JOURNAL OF BACTERIOLOGY, Sept. 1985, p. 1101–1108
0021-9193/85/091101-08$02.00/0

Vol. 163, No. 3

Thermoinducible Transcription System for *Bacillus subtilis* That Utilizes Control Elements from Temperate Phage φ105

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Received 5 April 1985/Accepted 14 June 1985

We describe a thermoinducible-expression system for *Bacillus subtilis* which utilized an early promoter-operator sequence from temperate phage φ105 and the thermolabile prophage repressor from the phage variant φ105 *cts23*. The system operated at the transcriptional level to control expression in *B. subtilis* of the *cat-86* gene derived from *Bacillus pumilis*. Details of the strategies used to isolate the early phage promoter are described. This promoter lay in close proximity to the prophage repressor gene on the φ105 genome. The sequence of the early promoter differed from that of the vegetative *B. subtilis* consensus promoter by 1 base pair in both the −10 and −35 regions. We also present evidence that our phage-derived expression system could function in *Escherichia coli* to effect thermoinducible expression of the *galK* gene.

The expression of foreign genes in *Bacillus subtilis* may in some cases be lethal or detrimental to the host cell. For this reason, we sought to develop an inducible system in which the timing of gene expression could be effectively controlled. An inducible system has been described for *B. subtilis* (28) which involves the regulatory elements of the *Escherichia coli lac* operon; transcription of the gene in question is normally repressed but is induced by the addition of isopropyl β-D-thiogalactopyranoside. Our aim was to develop a transcriptional system which could be rapidly induced by a temperature shift rather than by the addition of chemicals. Furthermore, to avoid potential problems caused by protease secretion in *B. subtilis* after the end of the exponential phase, we wished to use a promoter which was active during vegetative growth. Therefore, with the *E. coli* thermoinducible system comprising the p_L and *c*I857 controlling elements from lambda phage as a model (2), we developed a similar system based on *B. subtilis* temperate phage φ105. In our system, the *B. subtilis* host cell contained two compatible plasmids, one encoding the prophage repressor gene from the thermoinducible mutant φ105 *cts23* (1) and the other encoding the early promoter-operator sequence on which the repressor acts.

Previous work has indicated that the φ105 repressor gene lies on the 3.2-kilobase (kb) *Eco*RI F fragment (7). Our problem, therefore, was to isolate a DNA fragment containing the early promoter-operator sequence. In this paper we describe the strategies used to isolate this fragment, and we show that the *cat-86* gene (27), coding for chloramphenicol acetyltransferase (CAT), is regulated by the early promoter-operator sequence and the prophage repressor in a temperature-dependent fashion. We also demonstrate that this regulation is at the level of transcription. In addition, we present the map position of the early promoter-operator on the φ105 genome, the DNA sequence of this promoter, and a determination of its relative strength.

MATERIALS AND METHODS

Media and antibiotics. Difco Sporulation Medium (Difco Laboratories, Detroit, Mich.), for the storage of *B. subtilis* spores, and LB medium have been described previously (24). MacConkey galactose plates were used for *E. coli* selections as described by Miller (17). Antibiotics (Sigma Chemical Co., St. Louis, Mo.) were used in the following concentrations: for *E. coli*, ampicillin and kanamycin were present at 50 and 40 μg/ml, respectively: for *B. subtilis*, erythromycin, chloramphenicol, and neomycin were present at 5, 10 to 50, and 10 μg/ml, respectively.

Bacterial strains, phage, and plasmids. Strains, phage, and plasmids are listed, with their relevant properties, in Table 1.

Growth of φ105, preparation of phage stocks, and isolation of lysogens. Conditions for phage growth, phage stock preparation, and lysogen isolation have been described previously (21). To determine the immunity of a bacterial host to φ105, the clear-plaque mutant φ105 *c*30 was cross streaked against the bacterial host on LB or Difco Sporulation Medium plates containing the appropriate antibiotic. Known lysogens and nonlysogens were used as controls.

The absence of visible lysis was taken as an indication of lysogeny. In questionable cases, putative lysogens were assayed for the ability to produce phage spontaneously during growth in liquid medium or on plates containing sensitive indicator bacteria.

Isolation of phage and plasmid DNA. φ105 DNA was purified from phage stocks suspended in TE buffer (20 mM Tris [pH 7.5], 0.1 mM EDTA) by treatment of phage with 1% sodium dodecyl sulfate for 10 min at 50°C, followed by two phenol extractions, ethanol precipitation, suspension of DNA in TE buffer, and overnight dialysis of DNA against TE buffer.

B. subtilis plasmid DNA was prepared by the method of Gryczan et al. (9). *E. coli* plasmid DNA was prepared as described previously (13).

Transformations. Preparation and transformation of competent cells of *B. subtilis* was by the method of Sonenshein et al. (24). Competent cells were always used fresh. When selecting for colonies that were resistant to chloramphenicol, cells were allowed 1.5 h for phenotypic expression before being challenged with the drug. Transformation of *B. subtilis* protoplasts was by the method of Chang and Cohen (5). *E. coli* HB101 was transformed as previously described (6).

Recombinant DNA procedures. DNA restriction enzymes and T4 DNA ligase were purchased from New England BioLabs, Inc., Beverly, Mass., and used according to the directions of the manufacturer. Minipreps of both *E. coli* and

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TABLE 1. Bacterial strains, phage, and plasmids

Species (strain, plasmid, phage)	Relevant properties[a]	Source (reference)
E. coli		
HB101	*galK*	Biogen collection
pACYC177	Kan^r	Biogen collection
pKO300	Amp^r *galK*, promoter probe plasmid	M. Rosenberg (22)
pKO504	Amp^r *galK*+, ϕ105 early promoter precedes *galK* gene	This study
pIQF	Kan^r, encodes ϕ105 *c*ts23 repressor gene	This study
B. subtilis		
BR151	*tpC2, lys-3 metB10*	A. L. Sonenshein
168	*trpC2*	A. L. Sonenshein
ϕ105	Wild type	A. L. Sonenshein
ϕ105 *c*ts23	Thermoinducible	A. L. Sonenshein (1)
ϕ105 *c*30	Clear-plaque mutant	BGSC[b] (23)
pE194	Ery^r	G. Gray (26)
pUB110	Neo^r	A. L. Sonenshein (11)
pPL603	Neo^r Chr^s promoter-probe plasmid	P. Lovett (27)
p603-900	Neo^r Chr^r, ϕ105 early promoter precedes *cat-86* gene	This study
pM413-27		
p3-8	Neo^r Chr^r, early SPO1 promoter precedes *cat-86* gene	M. S. Osburne, and R. J. Craig, manuscript in preparation
p52-4	Neo^r Chr^r, *E. coli trc* promoter precedes *cat-86* gene	M. S. Osburne, and R. J. Craig, manuscript in preparation
pPL608	Neo^r Chr^r, an SPO2 promoter precedes *cat-86* gene	P. Lovett (27)
p1776	Ery^r, encodes ϕ105 *c*ts23 repressor gene	This study

[a] Abbreviations: r, resistant; s, sensitive; Kan^r, Amp^r, Ery^r, Neo^r, and Chr^r denote phenotypic resistance to the antibiotics kanamycin, ampicillin, erythromycin, neomycin, and chloramphenicol, respectively.
[b] Bacillus Genetic Stock Center, Ohio State University.

B. subtilis plasmids were done by a modification of the alkaline extraction procedure of Birnboim and Doly (described in reference 14). Agarose and acrylamide gel electrophoresis, electroelution of DNA fragments from agarose gels, and Southern blotting experiments were performed by standard procedures (14). DNA sequencing was performed by the method of Maxam and Gilbert (16).

RNA extraction and S1 nuclease experiments. RNA was isolated by a modification of the procedure outlined in reference 14. Cells were suspended in a 5 mM sodium citrate solution (pH 7.0) containing 5 M guanidinium isothiocyanate and 0.1 M β-mercaptoethanol and sonicated intermittently for a total of 2 min. Cell extracts were centrifuged through a salt cushion containing 5.7 M CsCl and 100 mM EDTA (pH 7.5). The RNA pellet was suspended in 0.3 M sodium acetate (pH 5.0)–1 mM EDTA buffer and stored at −70°C in 70% ethanol.

S1 nuclease digestions and promoter mapping experiments were performed by the method of Brosius et al. (3). A sample of RNA was precipitated and suspended in TE buffer, and the concentration was determined with a Gilford spectrophotometer at 260 nm. Hybridization reactions contained excess single-stranded probe to detect the amount of specific mRNA in each preparation. Concentrations of specific mRNA could be estimated by gel electrophoresis (6% acrylamide-urea gels). The band densities of autoradiograms, with several timed exposures, were compared. Alternatively, Cerenkov counts of ethanol-precipitated and ethanol-washed S1 reactions were measured. For the latter method, background counts were determined by conducting a mock hybridization and S1 reaction with the probe, and background counts were subtracted from the test results.

The probe used to measure transcription of the *neo* gene was a 293-base-pair (bp) *Hae*III fragment derived from the *neo* gene of plasmid pUB110, encoding what is believed to be the promoter region and the 5′ end of the structural gene (as defined in reference 15).

For labeling probes, [^{32}P]ATP (3,000 Ci/mmol) was obtained from Amersham Corp., Arlington Heights, Ill., or New England Nuclear Corp., Boston, Mass., and kinase was obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind.

RESULTS

Isolation of plasmid p1776 encoding the ϕ105 *c*ts23 repressor gene. As it is known that *Eco*RI fragment F of the ϕ105 genome encodes the prophage repressor gene (7), our object was to insert fragment F from the thermoinducible variant ϕ105 *c*ts23 into the Ery^r plasmid pE194. pE194 lacks an *Eco*RI site; therefore, fragment F was cloned in two steps: (i) Fragment F from a total *Eco*RI digest of ϕ105 *c*ts23 DNA was electroeluted from an agarose gel and inserted into the *Eco*RI site of Kan^r *E. coli* plasmid pACYC177, yielding recombinant plasmid pIQF (Fig. 1A); (ii) pIQF was digested to completion with endonuclease *Bam*HI and partially with *Pst*I, and a 3.95-kb piece of DNA containing fragment F was gel purified. This fragment was then ligated into plasmid pE194 which had been cut to completion with *Pst*I and partially with *Mbo*I (Fig. 1B). The ligation mix was transformed into protoplasts of strain 168, and Ery^r clones were

selected and shown to contain plasmid p1776 (which bears fragment F from ϕ105 *c*ts23). This result was confirmed both by patterns of restriction digests and by the fact that cells bearing p1776 were immune to superinfection by ϕ105 at a low temperature (30°C) but nonimmune at 45°C (see above).

Thus, we obtained an *E. coli* plasmid (pIQF) and a *B. subtilis* plasmid (p1776) both of which contained the ϕ105 *c*ts23 prophage repressor gene.

Isolation of the ϕ105 early promoter-operator sequence. We used two strategies to identify the ϕ105 early promoter-operator sequence. One approach involved direct cloning into *B. subtilis*, and the other involved using *E. coli* as an intermediate host. Both approaches were successful.

(i) Direct cloning into *B. subtilis*. For direct cloning into *B. subtilis*, we made use of multicopy Neo^r promoter-probe plasmid pPL603B (a derivative of plasmid pPL603 [27]). Plasmids pPL603 and pPL603B contain the *cat-86* gene without a promoter recognized in vegetative cells (19). pPL603B contains a *Bam*HI site in front of *cat-86*. Cells containing either of these plasmids are normally Chr^s but become Chr^r when a promoter-containing fragment is inserted upstream of *cat-86*. Partial and total *Sau*3A1 digests of ϕ105 DNA were ligated into the *Bam*HI site of pPL603B, and ligations were transformed into strain BR151(pUB110) by the resident plasmid recombination technique (10). A total of 44 colonies resistant to chloramphenicol at 25 μg/ml were isolated; they bore recombinant plasmids with DNA inserts of various sizes.

We reasoned that strains containing multiple copies of the early promoter-operator sequence (as on a multicopy plasmid) might be unable to maintain lysogeny by ϕ105 because multiple copies of the promoter-operator sequence might titrate repressor protein (whose gene would be present in only a single copy). We therefore attempted to isolate lysogenic derivatives of the 44 strains described above. Six derivatives were unable to form stable lysogens. Phage plaques were present in streaks of purified lysogens of these strains, indicating a very high rate of spontaneous induction and phage sensitivity in the population.

Plasmid p1776, containing the prophage repressor gene, was then transformed into competent cells of each of these six strains, and Chr^r Neo^r Ery^r colonies were selected at 45°C. One of these strains, containing a plasmid designated pM413-27 (Fig. 2), was Chr^r Neo^r Ery^r at 45°C and Chr^s Neo^r Ery^r at 30°C, indicating that the promoter driving the *cat-86* gene was regulated in a temperature-dependent fashion. Plasmid pM413-27 contained a 3-kb insert of ϕ105 DNA (Fig. 3, lane 2).

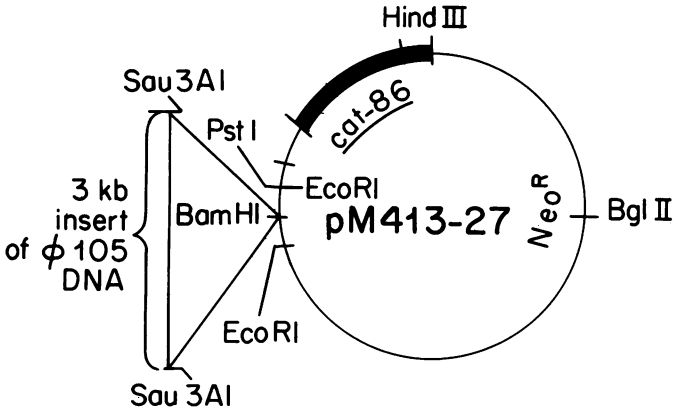

FIG. 2. Diagram of plasmid pM413-27. This plasmid, a derivative of plasmid pPL603B (see text), contains a 3-kb insert of ϕ105 DNA in front of the *cat-86* gene. This insert encodes a promoter-operator sequence which is regulated by the prophage *c*ts23 repressor gene.

(ii) Cloning promoter fragments into *E. coli*. In our second strategy for cloning the early ϕ105 promoter-operator, *E. coli* was used as an intermediate host. Plasmid pKO300, an *E. coli* promoter-probe plasmid, is Ap^r and a derivative of plasmid pKO-1 (22). It contains the *galK* structural gene without a promoter. *E. coli* HB101, itself *galK*, formed white colonies on MacConkey galactose plates in the presence or

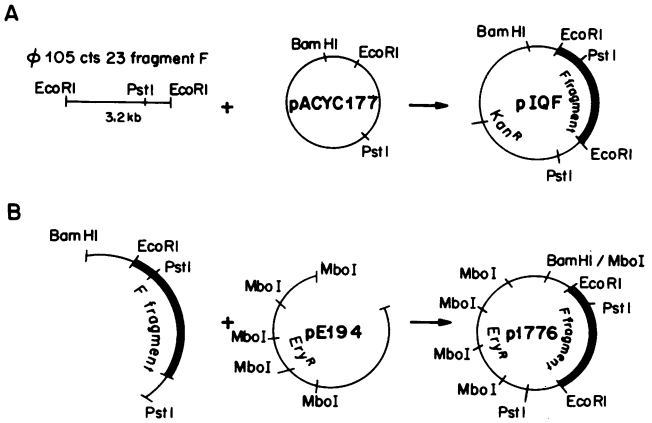

FIG. 1. Isolation of p1776, encoding the thermoinducible prophage repressor gene. (A) To obtain 3.2-kb fragment F from ϕ105 *c*ts23, 100 μg of phage DNA was digested with *Eco*RI. The fragment was gel purified, ligated into the *Eco*RI site of plasmid pACYC177, and transformed into *E. coli* HB101; Kan^r transformants were selected. These contained plasmid pIQF. (B) A 3.95-kb *Bam*HI-*Pst*I fragment from pIQF was ligated to a 3-kb *Mbo*I-*Pst*I fragment of plasmid pE194 (see text). The resulting Ery^r plasmid, p1776, contained the thermoinducible prophage repressor gene.

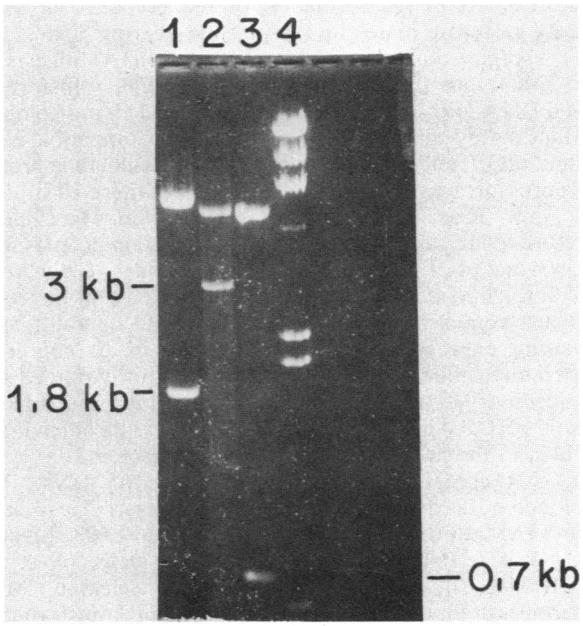

FIG. 3. Agarose gel electrophoresis of promoter-containing plasmids digested with *Eco*RI. A 1-μg sample of purified DNA from each plasmid was cut to completion with *Eco*RI, run through an 0.8% agarose gel, and stained with ethidium bromide. Lanes: 1, pKO504; 2, pM413-27; 3, p603-900; 4, λ *Hin*dIII fragments.

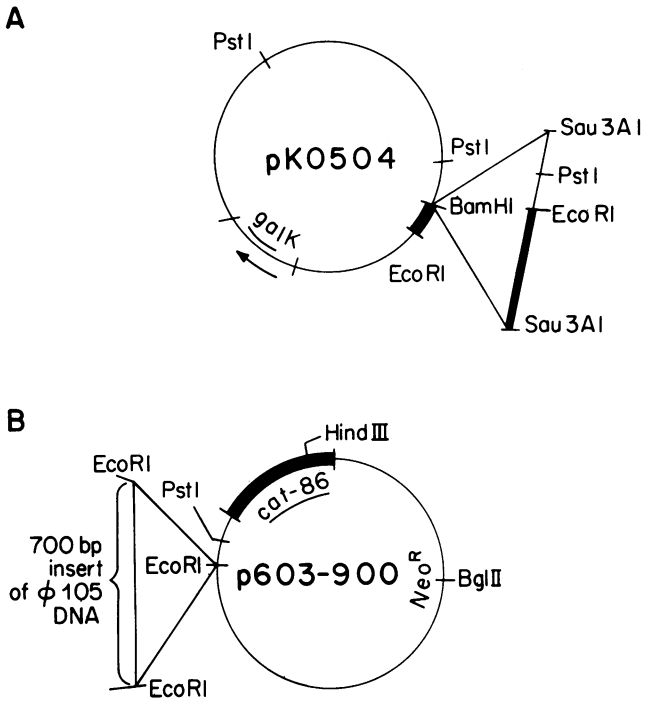

FIG. 4. Diagram of plasmids pKO504 and p603-900. (A) pKO504, a derivative of plasmid pKO300, contains a 2.4-kb insert of ϕ105 DNA. An 1,800-bp *Eco*RI fragment (heavy line), consisting mostly of ϕ105 DNA, was subcloned into the *Eco*RI site of *B. subtilis* promoter-probe plasmid pPL603. (B) The resulting plasmid, p603-900, containing a deleted fragment (~700 bp) of the 1,800-bp *Eco*RI fragment described above.

absence of plasmid pKO300. Promoter-containing inserts in the *Bam*HI site of pKO300, just upstream from the *galK* gene, resulted in the formation of red colonies on MacConkey galactose plates by the plasmid-bearing host.

We shotgun cloned partial and total *Sau*3A1 digests of ϕ105 DNA into the *Bam*HI site of pKO300, transformed ligated DNA into strain HB101, and selected 10 red colonies on MacConkey galactose-ampicillin plates. Compatible Kan^r plasmid pIQF, encoding the gene for the thermolabile prophage repressor, was transformed into each of these 10 strains. Red Amp^r Kan^r colonies were selected on MacConkey galactose plates at 42°C. One strain, containing plasmids pKO504 and pIQF, was Amp^r Kan^r and red at 42°C but Amp^r Kan^r and white at 30°C. This result suggested that the *c*ts23 prophage repressor gene was acting on its operator site, regulating expression of the *galK* gene in *E. coli* in a temperature-inducible fashion. To confirm this hypothesis, it was necessary to transfer the promoter-operator sequence in plasmid pKO504 into *B. subtilis* and to assess its behavior in this host in the presence of the repressor protein.

pKO504 contains a 2.4-kb insert of ϕ105 DNA. We subcloned a 1.8-kb *Eco*RI fragment of this insert (Fig. 3, lane 1; Fig. 4A) into the *Eco*RI site of plasmid pPL603. Ligated DNA was transformed into competent cells of strain BR151(pUB110), and Chr^r colonies were selected. After numerous attempts, we obtained a single Chr^r transformant. This transformant contained a plasmid (designated p603-900) consisting of plasmid pPL603 plus an insert of approximately 700 bp in the *Eco*RI site (Fig. 3, lane 3; Fig. 4B). Apparently the 1.8-kb *Eco*RI fragment of pKO504 underwent a spontaneous deletion.

Plasmid p1776, containing the gene for the thermolabile prophage repressor, was then transformed into competent cells of strain BR151(p603-900), and Chr^r Neo^r Ery^r cells were selected at 45°C. These cells were all Chr^s Neo^r Ery^r at 30°C. Cells bearing plasmid p603-900 alone were Chr^r Neo^r at both temperatures. The results suggested that the 700-bp insert in p603-900 contained a promoter-operator sequence regulated by the prophage repressor protein.

CAT activities of strains bearing various recombinant plasmids. As described above, we obtained two *B. subtilis* strains which expressed the Chr^r phenotype at 45°C but not at 30°C: strain BR151, containing plasmids p603-900 and p1776, and strain 168, containing plasmids pM413-27 and p1776. The CAT activities of these and other relevant strains are presented in Table 2. The CAT assays confirm that when a plasmid containing an early phage promoter-operator sequence and a plasmid containing the thermolabile repressor gene were present in the same cell, CAT activity was severely repressed at 30°C. However, activity was largely restored when cells were shifted to 45°C for 1 h. Table 2 also indicates that the activity of the phage promoters in pM413-27 and p603-900 was relatively weak compared with the activity of known strong promoters, such as a derivative of the *tac* promoter and an early SPO1 promoter (M. Osburne and R. Craig, manuscript in preparation) and the promoter (derived from phage SPO2) present in plasmid pPL608 (27).

Localization of the promoter-operator fragment on the ϕ105 genome. The 700-bp *Eco*RI insert in plasmid p603-900 was labeled with [^{32}P]ATP and tested for hybridization to various DNA fragments (Fig. 5). This fragment hybridized both to the 3-kb promoter-operator insert in plasmid pM413-27 and to the 1,800-bp insert in plasmid pKO504 (from which the 700-bp insert was derived). Interestingly, we found that the 700-bp insert hybridized to ϕ105 *Eco*RI fragment F (Fig. 5A), indicating that the promoter-operator sequence lies on the same *Eco*RI fragment as the repressor gene.

Next, we hybridized the 700-bp fragment to ϕ105 fragment F and to the 3-kb insert in plasmid pM413-27, both of which had been cut to completion with various restriction enzymes. The pattern of hybridization is presented in Fig. 5B. For the purposes of this analysis, we constructed a partial

TABLE 2. CAT activities of strains containing various plasmids

Plasmid(s) present in *B. subtilis* strain:	CAT activity[b] (mOD/min per mg of protein) at growth temp:			
	30°C	30–45°C[c]	45°C	37°C
168				
pM413-27^{-}[a]	2,021	4,111	5,937	
pM413-27, p1776^{+}	167	1,167	2,250	
pM413-27, pE194^{-}	1,818	2,950	3,500	
BR151				
p603-900^{-}	2,778	4,117	4,574	
p603-900, p1776^{+}	50	895	1,212	
p603-900, pE194^{-}	1,917	1,500	3,300	
p3-8^{-}				38,000
p52-4^{-}				19,256
pPL608^{-}				15,207

[a] +, Presence of ϕ105 cts 23 repressor; −, absence of repressor.
[b] Cells were grown at the indicated temperatures in LB broth with neomycin or with neomycin plus erythromycin. Chloramphenicol (10 μg/ml) was added 1 h before cells were harvested and assayed.
[c] Cells were shifted to 45°C for 1 h before harvesting.

restriction map of fragment F (Fig. 6). Our map is in general agreement with a recently published restriction map of ϕ105 (4). The hybridization data in Fig. 5B allow us to position the 700-bp promoter-operator fragment in close proximity to the ϕ105 repressor gene (Fig. 6). The positioning of the fragment is in agreement with the recent findings of Dhaese et al. (8), who located an early ϕ105 promoter with an alternate strategy. (It should be noted that because of the way the 700-bp fragment was derived, some of the base pairs in this fragment may be of plasmid [pKO300] origin.)

The pattern of hybridization of the 700-bp promoter-operator fragment to the 3-kb promoter fragment in plasmid pM413-27 is also shown in Fig. 5B. It is clear that the 3-kb piece of DNA is not a contiguous region of the ϕ105 genome but actually is composed of normally nonadjacent ϕ105 *Sau*3A1 fragments which were ligated together. The 3-kb piece apparently contains a small region derived from fragment F, because the restriction pattern in Fig. 5B is altered in every case compared with that of fragment F.

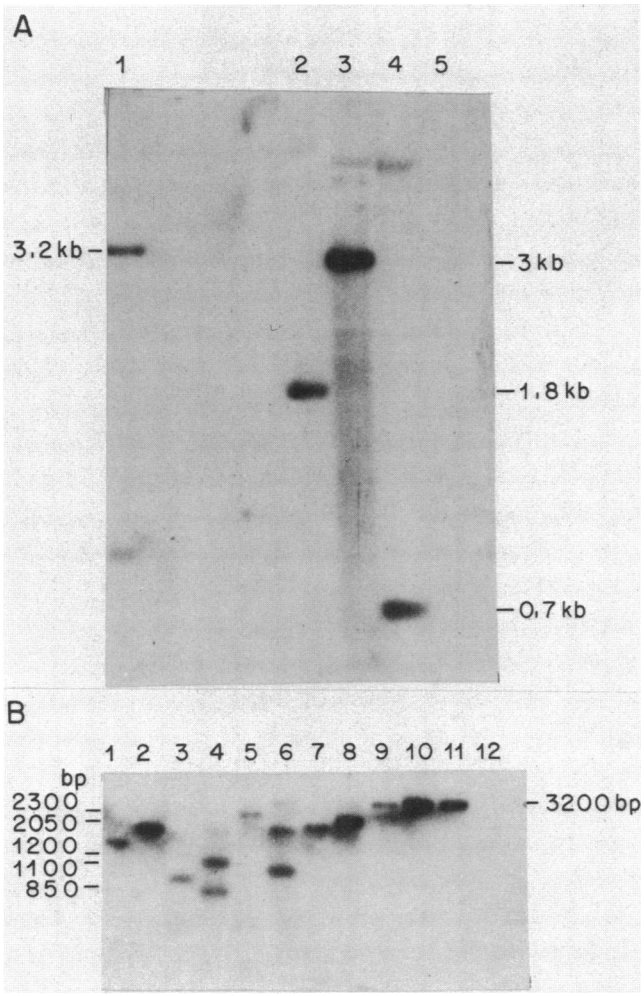

FIG. 5. Southern hybridization of ^{32}P-labeled 700-bp *Eco*RI promoter insert in plasmid p603-900 to various DNA fragments. (A) Lanes: 1, ϕ105 DNA, *Eco*RI digest; 2, plasmid pKO504, *Eco*RI digest; 3, plasmid pM413-27, *Eco*RI digest; 4, plasmid p603-900, *Eco*RI digest; 5, λ *Hin*dIII fragments. (B) Lanes: 1, 3, 5, 7, and 9, the 3-kb phage DNA insert in plasmid pM413-27 digested with *Pvu*II, *Hin*dIII and *Pvu*II, *Hin*dIII, *Pst*I, and no enzyme, respectively; 2, 4, 6, 8, and 10, ϕ105 *Eco*RI fragment F digested with *Pvu*II, *Hin*dIII and *Pvu*II, *Hin*dIII, *Pst*I, and no enzyme, respectively; 11, ϕ105 DNA, *Eco*RI digest; 12, DNA size markers.

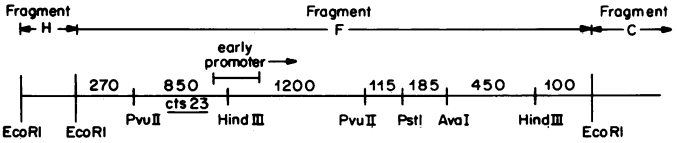

FIG. 6. Partial restriction map of ϕ105 fragment F, indicating the position of the early promoter and the direction of transcription.

Sequence of the early promoter. The DNA sequence of the promoter-operator fragment is presented in Fig. 7. The precise size of the fragment was 676 bp. The location of the early promoter on this sequence was determined as follows. Preliminary S1 nuclease transcriptional mapping studies revealed that the promoter was located upstream from the *Hin*dIII site (data not shown), which divides the 676-bp fragment into pieces of 285 and 391 bp. Next, 285- and 391-base single-stranded probes labeled at the *Hin*dIII site with [^{32}P]ATP were prepared, hybridized to RNA extracted from strain BR151(p603-900), and digested with S1 nuclease. A fragment of DNA approximately 50 bases in length, derived from the 285-bp *Hin*dIII-*Eco*RI fragment, was protected from nuclease digestion (Fig. 8). A sequencing gel of the 285-base fragment is shown in Fig. 9, with the portion protected from S1 nuclease digestion run in an adjacent lane. This allowed us to localize the promoter and to determine its sequence as

TTTACA——17 bp——TACAAT
−35 −10

(Fig. 7). The sequence differs by 1 bp in the −35 region and 1 bp in the −10 region from the consensus sequence for *B. subtilis* vegetative promoters (see above).

The thermoinducible system is controlled at the transcriptional level. Previous work has shown that transcription of the *cat-86* gene in *B. subtilis* is affected by translational as well as transcriptional control mechanisms (18). For this reason, it cannot be assumed that levels of CAT activity were strictly correlated to the efficiency of transcription of *cat-86*. In our system we expected that the *neo* message would be produced constitutively but that transcription of the *cat-86* gene would be regulated by temperature. We therefore compared levels of mRNA initiating from the ϕ105 promoter, produced by cells grown at high and low temperatures. The amount of *neo*-specific mRNA produced at the two temperatures was the control.

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  1-50   GAATTCGGAT CACCTXTCTC CTTTACAACA CATAGTGCCT CACTGTGCCA
 51-100  CTGTGTCTTG TGGCATGACA CAATTATAGT ATCCGAATGT CGGAAATACA
101-150  ATACTAAAAA AGACGGAAAT ACAAGTATTT TTTAGTAAAT TGACGGAAAT
151-200  ACAAGATAAA TACTCTCTGA ATCTTTAAAA TGCTTGAATT TCGTCAAATT
201-250  TCGACTTTTA CAAAATGTCG TGAATACCAT ACAATTTAGA CATACCTTAA
251-300  CGGGAGGTGA TAATCATGCT GGATGGGAAA AAGCTTGGGG CTTTAATTAA
301-350  GGACAAAAGA AAAGAAAAGC ACTTGAAACA GACAGAAATG GCGAAGGCAC
351-400  TGGGTATGTC CAGAACTTAT CTCTCTGATA TCGAAAACGG CAGATATCTG
401-450  CCGAGTACAA AAACACTTTC CAGAATAGCG ATTTTAATAA ATCTGGATTT
451-500  AAATGTGTTA AAAATGACGG AAATACAAGT AGTTGAGGAG GGTGGATATG
501-550  ATAGAGCTGC CGGCACATGT AGAAGACAGG CTTTATGAGA TTTTTATGAA
551-600  ACTATCAGTT CCAAGGTTGC TTGAGAAAGA AGCCCTGGAG AAAGGAGAGA
601-650  AGCCGAATGC GGAAAGAAAA GGCGCTTGAC CTCGCGGCCT TCTTCGCTGA
651-676  ATTTGAACAA ATGAXGATCC GAATTC
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FIG. 7. DNA sequence of the ϕ105 early-promoter fragment. The promoter sequence is underlined.

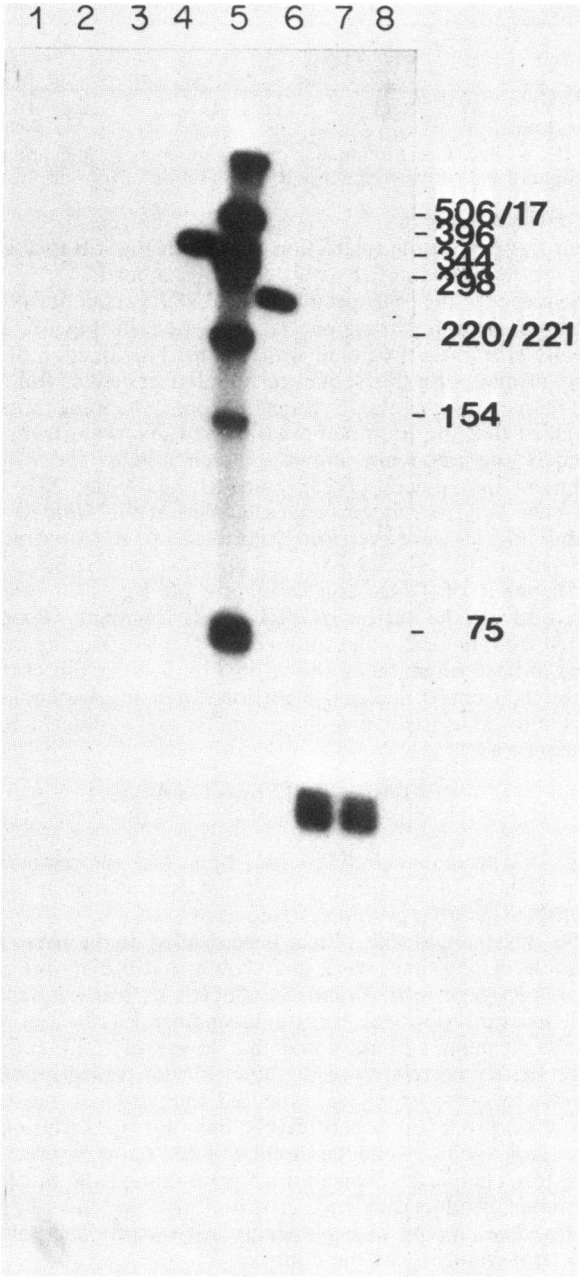

FIG. 8. S1 nuclease-protected fragments. Fragments consisting of 285 and 391 bases, derived from the 676-bp *Eco*RI promoter insert in plasmid p603-900 by digestion of the insert with *Hin*dIII, were labeled (see text), strand separated and hybridized to two different RNAs, digested with S1 nuclease, and electrophoresed as described previously (3). Lanes: 1, 391-base probe, 5 μg of RNA from BR151(pPL603), S1; 2, 391-base probe, 1 μg of RNA from strain BR151(p603-900, p1776) grown at 45°C, S1; 3, same as 2, with 5 μg of RNA; 4, 391-base probe (1,900 cpm), no RNA, no S1; 5, pBR322 *Hin*F markers; 6, 285-base probe (1,900 cpm) no RNA, no S1; 7, 285-base probe, 5 μg of RNA from strain BR151(p603-900, p1776) grown at 45°C, S1; 8, same as lane 7, with 1 μg of RNA.

Strain BR151(p603-900, p1776) (containing both the phage promoter-operator and the phage repressor sequences) was grown at either 45 or 30°C in LB broth containing erythromycin and neomycin. At a Klett reading of 100 (approximately 10^8 cells per ml) (green filter), chloramphenicol (10 μg/ml) was added, and cells were grown at their respective temperatures for an additional 1 h. At this time, the Klett reading of the 30°C culture had increased to 135, and that of the 45°C culture had increased to 154. Cells were harvested

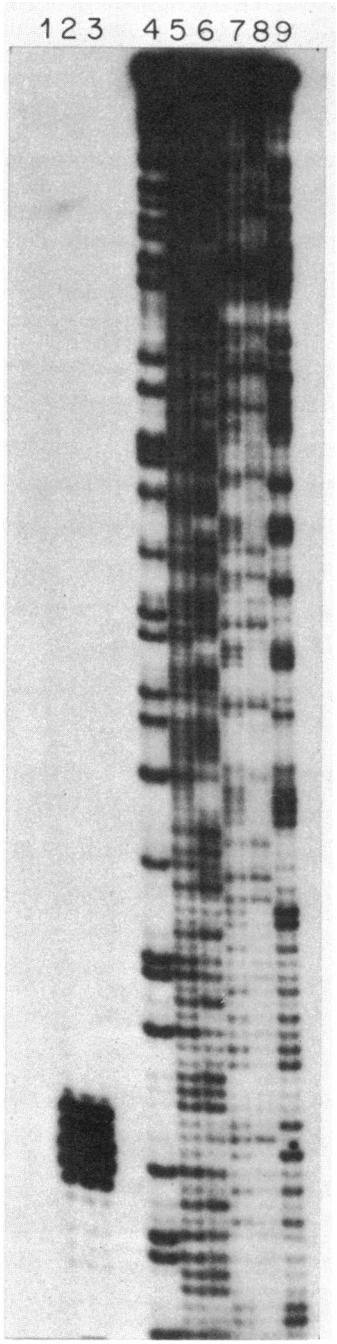

FIG. 9. S1 endonuclease mapping of the early φ105 promoter. The 285-base single-stranded probe from the *Eco*RI-*Hin*dIII DNA fragment containing the φ105 promoter was hybridized with RNA prepared from BR151(p603-900, p1776) grown at 45°C, treated with S1 nuclease, and electrophoresed next to the same single-stranded probe treated for Maxam-Gilbert sequencing. In lanes 1 through 3, the probe was hybridized to RNA and treated with S1 nuclease. Lanes 4 through 9 represent Maxam-Gilbert limited digests of probe at various bases as follows: 4, guanine; 5, adenine plus guanine; 6, adenine plus cytosine; 7, thymine plus cytosine; 8, cytosine; 9, thymine.

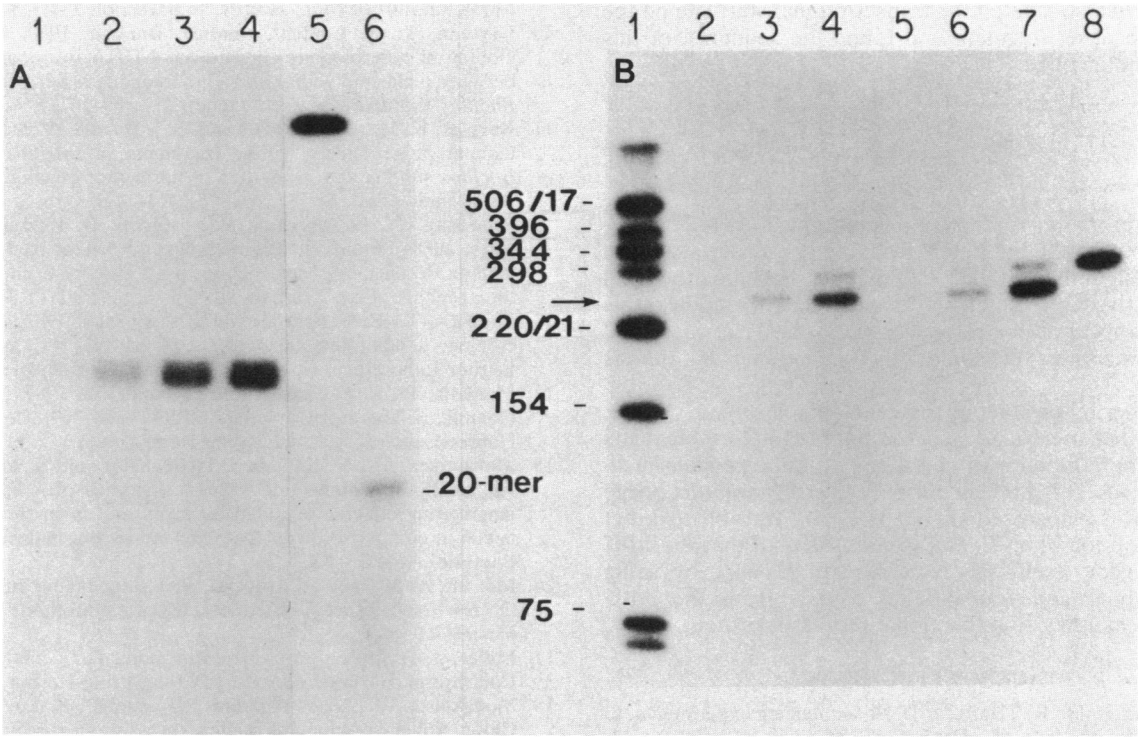

FIG. 10. S1 nuclease-protected fragments. (A) An excess of the 285-base single-stranded probe from the *Eco*RI-*Hin*dIII fragment containing the φ105 promoter was hybridized to RNA from strain BR151(p603-900, p1776) (grown at either 45 or 30°C), treated with S1 nuclease, and electrophoresed. Lanes: 1, 30°C cells, 5 μg of RNA; 2, 30°C cells, 20 μg of RNA; 3, 45°C cells, 1 μg of RNA; 4, 45°C cells, 2.5 μg of RNA; 5, untreated probe; 6, oligonucleotide marker (20-mer). (B) Same as panel A, except that the probe was the 293-base single-stranded probe from the *neo* gene in plasmid p603-900. Lanes: 1, size markers (pBR322 restricted with *Hin*F); 2, 30°C cells, 0.25 μg of RNA; 3, 30°C cells, 1 μg of RNA; 4, 30°C cells, 4 μg of RNA; 5, 45°C cells, 0.25 μg of RNA; 6, 45°C cells, 1 μg of RNA; 7, 45°C cells, 4 μg of RNA; 8, untreated probe. The arrow indicates the band protected from S1 nuclease digestion.

and RNA was extracted. RNA from the two cultures was hybridized to two different ^{32}P-labeled single-stranded DNA probes: the 285-base fragment containing the sequence complementary to the phage promoter sequence described above and the 293-base DNA fragment complementary to the 5′ end of the *neo* transcript (see above). DNA-RNA hybrids were then treated with S1 nuclease and run on acrylamide gels.

Transcription from the early phage promoter was very temperature dependent (Fig. 10A). In contrast, with RNA extracted from the same cultures, nearly equal amounts of the *neo* DNA probe were protected by *neo*-specific mRNA from cells grown at either temperature (Fig. 10B), indicating that transcription of the *neo* gene was not temperature dependent. These results show that the temperature-dependent expression of *cat-86* in strain BR151(p603-900, p1776) was effected at the transcriptional level; in the RNA preparations from cells grown at high temperature, the concentration of mRNA was at least 50 times that from cells grown at 30°C.

DISCUSSION

We described a thermoinducible-expression system which was used in *B. subtilis* to control transcription of a foreign gene. The system comprised two plasmids, one bearing the thermolabile prophage repressor from φ105 *c*ts23 and the other bearing the promoter-operator sequence on which the repressor acts. When the early promoter-operator sequence was inserted in front of the *cat-86* gene (from *B. pumilis*), expression of this gene was minimal at the nonpermissive temperature (30°C). Expression was induced at 45°C, the temperature at which lysogens of φ105 *c*ts23 undergo spontaneous induction (M. S. Osburne, Ph.D. thesis, Tufts University, Medford, Mass., 1977). We showed, by measuring levels of mRNA produced at both temperatures, that control of the system was at the transcriptional level. We expect that our system will prove useful to control the transcription of any particular foreign gene.

While these experiments were in progress, another interesting system which makes use of transcriptional controlling elements from φ105 was described by Dhaese et al. (8). This system is also composed of two plasmids, one encoding an early φ105 promoter-operator sequence, and the other, a derivative of plasmid pE194, encoding the wild-type prophage repressor gene. The thermoinducibility of this system depends on the natural temperature sensitivity of plasmid pE194. As several generations of growth at high temperature are required to dilute out the repressor-bearing plasmid, we believe that our system has the practical advantage that induction is rapid; approximately 50% of full expression was attained by 1 h after shifting the cells to growth at high temperature.

During the development of this system, we discovered several interesting properties of φ105. We mapped the early promoter-operator region next to the prophage repressor gene, in agreement with the results of Dhaese et al. (8). This positioning of an early promoter in close proximity to the prophage repressor gene is similar to that seen on the genetic map of bacteriophage lambda (12, 25). We do not yet know whether there is more than one early promoter on the φ105

genome. We also located the transcriptional start site on the promoter-bearing fragment and found the sequence of this early promoter to be

TTTACA——17 bp ——TACAAT ϕ105 early promoter

TTGACA —17 or 18 bp— TATAAT consensus promoter

−35 −10

The early promoter resembles a prototype *B. subtilis* vegetative promoter (20) but differs from the consensus sequence by 1 bp each in the −10 and −35 regions. Based on the level of CAT activity directed from the ϕ105 early promoter, this promoter appears to be relatively weak (Table 2) compared with the activities of some other functional *B. subtilis* promoters.

As another by-product of the development of our system, we found that the ϕ105 transcriptional regulatory elements described here functioned in a temperature-dependent fashion in *E. coli*. This implies not only that a *B. subtilis* phage promoter was recognized in vivo by *E. coli* and able to direct the transcription of an *E. coli* gene (*galK*) but that the ϕ105 repressor gene itself was expressed in *E. coli*, by using transcriptional and translational signals from the ϕ105 genome to synthesize a functional repressor protein.

ACKNOWLEDGMENTS

We are grateful to R. Tizard for DNA sequencing experiments, to P. Lovett for the gift of plasmids pPL603 and pPL608, to M. Rosenberg for the gift of plasmid pKO300, and to R. Cate for valuable advice concerning the S1 experiments and for the *neo* probe. We also thank P. Dhaese and C. Hussey for communication of results before publication.

LITERATURE CITED

1. **Armentrout, R. W., and L. Rutberg.** 1971. Heat induction of prophage ϕ105 in *Bacillus subtilis*: replication of the bacterial and bacteriophage genomes. J. Virol. **8**:455–468.
2. **Bernard, H.-M., E. Remaut, M. V. Hershfield, H. K. Das, D. R. Helinski, C. Yanofsky, and N. Franklin.** 1979. Construction of plasmid cloning vehicles that promote gene expression from the bacteriophage lambda p_L promoter. Gene **5**:59–76.
3. **Brosius, J., R. L. Cate, and A. P. Perlmutter.** 1982. Precise location of two promoters for the β-lactamase gene of pBR322; S1 mapping of ribonucleic acid isolated from *Escherichia coli* or synthesized *in vitro*. J. Biol. Chem. **257**:9205–9210.
4. **Bugaichuk, U. D., M. Deadman, J. Errington, and D. Savva.** 1984. Restriction enzyme analysis of *Bacillus subtilis* bacteriophage ϕ105 DNA. J. Gen. Microbiol. **130**:2165–2167.
5. **Chang, S., and S. N. Cohen.** 1979. High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. Mol. Gen. Genet. **168**:111–115.
6. **Cohen, S., A. Y. C. Chang, and L. Hsu.** 1972. Non-chromosomal antibiotic resistance in bacteria: transformation of *E. coli* by R-factor DNA. Proc. Natl. Acad. Sci. U.S.A. **69**:2110–2114.
7. **Cully, D. F., and A. J. Garro.** 1980. Expression of superinfection immunity to bacteriophage ϕ105 by *Bacillus subtilis* cells carrying a plasmid chimera of pUB110 and *Eco*RI fragment F of ϕ105 DNA. J. Virol. **34**:789–791.
8. **Dhaese, P., C. Hussey, and M. Van Montagu.** 1984. Thermoinducible gene expression in *Bacillus subtilis* using transcriptional regulatory elements from temperate phage ϕ105. Gene **32**:181–194.
9. **Gryczan, T. J., S. Contente, and D. Dubnau.** 1978. Characterization of *Staphylococcus aureus* plasmids introduced by transformation into *Bacillus subtilis*. J. Bacteriol. **134**:318–329.
10. **Gryczan, T., S. Contente, and D. Dubnau.** 1980. Molecular cloning of heterologous chromosomal DNA by recombination between a plasmid vector and a homologous resident plasmid in *Bacillus subtilis*. Mol. Gen. Genet. **177**:459–467.
11. **Keggins, K. M., P. S. Lovett, and E. J. Duvall.** 1978. Molecular cloning of genetically active fragments of *Bacillus* DNA in *Bacillus subtilis* and properties of the vector plasmid pUB110. Proc. Natl. Acad. Sci. U.S.A. **75**:1423–1427.
12. **Kourilsky, P., L. Marcaud, P. Sheldrick, D. Luzzati, and F. Gros.** 1968. Studies on the messenger RNA of bacteriophage lambda. I. Various species synthesized early after induction of the prophage. Proc. Natl. Acad. Sci. U.S.A. **61**:1013–1017.
13. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 90–91, 93–94. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 196, 368–369. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Matsumura, M., Y. Katakura, T. Imanaka, and S. Aiba.** 1984. Enzymatic and nucleotide sequence studies of a kanamycin-inactivating enzyme encoded by a plasmid from thermophilic bacilli in comparison with that encoded by plasmid pUB110. J. Bacteriol. **160**:413–420.
16. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labelled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
17. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 54. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Mongkolsuk, S., N. P. Ambulos, Jr., and P. S. Lovett.** 1984. Chloramphenicol-inducible gene expression in *Bacillus subtilis* is independent of the chloramphenicol acetyltransferase structural gene and its promoter. J. Bacteriol. **160**:1–8.
19. **Mongkolsuk, S., Y.-W. Chiang, R. B. Reynolds, and P. S. Lovett.** 1983. Restriction fragments that exert promoter activity during post-exponential growth of *Bacillus subtilis*. J. Bacteriol. **155**:1399–1406.
20. **Moran, C. P., N. Lang, S. F. J. LeGrice, G. Lee, M. Stephens, A. L. Sonenshein, J. Pero, and R. Losick.** 1982. Nucleotide sequences that signal the initiation of transcription and translation in *Bacillus subtilis*. Mol. Gen. Genet. **186**:339–346.
21. **Osburne, M. S., and A. L. Sonenshein.** 1976. Behavior of a temperate bacteriophage in differentiating cells of *Bacillus subtilis*. J. Virol. **19**:26–35.
22. **Rosenberg, M., A. B. Chepelinsky, and K. McKenney.** 1983. Studying promoters and terminators by gene fusion. Science **222**:734–739.
23. **Shapiro, J. A., D. H. Dean, and H. O. Halverson.** 1974. Low frequency specialized transduction with *Bacillus subtilis* bacteriophage ϕ105. Virology **62**:393–403.
24. **Sonenshein, A. L., B. Cami, J. Brevet, and R. Cote.** 1974. Isolation and characterization of rifampin-resistant and streptolydigin-resistant mutants of *Bacillus subtilis* with altered sporulation properties. J. Bacteriol. **120**:253–265.
25. **Taylor, K., Z. Hradecna, and W. Szybalski.** 1967. Asymmetric distribution of the transcribing regions on the complementary strands of coliphage lambda DNA. Proc. Natl. Acad. Sci. U.S.A. **57**:1618–1622.
26. **Weisblum, B., M. Y. Graham, T. Gryczan, and D. Dubnau.** 1979. Plasmid copy number control: isolation and characterization of high copy-number mutants of plasmid pE194. J. Bacteriol. **137**:635–643.
27. **Williams, D. M., E. J. Duvall, and P. S. Lovett.** 1981. Cloning restriction fragments that promote expression of a gene in *Bacillus subtilis*. J. Bacteriol. **146**:1162–1165.
28. **Yansura, D. G., and D. J. Henner.** 1984. Use of the *Escherichia coli lac* repressor and operator to control gene expression in *Bacillus subtilis*. Proc. Natl. Acad. Sci. U.S.A. **81**:439–443.