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Restriction Fragments That Exert Promoter Activity During Postexponential Growth of *Bacillus subtilis*

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Two restriction fragments of *Bacillus subtilis* DNA were identified which caused the *cat-86* gene present on the promoter cloning plasmid pPL703 to be activated predominantly during postexponential growth of host cells. The postexponential increase was observed in both sporulation-positive strains and in a *spo0A* mutant of *B. subtilis*. However, the postexponential increase in the *cat-86* gene product, chloramphenicol acetyltransferase, was diminished or not observed when the plasmid-containing cells were grown in the presence of excess glucose. The promoter-containing fragment, designated as 33, was mapped to a site on the *B. subtilis* chromosome adjacent to *hisA*. The other fragment, 14, mapped to a site adjacent to *ctrA*. When present on a high-copy vector, both fragments caused a reduction in the sporulation frequency of host cells. Fragment 33 in high copy number conferred on *B. subtilis* cells three additional phenotypic changes: brown colony color, intracellular inclusions, and, in a protease-deficient mutant, the production of extracellular protease activity. These activities were observed only in postexponential-phase cultures.

Analysis of the transcribing activity isolated from the spore-forming bacterium *Bacillus subtilis* has demonstrated several forms of RNA polymerase which differ in the sigma-like subunits that associate with an apparently common core enzyme (14, 15). The predominant RNA polymerase forms present in vegetatively growing cells contain sigma-like subunits of 55,000 daltons, the sigma-55 enzyme, and 37,000 daltons, the sigma-37 enzyme. Both of these forms have been reported to decrease as cells enter sporulation, and a third enzyme, sigma-29, has been found to become predominant. Lastly, a form of RNA polymerase designated as sigma-28 has been detected in vegetative cells, apparently in low concentration (26). In vitro transcription studies have provided evidence that the sigma-37 and sigma-29 enzymes may be active in the transcription of sporulation genes (14).

The sigma subunit of RNA polymerase is directly involved in promoter selection, and the nucleotide sequences of promoters recognized by the sigma-55, -37, and -28 enzymes differ (7, 13, 19, 20). The foregoing observations suggest that the *B. subtilis* chromosome may be organized as transcriptional units controlled by promoters which are recognized by one or more of the various forms of RNA polymerase present in cells during growth and sporulation.

Two plasmid vectors for *B. subtilis* contain *cat* genes lacking functional promoters (9, 27). *cat* specifies chloramphenicol acetyltransferase (CAT). Therefore, transcriptional activation of *cat* by cloning promoters enables both plasmids to confer a chloramphenicol (Cm)-resistance trait on cells of *B. subtilis*. In theory, either plasmid should be useful for cloning *B. subtilis* promoters that are regulated in vivo. Examples of regulated promoters include those responding to specific forms of repression or activation mediated in part by small molecules or those which respond to unique RNA polymerase forms appearing in cells at discrete points of the growth-sporulation cycle.

In the present study, we screened promoter fragments cloned from *B. subtilis* to identify those which activate plasmid gene expression predominantly during postexponential growth. It was anticipated that an approach might allow the identification of promoters that control genes whose expression seems to be normally observed in stationary phase, such as genes specifying extracellular enzymes (21) and genes involved in sporulation.

MATERIALS AND METHODS

Bacteria, plasmids, and media. Bacterial strains and plasmids used are listed in Table 1. Plasmids pPL703 and pPL603 are promoter cloning vectors whose constructions are described elsewhere (E. J. Duvall, D. M. Williams, P. S. Lovett, C. Rudolph, N. Vasantha, and M. Guyer, Gene, in press; 27, 28). Figure 1 shows a diagram of pPL603. Media used include tryptose blood agar base, Spizizen minimal medium (16), Penassay broth (Difco Laboratories), and SG

TABLE 1. Relevant properties of bacterial strains and plasmids

Bacterial strains and plasmids	Relevant properties	Source
B. subtilis		
BR151	*trpC2 metB10 lys-3*	F. E. Young
BD224	*trpC2 thr-5 recE4*	D. Dubnau
SR22	*trpC2 spo0A*	J. Hoch
1A31	*trpC2 hisA1 cysB3*	BGSC[a]
1A11	*trpC2 ctrA1*	BGSC
1S19	*trpC2 spo0F221 pheA1*	BGSC
1A422	*leuB6 recE4*	BGSC
E. coli HB101	*hsdM hsdR recA*	H. O. Smith; reference 2
Plasmids		
pBR322	Tet^r Amp^r	H. O. Smith
pPL703	Neo^r Cm^s	Duvall et al., in press
pPL603	Neo^r Cm^s	Reference 27

[a] BGSC, *Bacillus* Genetic Stock Collection, Ohio State University, Columbus.

broth and agar (4). Chloramphenicol (Cm) and neomycin sulfate were added, when appropriate, to a final concentration of 10 μg/ml. DM3 agar was prepared as described by Chang and Cohen (5).

Plasmid manipulations. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. The Klenow fragment of DNA polymerase I was purchased from Bethesda Research Laboratories. Each was used as indicated by the supplier. Electrophoresis of DNA fragments through horizontal agarose gels and other procedures were as previously described (23).

Genetic manipulations. Plasmid DNA was transformed into recipient cells at DNA concentrations of 1 to 3 μg/ml. Unless noted, plasmids were transformed into cells made competent by the Bott and Wilson method (1). Protoplasts were transformed as described by Chang and Cohen (5). PBS1 transduction was as previously described (17).

Enzyme assays. CAT was assayed by the colorimetric reaction (24, 28) and is expressed as micromoles of Cm acetylated per min per mg of protein at 25°C. Protein was measured by the method of Bradford (3).

RESULTS

Elimination of the postexponential promoter activity present in pPL603. The use of the *cat-86* gene as a probe for detecting cloned promoters depends on the absence of sequences preceding the gene that function as promoters during the growth-sporulation cycle of *B. subtilis*. We have previously demonstrated that pPL603 (Fig. 1) lacks sequences needed for transcriptional activation of *cat-86* during the vegetative growth phase of host cells (23). In contrast, assays of pPL603-specified CAT in extracts of *B. subtilis* BD224(pPL603) cells taken from the late stationary phase demonstrated a 10-fold increase in the specific activity of CAT over that detected when cells were harvested during exponential growth (Fig. 2A). This endogenous promoter activity was absent (Fig. 2B) from plasmid pPL703 which is a derivative of pPL603 in which the 203-base-pair (bp) *Eco*RI-to-*Pst*I fragment was replaced with a 21-bp synthetic linker from M13mp7 (Fig. 2B). This linker contains internal *Sal*I- and *Bam*HI-sensitive sites (18).

Insertion of a 0.3-kilobase (kb) promoter-containing fragment, from phage SP02 (27), into the unique *Eco*RI sites of pPL603 and pPL703 generated pPL608 and pPL708, respectively (Duvall et al., in press; 27). Analysis of CAT levels in extracts of BD224 harboring either plasmid demonstrated that the 0.3-kb fragment exerted strong promoter activity in both exponentially growing and late stationary-phase cells (Fig. 2).

Cloning *B. subtilis* restriction fragments that exert promoter activity predominantly during postexponential growth. *Eco*RI or *Bam*HI restriction digests of total cellular DNA of *B. subtilis* strain BR151 were ligated with the *Eco*RI- or *Bam*HI-generated linear form of pPL703. Cm^r transformants of BD224 protoplasts were selected on DM3 agar containing 10 μg of Cm per ml. We screened resulting transformants for CAT activity during the exponential growth phase and in the stationary phase.

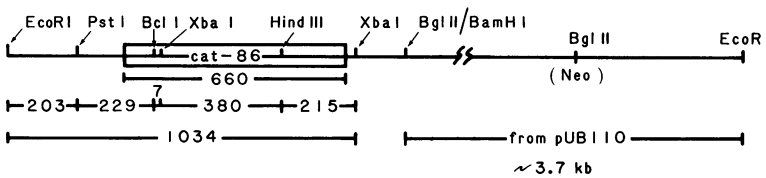

FIG. 1. Diagram of pPL603 opened at the single *Eco*RI site. The construction of this plasmid is described in reference 27. The region from the left-most *Eco*RI site through the *cat-86* gene has been sequenced (23; C. R. Harwood, D. M. Williams, and P. S. Lovett, Gene, in press; Duvall et al., in press). The plasmid specifies two drug-resistance traits. Neomycin resistance is specified by the 3.7-kb pUB110 segment, and Cm^r is due to the *cat-86* gene which encodes a Cm-inducible CAT. *cat-86* is transcriptionally activated by cloning promoters into the *Eco*RI site. pPL703 is a derivative of pPL603 in which the 203-bp *Eco*RI-*Pst*I segment has been replaced by a 21-bp *Eco*RI-*Pst*I synthetic linker from phage M13mp7 (18). This linker contains internal *Sal*I and *Bam*HI sites. The distances shown in the figure are in base pairs.

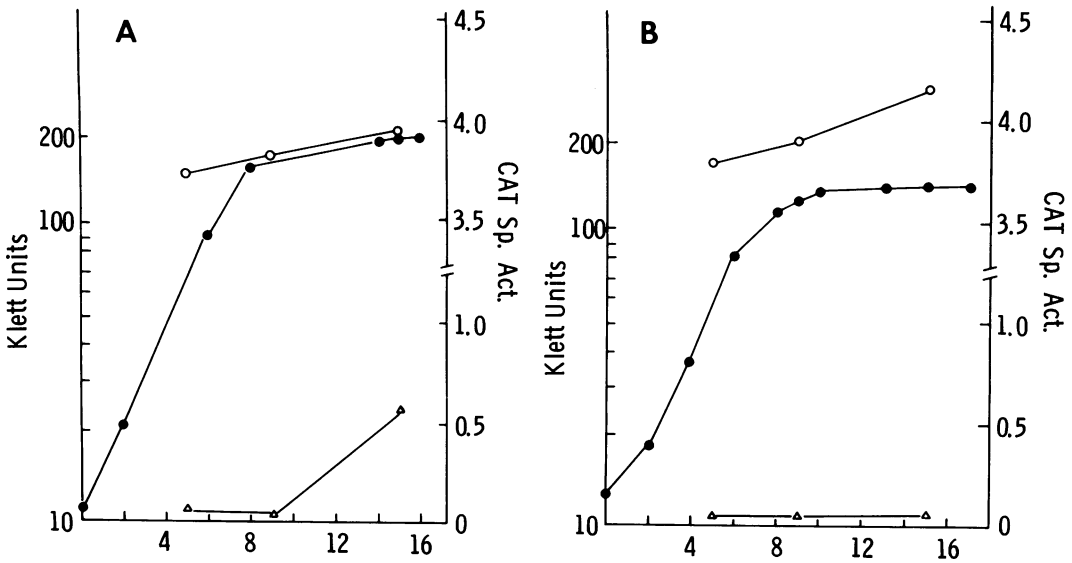

FIG. 2. Specific activity of plasmid-specified CAT during and after growth of *B. subtilis* strain BD224. (A) CAT activity specified by plasmid pPL603 (△) and pPL608 (○); (B) CAT activity specified by plasmid pPL703 (△) and pPL708 (○). Plasmid-containing cells were grown to mid-log phase in SG broth containing 2 μg of Cm per ml, diluted in the same medium and re-grown at 37°C. Growth was monitored in a Klett-Summerson colorimeter (●). Volumes (20 ml) were periodically removed and assayed for CAT as previously described (28).

Two general classes of plasmids constructed and selected in this manner contained promoter fragments that resulted in enhanced CAT levels in the stationary phase over that detected in exponentially growing cells. About 1% of the total Cm^r transformants contained plasmid inserts that reduced the sporulation frequency of host cells. A representative of this class is promoter fragment 14 which is present in pPL714 (Fig. 3). Fragment 14 was initially cloned as a *Bam*HI restriction fragment. This was placed into the unique *Bam*HI site of a synthetic oligonucleotide present in M13mp7 (18) and cloned back into pPL703 as an *Eco*RI fragment. During these manipulations, one of the *Bam*HI sites that should be present at one end of fragment 14 was lost.

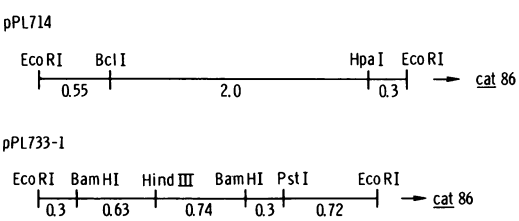

FIG. 3. Orientation of the insertion and restriction maps of fragments 14 and 33 in pPL703. pPL703 containing fragment 14 is designated pPL714, and pPL703 carrying fragment 33 is designated pPL733. Both cloned DNA segments are *Eco*RI fragments. In the figure, fragment 33 is in orientation 1 (pPL733-1). The plasmid carrying fragment 33 in the reverse orientation is designated pPL733-2.

A second class of plasmid present in about 1 Cm^r transformant per 1,000 caused BD224 colonies to acquire an intense brown color. The same *Eco*RI restriction fragment responsible for the brown colony was cloned in four separate experiments, suggesting the phenotype is due to a unique sequence within the *B. subtilis* genome. Fragment 33 (Fig. 3) is a representative of this class of promoter-containing fragment and is present in pPL733. ^{32}P-labeled, nick-translated pPL733 hybridized to a single fragment (ca. 2.7 kb) present in *Eco*RI digests of BR151 DNA as determined by the Southern hybridization method (25).

CAT activities specified by pPL714 and pPL733. The modulation of plasmid-specified CAT activity during the growth of host cells was somewhat similar for pPL714 and pPL733. Cells of BD224(pPL714) grown in SG medium containing 2 μg of CM per ml exhibited an 8- to 10-fold increase in the specific activity of CAT in the stationary phase over the minimal value detected during exponential growth (Fig. 4A). The increase was generally observed ca. 2 to 3 h after the cessation of exponential growth. Growth of the same cells in the presence of excess glucose (1%) eliminated the postexponential increase in CAT (Fig. 4A). If the increase in CAT were due to the appearance of a sporula-

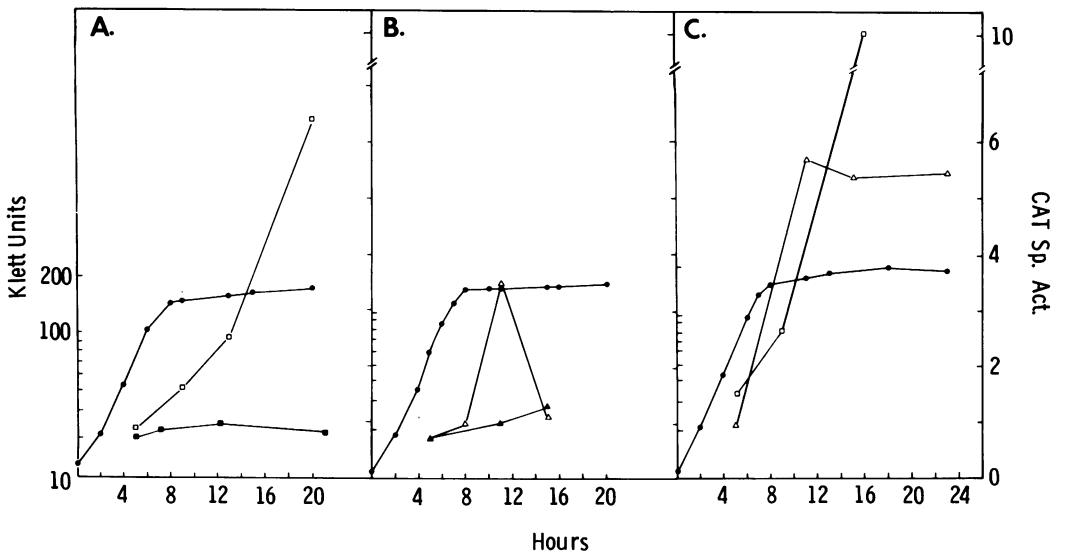

FIG. 4. CAT specified by pPL714 and pPL733 during the growth cycle of *B. subtilis*. (A) CAT activity specified by plasmid pPL714 in strain BD224 grown in SG (□) or SG + 1% glucose (■); (B) CAT activity specified by pPL733-1 in strain BD224 grown in SG (△) or SG + 1% glucose (▲); (C) CAT activity specified by pPL714 (□) and pPL733-1 (△) in strain SR22 (*spo0A*) grown in SG. Plasmid-containing cells were grown to mid-log phase in SG broth containing 2 μg of Cm per ml. Cells were diluted in the same medium and regrc wn at 37°C. Samples (20 ml) were periodically withdrawn, processed, and assayed for CAT as before (28). S lid circles indicate growth (Klett units).

tion-specific modified form of RNA polymerase, it would be expected that CAT would not exhibit the postexponential increase if pPL714 were introduced into an early-blocked sporulation mutant. However, pPL714 in SR22 specified a 6- to 8-fold increase in CAT during postexponential growth (Fig. 4C) as was observed in BD224.

Fragment 33 in pPL733 exerted bidirectional promoter activity. In orientation 1 (pPL733-1; Fig. 3), the plasmid specified a 5- to 6-fold increase in CAT activity during the early stationary phase (2 to 3 hours after cessation of exponential growth). This increased CAT level then decreased (Fig. 4B). Excess glucose (1%) in the SG medium prevented the postexponential increase (Fig. 4B). As with pPL714, pPL733 specified the same postexponential increase in CAT in the *spo0A* mutant SR22 (Fig. 4C) as seen in BD224. However, in SR22, the CAT activity was maintained at high levels throughout stationary phase, and did not decline during the period of the experiments. The reverse orientation of fragment 33, in pPL733-2, gave comparable results to those obtained with pPL733-1.

Location of the cloned promoter-containing fragments on the *B. subtilis* genetic map. Mapping of the cloned promoter containing fragments 14 and 33 was approached by the general method described by Haldenwang et al. (10). A mapping plasmid, pBR322C, was constructed by inserting the 2-kb *Eco*RI restriction fragment containing a functional *cat-86* gene from pPL531 (27) into the *Eco*RI site of pBR322. pBR322C replicates extrachromosomally only in *E. coli* and confers resistance to ampicillin, tetracycline and Cm. However, the Cm-resistance trait, owing to *cat-86*, can be expressed when pBR322C integrates into the *B. subtilis* chromosome. Integration of pBR322C into the *B. subtilis* chromosome was achieved by inserting all or part of the promoter-containing fragments into the plasmid, and transforming the chimeras into competent *B. subtilis* cells. Cm-resistant cells have been thought to arise by integration at the chromosomal site corresponding to the *B. subtilis* DNA present in the chimera (10). pBR322C-33 contained the 1.37-kb internal *Bam*HI region from fragment 33 (Fig. 3) inserted in the unique *Bam*HI site of pBR322C. pBR322C-14 contained the entire *Eco*RI fragment 14 (2.85 kb, Fig. 3), inserted adjacent to the *cat-86*–containing fragment. In control experiments, pBR322C generated no Cm^r transformants of BR151 or BD224, and pBR322C-33 and -14 generated no Cm^r transformants of BD224. However, both pBR322C-33 and -14 generated many Cm^r transformants when the recipient cells were strains BR151 or IS53. Two Cm^r transformants of both recipients for each of the two donor plasmids were shown to lack detectable supercoiled DNA by dye-buoyant density centrifugation. These eight Cm^r derivatives were used as propagating

hosts for PBS1. One donor lysate was used to map the location of each promoter. The other three donor lysates for each promoter-insert were used in confirmatory mapping experiments.

Mapping the location of the integrated form of pBR322C-33 by PBS1 transduction was approached by using Cm^r as the selected marker and scoring as nonselected markers auxotrophic mutations in various *B. subtilis* recipient strains. When strain 1A31 (*trpC2 hisA1 cysB3*) was the recipient, the His^+ phenotype was linked to Cm^r by 80 to 85% and Cys^+ was linked to Cm^r by 0.5 to 2%. The results of two three-factor crosses suggested the gene order *cysB hisA* Cm^r (Table 2). *hisA* is located at position 305 on the *B. subtilis* genetic map (11).

The location of integrated form of pBR322C-14 was determined as for pBR322C-33. In this case, the Cm^r marker was linked to *ctrA1* by 49% and to *spo0F* by 39%. The results of a three-factor cross suggested the gene order Cm^r *ctrA spo0F* (Table 3). *ctrA* is located at position 325 on the *B. subtilis* genetic map (11).

Plasmid-conferred host phenotypes. Both pPL733-1 and -2 conferred on host cells four distinct phenotypes which are due to the cloned promoter fragment, since none of the phenotypes was conferred by pPL703 or pPL708. First, colonies of cells, e.g., IA422, carrying pPL733, acquired an intense brown coloration. Second, introduction of the plasmid into the protease-deficient strain SR22 caused the cells to produce protease activity as detected on casein-containing agar (12; Fig. 5). However, the presence of pPL733 in cells that were already protease positive, such as IA422, did not significantly enhance protease levels as judged by the size of the halos surrounding cells patched to casein-containing agar. Third, the presence of pPL733 in IA422 Spo^+, SR22 (Spo^-), or in several other early-blocked sporulation mutants caused the cells to form internal inclusions when the cells were grown on a medium designed to promote sporulation, SG (Fig. 6). These inclusions were generally not detected when cells were grown on tryptose blood agar base nor minimal medium. Although the inclusions somewhat resembled spores in shape and refractility, the inclusions did not produce viable cells when cells containing the inclusions were heated at 80°C for 20 min. Similarly, dipicolinic acid was not detected in cells of SR22 (*spo0A*) exhibiting the inclusions. Lastly, the presence of pPL733 in IA422 reduced the sporulation frequency of cells by more than 90% (Table 4). Deletion of the internal 1.37-

TABLE 2. Three-factor PBS1-mediated crosses to map the location of the integrated form of pBR322C-33 in *B. subtilis*[a]

Selected marker	Recombinant classes	No.
Cm^r (n = 349)	His^+ Cys^+	1
	His^+ Cys^-	280
	His^- Cys^+	0
	His^- Cys^-	68
Cys^+ (n = 969)	His^+ Cm^r	14
	His^+ Cm^s	71
	His^- Cm^r	5
	His^- Cm^s	879

[a] The donor strain was BR151 (*trpC2 lys-3 metB10*), containing the integrated form of pBR322C-33. The recipient was 1A31 (*trpC2 hisA1 cysB3*).

TABLE 3. Three-factor PBS1-mediated cross to map the location of the integrated form of pBR322C-14[a]

Recombinant classes	No. (n = 187)
Ctr^+ Spo^+	18
Ctr^+ Spo^-	74
Ctr^- Spo^+	94
Ctr^- Spo^-	1

[a] The selected marker was Cm^r. The donor strain was 1S19 (*trpC2 pheA1 spo0F221*), containing the integrated form of pBR322C-14. The recipient was 1A11 (*trpC2 ctrA1*).

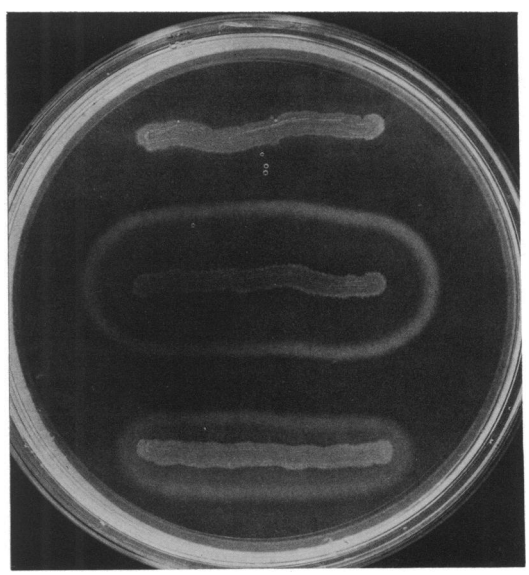

FIG. 5. Protease activity of SR22 and plasmid-containing derivatives on casein-containing agar. SR22 (top), SR22(pPL733-1) (middle), and SR22(pPL733-1ΔB; deleted for the internal 1.37-kb *Bam*HI fragment) (bottom) were streaked onto agar and incubated for 2 days at 37°C.

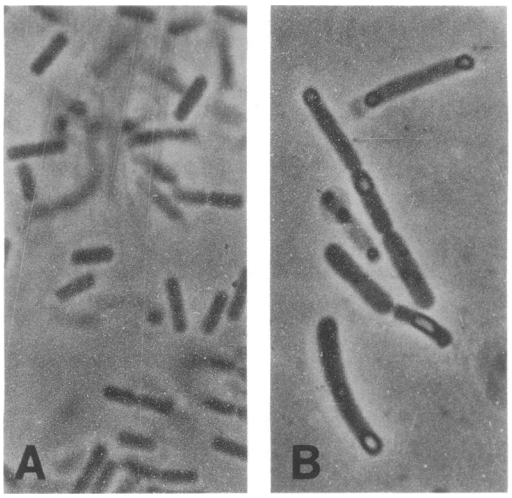

FIG. 6. Intracellular inclusions in *B. subtilis* cells harboring pPL733. SR22 (A) and SR22 harboring pPL733-1 (B) were grown for 48 h in SG and photographed under phase-contrast microscopy

kb *Bam*HI fragment from the promoter in pPL733 produced a derivative plasmid that lost the ability to confer the brown colony color and the inclusions. However, the *Bam*HI-deleted form did cause SR22 to produce protease, although the levels appeared reduced compared with the protease produced by SR22(pPL733) (Fig. 5). The *Bam*HI-deleted version of pPL733 retained the inhibitory effect on sporulation of IA422, and the fragment retained promoter activity in both orientations. In contrast to pPL733, pPL714 conferred on IA422 cells the single phenotype of sporulation inhibition (Table 4).

Evidence that fragment 33 encodes a protease activity. Although pPL733 caused SR22 cells to produce high-level protease activity, this could indicate that the fragment encodes a specific protease or regulates expression of a protease gene(s) present on the host chromosome. To distinguish between these possibilities, we attempted to introduce the fragment into a heterologous host, *E. coli* HB101, on a plasmid vector. Among several plasmid vectors tested, such as pBR322, none allowed transfer of the entire fragment 33 to *E. coli*. However, nine phages were identified in a charon 4A clone library of *B. subtilis* DNA (6) that shared DNA-DNA homology with ^{32}P-labeled, nick-translated pPL733 DNA. Five of these phages caused infected *E. coli* host cells to produce protease activity on casein-containing agar plates. Thus, we favor the view that fragment 33 encodes a gene for a protease.

TABLE 4. Sporulation of *B. subtilis* 1A422 harboring pPL708, pPL733, or pPL714[a]

Plasmid	Viable cells/ml		% Sporulation
	Before heating	After heating	
pPL708	6.8×10^5	3.1×10^5	45
pPL733	4.3×10^6	2.5×10^3	0.058
pPL714	6.33×10^5	1.42×10^4	2.2

[a] Plasmid containing cells were grown for 2 days at 37°C in SG containing 10 μg of neomycin sulfate and 5 μg of Cm per ml. Viable units were assayed on tryptose blood agar base containing 5 μg of Cm per ml before and after heating 0.5-ml portions at 80°C for 30 min.

DISCUSSION

The *cat* gene in plasmids pPL603 and pPL703 is transcriptionally activated by cloning restriction fragments upstream that contain promoters. We identified several restriction fragments of the *B. subtilis* chromosome that promote low-level expression of the *cat* gene in pPL703 when host cells are in the exponential growth phase, but these fragments promote high-level expression during postexponential growth. It is presumed that the level of *cat* expression reflects the level of transcription of the gene when *cat* is maximally induced with Cm. The goal of the present work was to clone *B. subtilis* restriction fragments that exert postexponential promoter activity, in the expectation that this general approach might identify chromosomal segments containing both promoters and structural genes that function preferentially in stationary phase cells; for example, genes specifying sporulation-related proteins or those determining any of several extracellular enzymes that appear in postexponential growth.

Fragment 33 (2.7 kb) was mapped to a site adjacent to *hisA*. The fragment was found to not complement two mutations that are reported to be closely linked to *hisA*, *gerF*, and *outE* (11). However, the presence of fragment 33 in high copy numbers in a protease-deficient *spoA* mutant, SR22, caused a major enhancement in the level of extracellular protease produced. Deletion of an internal 1.4-kb *Bam*HI segment from fragment 33 reduced the level of protease activity but did not abolish the activity. It seems likely that fragment 33 encodes a specific protease, since several phages in a charon 4A clone library of *B. subtilis* DNA which shared homology with fragment 33 caused infected *E. coli* cells to produce protease activity on casein agar plates.

Fragment 33, in high copy number, also determined two other host functions that were detected only during postexponential growth, and both of these phenotypes were eliminated by deletion

of the internal 1.4-kb *Bam*HI segment from fragment 33. One phenotype was the intense brown color of colonies carrying pPL733. In liquid cultures, the pigmentation was present in the growth medium of cells grown to stationary phase; the pigment was not detected in exponentially growing cultures. It is not known if the apparent extracellular state of the pigment in liquid culture is due to lysis of cells or to pigment excretion. The second phenotype specified by pPL733 was the appearance of inclusion structures within cells grown under conditions that normally promote sporulation. This phenotype, as well as the pigmentation phenotype, has been observed in both Spo^+ and early-blocked Spo^- mutants of *B. subtilis*. Morphologically, the inclusions resembled structures present within *E. coli* cells that are overproducing the products of cloned genes (8). The refractile structures in such *E. coli* cells are thought to represent the sites of deposition of the overproduced gene product.

Both fragments 14 and 33 greatly reduced the ability of host cells to form spores when each was present in cells at high copy number. Unfortunately, fragment 14 in pPL714 did not specify any additional detectable host phenotype. One approach to assigning a genetic function to fragment 14 involves introducing a deletion within the fragment into the *B. subtilis* chromosome and observing the phenotypic variation in the host cell (22). This has been tested by recombining a derivative of fragment 14, deleted for an internal 2-kb *Bcl*I-*Hpa*I segment (see Fig. 3), into the *B. subtilis* chromosome. The resulting mutation caused a sporulation-deficient phenotype, and the mutation showed 46% linkage by PBS1 transduction with *ctrA* (Y.-W. Chiang, unpublished data). However, the sporulation-negative phenotype has been highly unstable, due apparently to subsequent rearrangements in the host chromosome.

Further characterization of both restriction fragments will allow identification of the subregions which determine promoter activity. Similarly, analysis of the biological traits specified by the promoter-containing fragments will likely allow determination of the functions of genes present on the two fragments.

ACKNOWLEDGMENTS

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