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Sequence Analysis and Regulation of the *hpr* Locus, a Regulatory Gene for Protease Production and Sporulation in *Bacillus subtilis*†

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The hyperproduction of alkaline and neutral proteases is a phenotype of mutation at the *hpr* locus. This locus has been cloned and sequenced and has been found to code for a protein of 23,718 M_r. The mutations *hpr-1*, *scoC4*, and *catA7* were identified by sequencing as mutations within the *hpr* gene. The phenotype of mutations in the *hpr* gene is due to loss of the *hpr* gene product, and therefore we suggest that the *hpr* gene encodes a negative regulator of protease production. This negative regulator must control genes other than protease genes, and these genes must include at least one gene required for sporulation, since overproduction of the *hpr* gene product by cloning the locus on a multicopy vector results in the inhibition of sporulation as well as protease production. Truncated fragments of the *hpr* gene or its promoter do not have this phenotype. Transcription of the *hpr* locus is controlled by the *spo0A* gene. In an *spo0A* mutant the *hpr* gene transcript is constitutively overproduced, as determined by a transcription fusion to β-galactosidase. The results are consistent with the view that the *spo0A* gene may control sporulation and transcription by modulating the level and activity of several regulatory proteins.

Many microorganisms start to differentiate when an essential nutrient becomes scarce. Bacteria of the genus *Bacillus* initiate a developmental program that culminates with the production of heat-resistant spores. Closely associated with the onset of the sporulation process is the appearance of a wide variety of secondary metabolites and enzymes such as proteases, suggesting that their production might be regulated by the same mechanisms that control spore formation. *Bacillus subtilis* mutants blocked at the earliest stage of sporulation (stage 0 mutants) have long been known to have a pleiotropic negative effect on the production of stationary-phase-associated products, and on protease production (9, 14). On the other hand, a number of mutations of *B. subtilis* have been isolated that increase the production of secreted enzymes. *sacU*(Hy) mutants were characterized by hyperproduction of levansucrase, alkaline and neutral proteases, α-amylase, xylanase, and β-galactosidase (18). A similar phenotype was found for the *sacQ*(Hy) mutation, which mapped at a different locus (19). Increased production of levansucrase and alkaline and neutral proteases has been observed when a recently isolated gene, *prtR*, was overexpressed on a plasmid (24, 32). In the case of the *prtR* and *sacQ* genes, it has been shown that the hypersecretion phenotype is due to the overproduction of these two polypeptides, while the nature of the stimulation by the *sacU*(Hy) mutation is still unknown.

Other hyperproduction mutants were isolated, and mutations were mapped in a locus called *hpr* (13). These mutants show a more restricted phenotype, since they increase the production of alkaline and neutral protease only (it appears that they may have a small effect on the expression of intracellular serine protease; M. Ruppen, personal communication). Eighteen of these mutants were originally isolated for their ability to increase the production of extracellular proteases from 16- to 37-fold compared with the wild-type strain; the mutations were all mapped in a region of the chromosome linked to *argC* and *metC*. In the same chromosomal region two additional mutations leading to hyperprotease production and obtained by different selections were mapped. Mutants bearing *catA* mutations were isolated as insensitive to glucose repression of sporulation (17). These mutants produced five to six times more extracellular protease compared with the wild-type strain. Mutations affecting the quantitative regulation of sporulation-dependent proteins and leading to the overproduction of extracellular proteases were described for the spore control (*sco*) mutants (22). In particular, the *scoC* mutations were mapped in the same chromosomal region as *hpr* and *catA* mutations, and they were described as having the same catabolite-resistant phenotype as the *catA* mutants (7).

In this report we describe the isolation of the *hpr* locus from a Tn*917* insertion library. We show that the *hpr*, *scoC*, and *catA* mutations are all alleles of the *hpr* locus and that it is the lack of this gene product that results in the phenotype. The results are consistent with the *hpr* gene product serving as a negative regulator of protease production and sporulation.

MATERIALS AND METHODS

Bacterial strains and transformation. *B. subtilis* strains used in this study are shown in Table 1. Strains were grown in Schaeffer sporulation medium (28) or minimal medium and transformed by the method of Anagnostopoulos and Spizizen (1). Plasmid transformants in *B. subtilis* were selected on Schaeffer agar plates with 5 μg of chloramphenicol per ml. The level of protease production was tested on TBAB-milk plates (tryptose blood agar base plates supplemented with 0.5% Carnation powdered low-fat milk), and the size of the halo was measured.

Enzymes. Restriction enzymes used in this study were purchased from Bethesda Research Laboratories or New England Biolabs and used according to the suppliers' recommended assay procedures. T4 DNA ligase and the *Escherichia coli* large fragment of DNA polymerase I (Klenow)

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TABLE 1. *B. subtilis* strains used in this study

Strain	Genotype	Protease phenotype[a]
W168	Wild type	+
JH646	*trpC2 phe-1 spo0A12*	−
Hpr1	*trpC2 hpr-1*	++
Hpr2	*trpC2 hpr-2*	++
Hpr3	*trpC2 hpr-3*	++
Hpr5	*trpC2 hpr-5*	++
Hpr6	*trpC2 hpr-6*	++
Hpr7	*trpC2 hpr-7*	++
Hpr8	*trpC2 hpr-8*	++
Hpr9	*trpC2 hpr-9*	++
Hpr10	*trpC2 hpr-10*	++
Hpr11	*trpC2 hpr-11*	++
Hpr12	*trpC2 hpr-12*	++
Hpr13	*trpC2 hpr-13*	++
Hpr15	*trpC2 hpr-15*	++
Hpr16	*trpC2 hpr-16*	++
Hpr17	*trpC2 hpr-17*	++
Hpr18	*trpC2 hpr-18*	++
Hpr97	*trpC2 hpr-97*	++
BG97	*argC4 hisA phe-1 catA7*	++
BG213	*metC3 purB34 scoC4*	++

[a] Phenotype of protease production measured on TBAB milk plates by the size of the halo: +, wild-type producer; ++, hyperproducer; −, nonproducer.

were obtained from Bethesda Research Laboratories. *E. coli* DNA polymerase I was from New England Biolabs.

Nucleic acid isolation. Plasmid DNA was prepared from *E. coli* transformants by the alkaline lysis method of Birnboim and Doly (3). Minipreparations of plasmid DNA were obtained by the boiling method of Holmes and Quigley (15). *B. subtilis* chromosomal DNA was prepared by the method of Marmur (21) with some modifications.

Restriction fragments for subcloning or nick translation were purified after electrophoresis on 4% polyacrylamide gel (cross-linker 19:1; electrode buffer, 1× TBE [200 mM Tris–268 mM boric acid–8 mM EDTA]) by electroelution into dialysis membrane in 0.25× TBE.

Physical characterization of the cloned DNA. DNA fragments from restriction enzyme digests were resolved and analyzed on 0.75% agarose gel. For Southern blot analysis, agarose gels were treated as described by Maniatis et al. (20) and then transferred to nitrocellulose by the method of Southern (29). Hybridization and washing conditions were as described by Maniatis et al. (20). A ^{32}P-labeled probe was prepared by nick translation of the purified insert from plasmid pJM2471.

Plasmid constructions. A procedure described by Youngman et al. (33) was followed to obtain plasmid pJM2471. Strain CU4149zca-82::Tn*917* was transformed to Cm^r with plasmids pTV20 and pTV21Δ2 linearized by *Xba*I digestion. Chromosomal DNA was made from one transformed Cm^r colony containing pTV20 or pTV21Δ2, and 1 μg was digested to completion with *Eco*RI or *Sph*I, incubated with T4 ligase at a dilute DNA concentration (10 μg/ml), and then used to transform *E. coli* DH5α competent cells, selecting for ampicillin resistance. Transformants were obtained only from the ligation of *Sph*I-cut chromosomal DNA from the strain containing pTV20. Plasmids contained within these transformants were analyzed, and one of them was designated pJM2471. The basic vectors used in this study for subcloning constructions were the integrative vectors pJH101 (10) and pJM103 (a derivative of pUC19), the integrative vector pJM783 (Perego and Hoch, unpublished data),

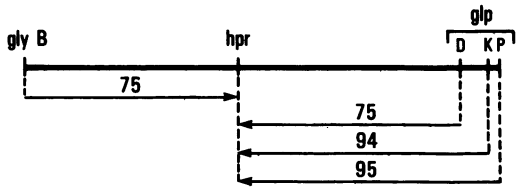


FIG. 1. Genetic map of the *glyB*-to-*glpDKP* segment of the *B. subtilis* chromosome. Numbers are the percentages of recombination in transformation between the markers.

which generates transcription fusions of β-galactosidase, and the multicopy shuttle vector pBS19 (Greg Gray, unpublished data), a derivative of pBS42 (2).

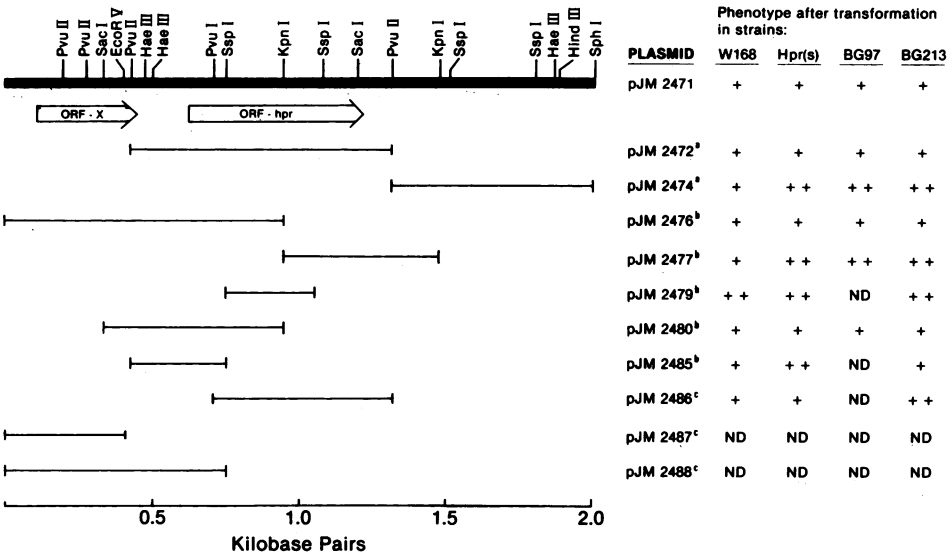
Sequence analysis. DNA fragments were cloned in M13mp18, M13mp19 (25), or pJM103, and sequencing was carried out by the dideoxynucleotide method of Sanger et al. (27) or the supercoil sequencing method of Chen and Seeburg (6). The 17-mer oligonucleotide primer 5′-TGATGCT CATTAATATT-3′, provided by the Genentech Organic Synthesis Group, was used to sequence the *scoC4* and *catA* mutations. The ^{35}Sequencing/Cloning Kit was purchased from Pharmacia Fine Chemicals and used as recommended by the supplier. [^{35}S]dATP (>1,000 Ci/mmol) was obtained from Amersham Corp.

Cloning of the *hpr* alleles. Chromosomal DNA (30 μg) from strains Hpr1, Hpr2, BG97, and BG213 was digested to completion with *Hin*dIII and electrophoresed in a 0.75% agarose gel. DNA fragments 2 kilobases long were electroeluted from the gel and ligated to *Hin*dIII-cut pJM103. After transformation in *E. coli* competent cells, selecting for ampicillin resistance on LB agar medium containing ampicillin (100 μg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (40 μg/ml), white colonies were analyzed by hybridization with a ^{32}P-labeled DNA probe prepared by nick translation of the gel-purified insert contained in plasmid pJM2471. Positive clones were analyzed, and the plasmids carrying the mutations were sequenced as described above.

RESULTS

Genetic location of the *hpr* locus. A number of mutants of *B. subtilis* producing high levels of extracellular proteases have been described (13). In genetic mapping analysis by PBS-1 transduction all the mutations leading to high protease production in the *hpr* mutants (Table 1) were found linked to the *argC4* and *metC1* markers; in particular, all appeared to lie closer to *argC4* than to *metC1*. We carried out a genetic linkage analysis by transformation to other markers in this region; the *hpr* locus mapped between the *glyB* and the *glp* markers (Fig. 1).

Cloning and characterization of the *hpr* locus. A system of transposon-mediated insertional mutagenesis in *B. subtilis* with the insertion element Tn*917* from *Streptococcus faecalis* developed by Youngman et al. (34) has been used to isolate a group of insertions at various chromosomal locations (31). One of these insertions in strain CU4149zca-82::Tn*917* mapped in the chromosomal region between *glyB* and *glpK*, at position 74°C on the genetic map, i.e., in the same region where the *hpr* locus was known to map. After integration of linearized plasmids pTV20 and pTV21Δ2 (33) into the chromosome of strain CU4149zca-82::Tn*917*, the genetic linkage of the integrated Cm^r marker to the *glyB* and *hpr* markers was checked by transformation analysis. Ap-



PLASMID	Phenotype after transformation in strains: W168	Hpr(s)	BG97	BG213
pJM 2471	+	+	+	+
pJM 2472[a]	+	+	+	+
pJM 2474[a]	+	+ +	+ +	+ +
pJM 2476[b]	+	+	+	+
pJM 2477[b]	+	+ +	+ +	+ +
pJM 2479[b]	+ +	+ +	ND	+ +
pJM 2480[b]	+	+	+	+
pJM 2485[b]	+	+ +	ND	+
pJM 2486[c]	+	+	ND	+ +
pJM 2487[c]	ND	ND	ND	ND
pJM 2488[c]	ND	ND	ND	ND

FIG. 2. Detailed restriction map of the chromosome region cloned in plasmid pJM2471 and containing the *hpr* locus. The extents of the ORFs identified in this region are shown. Single lines below the restriction map indicate the fragments subcloned in the integrative vector pJH101 (a), pJM103 (b), or pJM783 (c). The phenotype of protease production for Cm^r transformants obtained after integration of these plasmids in strains W168, Hpr(s), BG97, and BG213 is shown. The size of the halo was measured on TBAB-milk plates: +, wild-type level of protease production; ++, hyperproduction of protease; ND, not determined.

proximately 83 and 97% of cotransfer was found between the integrated *cat* gene and the *glyB* and *hpr* markers, respectively. Based upon these results, we decided to clone chromosomal sequences adjacent to either transposon insertion junction in an attempt to recover a fragment containing the *hpr* locus.

Chromosomal DNA from strain CU4149zca-82::Tn*917* containing an integrated copy of plasmid pTV20 or pTV21Δ2 was digested with *Eco*RI or *Sph*I, ligated at a dilute concentration, and transformed into *E. coli* competent cells as described by Youngman et al. (33). Colonies were obtained only with *Sph*I-digested DNA from strain CU4149zca-82::Tn*917* containing pTV20. The restriction map of the plasmid contained within these colonies, pJM2471, showed the presence of a 2.0-kilobase chromosomal insert (Fig. 2). Transformation of all of the *hpr* mutants with plasmid pJM2471, which integrates by a Campbell-type recombination, restored protease production to the wild-type level, indicating that the cloned fragment contained the *hpr* locus. Subsequent cloning of fragments of this insert in the integrative plasmid pJH101 or pJM103 and analysis by transformation revealed that all of the *hpr* mutations were transformed by the 900-base-pair (bp) *Pvu*II-*Pvu*II fragment contained in plasmid pJM2472 (Fig. 2). Furthermore, two mutations known as *scoC4* (22) and *catA* (17), characterized by a high protease production phenotype and genetically mapped in the same chromosomal region as the *hpr* mutations, were transformed by plasmid pJM2472 as well, suggesting the possibility that all of these mutations were different alleles at the same locus. Transformation by the other subclones of this insert (Fig. 2) showed that all of the *hpr* mutations known (except *hpr-2*) were localized to the 200-bp *Ssp*I-*Kpn*I fragment of the insert contained in plasmid pJM2476, whereas the *scoC4* mutation was transformed by the 330-bp fragment carried by plasmid pJM2485. The poor transformability of strain BG97 did not allow us to define the position of the *catA* mutation. Strains bearing the *hpr-2* allele did not transform with these subclones. This behavior results from the fact that the *hpr-2* allele is a deletion in this region.

Sequence analysis. Sequence analysis of the 2.0-kilobase fragment contained within plasmid pJM2471 was carried out as described in Materials and Methods. Two major open reading frames (ORFs) were identified (Fig. 2). One of them, ORF-X, codes for a protein of 119 amino acids with a calculated molecular weight of 13,066. The other open reading frame was identified as the ORF-*hpr* based upon the results of the transformation analysis. The *hpr* gene appeared to code for a protein of 203 amino acids with a calculated molecular weight of 23,718 (Fig. 3). Each ORF is preceded by a reasonable ribosome-binding site; according to the computer program of Brendel and Trifonov (4), a sequence for a potential stem-loop structure with similarity to a very strong rho-independent terminator is present between ORF-X and ORF-*hpr*. Nevertheless, no RNA studies have been done, so that nothing is known about the transcription of these two genes.

Cloning the *hpr*, *scoC4*, and *catA* alleles. Southern blot analysis on chromosomal DNA from strains W168, Hpr1, Hpr2, Hpr97, BG97, and BG213 digested with several restriction enzymes was carried out by using the insert contained within plasmid pJM2471 as a nick-translated probe. The probe hybridized to a 2-kilobase *Hin*dIII fragment for all the strains tested except for Hpr2, for which the positive band was an *Hin*dIII fragment with a deletion of about 400 bp (data not shown). Cloning of the chromosomal fragment carrying the mutations from strains Hpr1, Hpr2, BG97, and BG213 was done as described in Materials and Methods. Restriction analysis of the fragment containing the *hpr-2* mutation revealed the presence of a deletion covering the *Pvu*I and the *Kpn*I sites in the ORF-*hpr* (Fig. 3). The endpoints of the deletion were not determined. Sequence analysis was carried out for the *hpr-1*, *scoC4*, and *catA* alleles. The *hpr-1* mutation resulted from a transversion, T→A, that changed a phenylalanine into an isoleucine in the 59th codon; the *scoC4* allele was found to be a transition, C→T, that produced a chain-terminating codon, TAG, at the 22nd codon. The *catA* allele was due to a transversion, C→A, in the 21st codon that changed the amino acid alanine

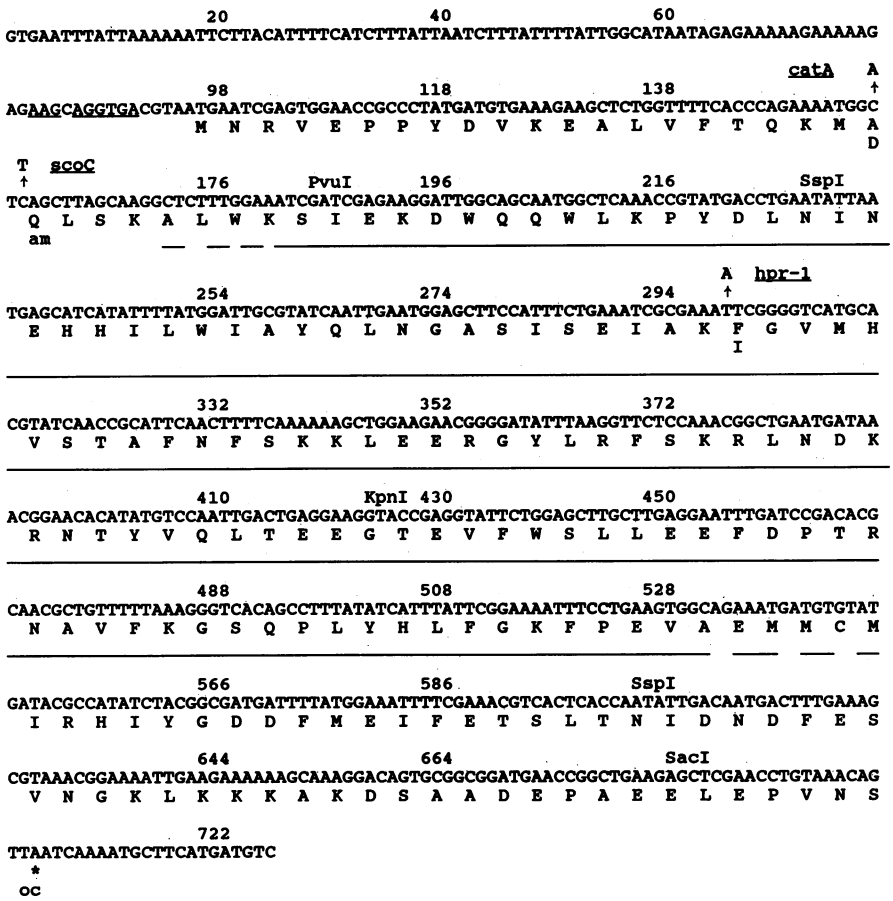


FIG. 3. Nucleotide and deduced amino acid sequence of the *hpr* gene. A putative ribosome-binding site is underlined. The locations of the *catA7*, *scoC4*, and *hpr-1* mutations are shown. The extent of the deletion identified in the Hpr2 mutant is underlined. am, Amber termination codon; oc, ochre termination codon.

into an aspartic acid residue. The fact that one of the mutations was a deletion in the structural gene coding for the *hpr* gene product and another one was a nonsense mutation implied that the phenotype of mutations in this locus must be due to the loss of the gene product of this locus and, moreover, that the missense mutations in strain BG97 and Hpr1 probably cause a change that impairs the biological activity of the protein.

Gene inactivation of the *hpr* locus. In order to further characterize the effect of the inactivation of the *hpr* gene in a wild-type strain, we constructed several plasmids that, after integration into the chromosome, interrupted the open reading frame for *hpr*. Plasmid pJM2479 (Fig. 2) contains the 370-bp *Ssp*I fragment internal to the coding sequence of *hpr*, cloned in the *Hin*cII site of the integrable vector pJM103. By transformation in the wild-type strain W168 with selection for Cm^r, the plasmid integrated into the chromosome via Campbell-type recombination inactivating the structural gene for *hpr*. Transformants were scored for protease production on TBAB-milk plates; 100% of them had a hyperprotease-producing phenotype. A pUC19 plasmid carrying the approximately 900-bp *Pvu*II fragment containing the *hpr* gene was constructed, and the *cat* gene from pC194 (16) was cloned in the *Kpn*I, *Sac*I, and *Pvu*I sites of the *hpr* sequence, producing plasmids pJM2499, pJM2500, and pJM2501, respectively (Fig. 4). Transformation in the wild-type strain was done with these linearized plasmids so that Cm^r transformants were the result of a double recombination event that replaced the wild-type copy of the *hpr* gene with a copy inactivated by the presence of the *cat* gene. The transformants were tested for protease production; the inactivation of the *hpr* locus at the *Pvu*I or at the *Kpn*I site resulted in the production of the hyperprotease phenotype in the wild-type strain, whereas the insertion of the *cat* gene in the *Sac*I site did not influence the production of protease. This may indicate that the carboxyl end of the *hpr* gene product is not directly involved in the mechanisms that regulate the expression of the protease producing genes.

Gene inactivation of ORF-X was undertaken by subcloning the chromosomal fragment from plasmid pJM2476 in pUC19 and then inserting the *cat* gene from pC194 in the *Sac*I and *Eco*RV sites, creating a deletion of 76 bp (Fig. 4). The plasmid pJM2502 was linearized and transformed in the wild-type strain. Colonies were checked on TBAB-milk plates; the level of protease production was the same as that of the wild type, suggesting that this gene has no role in the regulation of extracellular protease production. In addition, the transformants were not auxotrophic or sporulation defective.

***lacZ* transcription fusion analysis of the *hpr* locus.** The expression of the *hpr* gene during growth and sporulation has been analyzed by using *lacZ* transcription fusions made in the transcription fusion vector pJM783. pJM2488, the *hpr-lacZ* fusion plasmid, contains the fragment shown in Fig. 2

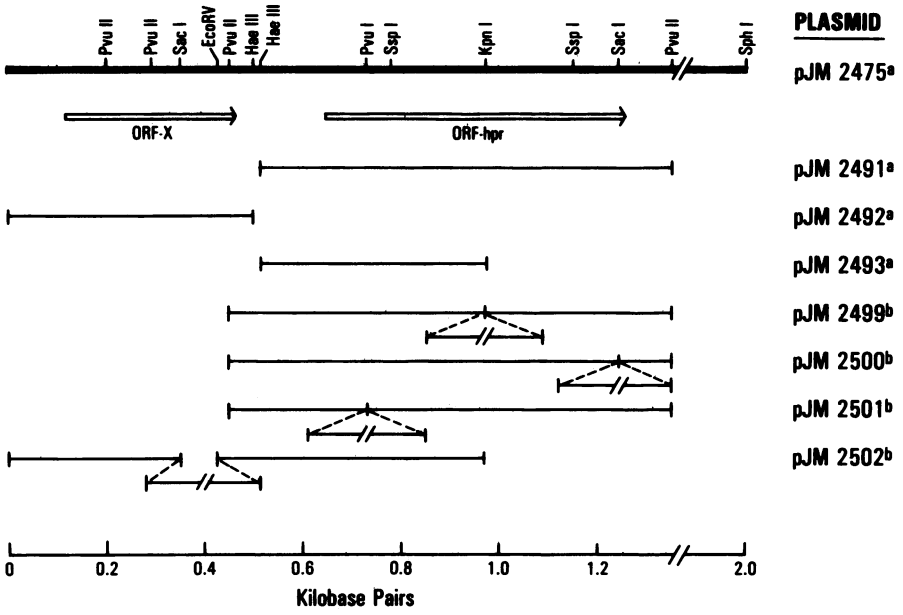


FIG. 4. Restriction map of the fragments subcloned in the multicopy vector pBS19 (a) or in the pUC19 vector (b) and used for multicopy effect analysis or gene inactivation analysis respectively. The sites of insertion of the 1-kb fragment carrying the *cat* gene from plasmid pC194 are indicated.

fused at its *Ssp*I end to the *lacZ* gene. Plasmid pJM2487 is the ORF-X-*lacZ* fusion, and the insert has been cloned in the vector with its *Eco*RV end in front of the *lacZ* gene. After integration of these plasmids into a wild-type strain and an *spo0A* strain, the level of β-galactosidase was measured as a function of growth (Fig. 5). In the wild-type strain, the specific activity of β-galactosidase expressed from the *hpr-lacZ* fusion increased in proportion to the growth curve, reached a maximum level at the early stationary phase, and remained at the same level during the stationary phase. On the contrary, in an *spo0A* mutant, the activity of the *hpr-lacZ* fusion was higher in vegetative growth and kept increasing during the first 3 h of the stationary phase, suggesting that in the absence of the *spo0A* protein the Hpr protein was overproduced. Synthesis of β-galactosidase directed by ORF-X increased in the earliest stage of vegetative growth and then remained at an almost constant level throughout the remainder of growth and stationary phase. Furthermore, its synthesis did not appear to be affected by the *spo0A* mutation.

The possibility of the presence of an ORF on the opposite strand of where the ORF-*hpr* lies was checked by constructing the transcription fusion plasmid pJM2486 (Fig. 2). The 630-bp *Pvu*I-*Pvu*II fragment was cloned in pJM783 in order to have the *Pvu*I site in front of the *lacZ* gene. After integration in the wild-type strain, β-galactosidase production was not detectable.

Effect of the *hpr* locus on a multicopy plasmid. In order to investigate the effect of a multicopy *hpr* gene, we constructed a series of plasmids in the multicopy vector pBS19. Plasmid pJM2475 was a derivative of the shuttle plasmid pBS19 containing the entire chromosomal fragment previously cloned in pJM2471, whereas pJM2491 contained the *Hae*III-*Pvu*II fragment carrying only the *hpr* ORF (Fig. 4). In order to test possible effects of the upstream region of the *hpr* locus, we constructed plasmid pJM2493, in which the *hpr* gene was truncated. The entire sequence of ORF-X was cloned in plasmid pJM2492. After transformation in the wild-type strain W168, the phenotype of the colonies obtained was checked. Transformants carrying plasmids pJM 2492 or pJM2493 sporulated normally and showed a wild-type level of protease production, but, surprisingly, colonies containing pJM2475 or pJM2491 plasmids had a sporulation-deficient phenotype. The strains carrying these latter two plasmids, along with the original vector without an insert, were subjected to sporulation conditions in order to quantitate their effects on sporulation. The efficiency of sporulation in strains carrying plasmids pJM2475 was 10^3 to 10^4 times less than in the wild-type strain (Table 2). However, only 10% of the colonies surviving the chloroform treatment showed a Spo^+ phenotype. This may be due to integration of the plasmid into the chromosome, which results in the release of the multicopy inhibitory effect (in fact we have not been able to recover a free plasmid from these strains). The remaining colonies still showed the sporulation-deficient phenotype. Colonies carrying plasmids pJM2475 and pJM2491 also showed a high rate of plasmid segregation compared with a strain containing the vector pBS19. Approximately 70 and 90% of the colonies originally containing plasmids pJM2475 and pJM2491, respectively, lost the Cm^r marker when grown in absence of chloramphenicol, whereas in the control strain the rate of plasmid segregation was less than 1%.

The multicopy plasmid pJM2475 was transformed in strain Hpr2, selecting for chloramphenicol resistance. Only approximately 40% of the transformants obtained were inhibited in sporulation, whereas the remaining colonies sporulated normally. We were able to isolate a plasmid from these Spo^+ colonies, and it had a deletion of about 400 bp. These Spo^+ colonies all maintained the Hpr phenotype, suggesting that they arose by gene conversion during the transformation event.

The level of protease production in strains carrying the *hpr* multicopy plasmid and showing a sporulation deficient phenotype was also checked on TBAB plates containing milk and chloramphenicol. Both W168 and Hpr2 strains contain-

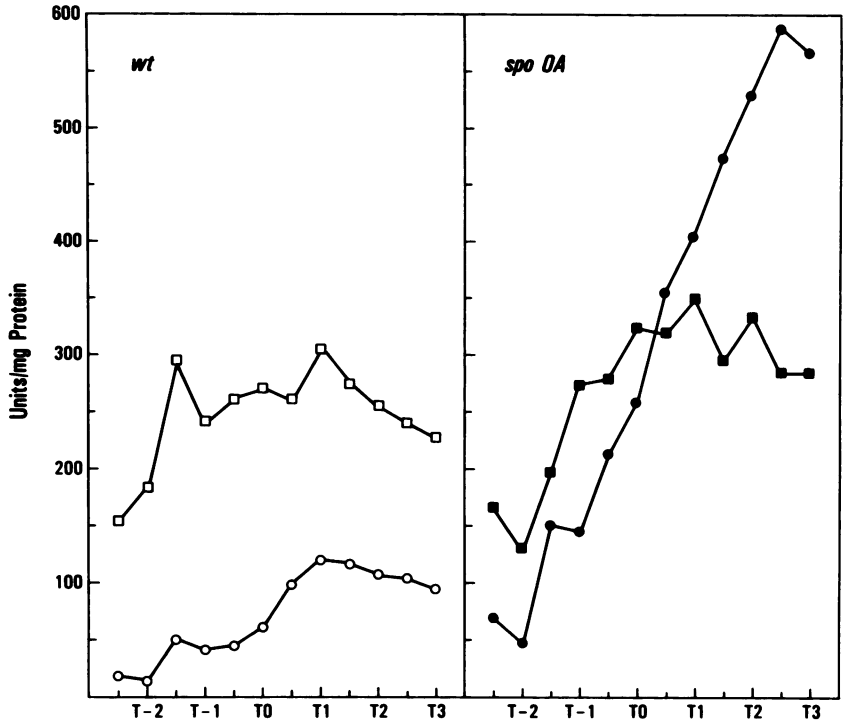


FIG. 5. Expression of β-galactosidase activity in the wild-type strain W168 or in the *spo0A* strain JH646 harboring *lacZ* transcription fusion constructs. Growth of the strains carrying pJM2487 (□, ■) or pJM2488 (○, ●) plasmids was in Schaeffer sporulation medium. Samples were taken at the indicated times, and optical density and β-galactosidase activity were measured as previously described (9). The specific activity of β-galactosidase is expressed in units per milligram of protein, which is a modified Miller unit (23) in which the optical density of the culture has been changed to milligrams of protein (9).

ing pJM2475 or pJM2491 appeared to have a level of extracellular protease production comparable to that observed in the stage 0 sporulation mutant *spo0A*, in which there was no detectable protease production in terms of a halo on TBAB-milk plates.

DISCUSSION

In this work we describe the cloning and sequencing of the *hpr* locus from *B. subtilis*. The deduced product of the *hpr* gene, obtained from the nucleotide sequence, is a protein of molecular weight 23,718 that shows no significant homology with any other protein whose sequence is known. The phenotype of mutations at this locus is the hyperproduction of extracellular proteases (alkaline and neutral protease). Characterization of some of the *hpr* mutations, the spore-control mutation *scoC4* (7), and the catabolite-repression resistant mutation *catA7* (17) by cloning and sequencing revealed that these mutations are different alleles of the same locus and that their hypersecretion phenotype is due to the loss of the *hpr* gene product or of its activity. Insertional inactivation experiments in the structural gene for *hpr* confirmed these results. These observations suggest that *hpr* plays the role of a negative regulator of protease production in *B. subtilis*, since it is in its absence that the level of extracellular protease is increased.

TABLE 2. Effect of the *hpr* gene on multicopy plasmids on sporulation

Strain[a]	Cm^r cells per ml	
	Total cells	Cells surviving $CHCl_3$
W168(pJM2475)	3.5×10^8	6.4×10^5
W168(pJM2491)	2.5×10^8	1.4×10^4
W168(pBS19)	2.0×10^8	1.2×10^8

[a] Strains carrying the multicopy plasmids were grown on Schaeffer sporulation medium containing chloramphenicol at 5 μg/ml for 24 h. Serial dilutions were plated on Schaeffer medium-chloramphenicol plates before and after treatment with $CHCl_3$.

We have recently shown that the *hpr* mutations increase the rate of β-galactosidase accumulation from a subtilisin–β-galactosidase gene fusion during the early portion of the stationary phase (8). Through the use of upstream deletions of the subtilisin promoter it was shown that the *hpr* mutations stimulate subtilisin production only if the region of the promoter upstream of position −200 from the transcription start point was intact. Furthermore, stimulation by the *hpr* mutations results in at least 10-fold higher levels of subtilisin mRNA, and the start site of this mRNA is identical to that found in a wild-type strain. How the absence of the *hpr* gene product could stimulate transcription from an upstream region of the subtilisin promoter is unclear. If we assume the upstream region is a site for either positive stimulation by another factor or perhaps a strong affinity site for RNA polymerase, then the role of the Hpr protein might be to inhibit the binding of these molecules to the upstream region. The fact that the deletion of the upstream region yields a promoter with wild-type characteristics in the presence or absence of the *hpr* gene product (12) suggests that the contribution to subtilisin transcription of this region is minimal under the conditions normally employed to assay sub-

tilisin production. It is only in the absence of the *hpr* gene product that this site is manifested by stimulation of transcription. It has been postulated that the *hpr* gene product along with the *sacU*, *sacQ*, and *prtR* gene products comprise a multifaceted mechanism for global response to a variety of nutritional conditions (12). Under laboratory conditions this mechanism may be virtually inactive, and therefore upstream deletion of both the *hpr* site and the *sacU-sacQ* site of action has little apparent effect on the production of subtilisin.

We have shown that deletion of the subtilisin promoter upstream of position −200 renders this promoter insensitive to the state of the *hpr* gene (12). Such a promoter is still exquisitely sensitive to the *abrB* gene product in an *spo0A* mutant. Thus protease production in a *spo0A hpr* double mutant resembles that of the *spo0A* mutant alone. That is, the potential overproduction of subtilisin by the *hpr* mutation cannot override *spo0A* control of this gene.

Placing the *hpr* locus on a multicopy plasmid results in inhibition of sporulation, inhibition of protease production, and extreme instability of the plasmid. This effect requires the entire gene since a 3′ truncated gene shows none of these effects. In this regard the *hpr* gene resembles the *spo0F* gene (5) or the *sin* gene (11) in its ability to inhibit sporulation. These properties are in contrast to the sporulation inhibition effect of the multicopy *spoVG* gene (35) or *spo0E* gene (26), which appear to result from the multicopy promoter of these genes. We assume that multicopy plasmids result in the overproduction of the *hpr* gene product and that it is this protein that produces the phenotype. One way to explain these observations is to postulate that overproduction of the Hpr negative regulator results in the inability to induce those genes under control of this regulator. This premise mandates that some of the genes under Hpr control are intimately involved in the sporulation process. Since neither subtilisin nor neutral protease is necessary for sporulation (30, 32) the putative "*spo*" gene has not been identified. The *hpr* gene product may represent another link between nutritional deprivation and sporulation in the global domain of genes it controls.

The inhibition of protease production by a multicopy *hpr* gene could indicate that the Hpr protein can effectively regulate subtilisin from the upstream region. It also seems possible that the Hpr protein might have additional binding sites closer to the promoter. Finally, the protease phenotype may result from the sporulation-deficient phenotype of these strains and may not be a direct consequence of Hpr protein action at the subtilisin promoter. The reason for the instability of plasmids bearing the *hpr* locus seems equally unclear. Overproduction of the *hpr* gene product might be deleterious to unimpaired growth of the organism, although it is not evident from the colony size of strains bearing the plasmid.

The transcription of the *hpr* locus is under control of the *spo0A* locus. In a *spo0A* mutant the *hpr* gene product is constitutively overproduced, whereas transcription of a linked gene is uneffected by the *spo0A* mutation. The constitutive phenotype in an *spo0A* mutant is also characteristic of the effect of *spo0A* mutations on the synthesis of the *abrB* gene product (Perego and Hoch, manuscript in preparation). Since we postulate that the *hpr* gene product is a negative regulatory protein controlling several genes, including at least one that is an important sporulation gene, the sporulation-defective phenotype of *spo0A* mutants may result from the constitutive overproduction of the Hpr and AbrB (36) negative regulators. A triple *spo0A hpr abrB* mutant is not sporulation sufficient, however, suggesting that, at a minimum, one more regulatory circuit is controlled by the Spo0A protein.

If the *hpr* gene product is a negative regulatory protein as postulated, then some of the genes under its control may be derepressed in an *hpr* mutant. This might account for the isolation of *hpr* mutations as *catA* (17) if mutation at this locus led to insensitivity to glucose repression of sporulation. This suggests that the glucose-sensitive step in sporulation might be under control of Hpr. Dod and Balassa (7) found that *catA* and *scoC* mutants developed resistance to glucose repression earlier during sporulation than did wild-type strains. This result is consistent with but does not prove this notion.

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