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Bacillus subtilis spo0H Gene

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A 2.8-kilobase fragment of the *Bacillus subtilis* chromosome containing a functional *spo0H* gene was cloned by using a modification of the helper system described by T. Gryczan and co-workers (T. Gryczan, S. Contente, and D. Dubnau, Mol. Gen. Genet. **177**:459–467, 1980). The chromosomal segment specifically complements *spo0H* mutations in *recE4* strains and when integrated into the chromosome of Rec$^+$ strains maps in the *spo0H* region of the *B. subtilis* genome. A deletion within the transcribed region of the cloned *spo0H* gene was constructed which abolishes its *spo0H*$^+$-complementing activity. DNA sequences containing this deletion were introduced into a *B. subtilis* Rec$^+$ strain containing the *spo0H75* mutation. The absence of recombination between the deletion and the *spo0H* mutation indicates that both reside in the same gene. There is homology between the *B. subtilis spo0H* gene and a 1.2-kilobase chromosomal fragment from *Bacillus licheniformis* which also complements *B. subtilis spo0H* mutations. In vivo transcription mapping experiments have shown that the *B. subtilis spo0H* gene is transcribed during vegetative growth as well as during sporulation.

We previously described the cloning of a 1.2-kilobase (kb) chromosomal fragment from *Bacillus licheniformis* which specifically complements *spo0H* mutations in *Bacillus subtilis* (6). Since the cloning technique utilized the *rec*-dependent helper system developed by researchers in the laboratory of David Dubnau (8), we used *B. licheniformis* to prepare DNA for the shotgun cloning of *spo* genes. We had shown that *B. licheniformis spo* genes would not integrate into the *B. subtilis* chromosome. Thus, we could rule out simple Rec$^+$ recombination into the chromosome of Spo$^-$ bacteria when selecting Spo$^+$ transformants. However, this lack of homology prevented us from genetically mapping the *B. licheniformis* gene by integration into the *B. subtilis* genome. As a result, we could not be certain that the cloned DNA fragment contained the *spo0H* gene.

A modification of the *rec*-dependent helper system has now enabled us to clone a chromosomal fragment of *B. subtilis* which complements *spo0H* mutations in *recE4* strains and maps in the *spo0H* region of the *B. subtilis* chromosome. We used this cloned fragment to show that the *B. licheniformis* 1.2-kb cloned gene is homologous to the *spo0H* gene of *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains and plasmids. Table 1 lists the strains and plasmids used in these experiments. Techniques relating to plasmid preparation, transformation of cells, restriction enzyme digestion, and electrophoresis have been previously described (6).

Measurement of sporulation in liquid culture by resistance to chloroform. Samples from cultures growing in nutrient sporulation medium (15) were diluted 100-fold in an inorganic salts solution, and 0.1 ml of chloroform was added to 5 ml of the diluted suspension (11). The suspension was mixed rapidly for 1 min, and dilutions were plated on tryptose blood agar base medium containing 5 μg of chloramphenicol (Cm) per ml. Chloroform resistance was calculated by determining the ratio of chloroform-resistant, viable colonies at the designated time point to the total viable count of the culture at the end of exponential growth. We did not use heat resistance to measure sporulation frequency in *recE spo0H* strains as these cells showed variable resistance to elevated temperatures.

Nick translation. Plasmid DNA and isolated DNA restriction fragments were labeled by nick translation to a specific activity of 2×10^8 cpm/μg with [α-^{32}P]dATP (Amersham Corp.) as described by Rigby et al. (14) but using the DNA polymerase buffer of Maniatis et al. (12). DNA polymerase I was from Bethesda Research Laboratories.

RNA isolation. RNA was isolated from cells harvested during exponential growth (2×10^8 to 5×10^8 cells per ml) in minimal medium (5) or VY (13) and from cells harvested at various times after transfer to minimal sporulation medium (19). Methods for the preparation of RNA and treatment with RNase-free DNase have been previously described (9).

DNA-DNA and RNA-DNA hybridization. For DNA-DNA hybridization, DNA restriction fragments were separated by electrophoresis on agarose gels. Denaturation of DNA, blotting of the gels, preparation of diazobenzyloxymethyl (DBM)-paper, and hybridization with nick-translated DNA were performed by the methods of Alwine et al. (2). For experiments in which DNA was transferred to nitrocellulose paper, the methods of Southern (17) were employed.

For Northern hybridization (2) experiments, DNase-treated RNA was denatured and electrophoresed through a 1.5% agarose–1.1 M formaldehyde gel in 20 mM morpholinepropanesulfonic acid–5 mM sodium acetate–1 mM EDTA (pH 7.0) buffer. RNA was visualized by staining with ethidium bromide and then transferred, without prior treatment, to nitrocellulose paper (17). Hybridization with nick-translated DNA was performed as described previously (2).

Berk-Sharp (3) experiments as modified by Weaver and Weissman (20) were carried out with RNA harvested from vegetative cells and from cells in the early stages of sporulation (1 and 2 h after transfer to minimal sporulation medium). RNA (25 μg) was mixed with 0.1 μg of pIS11 DNA which had previously been either 5′ end labeled with [α-^{32}P]dATP and polynucleotide kinase or 3′ end labeled with [α-^{32}P]dGTP and the DNA polymerase I large fragment (18) at the *Hin*dIII site. RNA and DNA samples were denatured at

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TABLE 1. Bacterial plasmids and strains

Plasmid or strain	Parent	Characters	Plasmid	Source
Plasmid				
pBD64		Cm^r Km^{ra}		D. Dubnau
pBD97		Cm^r		D. Dubnau
pUB110		Km^r		D. Dubnau
pIS1		Cm^r *B. licheniformis spo0H* gene		6
pIS2		Em^r *B. licheniformis spo0H* gene		6
pIS9a		Cm^r *B. licheniformis spo0H* gene		6
pIS11a		Cm^r *B. subtilis spo0H* gene		This paper
pIS11b		Cm^r *B. subtilis spo0H* gene		This paper
pIS20		Cm^r *B. subtilis spo0HΔHind*		This paper
pIS53		Cm^r *B. subtilis spo0H* internal fragment		This paper
Strain				
FD02[b]		Str^r Rif^r		6
IS72		*rif-29 strA spcA lin-2*		6
IS75		*met*(Am) *hisB*(Am) *leu*		16
IS120		*trpC2 thrA recE4*		D. Dubnau, BD224
IS160		*trpC2 pheA1 spo0H75*		J. Hoch, JH700
IS171	IS160	*trpC2 recE4 spo0H75*		6
IS191	IS75	*hisB*(Am) *glpD recE4*		Sequential transformation of IS75
IS193	IS160	*trpC2 pheA1 spo0H rif-29*		Transformation of IS160 with IS72 DNA
IS206	IS230	*trpC2 recE4*		Transformation of IS230 with IS191 DNA
IS230		*trpC2 pheA1*		J. Hoch, JH642
IS233	IS230	*trpC2 pheA1 spo0HΔHind*		This paper
IS234	IS233	*trpC2 recE4 spo0HΔHind*		Transformation of IS233 with IS191 DNA
IS1009	IS75	*met*(Am) *hisB*(Am) *leu*	pUB110	Transformation of IS75 with pUB110
IS1015	IS171	*trpC2 recE4 spo0H75*	pBD64	6
IS1078	IS171	*trpC2 recE4 spo0H75*	pIS11a	Transformation of IS171 with PIS11a
IS1084	IS171	*trpC2 recE4 spo0H75*	pIS11b	Transformation of IS171 with pIS11b
IS1124	IS206	*trpC2 recE4*	pBD64	Transformation of IS206 with pBD64
IS1142	IS234	*trpC2 recE4 spo0HΔHind*	pIS11a	Transformation of IS234 with pIS11a
IS1143	IS234	*trpC2 recE4 spo0HΔHind*	pIS11b	Transformation of IS234 with pIS11b

[a] Abbreviations: Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance. All other abbreviations are as described previously (6).
[b] *B. licheniformis* strain. All other strains are *B. subtilis*.

83°C for 15 min and then allowed to reanneal at 52°C for 3 h. Methods for hybridization, S1 nuclease digestion, and electrophoresis of samples on 7.5% acrylamide–7 M urea gels have been previously described (6).

RESULTS

Cloning of the *B. subtilis spo0H* gene. We previously reported the cloning and preliminary characterization of a 1.2-kb fragment from *B. licheniformis*, which complements several mutant alleles of the early sporulation locus *spo0H* (6). Since there is no detectable transformation for Spo$^+$ by *B. licheniformis* DNA with *B. subtilis spo* recipients, we were able to use the Rec-dependent host plasmid rescue system of Gryczan and co-workers (8). DNA taken up by competent cells of *B. subtilis* is generally nicked or otherwise damaged during uptake. This presumably explains the requirement for plasmid multimers in the transformation of plasmid-free recipients (4). When the host cell carries DNA homologous to the donor DNA, damaged DNA can be rescued by recombination between the recipient vector and the homologous portion of the donor DNA. This greatly increases the probability of obtaining transformants with cloned DNA (8).

Because this method requires the presence of the host recombination machinery, direct selection cannot be used for the cloning of *B. subtilis* chromosomal DNA fragments. Therefore, we modified the helper rescue technique of Gryczan and co-workers in an attempt to clone the homologous (*B. subtilis*) *spo0H* gene. DNA from an *spo*$^+$ strain of *B. subtilis* (IS72) was partially digested with *Mbo*I and ligated to vector pBD64, which had been restricted with *Bgl*II. The ligated DNA was used to transform a *rec*$^+$ strain of *B. subtilis* carrying pUB110 (IS1009). pUB110, which is homologous with a portion of pBD64, provides the host plasmid rescue function described by Gryczan and co-workers (8). The transformation mixture was diluted 10-fold into medium containing 5 μg of chloramphenicol per ml and incubated overnight. We hoped that newly transformed cells would accumulate sufficient levels of plasmid multimers by this initial passage, which selected for only the vector marker. Plasmid DNA was then prepared from this culture and used to transform a *recE spo0H75* host (IS171). Spo$^+$ Cm^r transformants were selected with chloroform vapors (10). One Spo$^+$ Cm^r clone was obtained and found to be Km^s, as expected from a cloning event in the *Bgl*II site of pBD64. Spo$^+$ Cm^r cells which were generated from the isolated clone had one species of plasmid DNA, of 7.6 kb, which was capable of simultaneously transforming IS171 to Cm^r and Spo$^+$. The pBD64 vector containing the insert DNA is called pIS11.

Spo$^+$ complementation by pIS11 is specific for *spo0H*; Cm^r transformants of strains carrying mutations in gene *spo0F*, *spo0J*, *spoIIC*, *spoIIIC*, or *spoIVA* remained Spo$^-$, whereas all Cm^r transformants became Spo$^+$ when the

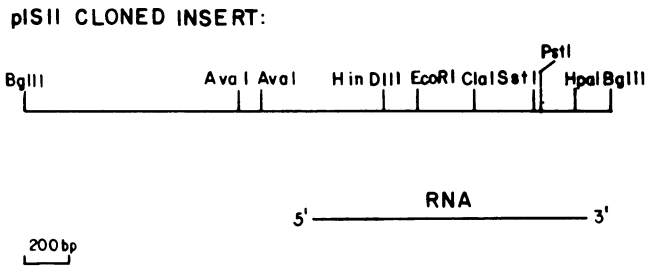

FIG. 1. Restriction map of pIS11 cloned insert. Restriction endonuclease cleavage sites on the pIS11 insert were mapped with respect to the *Bgl*II site of pBD64. Additional restriction sites not mapped on the 2.8-kb insert are *Bcl*I (four sites), *Dde*I (three sites), *Hae*II (four sites), *Hin*fI (five sites), and *Mbo*I (nine sites). Enzymes which do not have sites on the insert are *Bam*HI, *Pvu*II, *Xba*I, *Kpn*I, *Sal*I, *Ava*II, and *Nco*I. The size and polarity of the *spo0H* transcript homologous to the insert, indicated below the restriction map, were determined by S1 nuclease mapping techniques (see the text).

recipient carried the *spo0H75* mutation. pIS11 is quite unstable. When the *spo0H75 recE* strain carrying pIS11 (IS1078) was streaked on plates lacking chloramphenicol, 38% of the colonies became Cm^s and Spo^-.

Restriction map of pIS11. Restriction of pIS11 with *Bgl*II generated two fragments of 4.8 and 2.8 kb. Since the 4.8-kb fragment migrated with linearized vector pBD64 on agarose gels, it was concluded that the 2.8-kb fragment is the cloned insert. This result indicates that two *Bgl*II restriction sites were fortuitously generated by insertion of the *Mbo*I chromosomal fragment into the *Bgl*II site of pBD64.

A restriction map of the insert was constructed by performing a series of single or multiple restriction enzyme digestions on pIS11. Sizes of restriction fragments were determined by their electrophoretic mobilities on polyacrylamide gels. Restriction sites on the insert were oriented with respect to the *Bgl*II cloning site of pIS11 (Fig. 1).

Mapping the chromosomal origin of the pIS11 cloned insert. Although the pIS11 cloned insert specifically complements a *spo0H* mutation, it was necessary to determine whether the cloned fragment contained the *spo0H* gene rather than some second-site suppressor gene. To answer this question, the cloned insert was allowed to integrate into the chromosome of a rec^+ strain carrying the *spo0H75* mutation, and the genetic location of this integrated gene was determined.

pIS11 was restricted with *Xba*I, thus linearizing the plasmid at the pBD64 *Xba*I site but leaving the cloned insert DNA intact. This DNA, as well as uncut pIS11 DNA, was used to select Spo^+ recombinants in a rec^+ *spo0H75* strain (IS160). One hundred Spo^+ recombinants from each cross were purified and tested for Cm^r. All Spo^+ recombinants from the cross with *Xba*I-treated pIS11 DNA and 87% of the Spo^+ recombinants obtained from the cross with uncut pIS11 DNA were Cm^s. Therefore, when pIS11 is linearized and used to transform the Spo^+ phenotype, none of the resulting transformants carry the original Cm^r of the pIS11 plasmid, and even when the uncut pIS11 plasmid is used for transformation, most of the Spo^+ recombinants lack this plasmid marker. The Spo^+ phenotype appears to result from integration of the insert DNA into the chromosome.

One Spo^+ recombinant from the cross with *Xba*I-restricted pIS11 DNA was purified, and competent cells were prepared. These were used as recipients in a transformation cross, using DNA from a *spo0H75 rif* donor strain (IS193). Rif^r transformants were selected, and 100 were purified and tested for their Spo phenotype. A total of 59% had become Spo^- and therefore must have obtained the *spo0H75* mutation from the donor DNA. The *spo0H75* marker has been shown, by our previous work, to be linked by transformation to *rif* at a frequency of 30% (16). This result shows that the cloned insert can integrate into the chromosome at a site with similar linkage to *rif* as usually observed with *spo0H75*. We concluded that the cloned insert on pIS11 which complements the *spo0H75* allele originated from the $spo0H^+$ gene or a closely linked second-site mutation and not from an unlinked suppressor.

Introduction of a mutation in the clone into the chromosome. Our previous mapping experiments, described above, did not rule out the possibility of a closely linked suppressor causing the complementation of the *spo0H* mutation. To resolve this question, we created a deletion in the chromosomal fragment contained in pIS11 and integrated the deletion into the *B. subtilis* chromosome. The deletion plasmid was constructed by cleavage of pIS11 at its unique *Hin*dIII site (Fig. 1), digestion of the linearized plasmid with S1 nuclease, and blunt-end ligation. The religated DNA was used to transform a *recE spo0H* recipient strain, and plasmid DNA was purified from a Cm^r Spo^- transformant. This plasmid, called pIS20, is the same size as pIS11 but has lost its *Hin*dIII site. Because deletion of the *Hin*dIII site destroys the *spo0H*-complementing activity of the clone, this region of the pIS11 insert must be necessary for expression of the Spo^+ phenotype in *spo0H* strains carrying pIS11. Plasmid pIS20 was used to introduce the deletion into IS230, a Rec^+ spo^+ strain, as follows. pIS20 (2 μg) was linearized with restriction enzyme *Xba*I and used to transform IS230 along with 2 μg of uncut plasmid pBD97. Transformed cells were selected for by means of the chloramphenicol resistance determined by pBD97. Colonies were scored for their sporulation phenotype, and four putative Spo^- transformants were isolated from 600 Cm^r colonies. Spo^- colonies were passaged at 52°C on nonselective (tryptose blood agar base) solid medium to eliminate pBD97, whose replicon is nonfunctional at this temperature. A chloramphenicol-sensitive Spo^- strain, IS233, was thus obtained.

To ascertain the frequency of sporulation in IS233, cultures of IS233, IS160, an *spo0H75* strain, and the Spo^+ parent were plated on nonselective medium, and colonies appearing after 20 h were exposed to chloroform vapor. IS160 gave 40 Spo^+ revertants out of a total of 6.6×10^8 cells plated, whereas all 7.5×10^8 colonies of IS230 were Spo^+. IS233 gave <10 Spo^+ colonies out of a total viable count of 6×10^8.

IS233 was used as a source of chromosomal DNA to transform a Rec^+ *spo0H* strain, IS160, for Spo^+ (Table 2). No significant Spo^+ transformation was observed with IS233 DNA, indicating that the deletion in IS233 is extremely close to the *spo0H75* mutation carried by IS160 and both probably reside within the same gene. IS233 was also made competent and was transformed to Spo^+ with wild-type chromosomal DNA and with pIS9a DNA (Table 2). pIS9a contains the *B. licheniformis spo0H*-complementing 1.2-kb fragment (6). The *spo* lesion in IS233 is complemented in *trans* by the *B. licheniformis* cloned fragment and by wild-type *B. subtilis* DNA, as was the *spo0H* mutation carried by IS160.

Homology of the cloned insert with chromosomal sequences. DNA-DNA hybridization experiments were done to determine the chromosomal origin of the pIS11 insert and to identify any homology between this clone and the 1.2-kb *B. licheniformis* fragment in pIS1 (6). pIS11, pIS1, *B. subtilis*, and *B. licheniformis* DNAs were digested with *Bgl*II and

TABLE 2. *spo0H* deletion strain transformation experiments

Recipient	Transforming DNA[a]	Transformants per ml	
		Trp$^+$	Spo$^+$
IS160	IS160		110
	IS230		2×10^5
	IS233		20
	None		10
	pIS9a		5×10^3
	IS72		2.9×10^4
	pBD97		<10
IS233	pIS9a		5×10^2
	IS72	2×10^4	2×10^3
	pBD97		<10

[a] IS160, IS230, and IS233 DNA preparations also were used to transform IS75 for *leu*$^+$. All gave $>2 \times 10^5$ *leu*$^+$ transformants per ml.

then hybridized with nick-translated (14) ^{32}P-labeled pIS11 DNA by the Southern blot technique (17) (Fig. 2A). The pIS11 insert was found to have a low level of homology with the 1.2-kb insert of pIS1 and a 1.2-kb *Bgl*II restriction fragment of *B. licheniformis* DNA. These results are consistent with previous experiments (6), which indicated that the cloned insert of pIS1 contains the *B. licheniformis* counterpart of the *spo0H* gene. The pIS11 nick-translated probe hybridized to three *B. subtilis Bgl*II restriction fragments. This finding shows that the cloned insert is not derived from a contiguous segment of chromosomal DNA. Rather, the insert appears to have resulted from a rearrangement of DNA sequences such that noncontiguous regions of the chromosome were brought together in the cloning process. We have observed that the *spo0H*$^+$-transforming activity from *B. subtilis Bgl*II-restricted DNA migrates electrophoretically in the same region of 0.8% agarose gels as does the largest fragment which hybridized with the pIS11 probe (unpublished data).

Although the pIS11 insert contains noncontiguous chromosomal DNA sequences, our genetic experiments, described above, have shown that sequences near its *Hin*dIII site are derived from the *spo0H* gene. To determine whether the *B. licheniformis* clone shares homology with the *spo0H* sequences of pIS11, pIS53, a plasmid carrying the *Hin*dIII-*Pst*I restriction fragment of the pIS11 insert (Fig. 1), was used as a probe in DNA-DNA hybridization experiments (Fig. 2B). Nick-translated pIS53 DNA hybridized with a fragment of the same size as that from *B. subtilis* DNA restricted with *Hin*dIII and *Pst*I and with a single, large *Bgl*II restriction fragment from the *B. subtilis* chromosome thought to contain the *spo0H* gene. The probe also hybridized with the 1.2-kb *B. licheniformis* clone. We conclude that the *Hin*dIII-*Pst*I restriction fragment of the pSI11 insert is derived from contiguous chromosomal DNA sequences containing the *spo0H* gene. The *B. licheniformis* clone shares homology with this fragment.

Orientation studies. We examined the dependence of the Spo$^+$-complementing activity of the insert on its orientation in the pBD64 vector. pIS11 was restricted with *Bgl*II, religated, and used to transform a *recE spo0H* strain (IS171). Cm^r transformants were selected and screened for sensitivity to kanamycin and sporulation phenotype. Of 180 Cm^r transformants screened, 33 were Km^s, indicating the presence of insert DNA at the *Bgl*II site. Of these Km^s transformants, 19 showed the same Spo$^+$ phenotype as pIS11, appearing as brown colonies on minimal medium after 2 days of incubation at 37°C. The remaining 14 turned brown very slowly on minimal medium. Plasmids from six Cm^r Km^s strong Spo$^+$ transformants and six Cm^r Km^s weak Spo$^+$ transformants were purified and found to be the same size as pIS11.

Double digestions of the two groups of plasmids with restriction enzymes *Bam*HI and *Pst*I were performed to determine the orientation of the insert in the vector. *Bam*HI has a unique site in the pBD64 sequences of pIS11, whereas *Pst*I restricts pIS11 at a unique site within the insert (Fig. 1). The six strong Spo$^+$-conferring plasmids had restriction fragments of 5.25 and 2.25 kb. An identical restriction pattern was generated when pIS11 was digested with *Bam*HI and *Pst*I. In contrast, restriction of the six weak Spo$^+$ plasmids with these two enzymes gave fragments of 4.5 and 3.0 kb. This restriction pattern is identical to that predicted when the insert is situated in pBD64 in the reverse orientation. One strong Spo$^+$ plasmid and one weak Spo$^+$ plasmid were purified and used for subsequent studies. The plasmid carrying the insert in the strong Spo$^+$ orientation in pBD64 is called pIS11a. The plasmid carrying the insert in the alternate orientation is called pIS11b.

Sporulation phenotypes of strains carrying pIS11a or pIS11b. The abilities of pIS11a and pIS11b to confer chloroform resistance on IS171 were examined. Strains carrying pIS11a, pIS11b, or pBD64 were grown in a nutrient sporulation medium at 37°C. At the end of exponential growth and 22 h later, viable counts of the cultures before and after treatment with 2% chloroform were determined by plating appropriate dilutions of the cultures on tryptose blood agar base medium containing chloramphenicol. None of the strains examined showed greater than 0.005% chloroform resistance at the end of exponential growth. After 22 h,

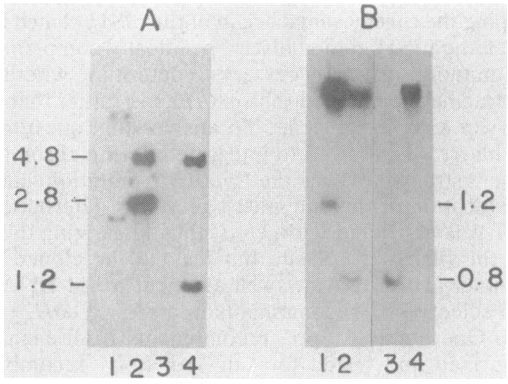

FIG. 2. (A) Chromosomal origin of cloned insert. DNA from *B. subtilis* IS72 (lane 1), *B. subtilis* pIS11 (lane 2), *B. licheniformis* FD02 (lane 3), and *B. licheniformis* pIS1 (lane 4) was restricted with *Bgl*II and hybridized with nick-translated ^{32}P-labeled pIS11 DNA by the Southern technique. An autoradiogram of the gel blot is shown. The larger band in lanes 2 and 4 is linearized pBD64 (4.8 kb). The smaller bands in lanes 2 and 4 are the excised inserts of pIS11 and pIS1 (2.8 and 1.2 kb, respectively). A 1.2-kb fragment is present in lane 3 but is too faint to appear in the reproduction. (B) Origin of internal fragment of the cloned insert. *Bgl*II-restricted pIS2 (lane 1) and *B. subtilis* IS72 (lane 4) DNAs and *Hin*dIII- and *Pst*I-restricted pIS11 (lane 2) and IS72 (lane 3) DNAs were hybridized with nick-translated ^{32}P-labeled pIS53 DNA as described in the text. An autoradiogram of the gel blot is shown. The smaller band in lane 2 is the *Hin*dIII-*Pst*I restriction fragment of the pIS11 insert (0.8 kb).

IS1124, the wild-type *B. subtilis* strain carrying pBD64, was 100% chloroform resistant, whereas IS1015, the *spo0H75* strain carrying pBD64, showed a 0.2% survival. The corresponding level for IS1078, the *spo0H75* strain with pIS11a, was 91%, and for IS1084, the *spo0H75* strain with pIS11b, the corresponding level was 24%. As pigmentation on solid medium had indicated, pIS11b complements the *spo0H75* mutation poorly compared with complementation by pIS11a. However, the ability of pIS11b to complement the mutation is significantly greater than for the pBD64 vector alone. Expression of the *spo0H*-complementing activity of the cloned insert is thus dependent on its orientation of insertion in the *Bgl*II site of pBD64.

In vivo synthesis of RNA homologous to the cloned insert. To study the in vivo expression of the *B. subtilis spo0H* gene in wild-type cells, in vivo transcription mapping experiments were performed. These experiments also allowed us to determine the size of the gene cloned in pIS11 by means of homology to an in vivo transcript.

Since DNA sequences near the *Hin*dIII restriction site of the pIS11 insert was required for complementation of *spo0H* mutations, it seemed probable that they reside within the *spo0H* gene. Therefore, pIS11 insert fragments labeled at the *Hin*dIII site should serve as probes for in vivo *spo0H* transcripts. Two ^{32}P-5′-end-labeled restriction fragments representing sequences on either side of the unique *Hin*dIII site of the pIS11 insert were generated as follows. pIS11 was digested with *Hin*dIII, ^{32}P-5′-end-labeled with polynucleotide kinase, and subsequently restricted with *Bgl*II. The fragments, 1.65 and 1.12 kb, were isolated and used separately in hybridization experiments with total *B. subtilis* RNA prepared from Spo$^+$ cells harvested during exponential growth or after transfer to a minimal sporulation medium. Each labeled DNA fragment was denatured and reannealed to RNA. The hybridization temperature used, 52°C, was chosen such that RNA-DNA hybridization would be favored. The annealed samples were treated with excess S1 nuclease (400 U/ml) for 30 min at 37°C, conditions under which a significant fraction of the hybrids should be nicked opposite DNA or RNA single-stranded loops (7, 20). The resulting RNA-DNA hybrids were electrophoresed on 7.5% acrylamide–7 M urea gels and analyzed by autoradiography (Fig. 3A).

At all stages of growth that were examined, the 1.65-kb 5′-end-labeled fragment was protected from S1 nuclease digestion by total cellular RNA. The level of *spo0H* transcript appears to increase as the cells sporulate. However, it should be noted that the hybridization techniques used do not allow accurate quantitations of relative RNA concentrations. Subsequent experiments have not consistently reproduced this pattern of band intensities. Since the 5′ label at the *Hin*dIII site of the 1.65-kb fragment is protected by in vivo cRNA, this fragment is identified as containing the promoter-proximal region of the *spo0H* gene. The length of DNA protected from S1 nuclease digestion is ca. 320 bases.

To determine the length of DNA on the promoter-distal *Hin*dIII-*Bgl*II fragment of the cloned insert homologous to in vivo RNA, pIS11 DNA was restricted with *Hin*dIII, ^{32}P 3′ end labeled by a fill-in reaction with the DNA polymerase I large fragment, and then digested with *Bgl*II. By this procedure, 1.65- and 1.12-kb fragments 3′ end labeled at the *Hin*dIII site were generated. When RNA prepared from exponentially growing *B. subtilis* cells was hybridized as above to each purified probe, about 950 bases of the 1.12-kb fragment were protected from S1 nuclease digestion (Fig. 3B). From these mapping experiments, a 1,270-nucleotide segment of the pIS11 insert has been identified which is homologous to in vivo RNA extending through the *Hin*dIII restriction site of the *spo0H* gene.

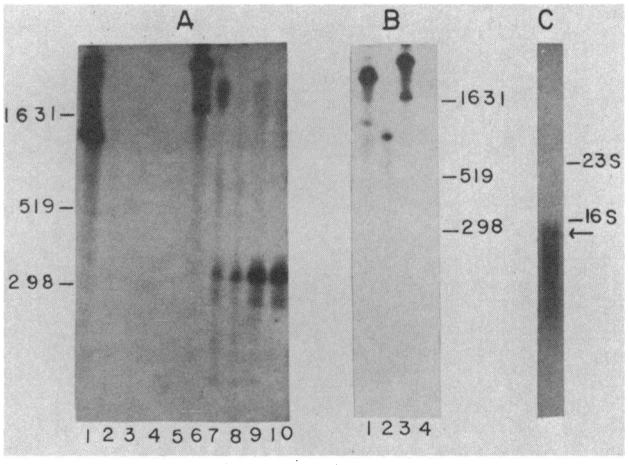

FIG. 3. Transcription mapping of the *spo0H* gene. (A) To map the 5′ terminus of the *spo0H* transcript, pIS11 DNA was restricted with *Hin*dIII, 5′ end labeled with polynucleotide kinase and [α-^{32}P]dATP, and subsequently restricted with *Bgl*II. Two labeled DNA fragments of 1.12 and 1.65 kb were isolated, and 0.1 μg of each (6,000 cpm) was hybridized with 25 μg of total RNA isolated from IS120 cells harvested during vegetative growth in VY (lanes 2 and 7) or minimal medium (lanes 3 and 8) and from IS120 cells harvested 1 h (lanes 4 and 9) or 2 h (lanes 5 and 10) after transfer to a minimal sporulation medium. After incubation at 52°C for 3 h, the samples were digested with S1 nuclease. S1 nuclease-resistant hybrids were analyzed by autoradiography after electrophoresis of the samples on a 7.5% acrylamide–7 M urea gel. Lanes 2 through 5 show the results of hybridization of the 1.12-kb *Bgl*II-*Hin*dIII restriction fragment with various RNA preparations. The results of hybridization of the 1.65-kb fragment with total RNA preparations are shown in lanes 7 through 10. Nondenatured samples of the 1.12- and 1.65-kb DNA restriction fragments were run in lanes 1 and 6, respectively, to serve as molecular weight markers. Unlabeled *Hin*fI-digested pBR322 DNA was run on the gel adjacent to the hybrids to provide additional size markers. (B) To map the 3′ terminus of the *spo0H* transcript, purified restriction fragments of pIS11 were generated by 3′ end labeling pIS11 DNA with [α-^{32}P]dGTP and the DNA polymerase I large fragment at the *Hin*dIII site and subsequently restricting the labeled DNA with *Bgl*II. Total RNA (25 μg) isolated from IS206 cells harvested during exponential growth in minimal medium was annealed to 0.1 μg (6,000 cpm) of the 1.12-kb 3′-end-labeled restriction fragment (lane 2) or 0.1 μg (6,000 cpm) of the 1.65-kb fragment (lane 4), digested with S1 nuclease, and analyzed. Nondenatured samples of the labeled 1.12- and 1.65-kb restriction fragments were run in lanes 1 and 3, respectively. (C) Northern hybridization experiments were performed to identify the full-length *spo0H* transcript present in wild-type *B. subtilis* cells. RNA (25 μg) isolated from IS206 cells harvested late in exponential growth was electrophoresed on an agarose-formaldehyde gel and transferred to nitrocellulose paper. This paper was hybridized with the *Hin*dIII-*Pst*I restriction fragment of the pIS11 insert, which had been isolated and nick translated to a specific activity of 4×10^7 cpm/μg. An autoradiogram of the Northern blot is shown. The positions to which 16S and 23S RNAs migrated were determined by ethidium bromide staining of the gel before transfer. An arrow indicates the position of RNA homologous to the *spo0H* probe.

RNA-DNA hybridization experiments by the Northern technique (Fig. 3C) showed that the *Hin*dIII-*Pst*I restriction fragment of the pIS11 insert was homologous to in vivo RNA from vegetatively growing IS206 cells of ca. 1,300 bases. Together with the results of the transcription mapping ex-

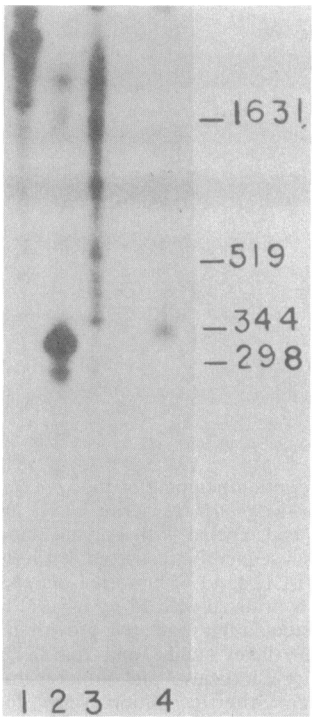

FIG. 4. Mapping of *spo0H* transcripts encoded by pIS11a and pIS11b in vivo. The 1.65-kb *Bgl*II-*Hin*dIII restriction fragment of the pIS11 insert, 5′ end labeled at the *Hin*dIII site, was hybridized with total RNA isolated from cells of strains IS206 (lane 2), IS1142 (lane 3), and IS1143 (lane 4) which were harvested during exponential growth in minimal medium. S1 nuclease-resistant hybrids were analyzed as described in the text. A nondenatured sample of the labeled 1.65-kb fragment was run in lane 1 as a size marker.

periments, this finding indicates that the *Hin*dIII-*Pst*I restriction fragment of the pIS11 insert lies within the *spo0H* transcribed sequences. It also strongly suggests that the pIS11 insert carries all or most of the *spo0H* structural gene.

***spo0H* RNA synthesized in vivo from pIS11a and pIS11b.** The *spo0H Hin*dIII deletion strain, IS233, does not encode RNA homologous to sequences at the *Hin*dIII restriction site of the pIS11 cloned insert (data not shown). Therefore, we used the IS233 genetic background to study *spo0H* transcripts made in vivo from pIS11a and pIS11b. RNA-DNA hybridization experiments were performed as described above, using RNA harvested from vegetatively growing cultures of strains IS206, IS1142 (pIS11a in the IS233 background), and IS1143 (pIS11b in the IS233 background). RNA was hybridized to the 1.65-kb *Hin*dIII-*Bgl*II restriction fragment of the pIS11 insert which had been 5′ end labeled with ^{32}P at the *Hin*dIII site. The hybrids were digested with S1 nuclease, and the protected fragments were analyzed (Fig. 4). As observed above with a different Spo$^+$ strain (Fig. 3A), RNA prepared from Spo$^+$ strain IS206 protected a labeled fragment of 320 bases. RNA from the strain containing pIS11b showed protection of a slightly larger fragment, ca. 340 bases. RNA from the pISS1a-containing strain gave a complicated pattern of protection, showing full-length and several smaller fragments. This pattern of protection was not affected by incubation of the hybrid with excess S1 nuclease (400 U/ml) at 37°C for up to 75 min. Thus, it is probably not the consequence of incomplete digestion. No protected fragment of the same size as that observed with either IS206 RNA or IS1143 RNA was observed.

The results indicate that a *spo0H* transcript is initiated from a site within the cloned insert of pIS11b. However, the initiation site in pIS11b may be different from that used when transcription is directed from the chromosomal *spo0H* gene. This could be due to a disruption of the natural *spo0H* promoter in the clone and may explain the low level of spore complementation by pIS11b. The full-length fragment protected by RNA of the pIS11a-containing strain suggests that initiation of a *spo0H* transcript occurs outside the cloned insert of pIS11a, possibly from the nearby vector promoter for the kanamycin nucleotidyltransferase gene, which is transcribed in the same direction as the *spo0H* gene in the pIS11a orientation (unpublished data). The strong Spo$^+$-complementing activity of pIS11a is apparently the result of read through from the upstream vector promoter. The absence of RNA initiated internally from the site mapped for pIS11b may be due to promoter occlusion (1).

Determination of the location of rearrangements within the pIS11 insert. The DNA-DNA hybridization experiments described showed that the pIS11 insert was derived from noncontiguous chromosomal DNA sequences. The boundaries of the cloned *spo0H* gene mapped by the modified Berk-Sharp techniques may merely represent sites of DNA rearrangement within the clone rather than the true initiation and termination sites of the *spo0H* transcript. Therefore, further DNA-DNA hybridization experiments were performed to determine whether a rearrangement had occurred in the region mapped by RNA-DNA hybridization as the 5′ boundary of the *spo0H* gene.

By S1 nuclease transcription mapping techniques, we have shown that the *spo0H* gene extends about 320 base pairs upstream of the *Hin*dIII site of the pIS11 insert (Fig. 3A). To probe the *B. subtilis* chromosome for the origins of sequences surrounding this 5′ boundary, the 0.56-kb *Ava*I-*Hin*dIII restriction fragment of the pIS11 insert, which spans the presumptive transcription initiation site of the *spo0H* gene, was used (Fig. 5). The nick-translated 0.56-kb probe hybridized to two fragments of *Ava*I-*Hin*dIII-digested *B. subtilis* DNA. This result indicates that the sequences within the probe are not derived from a contiguous chromosomal DNA segment. The *Ava*I-*Hin*dIII probe was also found to hybridize with two chromosomal *Bgl*II restriction fragments. These two *Bgl*II fragments are apparently the same as two of the three fragments which hybridized to the entire *Bgl*II insert (Fig. 2). The *Ava*I-*Hin*dIII fragment has homology with the large chromosomal *Bgl*II fragment thought to carry the *spo0H* gene.

These results indicate that the 0.56-kb *Ava*I-*Hin*dIII segment of pIS11 is the product of a DNA rearrangement. This finding is in accord with observations suggesting a damaged promoter in the insert, presumably due to a deletion or other rearrangement of DNA sequences which occurred at or very near the *spo0H* promoter during cloning.

DISCUSSION

We cloned a 2.8-kb fragment from the *B. subtilis* chromosome which specifically complements *spo0H* mutations in *trans*. Genetic studies, in which the cloned fragment was inserted into the *B. subtilis* chromosome, showed that the clone carries sequences derived from the chromosomal *spo0H* gene. An internal segment of the 2.8-kb fragment lying within the transcribed region of the *B. subtilis spo0H* gene shares homology with a 1.2-kb chromosomal fragment of *B. licheniformis* which also complements *spo0H* muta-

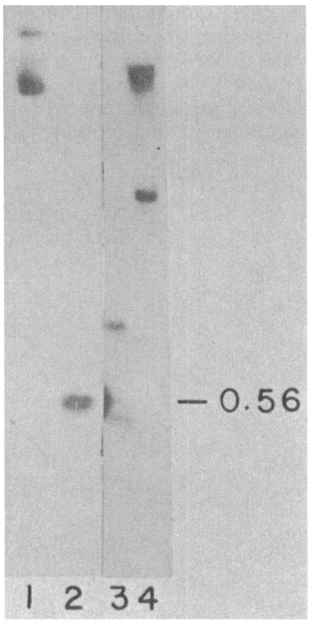

FIG. 5. Chromosomal origin of *Ava*I-*Hin*dIII restriction fragment of cloned insert. pIS11 DNA was restricted with *Ava*I and *Hin*dIII, and a 0.56-kb *Ava*I-*Hin*dIII restriction fragment of the pIS11 insert was isolated. The DNA fragment was nick translated to a specific activity of 2×10^8 cpm/μg and used in hybridization experiments with unrestricted pIS11 (lane 1), with *Ava*I- and *Hin*dIII-restricted pIS11 (lane 2) and IS72 (lane 3) DNAs, and with IS72 DNA which had been digested with *Bgl*II (lane 4). An autoradiogram of the Southern blot is shown. The band present in lane 2 is the *Ava*I-*Hin*dIII restriction fragment of the pIS11 insert.

tions in *trans*. These data show that both *B. subtilis* and *B. licheniformis* cloned fragments encode functional *spo0H* proteins.

Experiments presented in this communication suggest that the *B. subtilis spo0H* clone does not contain the natural *spo0H* promoter. This is presumably a consequence of the cloning event which created pIS11, the plasmid carrying the *B. subtilis spo0H* gene. Unlike the *B. licheniformis spo0H* clone in pIS1 (6), the cloned insert of pIS11 does not complement *spo0H* mutations equally well in both orientations. In vivo transcription mapping experiments indicate that the normal *spo0H* RNA initiation site may be altered in pIS11. In contrast, the *B. licheniformis* 1.2-kb cloned fragment, which has been fully sequenced, contains the natural *spo0H* promoter and RNA initiation site (submitted for publication). A 22,000-molecular-weight protein encoded by the pIS1 cloned insert has been observed in minicells (6). However, no specific *spo0H* protein has yet been identified in similar studies with pIS11 since several insert-specific proteins were observed (data not shown). This may result from the rearranged structure of the pIS11 *spo0H* clone.

The complicated nature of the pIS11 clone may limit its usefulness in studies of *spo0H* expression and gene product function. However, as described above, the pIS11 clone has enabled us to show that the *B. licheniformis* fragment in pIS1 is analogous to the *B. subtilis spo0H* gene. We are presently making antisera to the *B. licheniformis spo0H* protein. On the basis of the DNA sequence homology observed for the *B. subtilis* and *B. licheniformis spo0H* genes, we anticipate that this antisera will also react with the *B. subtilis spo0H* protein. We plan to use the *spo0H*-specific antibody to further study the regulation of *spo0H* gene expression and *spo0H* protein function.

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