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Characterization of a Cloned *Bacillus subtilis* Gene That Inhibits Sporulation in Multiple Copies

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We have isolated a 1.0-kilobase fragment of the *Bacillus subtilis* chromosome which, when present in high-copy-number plasmids, caused a sporulation-proficient strain to become phenotypically sporulation deficient. This is referred to as the sporulation inhibition (Sin) phenotype. This DNA fragment, in multicopy, also inhibited the production of extracellular protease activity, which normally appears at the beginning of stationary growth. The origin of the fragment was mapped between the *dnaE* and *spo0A* genes on the *B. subtilis* chromosome, and its complete DNA sequence has been determined. By analysis of various deletions and a spontaneous mutant the Sin function was localized to an open reading frame (ORF) predicted from the DNA sequence. Inactivation of this ORF in the chromosome did not affect the ability of cells to sporulate. However, the late-growth-associated production of proteases and alpha-amylase was elevated in these cells. The predicted amino acid sequence of the protein encoded by this ORF had a DNA-binding domain, typically present in several regulatory proteins. We propose that the *sin* ORF encodes a regulatory protein that is involved in the transition from vegetative growth to sporulation.

Sporulation in *Bacillus subtilis* is an adaptive response to certain adverse nutritional conditions. This process of endospore formation proceeds through a series of morphological changes and a temporally defined program of gene expression (20, 37). Approximately 10 genetic loci, the *spo0* genes, have been identified which control the onset of sporulation. Mutations in some of these genes have pleiotropic effects, which include blockage in production of late-growth-stage-appearing exoenzymes, acquisition of genetic competence, etc. Although these events are dispensable for sporulation, their appearance is temporally linked to the initiation of sporulation. Since it has been shown that many sporulation genes, when present in high copy number, inhibit sporulation (3, 22, 23), we reasoned that shotgun cloning of DNA fragments which, in multicopy plasmids, inhibit sporulation might allow us to clone new sporulation genes. Study of the sporulation inhibition (Sin) phenotype would also provide new information about the regulation of sporulation. This approach is very similar to that taken by Hartwell and co-workers in isolating genes from *Saccharomyces cerevisiae* which are involved in mitosis and cell division (30). This method has enabled us to clone and then characterize the *B. subtilis spo0F* gene (27).

In this report we describe the cloning of a DNA fragment from *B. subtilis* that inhibits sporulation and the production of extracellular protease when present in multicopy plasmid vectors. We have sequenced this fragment, and the predicted amino acid sequence determined by the gene encoding the spore inhibition function is similar to that of DNA-binding proteins. The phenotype resulting from inactivation of this gene in the chromosome suggests it might be a regulatory element involved in the transition from vegetative to stationary growth and sporulation.

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MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains used in this study are listed in Table 1. *Escherichia coli* strains were grown in LB medium and were made competent by the procedure described by Cohen et al. (11). *B. subtilis* strains were grown on tryptose blood agar base (TBAB; Difco Laboratories, Detroit, Mich.) or minimal glucose medium and were made competent by the procedure described by Anagnostopoulos and Spizizen (1). Sporulation proficiency was measured by exposing colonies on TBAB agar plates to chloroform vapors for 2 h. Spo^+ colonies survive this treatment, whereas Spo^- colonies are lysed.

Plasmid DNA manipulations. Isolation of plasmid DNA, restriction, ligation, and plasmid transformation were done as described by Gryczan and Dubnau (18) and Gryczan et al. (16). Transductions with generalized transducing bacteriophage AR9 were performed as previously described (47).

Formation of pIS19 and derivatives. Chromosomal DNA from strain IS190 was digested to completion with various restriction enzymes. The DNA was ligated to appropriately restricted pBD64 plasmid DNA. The ligated DNA was transformed into competent cells of strain IS75, which carried plasmid pUB110, by using the resident plasmid rescue system (17) modified for the cloning of homologous DNA fragments (51). The transformed IS75 cells were grown in the presence of chloramphenicol in liquid medium, and total plasmid DNA was prepared after overnight growth. The DNA was transformed to competent cells of IS120, a *recE* Spo^+ strain, and chloramphenicol-resistant (Cam^r) colonies were selected and screened visually for their Spo phenotype. Three Spo^- colonies were obtained from *Xba*I-cut DNA cloned into the *Xba*I site of pBD64. Plasmid DNA made from each of these colonies contained a 4-kilobase (kb) insert at the *Xba*I site. A restriction map of the insert in this plasmid (pIS19) is shown in Fig. 1. *Hin*dIII or *Fnu*DII deletions in pIS19, to give plasmids pIS37 and pIS92, respec-

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype[a]	Source[b] (strain designation)
B. subtilis strains		
IS75	*metB5 hisA1 leuA8*	I. Smith
IS120	*trpC2 thrA recE4*	D. Dubnau (BD224)
IS170	*metB5 hisA1 recE4*	I. Smith
IS190	*trpC2 leuA8 sacU^{h}100*	R. Dedonder (QB157)
IS149	*aroD120 lys-1 trpC2*	R. Dedonder (QB935)
IS211	*trpC2 pheA1 spo0A12*	BGSC (IS9)
BD359	*dnaE20 ilvA1 metB5*	D. Dubnau
IS353	*metB5 hisA1 leuA8* Cm^r	This work
IS354	*metB5 hisA1 leuA8* Cm^r	This work
IS383	*metB5 hisA1 leuA8* Cm^r	This work
Plasmids		
pBD64	Cm^r Km^r	D. Dubnau
pUB110	Km^r	D. Dubnau
pE194	Em^r	D. Dubnau
pBD337	Cm^r Em^r St^r	D. Dubnau
pCP115	Ap^r Tc^r Cm^r	R. Doi
pIS19	Cm^r Sin$^+$	This work
pIS21a	Em^r Sin$^+$	This work
pIS21b	Em^r Sin$^+$	This work
pIS37	Em^r Sin$^+$	This work
pIS100	Em^r Sin$^-$	This work
pIS92	Cm^r Sin$^-$	This work
pIS74	Cm^r Sin$^+$	This work
pIS87	Cm^r Sin$^-$	This work
pIS90	Cm^r Sin$^-$	This work
pIS91	Cm^r Sin$^-$	This work
pIS95	Ap^r Cm^r	This work
pIS96	Ap^r Cm^r	This work
pIS119	Cm^r Sin$^+$	This work
pIS120	Cm^r Sin$^+$	This work

[a] Abbreviations: Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Em^r, erythromycin resistance; St^r, streptomycin resistance; Ap^r, ampicillin resistance; Tc^r, tetracycline resistance; Sin$^+$, sporulation inhibition; Sin$^-$, no sporulation inhibition.

[b] BGSC, *Bacillus* Genetic Stock Center.

tively, were constructed by completely digesting pIS19 DNA with the respective restriction enzyme. After ligation, DNA was transformed into IS170, a *recE* and sporulation-proficient strain, and plasmid DNA from both Spo$^+$ and Spo$^-$ colonies was prepared and analyzed for deletions by restriction analysis. Plasmid pIS74 was constructed by subcloning *Xba*I-*Hin*dIII-digested pIS19 DNA into vector plasmid pBD337, which contains the *cop-6* mutation in its pE194 portion, which had also been digested with *Xba*I and *Hin*dIII. This separates the vector into two fragments, one of which contains the pE194 origin of replication and a chloramphenicol resistance gene. The second fragment contains the erythromycin resistance determinant but does not have an *ori* function. The streptomycin resistance gene is destroyed by the *Hin*dIII restriction. The ligated plasmid preparation was used to transform IS170 to chloramphenicol resistance. Spo$^+$ and Spo$^-$ colonies were purified, and their plasmids were analyzed by *Xba*I and *Hin*dIII double restriction. All Sin$^+$ (Spo$^-$) colonies had a 1.0-kb *Xba*I-*Hin*dIII insert. These plasmids had lost the ability to confer erythromycin and streptomycin resistance, as was expected from the cloning event. The *Bal*I-*Nru*I deletion in pIS74 to form pIS90 was constructed by ligating the blunt ends generated by *Bal*I and *Nru*I in pIS74. To form pIS100 and pIS91, small deletions at the *Bal*I site were constructed by restricting pIS37 and pIS74 with *Bal*I, ligating, and transforming into IS170 competent cells. Both Spo$^+$ (<5%) and Spo$^-$ colonies were obtained. Plasmids were made from both Spo$^+$ and Spo$^-$ colonies and analyzed for the presence of the *Bal*I site. Plasmids isolated from Spo$^+$ (Sin$^-$) colonies had lost the *Bal*I site, whereas plasmids isolated from Spo$^-$ (Sin$^+$) colonies retained this site.

Measurement of alkaline phosphatase activity. The culture was grown in nutrient sporulation medium (44), and alkaline phosphatase activity was measured at different times as described by Dancer and Mandelstam (12). A 10-ml sample was centrifuged, and the pellet was suspended in 1 ml of sterilized deionized water. Samples were vortexed for 1 min with 3 drops of toluene, and 1 ml of *p*-nitrophenylphosphate (1 mg/ml) in diethanolamine hydrochloride buffer (1.0 M, pH 10.0) was added to samples that had been heated to 37°C. After incubation at 37°C for 5 min, the reaction was stopped by adding 1 ml of 2 N NaOH. The suspension was centrifuged at 10,000 rpm for 20 min, and the optical density (OD) at 410 nm was measured against the reagent blank. Alkaline phosphatase activity was defined as the change in OD at 410 nm by cells from 1 ml of culture at 37°C in 5 min. One OD unit is equivalent to 1 U of activity. The specific activity was obtained by dividing the activity by the corresponding number of Klett units of the culture.

Measurement of extracellular protease activity. The protease activity in culture supernatants was assayed as described by Christison and Martin (10). A 25-mg amount of Azocoll (Sigma Chemical Co.) were added to suitable samples of cell supernatants in 0.1 M Tris hydrochloride (pH 7.5) and water to give a final volume of 5 ml. The reaction mixture was incubated at 37°C for 10 min with shaking. The digests were then filtered through Whatman filter paper, and the OD_{520} was measured in the filtrates. The blank was prepared the same way except that enzyme-free medium was added. Activity was defined as the change in OD_{520} by 1 ml of culture supernatant at 37°C in 10 min. One OD unit is equivalent to 1 U of activity. The specific activity was

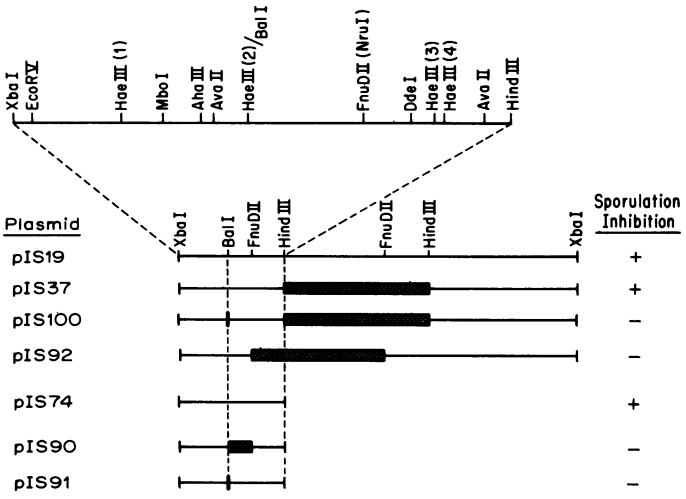

FIG. 1. Restriction map of the cloned region of *B. subtilis* DNA containing the Sin function. The deletion derivatives of pIS19 and pIS74 are shown, with their associated Sin phenotypes. The filled boxes indicate the deleted region in the original cloned insert. Sporulation inhibition in all cases was measured by the chloroform sensitivity of transformed cells on solid medium. Sin$^+$ colonies (Spo$^-$) were lysed by chloroform vapors, while Sin$^-$ (Spo$^+$) colonies were resistant and remained viable.

obtained by dividing the activity by the corresponding number of Klett units of the culture. To discriminate between neutral and serine protease activities, certain assays were performed in the presence of 5 mM EDTA, which inhibits the former enzyme, or 1 mM phenylmethylsulfonyl fluoride, which inhibits the latter.

DNA sequencing. The restriction fragments to be sequenced were cloned into appropriate cloning sites in mp10 and mp11 vectors derived from bacteriophage phage M13 (31). DNA sequencing was performed by the dideoxy chain termination method (43) with the M13 sequencing kit obtained from Amersham Corp. Sequencing gels were 0.4 mm thick and 40 cm long and were cast with 6% polyacrylamide containing 8 M urea.

RESULTS

Cloning of a *B. subtilis* restriction fragment that exerts sporulation inhibition (Sin) activity. By transforming sporulation-proficient, *recE B. subtilis* strains with multicopy plasmid vectors containing restriction fragments from the *B. subtilis* chromosome, we were able to isolate clones which inhibited sporulation. One of these clones, pIS19, was obtained by cleaving plasmid pBD64 with *Xba*I and ligating it to *Xba*I limit restriction chromosomal digests (Fig. 1). To analyze the 4.0-kb *Xba*I insert in pIS19, we first recloned the insert into the *Xba*I site of plasmid pE194 to generate pIS21a and pIS21b. These plasmids, which contained the insert in two orientations, both inhibited sporulation.

To find out which portion of the 4.0-kb fragment was necessary for the spore inhibition (Sin) phenotype, various restriction deletions were made (Fig. 1). The tandem *Hin*dIII deletion in pIS37 did not affect the Sin phenotype, but the *Fnu*DII tandem deletion (pIS92) or small deletions around the *Bal*I restriction site (pIS91 and pIS100) caused a Sin^- phenotype (not inhibitory of sporulation). The 1.0-kb *Xba*I-*Hin*dIII leftmost fragment was then subcloned into the high-copy-number plasmid pBD337 to form pIS74, which as expected maintained the Sin function.

Southern gel analysis of chromosomal DNA isolated from strain IS190, with this *Xba*I-*Hin*dIII fragment as a radioactive probe, demonstrated the presence of an *Xba*I fragment which migrated identically with the 4.0-kb *Xba*I insert in pIS21. An *Xba*I-*Hin*dIII chromosomal fragment which comigrated with the 1.0-kb insert found in pIS74 was also observed (data not shown). This indicates that no DNA rearrangements occurred during the initial cloning of the 4.0-*Xba*I fragment and its subcloning.

Characterization of the Sin phenotype. The frequency of sporulation in liquid medium of IS170 (*recE* Spo^+) carrying pIS74 was compared with that of IS170 cells carrying pIS87, a spontaneous mutant of pIS74 which has lost the Sin function (see below). Total viable and spore (heat-resistant) counts were made at intervals during the sporulation process. Sporulation was inhibited by 2 to 3 orders of magnitude when pIS74 was present (Table 2). Cells carrying pIS87 sporulated as efficiently as plasmid-free Spo^+ cells (data not shown).

TABLE 2. Sporulation inhibition by pIS74[a]

Time (h)[b]	IS170(pIS74)		IS170(pIS87)	
	Cells/ml	Spores/ml	Cells/ml	Spores/ml
2	3.42×10^8	8.10×10^3	2.94×10^8	1.68×10^5
8	4.66×10^8	1.10×10^5	1.74×10^8	2.32×10^8
20	3.40×10^8	7.70×10^5	3.69×10^8	3.60×10^8

[a] Cells were grown in nutrient sporulation medium at 37°C, and at the indicated times appropriate dilutions of the cell cultures were made and plated on TBAB agar, containing 5 μg of chloramphenicol per ml. This gave total viable cell counts (cells). Diluted samples were heated at 80°C for 30 min before plating. This gave heat-resistant counts (spores).

[b] Hours after the beginning of stationary phase.

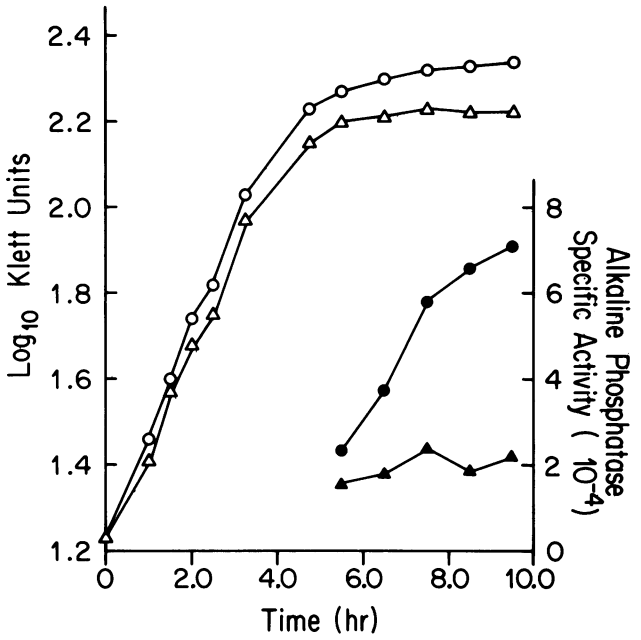

FIG. 2. Effect of the Sin function on alkaline phosphatase activity. Alkaline phosphatase levels were measured in IS170 carrying pIS74 (▲) or pIS87 (●) during growth in nutrient sporulation medium containing chloramphenicol (5 μg/ml). A 10-ml amount of culture was removed and centrifuged at the indicated times, and the pellet was suspended in 1 ml of deionized water. The samples were assayed for alkaline phosphatase after cells were permeabilized with toluene as described in the text. The growth of IS170 carrying pIS74 (△) and pIS87 (○) is also shown. Specific activity was obtained by dividing alkaline phosphatase activity, as defined in Materials and Methods, by the OD (Klett units) of the culture.

Alkaline phosphatase activity dramatically increases when sporulating bacilli pass spore stage II, and its appearance is blocked in early *spo* mutants (carrying *spo0* and *spoII*) (37). We measured alkaline phosphatase activity in IS170 cells carrying pIS74 or pIS87 grown in liquid sporulation medium and found that cells carrying the former plasmid did not show the increase in enzyme activity observed in the control cells (Fig. 2).

Two major extracellular proteases, the calcium-dependent neutral protease and the serine protease subtilisin, appear at the onset of sporulation (20, 37). We assayed the production of total extracellular protease activity in strains carrying pIS74 and pIS87. Little or no extracellular protease activity was observed in pIS74-containing cells, while normal amounts were observed in the controls (Fig. 3). The presence of a *sacU*^{h} mutation, which causes overproduction of levansucrase and several growth-regulated exoenzymes (26), had no effect on the inhibition of protease production caused by pIS74 (data not shown). α-Amylase production was not affected by the presence of pIS74 (data not shown).

Chromosomal origin of the cloned fragment. To map the origin of the cloned fragment in the *B. subtilis* chromosome, we used the integrative plasmid pCP115, a derivative of pBR322 carrying a chloramphenicol resistance determinant which can be expressed in *B. subtilis* (40). The plasmid is

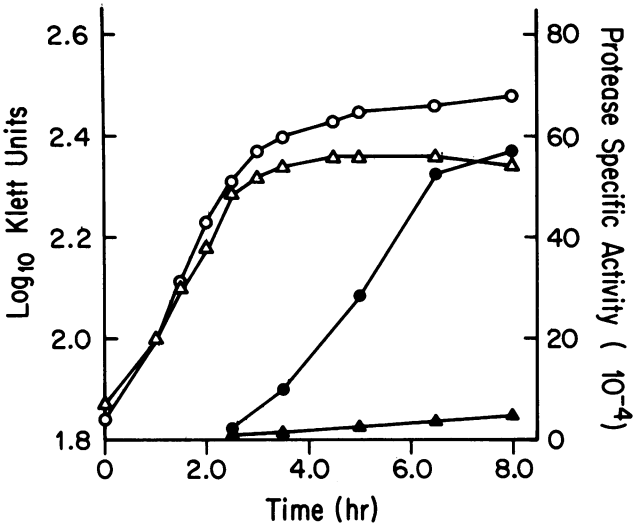

FIG. 3. Effect of Sin function on extracellular protease activity. Extracellular protease activity was measured in cell-free supernatants of IS170 carrying pIS74 (▲) and pIS87 (●) grown in nutrient sporulation medium containing chloramphenicol (5 μg/ml). At the indicated times, 5.0-ml samples were removed from cultures, and the supernatants were assayed for protease activity as described in the text. The growth of the culture carrying pIS74 (△) and pIS87 (○) is also shown. Specific activity was obtained by dividing protease activity, as defined in Materials and Methods, by the OD (Klett units) of the culture.

incapable of replicating in *B. subtilis* but can transform *B. subtilis* to Cm^r if it carries a fragment of the *B. subtilis* chromosome, which allows the plasmid to integrate into the homologous site of the cloned fragment (19). Mapping of the Cm^r marker provides the chromosomal location of the fragment.

We cloned the 161-base-pair (bp) *Aha*III-*Hae*III fragment from pIS74 into the *Bam*HI site of pCP115 after filling in the 5′ overhang ends created by *Bam*HI. The resultant plasmid, pIS95, was used to transform IS75 to Cm^r. The plasmid integrated into the *B. subtilis* chromosome at the site of homology provided by the cloned *Aha*III-*Hae*III fragment, as shown by Southern blot analysis (data not shown). The strain carrying integrated pIS95 is referred to as strain IS353. Strain IS354 was constructed in a similar fashion by using the *Bal*I-*Nru*I fragment cloned into pCP115 to form plasmid pIS96. AR9 transducing bacteriophage was grown on IS353 and IS354, and the lysates were used as donors in transductional crosses with multiply marked strains of *B. subtilis*. Cm^r transductants were selected, and linkage to various unselected markers was determined (Table 3). The three-factor data suggest that the plasmids have integrated into the *B. subtilis* chromosome between *aroD* and *lys*, and two-factor data suggest that the sites of integration are equidistant to and map between *dnaE* and *spo0A*. We were unable to map these sequences relative to *spo0G*, which also lies between *dnaE* and *spo0A*, because of an unusually rough phenotype observed with the integrated plasmid in *spo0G* strains. (This was not observed in a *spo0A* background.) However, transformation experiments indicated that the cloned DNA did not contain *spo0G*. The order of genes in this part of the chromosome is thus deduced to be *aroD-dnaE-sin-spo0A-lys*.

TABLE 3. Transduction crosses involving Cm^r, *aroD*, *lys*, *dnaE*, and *spo0A*[a]

Donor	Recipient	Recombinant classes[b]						Suggested order[c] or linkage (%)
		spo0A	*dnaE*	Cm^r	*lys*	*aroD*	No.	
IS353	IS149			1	1	1	12	*aroD*-Cm^r-*lys*
(Cm^r)	(*aroD lys*)			1	1	0	23	
				1	0	1	60	
				1	0	0	103	
IS354	IS149			1	1	1	10	*aroD*-Cm^r-*lys*
(Cm^r)	(*aroD lys*)			1	1	0	38	
				1	0	1	70	
				1	0	0	68	
IS353	IS211	0		1			70	49
(Cm^r)	(*spo0A*)	1		1			67	
IS353	BD359		0	1			47	68
(Cm^r)	(*dnaE*)		1	1			81	
IS354	BD359		0	1			47	66
(Cm^r)	(*dnaE*)		1	1			92	

[a] Selection was for Cm^r in each case.
[b] 1 and 0, donor and recipient phenotypes, respectively.
[c] The order is tentative, since Cm^r was the only marker selected for in the three-factor crosses.

Nucleotide sequence analysis. The 1.0-kb *Xba*I-*Hin*dIII fragment in pIS74 was sequenced completely by the strategy outlined in Fig. 4. We determined the sequence on both DNA strands and through all restriction sites used in subcloning by the dideoxy chain terminating method (43). The DNA sequence of one strand of the 1037 bp fragment is presented in Fig. 5. Three open reading frames (ORFs) were encoded by this DNA strand which had appropriately spaced putative translational signals. The DNA sequence of the other strand predicted two ORFs (ORF 4 and ORF 5), which had no known consensus translational signals (Fig. 6). ORF 1, encoding 57 amino acid residues, had an ATG codon at position 283 and was preceded by a potential ribosome-binding site, an AGGAGGAG sequence, which was complementary to the 3′ terminus of the 16S rRNA (45). The 111-amino-acid ORF 2 had TTG, a rare start codon in *B. subtilis* (29), and was preceded 8 bp upstream by an AAGGAAGG sequence. ORF 3 coded for 35 amino acid residues and had an ATG start codon at position 840 which was preceded by a relatively weak ribosome-binding site, GAGACGG, 8 bp upstream. The sequence from 813 to 902 contained two regions of dyad symmetry which, if transcribed, would produce two potential hairpin structures in the RNA. The first hairpin structure, which extends from 813 to 855, has 17 bp in the stem with a single mismatch and 6

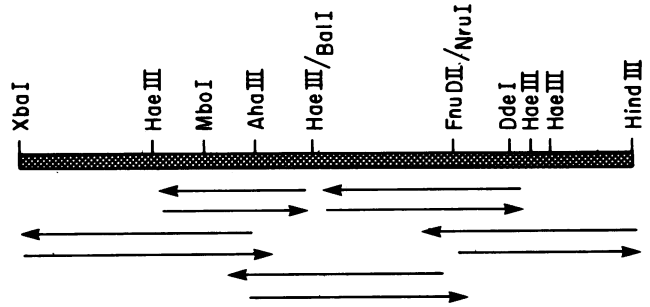

FIG. 4. Sequencing strategy for the cloned insert in pIS74 bearing the *sin* gene. The arrow represents the direction and extent of the sequence read. The DNA sequence was determined by the dideoxy chain termination method described by Messing and Viera (31). Each strand was sequenced at least twice, and all restriction sites were overlapped.

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XbaI
TCTAGAAAAACAGGCGCTGAAAACCTTGTATCAACCAAAGATATCAGTTGAAATTGAAAATGGCGGATTGTTTTATCTGCAAAATAATATTTCAAGCTA   99

ACTTTTTTACCATTCGACATCATTCTCGTTTTTTTTGAGAAAATACGATTATAATAAAGGTATATTGGAAAAAAATTCTGGTGATTTAATGGCAAATGA  198

                                    HaeIII
CTTCCAGAGACTAATGAAGCATACAATAAGTCATGGCCGGACTGGCTGAAATACATAAACAAGTATTTTAGGAGGAGAAACTGC   ATG AAG AAT  291
                                                                    S.D. 1          f-Met Lys Asn

                          MboI
GCA AAA CAA GAG CAC TTT GAA TTG GAT CAA GAA TGG GTT GAA TTA ATG GTG GAA GCC AAA GAG GCA AAT ATC AGC  366
Ala Lys Gln Glu His Phe Glu Leu Asp Gln Glu Trp Val Glu Leu Met Val Glu Ala Lys Glu Ala Asn Ile Ser

                    AhaIII
CCG GAA GAA ATA CGA AAA TAT TTA CTT TTA AAC AAA AAG TCT GCT CAT CCT GGT CCG GCA GCC AGA AGT CAT ACC  441
Pro Glu Glu Ile Arg Lys Tyr Leu Leu Leu Asn Lys Lys Ser Ala His Pro Gly Pro Ala Ala Arg Ser His Thr

                                                BalI(HaeIII)
GTA AAT CCT TTC TGA ATGTGCTATAATATCACAAGGAAGGTGATGACA   TTG ATT GGC CAG CGT ATT AAA CAA TAC CGT AAA  522
Val Asn Pro Phe ***          S.D.2          f-Met Ile Gly Gln Arg Ile Lys Gln Tyr Arg Lys

GAA AAA GGC TAC TCA CTA TCA GAA CTA GCT GAA AAA GCT GGG GTA GCG AAG TCT TAT TTA AGC TCA ATA GAA CGA  597
Glu Lys Gly Tyr Ser Leu Ser Glu Leu Ala Glu Lys Ala Gly Val Ala Lys Ser Tyr Leu Ser Ser Ile Glu Arg

AAT CTA CAA ACG AAC CCC TCC ATT CAA TTT CTC GAA AAA GTC TCC GCT GTT CTG GAC GTC TCG GTT CAT ACT TTG  672
Asn Leu Gln Thr Asn Pro Ser Ile Gln Phe Leu Glu Lys Val Ser Ala Val Leu Asp Val Ser Val His Thr Leu

                                                NruI(FnuDII)
CTC GAT GAG AAA CAT GAA ACC GAA TAC GAT GGT CAA TTA GAT AGT GAA TGG GAG AAA TTG GTT CGC GAT GCG ATG  747
Leu Asp Glu Lys His Glu Thr Glu Tyr Asp Gly Gln Leu Asp Ser Glu Trp Glu Lys Leu Val Arg Asp Ala Met

ACA TCC GGG GTA TCG AAA AAA CAA TTT CGT GAA TTT TTA GAT TAT CAA AAA TGG AGA AAA TCC CAA AAA GAG GAG  822
Thr Ser Gly Val Ser Lys Lys Gln Phe Arg Glu Phe Leu Asp Tyr Gln Lys Trp Arg Lys Ser Gln Lys Glu Glu

      DdeI                                        HaeIII                          HaeIII
TAG TGCCTGAGCAGAGGCACTAACTCCTCTTTTGTCAATAACCATCGAGACGGCCCAGTAT  ATG AAT ACT GGG CCG TCT CTT TTT TTT  910
***                                             S.D. 3          f-Met Asn Thr Gly Pro Ser Leu Phe Phe

GCT GTT ATT AAT TTT TAT CCT CGC TAT GCG CTT TTT CAT TTT CTT TCA CGT AAC CAT CTT TAT CAG TAT GGT CCT  985
Ala Val Ile Asn Phe Tyr Pro Arg Tyr Ala Leu Phe His Phe Leu Ser Arg Asn His Leu Tyr Gln Tyr Gly Pro

                                                HindIII
TTT TGA TTGTCAAGCCGTTCCACTGTGTAGCTTCGAATGAGAATTGAAGCTT  1037
Phe ***
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FIG. 5. Nucleotide sequence of the insert in pIS74. The DNA sequence of one strand of the *Xba*I-*Hin*dIII insert in pIS74 is illustrated. The predicted amino acid sequences of three ORFs with putative ribosome-binding sites (S.D. 1, S.D. 2, and S.D. 3) are also shown. The two regions of dyad symmetry are overlined. Relevant restriction sites are also indicated.

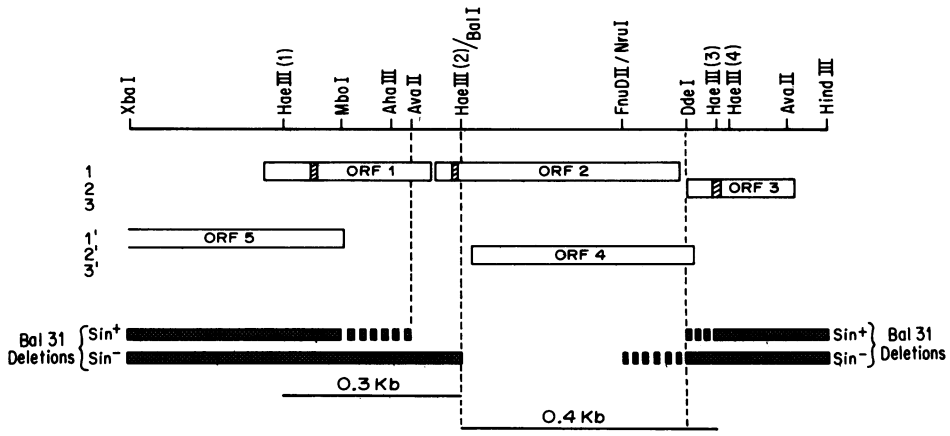

FIG. 6. Predicted ORFs from nucleotide sequence and the effect of Bal 31 deletions on the Sin phenotype. The ORFs are shown as boxes with respect to restriction sites. The shaded vertical line in the box represents the position of the putative translational signal (ribosome-binding site and appropriately spaced initiation codon). The lower two boxes are the ORFs deduced from the sequence of the other DNA strand and are devoid of any known translational signal. The Bal 31 deletions are shown with their associated Sin phenotypes. The dotted line represents the endpoints of the various deletions. The positions of the 0.3-kb *Hae*III (*Hae*III sites and 1 and 2) and 0.4-kb *Hae*III (*Hae*III sites 2 and 3) fragments are also shown. To construct Bal 31 deletions, plasmid pIS74 DNA (ca. 10 μg) was linearized at the unique *Hin*dIII or *Xba*I site and incubated with 10 U of Bal 31 (Boehringer Mannheim) for different times. The incubation was done at 24°C under the following conditions: 20 mM Tris hydrochloride (pH 8.0), 600 mM NaCl, 12.5 mM $CaCl_2$, and 12.5 mM $MgCl_2$ in a 100-μl total reaction volume. The reaction was stopped by adding 10 μl of 0.2 M EGTA (pH 8.0) and immediately extracting with phenol. The ligated DNA was transformed into IS170 competent cells, and the plasmid DNAs from both Spo^+ and Spo^- colonies were analyzed by restriction analysis.

TABLE 4. Homology between ORF 2, sigma factors, and DNA-binding proteins[a]

Protein	Amino acid sequence	No. identical
	HA HGH I/L/V	
ORF 2	L S E L A E K A G V A K S Y L S S I E R	
E. coli σ^{32}	L Q E L A E R Y G V S A E R V R Q L E K	8
B. subtilis σ^{43}	L E E V G K V F G V T R E R I R Q I E A	6
λ cII	T E K T A E A V G V D K S Q I S R W K R	8
LacR	L Y D V A E Y A G V S Y Q T V S R V V N	7
TetR Tn*10*	T R K L A Q K L G V E Q P T L Y W H V K	6
λ Rep	Q E S V A D K M G M G Q S G V G A L F N	4
λ Cro	Q T K T A K D L G V Y Q S A I N K A I H	4
P22 Rep	Q A A L G K M V G V S N V A I S Q W Q R	5
P22 Cro	Q R A V A K A L G I S D A A V S Q W K E	3
CAP	R Q E I G Q I V G C S R E T V G R I L K	3
GalR	I K D V A R L A G V S V A T V S R V I N	5
γδ resolvase	A S H I S K T M N I A R S T V Y K V I N	3

[a] Comparison of homology between ORF 2 (amino acids 17 through 36), σ^{32} (25), σ^{43} (15), and DNA-binding proteins (35). Conventional one-letter amino acid codes are used. The overlined amino acid in the DNA-binding proteins is identical to the corresponding amino acid in ORF 2. The numbers at the right side of each line indicate the number of identical amino acids shared by ORF 2 and the particular protein. The overlined amino acids in ORF 2 are homologous with amino acids in at least one of the DNA-binding proteins and σ factors. The first line across shows the consensus amino acids. Hydrophobic residues are denoted by H.

nucleotides in the loop and would have a predicted ΔG value of −38.0 kcal. The second hairpin structure, which spanned the region from 869 to 902, resembled a typical rho-independent transcription terminator (42). This hairpin structure was immediately followed by a run of eight U's and had a perfect GC-rich 15-bp stem with a ΔG value of −32.5 kcal, with four nucleotides in the loop.

Homology with other proteins. DNA-binding proteins share a structural similarity in that they all have a helix-turn-helix structure which is required for protein-DNA interactions (35). They generally contain a sequence of 20 amino acids which includes the virtually invariant pattern Ala $(N)_3$-Gly-$(N)_5$-Val/Ile/Leu. ORF 2 had a region, amino acids 17 through 36, that was similar to the 20-amino-acid consensus sequence, containing residues at 18 positions that were identical to those found in the sigma factors and DNA-binding proteins listed in Table 4. The ORF 2 sequence most closely resembled segment C, one of the two DNA-binding domains of *E. coli* σ^{32} (25), having eight identical amino acids with five conservative replacements. Chou-Fasman analysis (9) of the predicted amino acid sequence revealed that the region had two α-helices which were connected by a turn at which the conserved glycine was present.

The amino acid sequence comparison of *E. coli* σ^{70} (8) and σ^{32} (25) revealed another highly conserved region with 14 identical amino acids. Homologous sequences are found in *B. subtilis* σ^{43} (15) and σ^{29} (48). We compared predicted amino acid sequences from ORF 1 with this conserved region and found partial homology between ORF 1 (amino acids 10 to 22), σ^{70} (amino acids 403 to 416), and σ^{32} (amino acids 77 to 90). Five amino acids were identical and three were conservative replacements in this 14-amino acid-region (Table 5).

Deletion analysis of Sin function. Knowledge of the nucleotide sequence allowed us to construct various deletions in pIS74 to further localize regions determining the Sin function. A 240-bp deletion was constructed in pIS74 by restricting it at the unique *Bal*I and *Nru*I sites. The resulting plasmid, pIS90, had a deletion in the 0.4-kb *Hae*III fragment (delimited by *Hae*III sites 2 and 3) and was not capable of inhibiting sporulation (Fig. 1 and 6). Two spontaneous deletions (<50 bp) were also identified in the 0.3-kb *Hae*III fragment (delimited by *Hae*III sites 1 and 2) which also caused loss of the ability to inhibit sporulation. The requirement for both 0.3-kb and 0.4-kb *Hae*III fragments for sporulation inhibition was also demonstrated by partially restricting pIS74 with *Hae*III and transforming ligated DNA into IS170 competent cells. The Spo$^-$ colonies always carried plasmids having both 0.3- and 0.4-kb *Hae*III fragments, indicating that both fragments are required for Sin function. Small deletions around the *Bal*I site, in pIS100 and pIS91, also inactivated the Sin function (Fig. 1).

To further define the boundary of Sin function, we constructed a series of deletions in pIS74. The plasmid was linearized at the unique *Hin*dIII site and incubated with Bal 31 exonuclease for different times. Comparable numbers of Spo$^+$ and Spo$^-$ colonies were obtained after a 1-min incubation with Bal 31. All 13 Sin$^-$ plasmids (prepared from Spo$^+$ colonies) had lost the *Dde*I and *Hae*III sites denoted by the numbers 3 and 4 (Fig. 6). All 31 Sin$^+$ plasmids (prepared from Spo$^-$ colonies) retained their *Dde*I site, with the exception of ΔH133, which had lost the site. *Hae*III sites 3 and 4 were present in 24 of the Sin$^+$ plasmids and absent in the remaining 7. Figure 6 shows the approximate endpoints of deletions from the *Hin*dIII site and the phenotype associated with them. The results suggest that the *Dde*I site should be very close to one end of the Sin function. These results also indicate that ORF 3 and the region of dyad symmetry between ORFs 2 and 3 are not essential for Sin

TABLE 5. Homology between ORF 1 and sigma factors[a]

Protein	Amino acid position	Amino acid residues
ORF 1	10	Glu Leu Asp Gln Glu Tyr — Val Glu Leu Met Val Glu Ala
		+ * * * + * * +
σ^{70}	403	Asp Leu Ile Gln Glu Gly Asn Ile Gly Leu Met Lys Ala Val
		* * * * * * * * * * * * * *
σ^{32}	77	Asp Leu Ile Gln Glu Gly Asn Ile Gly Leu Met Lys Ala Val

[a] The amino acid sequences of the σ factors have been previously published (8, 25). A gap was introduced in the derived amino acid sequence of ORF 1 to allow better alignment. Symbols: * and +, identical and conserved amino acids, respectively.

activity. This deletion analysis and the necessity of the *BalI* site for the Sin function (see above) rule out the involvement of ORF 4 in this phenomenon.

Similarly, Bal 31 deletions were produced from the *XbaI* site of pIS74. Of 92 clones resulting from the transformation of Spo$^+$ cells by plasmids that had been restricted with *XbaI*, Bal 31 exonuclease treated, and ligated, 35 were Sin$^-$ and 57 were Sin$^+$. Forty-three of the Sin$^+$ plasmids kept *HaeIII* site 1, whereas 14 of the Sin$^+$ plasmids lost this *HaeIII* site. Of these, five had also lost the *MboI* site. Thirty-two of the Sin$^-$ plasmids lost *HaeIII* site 2, and three retained this site. These deletions place the other end of the Sin function between the *MboI* site and *HaeIII* site 2. The results from both sets of Bal 31 deletions indicate that the Sin function is localized between the *MboI* and *DdeI* restriction sites on pIS74 and that like ORF 3, ORF 1 is not required for Sin activity.

To provide more evidence for this localization of Sin function, the *MboI-HindIII* fragment containing ORF 2 and the unnecessary ORF 3 (see above) was subcloned into two high-copy-number *B. subtilis* plasmids, pIS26 and pMM37. The resulting plasmids, pIS120 and pIS119, respectively, both inhibited sporulation. (The noninvolvement of the *XbaI-MboI* region in the Sin function also demonstrates that ORF 5 is not necessary for the Sin phenotype.) pIS26 was derived from pIS9A, and there was no promoter upstream reading through the pIS9A *BglII* (*MboI*) site (41). This suggests that ORF 2, if its gene product is responsible for the Sin phenotype, has its own promoter. More evidence for the involvement of ORF 2 in the Sin phenotype was obtained from the DNA sequence of pIS87, a plasmid that was isolated as a spontaneous Sin$^-$ mutant of pIS74. The sequence was identical to the *XbaI-HindIII* pIS74 insert except for six positions (Fig. 7). These changes lay in the first hairpin structure but were such that they do not change its stability. One of these changes was the insertion of a T at bp 822, just before the UAG termination codon of ORF 2. This would remove this termination site and place a new termination codon (UAA) at position 918, adding 32 amino acids at the C-terminal end (Fig. 7). Since the deletion analysis

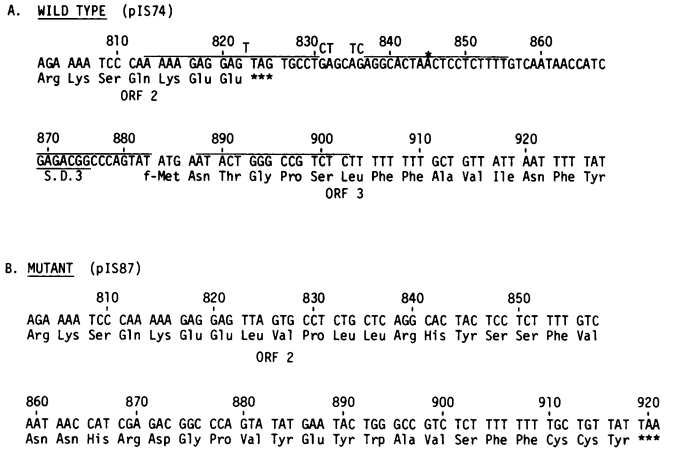

FIG. 7. Nucleotide and deduced amino acid sequences of a spontaneous mutation affecting Sin function. (A) Sequence of the C-terminal end of ORF 2 and the N-terminal end of ORF 3 in pIS74. The mutations found in pIS87 are indicated above the nucleotide sequence. The asterisk over bp 845 indicates the deletion of this adenine residue found in pIS87. (B) Sequence of the cloned insert in pIS87, a spontaneous mutant with an inactivated Sin function. ***, Stop codon.

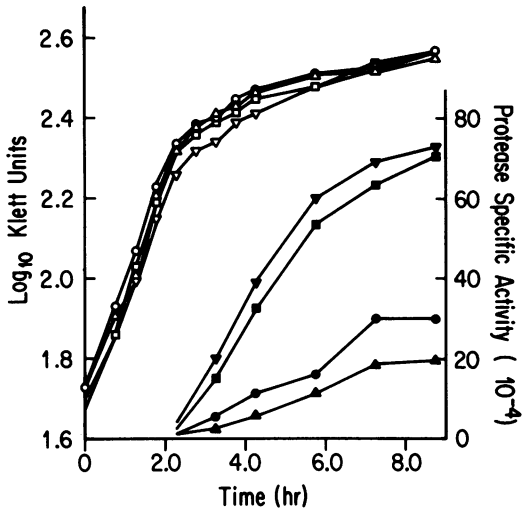

FIG. 8. Effect of ORF 1 and ORF 2 disruptions on exoprotease production. Protease activity was measured in the supernatants of IS75 (●), IS383 (▲), IS353 (■), and IS354 (▼) grown in nutrient sporulation medium. All strains were grown in the medium containing 5 μg of chloramphenicol per ml except IS75, which was grown without antibiotic. IS383 contains a plasmid conferring Cm^r which has integrated near *cysB*, an unlinked region of the *B. subtilis* chromosome. Activity was determined in the supernatants as described in Materials and Methods. The growth of IS75 (○), IS383 (△), IS353 (□), and IS354 (▽) is also shown. Specific activity was obtained by dividing the protease activity by the OD (Klett units) of the culture.

reported above indicated that the region of dyad symmetry was not necessary for the Sin phenotype, it is likely that the alteration of the C-terminal end of ORF 2 is responsible for the loss of Sin function.

Since cells containing pIS87 showed normal exoprotease production (Fig. 3), ORF 2 also must be involved in the repression of this late growth function. Plasmids pIS119 and pIS120, described above, which had a disrupted ORF 1, inhibited exoprotease production when present in *B. subtilis* cells (data not shown), ruling out the involvement of ORF 1 in this repression. Similar experiments with two plasmids with Bal 31 deletions from the *HindIII* site, both of which removed ORF 3, showed that the plasmid which was Sin$^+$, ΔH149, repressed extracellular protease production, while ΔH137 (Sin$^-$) did not (data not shown). This indicates that ORF 3 is also not involved in the repression of exoprotease production.

Disruption of Sin sequences in the chromosome. To see whether the DNA sequences containing ORF 1 and ORF 2 were necessary for sporulation, we generated disruptions in these sequences. Restriction fragments internal to ORF 1 and ORF 2 DNA sequences were cloned into the integrative vector pCP115, and the resulting plasmids were integrated into the *B. subtilis* chromosome (see above). Strains carrying disruptions in either ORF 1 (IS353) or ORF 2 (IS354) sporulated normally but displayed an unusual aggregation of cells in the form of long chains, and their colonies were extremely rough. The strains were transformed poorly by both chromosomal DNA and plasmid DNA. (These strains were Rec$^+$, as evidence by their mitomycin resistance.) Both strains had about threefold more extracellular protease activity (both neutral and alkaline proteases showed the same relative increase) than did control strains IS75 and IS383 (Fig. 8). IS75 contained no plasmid, and IS383 contained a

plasmid conferring Cm^r which had integrated into the *cysB* region of the *B. subtilis* chromosome, which is far from the location of the *sin* gene. Strains IS353 and IS354 also had twofold more extracellular α-amylase activity than the control strains (data not shown).

DISCUSSION

We have cloned a 4.0-kb DNA fragment from *B. subtilis* which inhibits sporulation and alkaline phosphatase and extracellular protease production in wild-type strains of *B. subtilis* when present on multicopy plasmids. Deletion analysis and subcloning have identified a 1.0-kb fragment responsible for sporulation inhibition, which we have sequenced. Further deletion analysis and subcloning demonstrated that a DNA sequence containing an ORF, ORF 2, caused the inhibition of sporulation and exoprotease production.

The fragment was mapped on the *B. subtilis* chromosome between the *dnaE* and *spo0A* genes, a region which has the *spo0G* and *crsC* loci (38). The cloned fragment did not complement the *spo0G* mutation, indicating that the insert does not have the *spo0G* gene in it. Sporulation in IS353 and IS354, strains with disrupted ORF 1 and ORF 2, remained sensitive to glucose, unlike strains with *crs* mutations (49), which indicates that the ORF 1 and ORF 2 disruptions are not the same as the *crsC* mutation. ORF 1 and ORF 2 disruptions did not cause the same phenotype as mutations in *mpo*, another gene found in this region which affects the synthesis of membrane proteins and causes temperature-sensitive sporulation (28).

The inactivation of chromosomal DNA sequences containing either ORF 1 or ORF 2 by the gene disruption technique did not prevent sporulation; however, it resulted in the hyperproduction of protease and amylase, which are late-growth-associated events. Both chromosome disruptions also resulted in the appearance of long chains of cells which increased in number as the cells entered the stationary phase of growth. The associated phenotypes of elevated exoprotease production and the formation of long bacterial chains have been observed previously in *B. subtilis* (21, 34). These two phenomena may be related by high levels of protease interfering with the turnover of cell wall peptidoglycan (21).

Several other *trans*-acting mutations which raise the production of exoprotease have been described in *B. subtilis* genes *sacU*, *sacQ*, *prt*, and *scoA*, *B*, *C*, and *D*, which are unlinked to the *dnaE*, *spo0A* region (39). The *sacU* and *sacQ* genes have been cloned (2, 52), and it was shown that the $sacU^h$ and *sacQ* mutations cause overproduction of their respective gene products. A cloned DNA fragment from *Bacillus natto* with a single translated ORF also causes increased exoprotease synthesis in *B. subtilis* when present on multicopy plasmids (34). These cases are examples of positive regulation of exoprotease production. Consistent with this is the fact that cells with the *sacU* mutation do not produce most extracellular enzymes (26). Presumably, *sacU* is a null mutation for the *sacU* gene product. *spo0* gene products, especially that of *spo0A*, may also be positive regulatory factors for exoprotease production, as *spo0* mutations drastically lower subtilisin production (14). The Sin phenotype described here, on the other hand, is different, as multiple copies of ORF 2 repress exoprotease production and disruption of ORF 2 or ORF 1 in the chromosome, which destroys their ability to encode polypeptides, results in elevated levels of exoprotease. This suggests that ORF 2 and possibly ORF 1 function as negative regulatory elements (see below).

The predicted amino acid composition of ORF 1 and ORF 2 is rich in charged amino acids (34.9 and 36.0%, respectively), a property of RNA polymerase proteins σ (8). Interestingly, ORF 1 has partial homology with a highly conserved segment of sigma proteins, and ORF 2 has a domain typical of DNA-binding proteins (35). Although ORF 1 and ORF 2 have their own putative translational signals, they are present in the same phase and are separated by a single UGA termination codon. Possible readthrough at UGA would merge ORF 1 with ORF 2 and result in the formation of a 180-amino-acid polypeptide. Readthrough of UGA followed by A has been observed in *E. coli* and has been implicated in gene regulation (13, 24).

There seems to be a contradiction in the fact that ORF 2 is necessary and sufficient for *trans* spore and exoprotease inhibition, yet disruption of either ORF 1 or ORF 2 in the chromosome results in a similar phenotype. The ORF 1 and ORF 2 gene products may normally function together as two polypeptides or as two domains of the potential readthrough protein discussed above. The phenotype of inhibition caused by multiple copies of ORF 2 might then be due to the presence of high levels of the ORF 2 polypeptide alone, which is quantitatively and qualitatively different from the normal situation. Another possibility is that ORF 2 is the negative regulator but that ORF 1 and ORF 2 are normally in the same transcription unit; thus, disruption of either ORF 1 or ORF 2 in the chromosome could cause a similar phenotype. The ability of ORF 2 to inhibit in *trans* could be due to readthrough from an upstream plasmid promoter or to the utilization of a normally inactive secondary promoter just upstream from ORF 2. We are currently performing in vivo transcriptional mapping experiments to decide which of these alternatives may be occurring.

Multicopy inhibition of sporulation has been observed when *spo0F* (22), *spoVG* (3), and other *spo* genes (23) are present on high-copy-number plasmids. The sporulation inhibition caused by the cloned *spo0F* gene seems to be associated with amplification of the *spo0F* gene product, since all mutations which disrupt Sin function are found in the *spo0F* protein-coding sequence (27, 36, 50, 53, 54). On the other hand, *spoVG*-dependent sporulation inhibition is caused by the presence of the *spoVG* promoter on a high-copy-number plasmid and is thought to be due to titration of a regulatory protein (3). Multicopy inhibition of gene expression has also been observed in other organisms. The *nifL* gene of *Klebsiella pneumoniae* codes for a negative regulatory protein of nitrogen fixation. The presence of the functional *nifL* gene on multicopy plasmids represses *nif* genes under normal derepressing conditions (6). Multiple copies of *nifH* and *nifU* promoters and their upstream regions also inhibit nitrogen fixation, presumably by titrating out positive regulators for this process (6, 7). The presence of the *micF* gene in a multicopy plasmid represses *ompF* expression and is due to overproduction of *micF* RNA, which inhibits *ompF* transcription (33). Similar multicopy inhibition through repressor RNA has been observed in the expression of the transposase of transposon Tn*10* (46). Multicopy inhibition of mitotic chromosome transmission has enabled Hartwell and co-workers to clone two genes in *S. cerevisiae* which are involved in mitosis (30).

We have shown that inhibition of both sporulation and exoprotease production is associated with the region which encodes ORF 2. It is possible that the ORF 2 protein or a larger complex containing the ORF 2 gene product is a normal repressor of sporulation and exoprotease production and that abnormally high levels of this protein would keep

these functions repressed even under derepressing conditions. Such a repressor would not necessarily be required for sporulation, and inactivation of its genetic determinant in the chromosome by gene disruption might not be expected to give a Spo$^-$ phenotype. Inactivation of the chromosomal gene for the repressor would also not necessarily derepress sporulation, as there might be more than one negative regulatory factor for sporulation. It is also possible that *sin* gene inactivation might derepress sporulation under certain conditions which we have not as yet ascertained. Extracellular protease production might have fewer negative regulatory elements, so that disruption of ORF 2 might result in a measurable derepression of exoprotease. In this regard, Ferrari et al. (14) have recently postulated the existence of a repressor of subtilisin production, which can be titrated by multiple copies of the subtilisin regulatory sequences.

Other explanations for the sporulation inhibition caused by multiple copies of ORF 2 sequences, such as DNA sequence titration of a positive regulatory protein or inhibition by RNA transcribed from ORF 2, cannot be ruled out. These are unlikely, however, since mutations throughout the ORF 2 sequence, i.e., the *Bal*I small deletion and the alteration of the C-terminal region in pIS87, destroyed the Sin phenotype. The presence of the highly conserved DNA-binding domain in ORF 2 found in many regulatory proteins (Table 4) also strongly suggests that the protein potentially encoded by ORF 2 is responsible for the Sin phenotype.

The role of negative regulator of sporulation that we hypothesize for the *sin* gene is analogous to the function of the *S. cerevisiae* gene *RME1*. This gene inhibits meiosis and sporulation and must be repressed for the initiation of these processes (32). Like ORF 2, the *RME1* gene, when present in multiple copies, inhibits normal sporulation.

Proof that the *sin* gene we have described is truly involved in regulating late growth functions, however, awaits further experimentation, now in progress, on the nature and regulation of the ORF2 gene product and more specific knowledge on the mechanism of inhibition of sporulation and exoprotease secretion. The relationship, if any, between the ORF 2 protein and DNA-binding proteins observed in *B. subtilis* by Hoch and co-workers (4), some of which are altered by *spo0* mutations (5), is presently not known and is also under investigation.

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