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# *Bacillus* Sporulation Gene *spo0H* Codes for $\sigma^{30}$ ($\sigma^{H}$)

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**The DNA sequences of the *spo0H* genes from *Bacillus licheniformis* and *B. subtilis* are described, and the predicted open reading frames code for proteins of 26,097 and 25,447 daltons, respectively. The two *spo0H* gene products are 91% identical to one another and about 25% identical to most of the procaryotic sigma factors. The predicted proteins have a conserved 14-amino-acid sequence at their amino terminal end, typical of sigma factors. Antibodies raised against the *spo0H* gene product of *B. licheniformis* specifically react with RNA polymerase sigma factor protein, $\sigma^{30}$, purified from *B. subtilis*. We conclude that the *spo0H* genes of *B. licheniformis* and *B. subtilis* code for $\sigma^{30}$, now known as $\sigma^{H}$.**

Sporulation is one of several biological responses of the bacilli and other microorganisms to an environment poor in nutrients. It is an ordered process with early, middle, and late functions. The transition of cells from the vegetative state of growth to a differentiated or specialized condition is a basic property of all higher organisms as well as many microorganisms, and for this reason interest has centered on the initiation of sporulation and upon the functions of the *spo0* genes which control the early processes. There are seven known *spo0* genes, of which five, *spo0A*, *B*, *E*, *F*, and *H*, have been cloned and sequenced (4, 8, 10, 11, 19, 29, 38, 44), yet nothing is understood about their functions. Recently, Ikeuchi et al. (20) have shown that the *spo0A* gene codes for a DNA-binding protein. Regulation studies with *lacZ* fusions have revealed that although *spo0* genes are expressed during vegetative growth, they exhibit different patterns of expression during growth and sporulation and exhibit different epistatic dependencies. The expression of the *spo0A* and *spo0F* genes increases at $T_0$ (defined as the time at which the cells enter stationary phase), and full expression is dependent upon the presence of functional *spo0B*, *A*, *E*, *F*, and *H* (43). *spo0B* is expressed maximally during vegetative growth (4, 10), and its expression is not altered when the cells are grown in glucose. *spo0H* expression (7) shows a slight rise at or before $T_0$ and is dependent upon the *spo0A* gene product.

The nucleotide sequences of the *spo0A* and *spo0F* genes have revealed (38, 44) that the N-terminal portions of their predicted protein sequences have strong homologies with each other as well as with an N-terminal domain found in several proteins which are involved with sensing environmental stimuli (25, 42). Therefore, these *spo* gene products are thought to function as part of an environmental sensory system of *Bacillus subtilis*.

The *spo0H* gene, whose activity is required for full expression of several *spo0* genes, all late *spo* genes, *com* (competence) genes (1), and the *aprE* (alkaline protease) gene (9) have been proposed to be positive regulatory factors (45). In this paper we will show that the *spo0H* gene codes for the RNA polymerase $\sigma^{30}$ ($\sigma^{H}$), described by Carter and Moran (5).

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## MATERIALS AND METHODS

**Bacterial strains and plasmids.** Plasmid pIS9A (8) contains the *Bacillus licheniformis spo0H* gene on a 1.2-kilobase (kb) *Bgl*II restriction fragment cloned into the plasmid vector pBD97 (14). pIS9A was restricted with *Hin*dIII and religated to generate pIS26, a *spo0H* deletion derivative missing a 356-base-pair (bp) *Hin*dIII fragment from within the *spo0H* gene (see Fig. 2B). From the derived amino acid sequence of *spo0H* (see below), pIS26 is predicted to encode a truncated *spo0H* gene product of 10,765 daltons. Plasmid pIS89 was constructed by a *Bcl*I digestion and religation of pIS9A, which deleted a 667-bp *Bcl*I restriction fragment from the 3′ end of the *spo0H* gene (see Fig. 2B). pIS89 is predicted to encode a 17,623-dalton truncated *spo0H* polypeptide. Isogenic sets of *B. subtilis trpC2 pheA1* strains bearing either the *spo0H75* (17) or the *spo0HΔHin*d (41) mutation were constructed which contained the plasmids described above.

Plasmid pIS118 is pUC18 containing a 1,439-bp *Ava*I-*Pst*I DNA fragment containing the *B. subtilis spo0H* gene (41).

**Recloning the *B. subtilis spo0H* gene and its upstream regulatory sequences.** We previously reported the cloning and characterization of the *B. subtilis spo0H* gene (41). Analysis of the *spo0H* clone revealed the presence of rearranged DNA sequences near its 5′ end which disrupted the *spo0H* promoter region. To reclone the intact *spo0H* gene and its 5′-flanking sequences from the *B. subtilis* chromosome, we followed the strategy outlined below.

Plasmid pIS133 was constructed by inserting a 700-bp *Hin*dIII-*Pst*I restriction fragment from pIS11A (41), containing the 3′ half of the *B. subtilis spo0H* gene, into the polylinker of pSP65 (Promega Biotec) and subsequently inactivating the *Pst*I site with T4 DNA polymerase (23). We have previously shown that the *Pst*I site is not essential for *spo0H* function and presumably is 3′ distal to the *spo0H* gene. pCP115 (31) is a pBR322 derivative bearing a chloramphenicol resistance ($Cm^r$) determinant which can be expressed in *B. subtilis* but is unable to replicate in this organism. pIS133 and pCP115 were each digested with *Eco*RI, ligated, and used to transform *E. coli* cells to ampicillin resistance ($Amp^r$). The product was plasmid pIS136, which carries a 600-bp *spo0H*-containing *Eco*RI fragment from pIS133 at the *Eco*RI site of pCP115 oriented

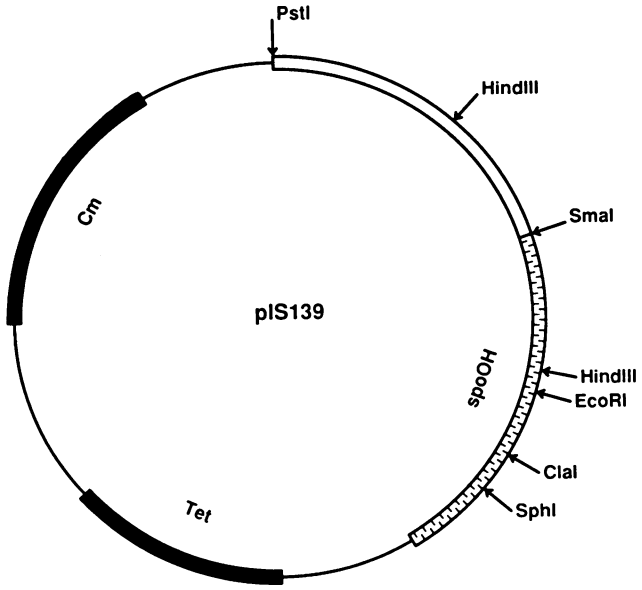


FIG. 1. Structure of pIS139. This plasmid contains the entire *B. subtilis spo0H* gene. The region from the *Pst*I through the *Sph*I site was cloned as described in Materials and Methods. The stippled segment from the *Sma*I site through the *Sph*I site was sequenced as described below. The rest of the plasmid is derived from pCP115. The plasmid is 6.1 kb in size.

so that the *spo0H* gene and the tetracycline resistance (Tet$^r$) gene of pBR322 are transcribed in the same direction.

pIS136 was integrated into the *B. subtilis* chromosomal *spo0H* locus by transforming cells of a Rec$^+$ *spo0H*$^+$ strain for Cm$^r$. Since pIS136 can not replicate in *B. subtilis*, any Cm$^r$ transformants obtained must result from homologous recombination between *spo0H* sequences present in pIS136 and those in the chromosome. A Cm$^r$ transformant (which remained Spo$^+$, as would be expected if the 600-bp *Eco*RI DNA fragment contained the intact 3′ end of the *spo0H* gene) was purified and designated IS418. Chromosomal DNA prepared from IS418 was capable of simultaneously transforming a *spo0H75* strain to Cm$^r$ and Spo$^+$ with 96% linkage.

To retrieve the *spo0H* gene and its 5′-flanking sequences from IS418, chromosomal DNA was restricted with *Pst*I, religated, and used to transform *Escherichia coli* cells to Tet$^r$. We had previously shown that the entire *spo0H* gene resided on a 2.5-kb *Pst*I chromosomal fragment (J. Weir and I. Smith, unpublished results). By cutting the chromosome of IS418 with *Pst*I and self ligating we expected to obtain a DNA fragment containing the *spo0H* gene (with the nonessential *Pst*I site at its 3′ end inactivated) and all of pCP115 to the *Pst*I site in the Amp$^r$ gene. A 6.1-kb plasmid, pIS139 (Fig. 1), was isolated from a purified transformant and found to carry a 2.5-kb insert from the *B. subtilis* chromosome. Restriction analysis confirmed that the insert carries DNA sequences with restriction sites identical to those of the *spo0H* gene of pIS11A, and, in addition, contains approximately 1 kb of DNA upstream of the *spo0H* gene. Southern blot hybridizations showed that the 2.5-kb fragment was comprised of contiguous, nonrearranged chromosomal DNA sequences (data not shown).

pIS144, a bifunctional plasmid capable of replicating in both *B. subtilis* and *E. coli*, was constructed by joining pIS139 and pE194 at their unique *Pst*I sites. pIS144 can complement the *spo0H75* mutation in *trans*.

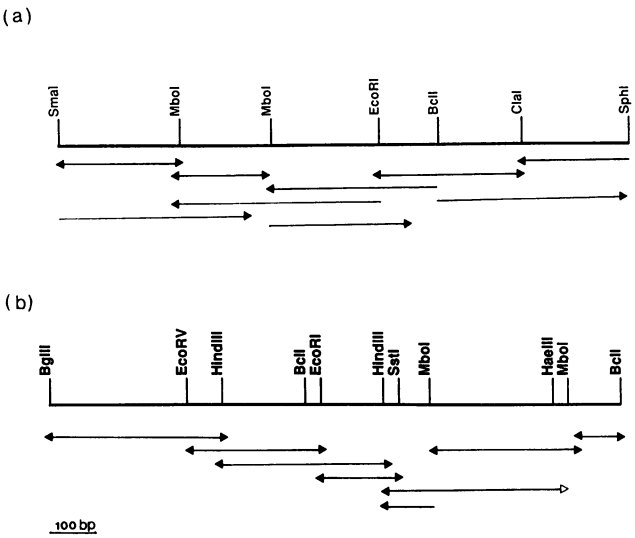


FIG. 2. Sequencing strategy for *spo0H* genes of *B. licheniformis* and *B. subtilis*. Subclones of the *B. licheniformis spo0H* gene were prepared from pIS9A (8), and subclones of the *B. subtilis spo0H* gene were prepared from pIS118 and pIS139 (see Materials and Methods). The arrows represent the direction of sequence read and the restriction sites used in the subcloning. The empty arrow head indicates that the sequence was read from a double-stranded plasmid by using the M13 reverse sequencing primer. This method was used when difficulty was encountered obtaining the clone in one of the orientations. The *B. licheniformis* gene was sequenced in both directions, except for about 100 bp from the *Bgl*II site. That had been sequenced previously (32), and we found no differences with that sequence. All restriction sites were crossed except the last *Mbo*I site in the 3′ end, which is beyond the open reading frame. The *Sma*I site through the first *Mbo*I site of the *B. subtilis* gene, which contains the promoter site and upstream regions, were subcloned from pIS139, and the rest was subcloned from pIS118. A *Sma*I-*Eco*RI subclone from pIS139 was made in mp18, and the sequence was determined through the first *Mbo*I site; this was the same as the sequence obtained from the pIS118 subclone. The *Eco*RI-*Cla*I fragment from pIS139 and pZOH13Δ*Hin*dIII (obtained from J. Healy and R. Losick) was also subcloned and sequenced. The gene was sequenced fully in both directions except for about 100 bp at the 3′ end of the gene, which was done in only one direction and which is well past the open reading frame, and all restriction sites were crossed.

**DNA sequencing.** The restriction fragments to be sequenced were cloned from pIS118 and pIS139 into appropriate cloning sites in mp18 and mp19 vectors derived from bacteriophage M13 (40). DNA sequencing was performed by the dideoxy chain termination method (33). The sequencing strategy for the *B. subtilis* and *B. licheniformis spo0H* genes is illustrated in Fig. 2.

The sequence of the *Eco*RI-*Cla*I fragment from pIS118 differed from that derived from pIS139, a plasmid which contains a *spo0H* DNA fragment derived independently from that of pIS118. At 795 bp the sequence from pIS118 reads AAT, whereas the sequence from pIS139 reads AAAT. The latter sequence predicts a protein which is completely identical to the C-terminal end of the *B. licheniformis spo0H* gene (see below) terminating 25 codons downstream from bp 795 to 797, whereas the sequence from pIS118 contains a termination signal 9 codons from bp 795 to 797, which would result in a *B. subtilis spo0H* protein with a predicted molecular weight of 23,442. The predicted molecular weight of the *B. licheniformis* protein is 26,097, and results described below show that the *spo0H* gene products

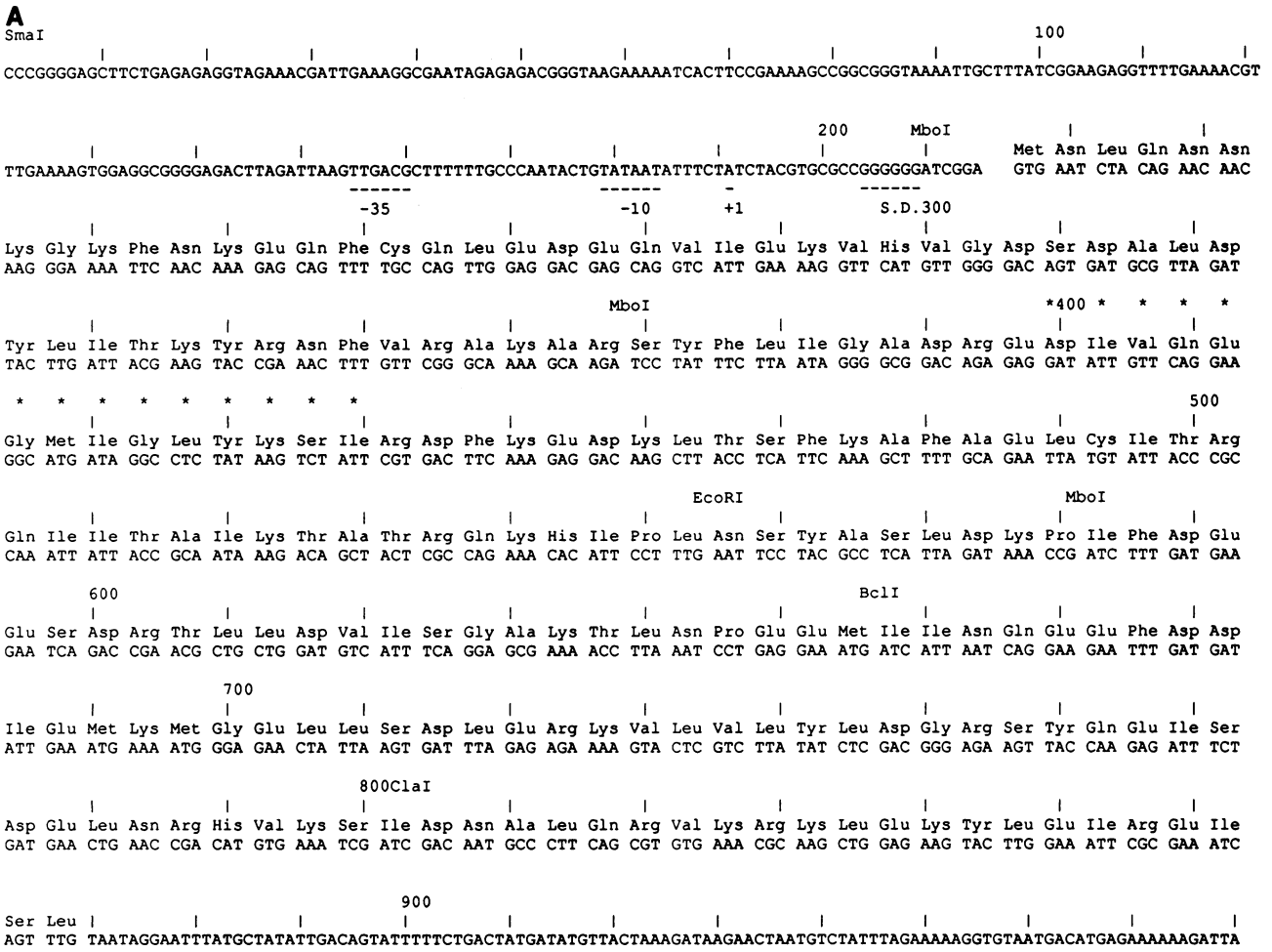


FIG. 3. DNA sequence of the *spo0H* gene of (A) *B. subtilis* and (B) *B. licheniformis*. The coding strand of the nucleotide sequence is shown as well as the single open reading frame; the 14-amino-acid sequence highly conserved in all known sigma factors is indicated with asterisks (*). The presumed ribosome-binding site (GGGGGG) and the promoter region (position −10, TATAAT; position −35, TTGACG) are underlined. A few relevant restriction sites are indicated.

of both bacilli are of similar size. To confirm that the sequence derived from pIS139 was correct, we sequenced the *Eco*RI-*Cla*I fragment from pZOH13Δ*Hin*dIII (kindly provided by J. Healy and R. Losick), which contains the 3′ portion of another independently derived *spo0H* clone. The sequence of this fragment was identical to that obtained from pIS139.

**Immunological techniques.** The generation of antiserum against a *B. licheniformis spo0H-lacZ* fusion protein has been described previously (34). Antiserum was enriched for spo0H-specific antibodies by one of the following methods.

(i) Proteins of a total cell lysate from the *E. coli lac* deletion strain MC1060 (6) carrying the plasmid pBD246 (15) were coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals) according to the directions of the manufacturer. pBD246 encodes an *ermC-lacZ* fusion protein. Antiserum was passed through the column to adsorb out antibodies specific for β-galactosidase or other *E. coli* proteins. The flow-through material is referred to as Spo0H-enriched antiserum.

(ii) Spo0H-specific antibodies were blot affinity purified from immunoblots by the protocol of Olmsted (27). A protein extract from a *B. subtilis trpC2 pheA1 spo0HΔHin*d strain carrying pIS9A was prepared, electrophoresed through a sodium dodecyl sulfate-polyacrylamide gel, and transferred to nitrocellulose paper (37). The blot was incubated with anti-*spo0H-lacZ* fusion protein antiserum and washed thoroughly. Bound antibody was localized by subsequent treatment of strips from the left and right ends of the blot with goat anti-rabbit immunoglobulin G gold conjugate (Bio-Rad Laboratories). Antibodies bound to the 27-kilodalton *spo0H* protein were eluted from the center of the blot with 0.2 M glycine (pH 2.8).

Protein blotting and immunodetection techniques were performed as described by Blake et al. (3) with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G antibodies (Calbiochem).

**RNA polymerase purification and assay.** $\sigma^{30}$-containing RNA polymerase was purified from cells of *B. subtilis* MLI, containing a *sigB* deletion, as previously described (5). The final step in the purification involved fractionation of RNA polymerase by chromatography through a calf thymus DNA-cellulose column with a 60-ml linear gradient of KCl (0.4 to 1.0 M). Fractions were collected and assayed for transcriptional activity in mixed-template competition reactions (5). Plasmids pCB1291, containing the *spoVG* promoter (24) and pUC31, containing the *ctc* promoter (36), were linearized

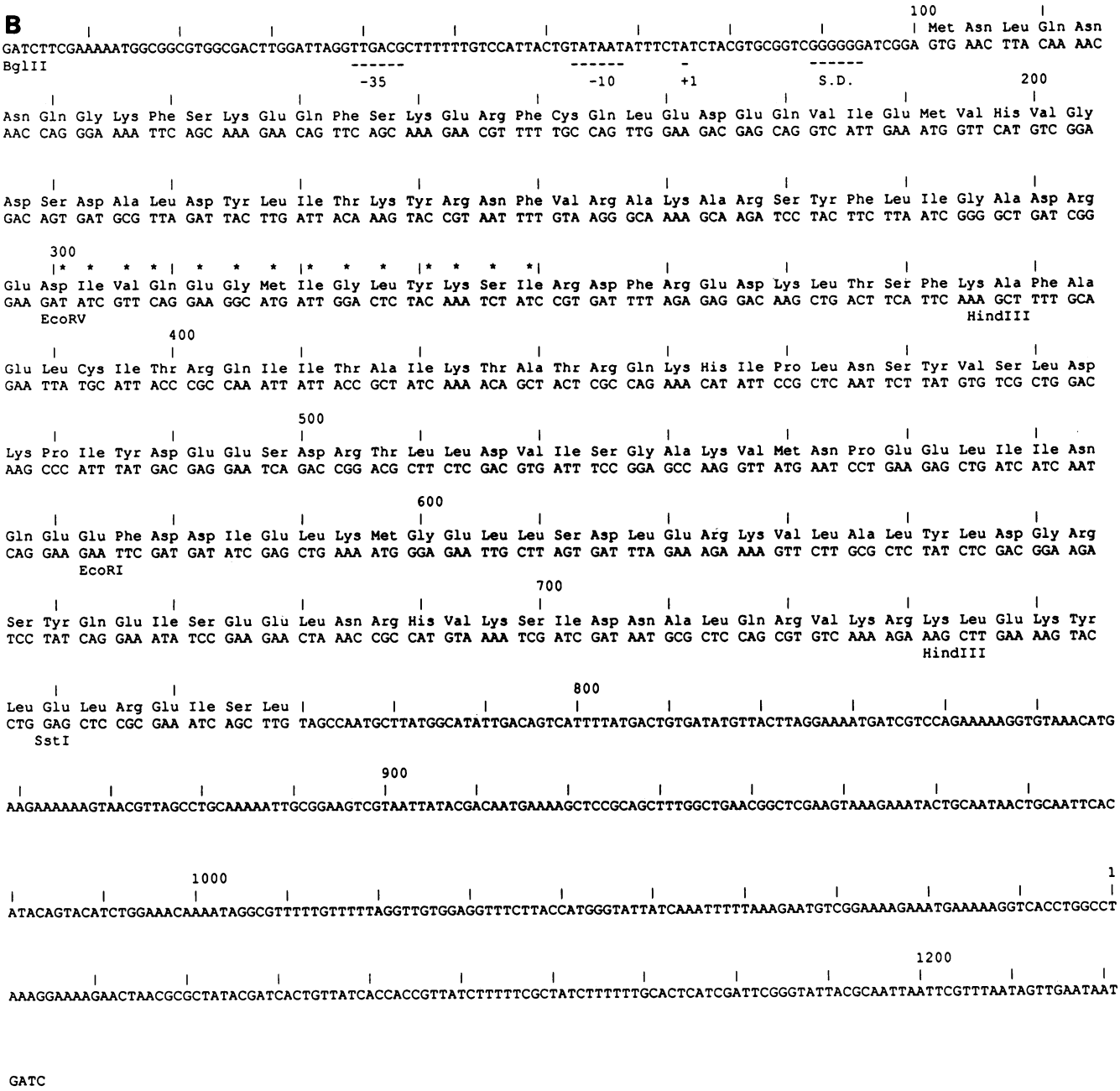


FIG. 3. *Continued*

with *Eco*RI and *Hin*dIII, respectively, to serve as DNA templates in these reactions.

## RESULTS

**DNA sequence of the *B. subtilis spo0H* gene.** The *B. subtilis spo0H* gene which we had originally cloned (41) did not include its promoter. A new clone containing the entire *spo0H* gene was constructed (see Materials and Methods) that could fully complement in *trans* a strain containing a deletion in the *spo0H* gene. This 2.5-kb DNA fragment, unlike the previous cloned fragment, had no DNA rearrangements detectable by Southern blot hybridization (data not shown) and contained 1 kb of DNA upstream of the region required to specify the 1,300-base *spo0H* mRNA observed in Northern hybridization experiments (41). It was presumed to contain all necessary sequences for promoter activity as well as regulatory sequences. Restriction fragments from pIS139, which contains the 2.5-kb fragment with the *B. subtilis spo0H* gene insert, and from pIS118, which contains the *B. subtilis spo0H* gene without its promoter, were used for the construction of M13 clones needed to sequence the gene (Fig. 2A). These clones were also used in recombinational crosses to determine that mutations *spo0H81* (17) and *spo0H12* (obtained from J. Hoch) lie between the *Mbo*I site at 210 bp and the *Hin*dIII site at 460 bp, a region which

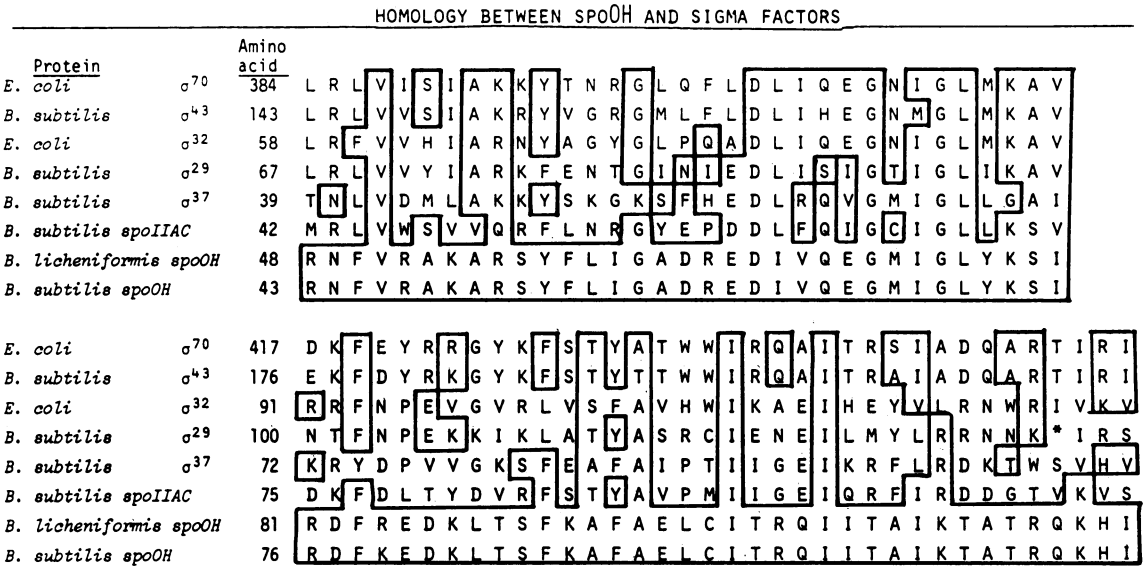


FIG. 4. Homology among procaryotic sigma factors. The boxed areas indicate positions at which the amino acids of the *spo0H* proteins are identical or chemically equivalent to one or more of the indicated sigma factors. Amino acids are identified by the single-letter code. The position of the first residue in each line of the proteins is indicated by the column of numbers.

includes the presumed core binding domain (see below). The mutations *spo0H75* (17) and *spo0H17* (30) lie between the *Eco*RI site at 460 bp and the *Bcl*I site at 660 bp.

We also determined the nucleotide sequence of *spo0H* from *B. licheniformis*, since recent studies with *B. licheniformis spo0H-lacZ* fusions (7) indicated that our previously published *spo0H* sequence (32) was wrong. Subclones of the *B. licheniformis spo0H* gene from plasmid pIS9A were prepared in mp18 and mp19 before sequencing (Fig. 2B). We discovered several errors in our published sequence of the *B. licheniformis* gene (32), which resulted in a completely different open reading frame from the one obtained with the new sequence.

The *B. subtilis* sequence (Fig. 3A) indicated a single long open reading frame coding for a protein of 218 amino acids (25,447 daltons), and the equivalent long open reading frame of the *B. licheniformis* sequence coded for a protein of 223 amino acids (26,097 daltons). The amino acid sequences of the *B. subtilis* and *B. licheniformis spo0H* proteins were 91% identical, and their DNA sequences were 75% identical.

TABLE 1. Sigma factor amino acid sequence homologies

| Sigma factor | Compared with *B. subtilis* $\sigma^{29}$ | | Compared with *B. subtilis spo0H* protein | |
|---|---|---|---|---|
| | % Identity | Optimized score[a] | % Identity | Optimized score |
| *B. subtilis* $\sigma^{29}$ ($\sigma^{E}$) | 100 | 1,168 | 23 | 145 |
| *E. coli* $\sigma^{32}$ | 45 | 200 | 25 | 113 |
| *B. subtilis* $\sigma^{43}$ ($\sigma^{A}$) | 33 | 175 | 22 | 126 |
| *B. subtilis* $\sigma^{37}$ ($\sigma^{B}$) | 30 | 124 | 31 | 108 |
| *B. coli* $\sigma^{70}$ | 29 | 161 | 22 | 113 |
| *B. subtilis spoIIAC* | 30 | 208 | 27 | 115 |
| *B. licheniformis spo0H* | 25 | 145 | 91 | 985 |
| *B. subtilis spo0H* | 23 | 145 | 100 | 1,051 |

[a] FASTP analysis according to the FASTP algorithm of Lipman and Pearson. The optimized scores were calculated with a $K_{tup}$ (defined in reference 21) of 1 for the *B. subtilis spo0H* protein and a $K_{tup}$ of 2 for the *B. subtilis* $\sigma^{29}$.

The amino acid sequence of the protein coded for by either gene showed a 14-amino-acid sequence (indicated by asterisks in Fig. 3) which is highly conserved in most procaryotic sigma factor proteins and is thought to be a core binding domain (12, 35). This conserved amino acid sequence, over a more extended part of the N-terminal region, is shown for a number of sigma factors (Fig. 4). Using the FASTP comparison algorithm (21), we found that the *spo0H* proteins of *B. licheniformis* and *B. subtilis* demonstrated significant amino acid sequence homology with several procaryotic sigma factors (Table 1). This homology is similar to that shown by $\sigma^{E}$ (formerly $\sigma^{29}$) of *B. subtilis* to the other sigma factors (Table 1) and extends over the whole length of the proteins (Fig. 5 compares the entire *B. subtilis spo0H* protein sequence with that of $\sigma^{E}$). In addition to the conserved core binding domain, both proteins have a region similar to that contained in many DNA-binding proteins (28). In the *B. licheniformis* sequence this region is centered at Gly-184 (bp

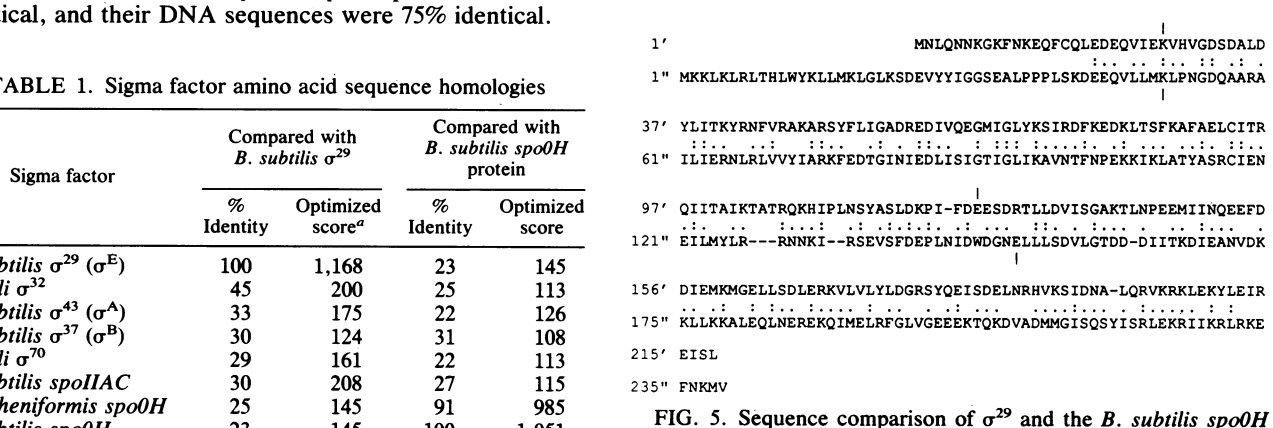


FIG. 5. Sequence comparison of $\sigma^{29}$ and the *B. subtilis spo0H* proteins. The complete amino acid sequences of the *B. subtilis* $\sigma^{29}$ ($\sigma^{E}$) and *spo0H* proteins are displayed. Two dots indicate identity, and one dot signifies chemical equivalence according to Lipman and Pearson (21). The top sequence shown is the *spo0H* protein amino acid sequence, and the bottom is the $\sigma^{29}$ amino acid sequence.

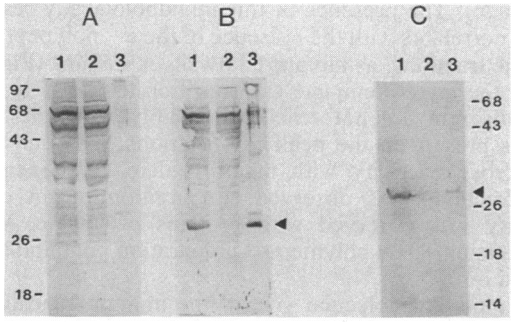


FIG. 6. Detection of $\sigma^{30}$ with affinity-purified Spo0H-specific antibodies. Total protein extracts from cultures of *B. subtilis spo0HΔHind* strains carrying pIS9A (lane 1) or pBD97 (lane 2). prepared as described in the legend to Fig. 7, were electrophoresed through 12.5% polyacrylamide gels alongside sample of a peak $\sigma^{30}$ RNA polymerase-containing DNA-cellulose elution fraction from a *B. subtilis sigB* strain containing a *spo0H* missense mutation, *spo0H81* (Zuber *et al.*, personal communication) (lane 3). After transfer to nitrocellulose, blot A was incubated with preimmune serum, blot B was incubated with antiserum raised against the *spo0H-lacZ* fusion protein, and blot C was incubated with Spo0H-specific antibodies but affinity purified from crude anti-fusion protein antiserum. Detection of bound antibody was carried out as described above. The samples of blots A and B were run on adjacent lanes of the same gel. Electrophoresis of the samples on blot C was carried out separately. Positions of molecular size markers run adjacent to the samples on each gel are indicated to enable direct comparisons of the blots. The position of the $\sigma^{30}$ polypeptide in lane 3 is shown with an arrowhead.

650), and in the *B. subtilis* sequence the region is centered at Gly-179 (bp 750). These regions contain the amino acid sequence AL(N3)G(N5)I and VL(N3)G(N5)I, which is similar to the conserved DNA binding domain, (hydrophobic amino acid) A(N3)G(N5)I/L/V, of DNA-binding proteins (28). This region, as is typical for sigma factors (regions 3 and 4 of Gribskov and Burgess [12]), is in the carboxyl part of the protein.

**Identification of the *spo0H* protein.** We have previously described the generation of polyclonal antiserum against a *B. licheniformis spo0H-lacZ* translational gene fusion protein (34). This antiserum (Fig. 6B), but not preimmune serum (Fig. 6A), reacted strongly with a 27-kilodalton polypeptide present in an extract prepared from a *B. subtilis* strain harboring the cloned *B. licheniformis spo0H* gene on plasmid pIS9A (lanes 1) but absent from an extract of an isogenic strain carrying the vector pBD97 (lanes 2). To minimize background immunological reactions not specific for *spo0H*, we enriched the antiserum for Spo0H-specific antibodies (method i, Materials and Methods) and used it for additional immunoblot analyses. The Spo0H-enriched antiserum reacted with a single protein, with a molecular size of approximately 27 kilodaltons, in the pIS9A extract (Fig. 7, lanes 2 and 5) but did not detect a protein of this size in extracts from strains carrying pBD97 (lane 1) or the pIS9A deletion derivatives pIS26 (lane 3) and pIS89 (lane 4) (see Materials and Methods for construction of these plasmids). Instead, the pIS89 extract possessed a single reactive protein of 16 kilodaltons, about the size predicted for the truncated *spo0H* gene product encoded by this plasmid. In contrast, no reactive protein was seen in the pIS26 extract, an observation consistent with the prediction that this plasmid would encode a 10-kilodalton *spo0H* product, too small to be detected under the gel electrophoresis conditions used. From these experiments, we conclude that the 27-kilodalton polypeptide detected with the Spo0H-enriched antiserum is the product of the cloned *B. licheniformis spo0H* gene. There was no detectable reaction of the Spo0H-enriched antiserum with any protein of a *spo0H*$^+$ *B. subtilis* strain carrying the vector pBD97 (lane 1), despite the presence of a wild-type chromosomal copy of the *B. subtilis spo0H* gene in this strain. This finding may reflect the low level of *spo0H* protein encoded by the single-copy chromosomal gene as compared with that expressed by the multicopy plasmid pIS9A (unpublished results) (7). DNA sequence analysis of the *B. subtilis* and *B. licheniformis spo0H* genes predicts 91% identity between the two proteins, and they would be expected to be immunologically cross-reactive.

Carter and Moran (5) have recently described the purification of a new sigma factor from *B. subtilis*, called $\sigma^{30}$, capable of directing core RNA polymerase to use the *spoVG* promoter in an in vitro runoff transcription assay. Led by the observations that the derived *spo0H* amino acid sequence bears striking homologies to known sigma factors and that *spo0H* is essential for transcription of *spoVG* (5, 26, 46), we sought to determine whether *spo0H* is the structural gene for $\sigma^{30}$ by using Spo0H-specific antiserum. RNA polymerase was isolated from early-stationary-phase *B. subtilis* MLI (which contains a deletion in the *sigB* gene, coding for $\sigma^{37}$ [2]) and was fractionated into the different holoenzymes by salt elution from a DNA-cellulose column. Samples of fractions were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels for silver staining and subjected to immunoblot analysis with anti-Spo0H antiserum. Each fraction was also used to transcribe a mixture of *spoVG* and *ctc* templates in an in vitro mixed-template competition assay. In such an assay, $\sigma^{30}$ RNA polymerase directs transcription exclusively from the *spoVG* promoter, despite the presence of the *ctc* template, whose promoter is efficiently utilized in vitro by $\sigma^{32}$- and $\sigma^{37}$-containing polymerases (5).

Figure 8A shows results of the immunoblot analysis. Spo0H-enriched antiserum reacted most strongly with a single protein of about 27 kilodaltons present in the lanes representing fractions 22 and 24 (and present very faintly in

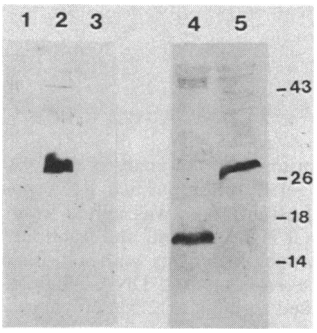


FIG. 7. Immunoblot analysis of proteins encoded by plasmids bearing deletions in the *spo0H* gene. Extracts were prepared from late-vegetative-phase cultures of *B. subtilis* Spo0H$^+$ strains carrying the plasmids pBD97 (lane 1), pIS9A (lane 2), or pIS26 (lane 3), and *spo0hΔHind* strains harboring the plasmids pIS89 (lane 4) or pIS9A (lane 5), by incubation with lysozyme in the presence of RNase and DNase, followed by boiling with sodium dodecyl sulfate. Proteins were separated by electrophoresis through a 15% polyacrylamide–sodium dodecyl sulfate gel and transferred to nitrocellulose. Immunodetection was carried out as described in Materials and Methods with Spo0H-enriched antiserum. Commercially prepared protein size standards (Bethesda Research Laboratories, Inc.) were run adjacent to lane 5, as indicated.

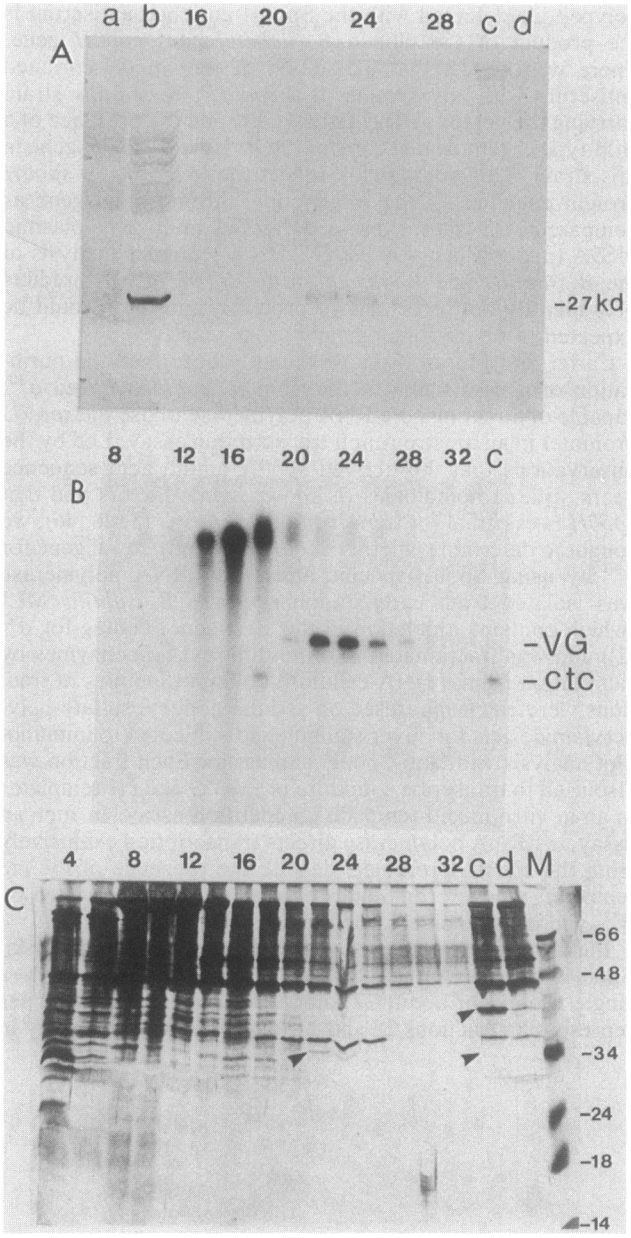


FIG. 8. Western immunoblot analysis of RNA polymerase fractions. Phase and gel filtration-purified RNA polymerase from *B. subtilis sigB* (MLI strain) cells was eluted from a DNA cellulose column with a linear KCl gradient, and fractions were analyzed as follows. (A) Immunoblot analysis with anti-fusion protein antiserum. Samples of even-numbered DNA-cellulose elution fractions were electrophoresed through a 12.5% polyacrylamide–sodium dodecyl sulfate gel, transferred to nitrocellulose, and incubated with Spo0H-enriched antiserum. Bound antibody was detected as described in Materials and Methods. Numbered lanes refer to fraction numbers. Lanes a and b contain extracts prepared from *B. subtilis spo0H75* strains carrying pBD97 and pIS9A, respectively. Lane c represents a fraction from an RNA polymerase preparation eluted from a DNA cellulose column, containing $\sigma^{43}$, $\sigma^{37}$, and $\sigma^{32}$, made from a *B. subtilis spo0HΔHind* strain which does not contain $\sigma^{30}$. Lane d represents another DNA-cellulose elution fraction from the same *spo0HΔHind* preparation which corresponds to the peak salt elution fraction from a Spo0H$^+$ strain containing $\sigma^{30}$. No $\sigma^{30}$ is visible in the fraction run in lane d (see lane d of panel C, below). (B) Runoff transcription assays. Even-numbered fractions 8 through 32 were used in mixed template transcription assays as previously described (5). Each reaction contained 1 μg of a *spoVG* promoter template cut at an *Eco*RI site 120 bp downstream from the promoter and 1 μg of a *ctc* promoter template cut at a *Hin*dIII site 95 bp downstream from the promoter. $^{32}$P-labeled runoff transcripts were visualized by autoradiography after electrophoresis through a 7 M urea–9% polyacrylamide gel. Lane numbers indicate the fractions assayed. Lane c represents runoff transcripts generated from the RNA polymerase preparation described for lane c of panel A. Only the *ctc*-derived transcript is observed, as expected. Positions of the 120-nucleotide *spoVG* run-off transcript and the 95-nucleotide *ctc* run-off transcript are shown. (C) Direct staining of proteins. The proteins in each even-numbered fraction were visualized by staining with silver (Bio-Rad) after electrophoresis through a polyacrylamide–sodium dodecyl sulfate. Lane numbers reflect fractions examined. Lanes c and d are as in panel A. Molecular weight markers were run in lane M. The position of $\sigma^{30}$ in fraction 22 is indicated with an arrowhead. The upper and lower arrowheads beside lane c indicate the positions to which $\sigma^{37}$ and $\sigma^{30}$, respectively, should migrate.

fraction 26). The presence of this immunologically reactive protein correlates with the presence of the $\sigma^{30}$ polypeptide in the same fractions, as monitored by silver staining (Fig. 8C) and by the mixed-template transcription assays (Fig. 8B). The antiserum did not react appreciably with any other proteins present in the peak $\sigma^{30}$ fractions, although some slight cross-reactivity with major protein components of other fractions was observed. In addition, slight cross-reactivity was observed with proteins of lane c, which represents an RNA polymerase preparation containing $\sigma^{43}$, $\sigma^{37}$, and $\sigma^{32}$.

The apparent molecular size of the immunologically detected protein (27 kilodaltons) is similar to that previously assigned to $\sigma^{30}$ (30 kilodaltons) (5). The slight discrepancy in size may be attributable to the use of different gel systems and molecular weight standards for the immunoblot and silver-staining experiments. Significantly, no immunologically reactive protein was observed corresponding to $\sigma^{30}$ in lane d of the Western immunoblot analysis. This RNA polymerase, also isolated after DNA cellulose purification, was prepared from a strain carrying the *spo0HΔHind*III deletion. No $\sigma^{30}$ holoenzyme was detected from this strain; the sample run in lane d was from a column fraction which would contain peak $\sigma^{30}$ holoenzyme in a wild-type strain.

Further immunoblot experiments were performed to determine whether immunological cross-reactivity of our antiserum with $\sigma^{30}$ is due to shared *spo0H* antigenic determinants or to the reactivity of nonspecific antibodies contaminating our antiserum (Fig. 6). A comparison of Fig. 6A and B reveals that whereas several proteins in the $\sigma^{30}$ peak DNA cellulose fraction (lane 3) were detected with both the preimmune serum and immune serum, only the latter reacted with $\sigma^{30}$. Antibodies which had reacted with the 27-kilodalton *spo0H* protein were isolated by Western blot affinity purification (method ii, Materials and Methods), and they reacted exclusively with the $\sigma^{30}$ protein in the peak fraction (Fig. 6C).

## DISCUSSION

The DNA sequences of the *spo0H* genes of *B. licheniformis* and *B. subtilis* predict polypeptides whose amino acid sequences show 91% identity and contain a 14-amino-acid region which is very homologous to the highly conserved core binding domain of most procaryotic sigma factors (12, 35). In addition to this region, there are other portions of the

predicted amino acid sequence which have similarities to sigma factors; in fact, the *spo0H* proteins show approximately 25% identity and approximately 40% conservative homology to most of the sigma factors whose sequences have been identified. These data strongly suggest that the *spo0H* gene product is a sigma factor.

Several lines of evidence support the idea that *spo0H* is the structural gene for $\sigma^{30}$, recently described by Carter and Moran (5). Immunological analyses show that antibodies raised against the *spo0H* gene product of *B. licheniformis* specifically react with purified $\sigma^{30}$ from *B. subtilis*. $\sigma^{30}$ holoenzyme was not observed in a strain carrying a deletion in the *spo0H* gene (Fig. 8). Moreover, a *spoVG* promoter mutation is specifically suppressed by an amino acid change in the *spo0H* structural gene, and it has been shown recently that the $\sigma^{30}$-containing holoenzyme from this *spo0H* mutant (*spo0H81*) can use the mutant *spoVG* promoter more efficiently in vitro than the wild-type $\sigma^{30}$ holoenzyme (P. Zuber et al., personal communication). Carter and Moran (5) found that $\sigma^{30}$ holoenzyme and activity, as measured by *in vitro* transcription of the *spoVG* gene, was lacking in strains with mutations in *spo0A* but restored in strains carrying mutations in both *spo0A* and *abrB* (39). This correlates with the observation that the expression of the *spo0H* gene depends upon the *spo0A* gene and that this dependence is bypassed in strains with an *abrB* mutation (7). Henceforth, we refer to $\sigma^{30}$ as $\sigma^{H}$ according to the nomenclature of Losick et al. (22).

S1 RNA mapping experiments show that the −10- and −35-bp regions of the *B. licheniformis* gene (32) as well as the *B. subtilis* gene (J. Weir and I. Smith, unpublished results) have the consensus sequence for promoters recognized by the major $\sigma^{43}$ of *B. subtilis*. This is consistent with the fact that *spo0H* is expressed in vegetative growth (7, 8, 41) and that the major RNA polymerase containing $\sigma^{43}$ can transcribe the *spo0H* gene in vitro (32).

The *spo0H* gene product, $\sigma^{H}$, has an obligate role in the initiation of sporulation. Thus far, only two promoters, those of *spo0VG* (5) and P3 of the *rpoD* gene (H. L. Carter et al., personal communication), are known to be transcribed in vitro by holoenzyme-containing $\sigma^{H}$ and are *spo0H* dependent in vivo. These promoters are not transcribed during vegetative growth but are turned on at the beginning of the sporulation process ($T_0$) (H. L. Carter et al., personal communication) (46).

The expression of $\sigma^{H}$-dependent promoters, such as *spoVG* and *rpoD* P3, which are growth stage dependent, could be temporally regulated either by (i) the increased production of $\sigma^{H}$ itself, (ii) the appearance of a transcriptional activator, or (iii) the inactivation of a transcriptional repressor. The transcription of heat shock promoters in *E. coli* is an example of the first type of control, in which the expression of $\sigma^{32}$ (product of the *htpR* gene), an extremely unstable protein, is increased by heat shock (13). Overproduction of this protein without stimulation by heat shock is sufficient to turn on the heat shock promoters (13). The second type of control system is exemplified by the alternative sigma factor, $\sigma^{N}$, whose gene, *ntrA*, is constitutively expressed but whose activity requires the environmentally regulatable modification of positive regulator NRI, the product of *ntrC* (16, 18). We favor the second model for control of *spo0H*-dependent promoters, because, according to data obtained with *spo0H-lacZ* translational fusions, the *B. licheniformis* and *B. subtilis spo0H* genes are expressed throughout vegetative growth (7) (J. Weir et al., unpublished results). However, it remains possible that the $\sigma^{H}$ protein or its RNA is unstable during vegetative growth and therefore that functional levels of this minor sigma factor change at different stages of growth.

If indeed, $\sigma^{H}$ requires a factor or factors to become activated at $T_0$, such controls could be exerted at various levels: i.e., the processing of $\sigma^{H}$, its stability, its binding to the core polymerase, or the activity of the new RNA polymerase might require auxiliary factors.

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