The 5′ ends of the transcripts were determined by comparison with a DNA-sequencing ladder generated with the same primer and run in parallel on the same gel (lanes A, C, G, and T) and were labeled with arrowheads.

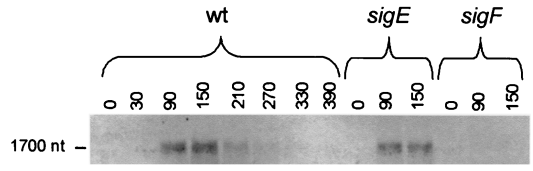

FIG. 4. Northern blot analysis of *lonB* transcription during sporulation. Sporulation of wild-type (wt) strain JH642 and the *sigE* and *sigF* mutants MO512 and MO1073, respectively, was induced by the resuspension method (see Materials and Methods). Total RNA was prepared from 10-ml samples collected immediately after resuspension and at the indicated times (in minutes) thereafter. The RNA samples were electrophoretically resolved on agarose-formaldehyde gels, vacuum transferred to neutral nylon membranes, and immobilized by UV cross-linking. The RNA blot was then probed with a 1,605-bp fragment internal to the *lonB* coding sequence, which was digoxigenin labeled. The position of the *lonB* transcript is indicated, as well as size estimation, based on the relative position of appropriate size markers. nt, nucleotides.

early sporulation did not include the complete *orf61* coding region.

A second, much weaker extension product that was present prior to the onset of σ^F activity was found to correspond to a G 101 bp upstream of the *lonB* translational start site. This position is just downstream of a region of dyad symmetry that could represent a transcription termination signal of *clpX*. Thus, the possibility exists that some *lonB* transcripts originate from a promoter upstream of *orf61*, perhaps in the *tig-clpX* region (Fig. 1A). In support of this view, we note that the β-galactosidase activity of a *lonB-lacZ* fusion observed prior to activation of σ^F was slightly higher when the fusion was integrated into the *lonB* region (strain AH2435 [data not shown]) instead of the *amyE* locus (Fig. 2). The suggestion that *clpX* and *lonB* are cotranscribed prior to the asymmetric division of sporulation is compatible with previous work implicating LonB in the regulation of σ^H activity at the entry into the stationary phase of growth (26).

The σ^F-dependent *lonB* transcript is monocistronic and *lonB-gfp* expression is confined to the prespore compartment. Analysis of the 5′ ends of the *lonB* transcript showed that during sporulation *lonB* is transcribed mainly from a single promoter that is utilized by the σ^F form of RNA polymerase and that *orf61* is not part of that transcript (Fig. 1 and 3; see above). It further indicated that transcripts originating upstream from *lonB* could accumulate in the predivisional cell, prior to the activation of σ^F. Northern blot analysis was performed to establish whether *lonB* was cotranscribed with its flanking genes (*clpX* and *lonA*) during sporulation. Probing of blots of RNA samples prepared from sporulating cells of the wild-type strain JH642 and of the *sigE* (MO512) and *sigF* (MO1073) mutants with a *lonB*-specific probe revealed a single specific signal that corresponded to a message size of about 1,700 nucleotides (Fig. 4). The size of the transcript is in agreement with the distance between the *lonB* transcriptional start site and the stop codon of the gene (1699 bp). In consonance with the analysis of *lonB-lacZ* expression in resuspension medium (Fig. 2), synthesis of the 1,700-nucleotide-long message could be detected 30 min after the initiation of sporulation and reached a maximum some 2 h later. Moreover, its synthesis was completely dependent on *sigF* but not on *sigE* expression (Fig. 4). The results of the Northern blot analysis were corroborated by DNA array experiments with RNA from sporulating wild-type bacteria and *sigF* or *sigE* mutants. In these DNA array analyses, *lonB* was independently discovered as a σ^F -dependent gene (L. Steil et al., unpublished data). While *lonB* displayed a strong *sigF*-dependent increase in expression, the genes flanking *lonB* (*clpX* and *lonA*) did not reveal such an induction during sporulation (Steil et al., unpublished).

Heat, ethanol, and other stresses induce the synthesis of the *lonA mRNA* from a σ^A-dependent promoter located between the two *Nde*I sites in front of the *lonA* gene (Fig. 1A and reference 39). To determine whether *lonB* might also respond to stress, RNA was isolated from the wild-type strain JH642 before and after exposure to 4% ethanol and subjected to slot blot analysis. These experiments failed to reveal any induction of *lonB* by ethanol stress (data not shown). Furthermore, production of active σ^B during exponential growth of JH642(pDG1481) by induction of the P_{spac} promoter with IPTG did not result in any significant induction of *lonB* (Fig. 3). Our results indicate that during sporulation *lonB* is transcribed mainly as a monocistronic message that includes neither *clpX-orf61* nor *lonA*. Together with those of Riethdorf et al. (39), our results further suggest that *lonA* and *lonB* respond to entirely different environmental cues.

To directly localize the cellular compartment of *lonB* transcription, we fused *lonB* to the *gfp* gene of *Aequorea victoria*, encoding the green fluorescent protein (GFP) (44, 53), and integrated the fusion into the *lonB* region of a wild-type strain and its isogenic *sigF* mutant. Samples of both strains were collected at various times after the onset of sporulation following nutrient exhaustion in DSM and analyzed by fluorescence microscopy. Whereas the *sigF* mutant strain (BSM111) did not display any signal (not shown), the green fluorescence that resulted from GFP accumulation was restricted to the forespore in the wild-type strain, BSM110 (Fig. 5). This fluorescence peaked about 3 hs after the onset of sporulation, consistent with the analysis of *lonB-lacZ* transcription in resus-

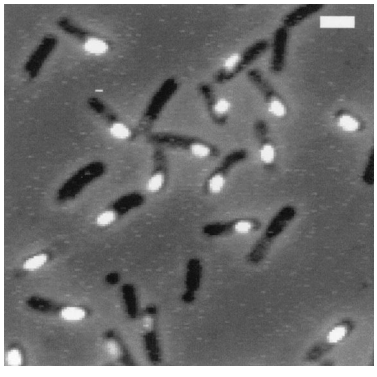

FIG. 5. Localization of *lonB-gfp* expression. A *lonB-gfp* transcriptional fusion was introduced into the *lonB* region of wild-type strain JH642. Bacteria were induced to sporulate in DSM, and samples were collected around 150 min after the onset of sporulation (defined as the end of the exponential phase of growth). The cells were mounted on a microscope slide without fixation and observed in a Zeiss fluorescence microscope in either phase-contrast or fluorescence mode. An overlay of the phase-contrast and fluorescence pictures is displayed. Bar = 2 μm.

TABLE 2. Effect of multicopy plasmids bearing *lon* alleles on sporulation

Strain	Plasmid	*lon* allele[a]	Sporulation (CFU/ml)		% Sporulation[b]
			Viable cells	Spores	
MB24		wt	7.01×10^8	8.09×10^8	100
AH2350	pMK3	wt	4.53×10^8	7.96×10^8	100
AH2351	pMS56	*lonB*	2.60×10^8	2.30×10^8	88
AH2433	pMS76	P_{lonB}	4.26×10^8	4.21×10^8	100
AH2368	pMS72	P_{lonB}-*lonA*	1.11×10^8	3.74×10^6	3
AH2427	pMS72	P_{lonB}-*lonA* (*lonB*::*spc*)	3.80×10^8	7.60×10^5	0.2
AH2434	pMS94[c]	*orf61*(K27Stop) *lonB*	1.7×10^8	1.65×10^8	97

[a] In all strains except AH2427, the chromosome contains the wild-type allele of the *lonB* gene.
[b] Defined as the ratio between the heat-resistant spore count and the total (viable) cell count.
[c] Similar to pMS56 but carrying a nonsense mutation in *orf61* (codon 27).

pension medium (Fig. 2) and in DSM (not shown). The failure of observing fluorescence in the mother cell compartment of wild-type cells or in *sigF* mutants proved that transcriptional readthrough into *lonB* from *clpX* contributed to a very minor extent to the overall expression of *lonB* during sporulation. We conclude that expression driven by the promoter upstream of *lonB* is restricted to the prespore compartment of sporulating cells.

Expression of *lonA* but not of *lonB* from a multicopy plasmid arrests sporulation at the engulfment stage. A comparison of wild-type strain JH642 and its isogenic *lonB* insertion mutant strain BSM105 revealed that the *lonB*::*spc* mutation did not impair the frequency of formation of heat-resistant spores at 37 or 50°C, nor did it significantly affect the expression of genes belonging to the different sporulation-specific regulons (data not show). The *lonB*::*spc* mutation is not expected to have a polar effect on *lonA* expression, since unlike a *lonA* mutant allele (42), it did not enhance σ^G-dependent gene expression in nonsporulating cells (data not shown).

Even though we failed to observe a sporulation phenotype upon its disruption, the *lonB* gene could still play a role in controlling the level of regulatory factors or proteins otherwise involved in morphogenesis. To explore these possibilities, we decided to overexpress the *lonB* gene, as well as several other *lon* alleles, from a multicopy plasmid (Fig. 1A and Table 2). We introduced the *lonB* gene (pMS56), its promoter region (pMS76), or the *lonB* promoter fused to the *lonA* gene (pMS72) into the multicopy plasmid pMK3 (50). To determine whether *orf61* had a role in the control of *lonB* activity, we also constructed a pMK3 derivative bearing a copy of the *lonB* gene in which a nonsense mutation was created at codon 27 of *orf61* (pMS94). These plasmids were introduced into competent cells of the wild-type host MB24. The strains bearing a multicopy *lonB* gene with or without a nonsense mutation in *orf61* or bearing the *lonB* promoter region were shown to sporulate with the same efficiency as the wild-type parental strain or the strain (AH2350) carrying the pMK3 vector (Table 2). However, cells harboring pMS72 (P_{lonB}-*lonA*; strain AH2368) were severely impaired in the ability to sporulate (Table 2), suggesting that *lonA* expression from the *lonB* promoter was interfering with a function essential for sporulation. Because of the previously characterized effect of *lonA* in preventing inappropriate σ^G-directed transcriptional activity (42), we hypothesized that expression of *lonA* in the prespore was similarly diminishing the ability of σ^G to utilize its cognate promoters. To test this hypothesis, we monitored the expression of reporter genes for σ^F, σ^E, σ^G, and σ^K activity in cells harboring pMS72 (P_{lonB}-*lonA*) or the parental plasmid pMK3. The presence of plasmid pMK3 had no noticeable effect on the expression of the different sporulation regulons (Fig. 6). In contrast, the forespore-specific expression of the P_{lonB}-*lonA* allele (pMS72) severely interfered with the ability of σ^G to utilize the *sspE* promoter (Fig. 6D). The decrease in *sspE-lacZ* transcription caused by pMS72 was not due to impaired transcription of *spoIIIG* in the presence of the multicopy P_{lonB}-*lonA* allele (Fig. 6B). Thus, it is likely that the forespore-specific expression of *lonA* from the *lonB* promoter interfered with the activity of σ^G. In addition, because σ^G is required for the activation of σ^K at late stages of development, expression of the σ^K-dependent *gerE-lacZ* fusion was also severely curtailed (not shown). The observation that the expression of *lonA* in the forespore interfered with σ^G activity suggested that one role of *lonB* could be to prevent the accumulation or activity of LonA in the forespore. However, this did not seem to be the case because expression of the P_{lonB}-*lonA* allele in the *lonB*::*spc* mutant (strain AH2427 [Table 2]) did not aggravate the sporulation phenotype of strain AH2368 (in which the multicopy P_{lonB}-*lonA* allele is propagated in a $lonB^+$ background [Table 2]). Moreover, a c-Myc epitope-tagged allele of *lonA* allowed the prompt detection of the epitope during growth and early sta-

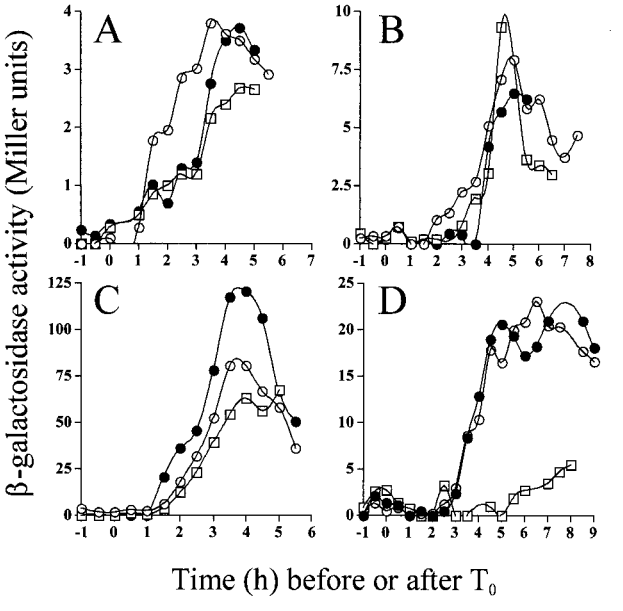

FIG. 6. Effect of expression of a P_{lonB}-*lonA* fusion (see text) in a multicopy plasmid on expression of *lonB-lacZ* (A), *spoIIIG-lacZ* (B), *spoIID-lacZ* (C), and *sspE-lacZ* (D) fusions during sporulation in DSM. Strains bearing the indicated fusions were transformed with the pMK3 vector (open circles) or with its derivative pMS72 (in which the *lonB* promoter was fused to the *lonA* gene; squares). Close circles represent expression of the different fusions in a wild-type strain without plasmids. Samples were collected every 30 min after the end of the exponential phase of growth (defined as the onset of sporulation) and assayed for β-galactosidase activity. Enzyme activity is expressed in Miller units (see Materials and Methods). Background levels of enzyme activity in the wild-type strain MB24 were subtracted in all cases.

tionary phase in 2xYT but not during sporulation in DSM, suggesting that the levels of LonA may be extremely low (data not shown). The results suggest that even in a multicopy situation, *lonB* could not interfere significantly with sporulation (Table 2), whereas *lonA* could reduce σ^G- but not σ^F-dependent activity when expressed in the forespore compartment (Table 2 and Fig. 6).

Both *lonA* and *lonB* can reduce σ^F-dependent activity in a *spoIIIE* mutant. In wild-type cells, both σ^F and SpoIIAB disappear at approximately equal rates from the two-sporangium compartments (23). However, in class I *spoIIIE* mutants, σ^F and SpoIIAB disappear at a much greater rate from the mother cell than from the prespore, causing the prevalence of cells with increased σ^F- and SpoIIAB-specific immunofluorescence signals in the prespore (25). Moreover, the transcription of several σ^F-dependent transcriptional fusions to *lacZ* has been shown to be higher in *spoIIIE* mutants than in wild-type cells (59). In those mutants, only about 30% of the chromosome centered around *oriC* is enclosed in the prespore, but σ^F activity correctly localizes to this compartment (59). The *lonB* locus is located at about 246° on the genetic map and is therefore outside the region of the chromosome present in the prespore compartment of a *spoIIIE* mutant (22). In agreement with this observation, we found no σ^F-dependent increase in the activity of a *lonB-lacZ* fusion integrated at the *lonB* locus in a *spoIIIE47* mutant (data not shown). In contrast, the integration of a similar fusion at the *amyE* locus of a *spoIIIE47* recipient (close to *oriC* at 8° on the genetic map) resulted in its σ^F-dependent induction (Fig. 7A; see also below). We also monitored expression of the σ^F-dependent *spoIIIG-lacZ* fusion (integrated at the *amyE* locus) in cells of a *spoIIIE47 spoIIIG*::*spc* double mutant. The *spoIIIG*::*spc* mutation was introduced to eliminate the contribution of σ^G (whose promoter specificity partially overlaps that of σ^F) to the transcription of certain σ^F-dependent promoters (51). The results in Fig. 7B confirm that as in the case of the *lonB-lacZ* fusion, *spoIIIG-lacZ* was overexpressed in the mutant compared to a congenic wild-type strain. To test the idea that the increased activity of σ^F in the prespore of *spoIIIE47* cells could be caused by the absence of the *lonB* gene product, we introduced pMS56 (which carries the *lonB* gene) in cells of the mutant. The results show that reintroduction of *lonB* into the prespore via a replicative plasmid restored expression of *lonB-lacZ* and *spoIIIG-lacZ* to wild-type levels (Fig. 7). Neither the pMK3 vector nor its derivative carrying the *lonB* promoter region (pMS76, which was analyzed only in the case of *spoIIIG-lacZ*), caused a similar effect (Fig. 7). Interestingly, the introduction of pMS72 (carrying the P_{lonB}-*lonA* allele) also restored wild-type levels of expression of the indicated fusions (Fig. 7). It should be noted that expression of *lonA* from the *lonB* promoter in a wild-type strain did not reduce expression of the σ^F-dependent *lonB*- and *spoIIIG-lacZ* fusions (Fig. 6A and B). Moreover, the introduction of pMS56 (multicopy *lonB* gene) in a wild-type strain did not significantly interfere with *spoIIIG-lacZ* expression (not shown). Therefore, it is unlikely that the reduction of *lonB-lacZ* and *spoIIIG-lacZ* activity caused by the introduction of multicopy alleles of *lonB* or *lonA* in the *spoIIIE47 spoIIIG*::*spc* double mutant was due to degradation of the reporter enzyme. The results suggest that in the *spoIIIE47* mutant both LonA and LonB can act to reduce the levels of σ^F-directed gene expression, even though neither LonA nor LonB appeared to interfere with σ^F-dependent transcriptional activity in otherwise wild-type cells.

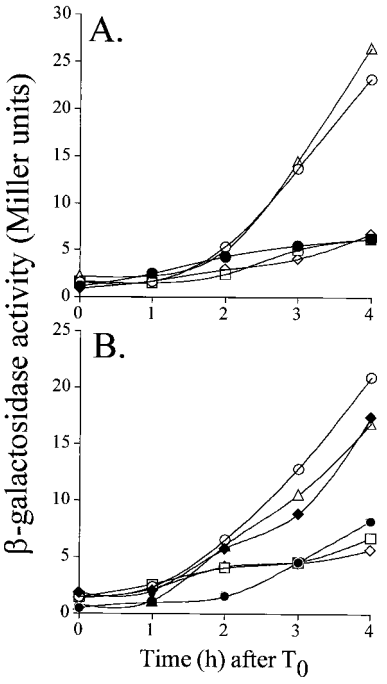

FIG. 7. Both *lonB* and *lonA* can negatively regulate σ^F activity in a class I *spoIIIE* mutant. Expression of *lonB-lacZ* (A) and *spoIIIG-lacZ* (B) was determined in a wild-type strain (closed circles), a *spoIIIE47* Δ*spoIIIG*::*spc* double mutant (open circles), or derivatives bearing plasmids pMK3 (triangles), pMS56 (squares), and pMS72 (diamonds). Expression of *spoIIIG-lacZ* in the *spoIIIE47* Δ*spoIIIG*::*spc* strain was also monitored in the presence of pMS76, which carries only the *lonB* promoter (B, close diamonds). Sporulation was induced in DSM, and samples were collected every 60 min after its onset (defined as the end of the exponential phase of growth) to assay for β-galactosidase activity. Enzyme activity is expressed in Miller units (see Materials and Methods). Background levels of enzyme activity in the wild-type strain MB24 were subtracted in all cases.

DISCUSSION

The results described herein show that the *lonB* gene of *B. subtilis* is transcribed during sporulation exclusively in the forespore compartment of the sporulating cell. Transcription of *lonB* is dependent on the expression of the *sigF* gene and likely occurs from a promoter directly recognized by σ^F, located just upstream from the *lonB* coding region. Transcription from this promoter results in the production of a monocistronic message which does not include the downstream *lonA* gene. This is of importance, as expression of *lonA* in the forespore compartment interfered with the activity (but not with the production) of σ^G and strongly impaired sporulation (Fig. 6 and Table 2). *lonB* belongs to a first temporal class of σ^F-dependent genes, which does not require the additional activity of σ^E in the mother cell for transcription. Moreover, transcription of *lonB* seems to be exclusively under σ^F control, with only a negligible contribution of σ^G (Fig. 2). Thus the *lonB*-encoded product is expected to be present in the forespore since its synthesis required the production and subsequent activation of

σ^F (16, 24, 29, 34, 49). The early prespore-specific transcription of *lonB* suggests that it may function early in the prespore developmental program. The observation that LonB can, under certain genetic conditions, act to reduce σ^F-dependent activity implies that it may be part of a feedback mechanism designed to keep σ^F activity within certain limits. If such a mechanism exists and is relevant for sporulation, then *lonB* must be redundant, because we failed to detect a phenotype associated with loss of LonB in wild-type cells. Alternatively, LonB could also contribute to the removal of σ^H and/or σ^E from the prespore compartment. An influence of LonB on restricting σ^E levels in the prespore seems unlikely since pro-σ^E did not accumulate in the prespore compartment of a *lonB* mutant (W. G. Haldenwang, personal communication). Moreover, σ^E activity correctly localizes to the mother cell compartment in a strain able to activate processing of pro- σ^E to its active form in the absence of σ^F (27, 61).

Two other proteins that appear to be specifically removed from the prespore compartment are the SpoIIE phosphatase and the SpoIIAB kinase, both by mechanisms that at least in part depend on *spoIIIE* function (25, 37). We did not specifically address this question here, but our results suggest that at least in the *spoIIIE47* strain, both LonB and LonA can reduce σ^F-dependent transcriptional activity. Interestingly, this reduction is only to a point where the levels of σ^F-dependent gene expression are comparable to those observed in a wild-type strain. Expression of *lonB* of the P_{lonB}-*lonA* allele in wild-type cells did not interfere with the levels of σ^F-directed gene expression, again suggesting that the capacity of LonA and LonB to act on σ^F is somehow regulated. Schmidt et al. (42) have shown that LonA can contribute to prevent inappropriate expression of σ^F activity under nutritional conditions that do not support efficient sporulation. In extension of these studies, we found that transcription of *lonA* in the forespore can significantly reduce σ^G-directed gene expression and the frequency of sporulation. Surprisingly, even though both proteases reduced σ^F-directed gene expression in the *spoIIIE47* mutant, only LonA appeared to be capable of interfering with σ^G activity during sporulation. Either LonB is not active at the time during sporulation when σ^G accumulates in the prespore or LonB differs from LonA in its substrate specificity at least toward σ^G. In this respect it is interesting that LonA and LonB particularly differ in their N-terminal regions, which consist of 250 and 78 residues, respectively. Recent reports have implicated residues in this N-terminal region of Lon in the discrimination between substrates. For example, a single amino acid change, of aspartate 240 to a lysine in LonA from *E. coli*, prevents it from interacting with its specific substrate RcsA but does not impair its ability to interact with and degrade the cell division inhibitor SulA (10). Mutant forms of the Lon protease from *Mycobacterium smegmatis* lacking its 277 N-terminal residues showed neither peptidase nor ATPase activity despite the fact that the deleted region included neither the catalytic serine residue (at residue 675) nor the ATP binding motifs (40). Shorter deletions (of 90 and 225 residues) resulted in proteins with peptidase activity against small unstructured peptides but severely impaired in their ability to degrade the protein substrates alpha-casein in vitro or RcsA in vivo (40). A different study has revealed a second region involved in substrate recognition in Lon and the Clp proteins (46). These proteins share a common design (see reference 46 and references therein) and have a homologous sensor and substrate discrimination domain of about 100 amino acids, located downstream of the ATPase domain. LonB, which lacks an extended N-terminal domain as well as the sensor and substrate discrimination domain, may need to interact with a second protein to form a functional protease; alternatively, the recognition of specific substrates by LonB may follow different rules. If LonB needs to associate with a second protein to form an active protease, then synthesis of this putative subunit may be under σ^F control. This putative polypeptide should be encoded by a gene located close to the *oriC* marker (58), because LonB was able to reduce σ^F-dependent gene expression in a *spoIIIE47* mutant. The substrate specificities of LonA and LonB have not been studied in detail and so far in *B. subtilis* include only σ^G and σ^H, respectively (26, 42; this work). More detailed studies will be necessary to extend the range of in vivo substrates for both proteases and to unravel their role in *B. subtilis*.

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