JOURNAL OF BACTERIOLOGY, Nov. 1982, p. 780–785
0021-9193/82/110780-06$02.00/0

Vol. 152, No. 2

Chromosomal Location of a *Bacillus subtilis* DNA Fragment Uniquely Transcribed by Sigma-28-Containing RNA Polymerase

FRANCO A. FERRARI, EUGENIO FERRARI,† AND JAMES A. HOCH*

Department of Cellular Biology, Research Institute of Scripps Clinic, La Jolla, California 92037

Received 3 May 1982/Accepted 7 July 1982

A fragment of the *Bacillus subtilis* chromosome containing a gene whose transcription is dependent on sigma-28-containing RNA polymerase has been genetically mapped by means of an integrable plasmid. This gene resides on the chromosome adjacent to the stage 0 sporulation locus *spo0E* between *metC* and *citL*. The gene was insertionally inactivated by cloning an internal *Eco*RI-*Hin*dIII fragment in the integrable plasmid pJH101 and by inserting the plasmid into the chromosome by transformation. Transformants bearing an inactivated gene were indifferent to this inactivation for both growth and sporulation.

The first step in the transcription of bacterial genes is the recognition of DNA promoter sites by RNA polymerase. The specificity of transcription may be controlled by the sigma subunit of RNA polymerase, which is responsible for the sequence specificity of promoter-RNA polymerase interactions (4, 17). *Bacillus subtilis* bacteriophage SPO1 directs the temporal order of gene transcription during infection by changing the sigma-like factors that associate with RNA polymerase during infection (8, 10, 23). A variety of sigma-like subunits is found in RNA polymerase purified from uninfected *B. subtilis*. One of these, a 37,000-dalton polypeptide, has been shown to confer novel promoter recognition specificities upon RNA polymerase (14). In addition, this form of RNA polymerase transcribes a gene known to be under sporulation control (13, 21). The other forms of RNA polymerase that have been identified may have unique roles in controlling aspects of the sporulation process.

Recently, a previously unidentified sigma-like subunit of 28,000 daltons was purified and characterized (24). RNA polymerase containing this polypeptide has very stringent promoter requirements, and the promoters differ in sequence from those transcribed by other forms of the enzyme (11, 24). A *B. subtilis* chromosomal gene uniquely transcribed by RNA polymerase containing the 28,000-dalton sigma protein has been cloned and characterized (11). In the studies reported below, this gene was mapped on the *B. subtilis* chromosome, and the characteristics of strains having mutations in this gene were determined.

† Present address: Istituto di Genetica, University of Pavia, Pavia, Italy.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains used in this study are described in Table 1. *Escherichia coli* strains were made competent and were transformed by the method of Cohen et al. (6) or of Dagert and Ehrlich (7). *B. subtilis* strains were cultured on tryptose blood agar base (Difco Laboratories) or minimal glucose medium and were brought to competence and transformed by the method of Anagnostopoulos and Spizizen (1).

Construction of the *Pst*I library in plasmid pHV14. High-molecular-weight chromosomal DNA was prepared from *B. subtilis* 168 and was digested to completion with the restriction enzyme *Pst*I. After purification, approximately 28 μg of digested DNA was mixed with 7 μg of *Pst*I-digested plasmid pHV14 (9) in ligation buffer (66 mM Tris-hydrochloride [pH 7.5], 6.6 mM $MgCl_2$, 10 mM dithiothreitol, 4.0 mM ATP) to a final volume of 100 μl. After the addition of T4 DNA ligase, the mixtures were incubated for 14 h at 12°C. The ligated DNA was used to transform *E. coli* SK1542 with selection for resistance to chloramphenicol (Cm^r; 5 μg of chloramphenicol per ml), and the clones were subsequently replica plated on ampicillin (150 μg/ml).

All the ampicillin-sensitive (Amp^s) colonies were hybridized by the method of Hanahan and Meselson (15) with nick-translated (19) chromosomal DNA. From this initial screening, 1,260 colonies were isolated which contained *B. subtilis* DNA. These colonies were pooled in groups of 20 and stored at −70°C.

Construction of plasmid pJF501. Plasmid pCD4322 (Fig. 1; 24) was digested to completion with *Eco*RI and *Hin*dIII. The digested plasmid DNA was purified from agarose gel by the procedure of McDonell et al. (18). The purified *Eco*RI-*Hin*dIII fragment of 0.6 kilobases

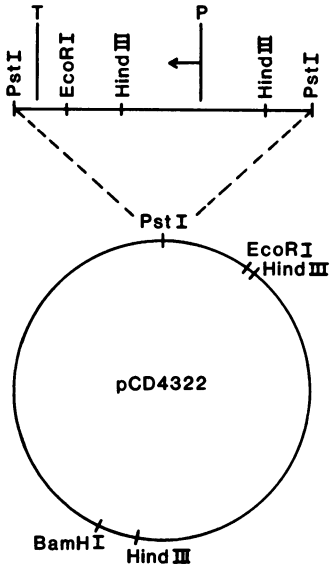

FIG. 1. Restriction endonuclease map of plasmid pCD4322. P shows the location of the sigma-28-dependent promoter and the direction of transcription from it. T locates the termination site of the transcript from the promoter.

(kb) was cloned in plasmid JH101 (Fig. 2; Nguyen et al., manuscript in preparation) and was scored by insertional inactivation of tetracycline resistance. Transformants were screened by the method of Birnboim and Doly (3). Plasmid DNA was prepared from these clones by the same procedure with few modifications. The sizes of the insertions were estimated by electrophoresis on horizontal 1% agarose gels.

Isolation and analysis of chromosomal DNA. A number of transformants of *B. subtilis* 168 harboring plasmid pJF501 were grown to the late logarithmic stage of growth in 50 ml of tryptose blood agar base supplemented with chloramphenicol (5 μg/ml). Cells were harvested by centrifugation, washed twice with minimal media, and suspended in 4 ml of 50 mM Tris-hydrochloride (pH 7.5)–5 mM EDTA. Next, 1 mg of lysozyme per ml and 20 μg of RNase per ml were added, and the suspension was placed at 37°C for 30 min. Complete cellular lysis was accomplished by the addition of sodium dodecyl sulfate to a final concentration of 1%. After further incubation at the same temperature for 30 min, an equal volume of phenol (water saturated) was added, and the mixture was shaken for 10 min. The phenol and water layers were separated by centrifugation, and the aqueous layer was subjected to another phenol extraction. An equal volume of $CHCl_3$-isoamyl alcohol (24:1) was added to the upper phase, and the mixture was shaken for 15 min. After separation by centrifugation, two volumes of cold ethanol were added to the aqueous layer to precipitate the DNA. The precipitated DNA was collected by centrifugation at 10,000 × *g*. The precipitate was dissolved in DNA buffer (10 mM Tris [pH 7.2], 5 mM NaCl, 0.2 mM EDTA).

The digested DNAs were electrophoresed in horizontal gels of 0.7% agarose stained with ethidium bromide and were photographed. The DNAs were then transferred to nitrocellulose paper (22) and were hybridized with nick-translated plasmid pJF501 (15).

TABLE 1. Strains used in this study

Strain	Genotype
B. subtilis JH163	*trpC2 metC3 spo0EII*
B. subtilis RB1144	*pyrD ilvAI thyA thyB* Δ(PBSX-*pro-metC*)
B. subtilis JH422	*trpC2 citL22*
B. subtilis JH342	*trpC2 argE342*
E. coli SK1592	F^- *hsdR4 hsdM*$^+$

RESULTS

Construction of plasmid pCD4322. A plasmid library of the *B. subtilis* chromosome was prepared by digesting purified *B. subtilis* chromosomal DNA with restriction endonuclease *Pst*I. The *Pst*I fragments were ligated to *Pst*I-digested plasmid pHV14. Plasmid pHV14 is a chimera between the *E. coli* plasmid pBR322 and the *Staphylococcus aureus* plasmid pC194 (9) and codes for both ampicillin resistance (Amp^r) and Cm^r. The ligation mixture was used to transform *E. coli* strain SK1542, and Cm^r transformants were selected. Presumptive insert-containing plasmids were identified by screening the Cm^r transformants for Amp^s. Plasmids were extracted from Cm^r Amp^s colonies and were subjected to further analysis.

The plasmid library was screened by in vitro transcription with purified RNA polymerase containing the sigma-28 sigma subunit for inserts

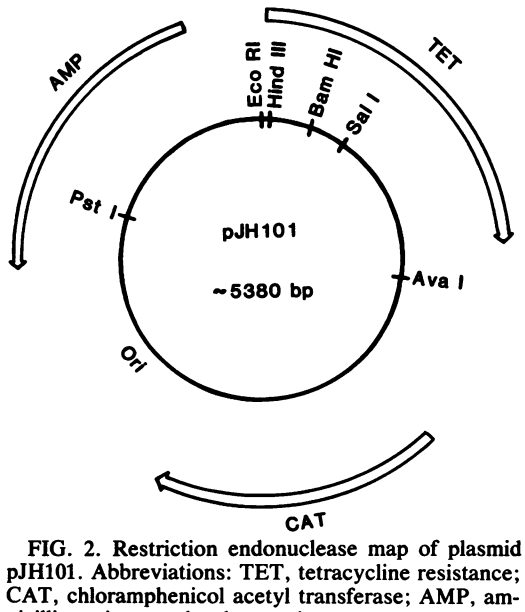

FIG. 2. Restriction endonuclease map of plasmid pJH101. Abbreviations: TET, tetracycline resistance; CAT, chloramphenicol acetyl transferase; AMP, ampicillin resistance. bp, base pairs.

that would promote transcription with the sigma-28-containing RNA polymerase (24). One plasmid, pCD4322, contained a 3.2-kb DNA insert which was actively transcribed by this RNA polymerase. Transcription of insert DNA from plasmid pCD4322 after cleavage with *Pst*I gave two overlapping transcripts from the same promoter which resulted from a partially effective terminator. The transcription and restriction map of this insert determined by Wiggs et al. (24) is shown in Fig. 1.

Genetic location of the chromosomal fragment cloned in plasmid pCD4322. Since genes transcribed by unique species of RNA polymerase were found first to be under sporulation control, it was of particular interest to find the chromosomal location of the DNA insert of plasmid pCD4322 and to determine if it was associated with genes for sporulation. To determine the chromosomal location of the DNA fragment cloned in plasmid pCD4322, the Cm^r phenotype of the integrated plasmid was used as a genetic marker in phage PBS1 transduction (12). Although pCD4322 and other plasmids derived from pHV14 can replicate in *B. subtilis*, they do not partition correctly and are lost at a high frequency in the absence of chloramphenicol selection. However, the DNA fragment contained in plasmid pCD4322 is homologous to the chromosome, and this allows such plasmids to integrate into the chromosome by a Campbell-type recombination, giving rise to stable Cm^r strains (12). Plasmid pCD4322 was used as the donor to transform strain 168 for Cm^r. A stable Cm^r transformant (168::pCD4322) was single colony isolated three times, and a phage PBS1 transducing lysate was prepared on it. This lysate was used to transduce a variety of strains carrying genetic markers from all areas of the chromosome. Transductants were patched on chloramphenicol-containing plates to test for cotransfer of the integrated plasmid. Such Cm^r recombinants were found with *metC3* and *ura-1* recipients. The data suggest that the plasmid was integrated between these two markers since these markers are only rarely cotransduced.

A more precise localization of the integrated plasmid pCD4322 was determined by transformation analysis. Transformation of an *spo0E* recipient with 168::pCD4322 DNA gave tight linkage of the incoming Cm^r phenotype with *spo*$^+$. To find the position of plasmid pCD4322 with respect to its linked markers, 168::pCD4322 DNA was used to transform strain JH163 (*trpC2 metC3 spo0E11*) in three-factor analyses (Table 2). Since the Cm^r phenotype is more closely linked to *spo* than to *met* (Table 2), we considered two likely orientations: *metC3*–pCD4322–*spo0E11* and *metC3*–*spo0E11*–pCD4322. If plasmid pCD4322 had integrated between *metC3* and *spo0E11*, 10.6 kb of integrated plasmid would have been between these markers, and the linkage between them should have decreased markedly. Instead, there was no reduction of linkage between *metC3* and *spo0E11* when the 168::pCD4322 donor was compared with wild-type DNA. This result suggests the order *metC3*–*spo0E11*–pCD4322. The three-factor analysis with *met*$^+$ selection was uninformative because of the weak linkage of both *spo0E11* and pCD4322 to *metC3*. However, selection in the same cross for Cm^r was informative. Since in this case we required the entire plasmid to integrate as part of the transforming DNA, the absolute number of Cm^r transformants was about 10-fold less than the *met*$^+$ transformants. Among the Cm^r transformants, the cotransfer class increased to 23% of the total transformants

TABLE 2. Transformation crosses to locate integrated plasmid pCD4322 in the chromosome[a]

Donor	Recombinants				Cotransfer		
	Class[b]			No.	Type	No.[c]	%
	met	Cm^r	*spo*				
168::pCD4322	1	1	1	9			
	1	1	0	10	*spo*$^+$/*met*$^+$	25/160	16
	1	0	0	125			
	1	0	1	16	Cm^r/*met*$^+$	19/160	12
168::pCD4322	1	1	1	38			
	0	1	1	63	*met*$^+$/Cm^r	51/168	30
	1	1	0	13			
	0	1	0	54	*spo*$^+$/Cm^r	101/168	60
Wild-type	1		1	22	*spo*$^+$/*met*$^+$	22/171	13
	1		0	149			

[a] Recipient: JH163 (*trpC2 metC3 spo0EII*).
[b] Donor and recipient phenotypes are indicated by 1 and 0, respectively.
[c] Number cotransferred/number tested.

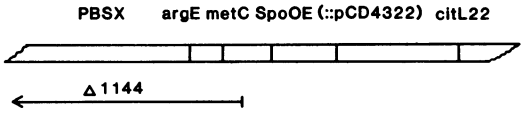

FIG. 3. Genetic map of the region of the *B. subtilis* chromosome surrounding integrated plasmid pCD4322.

compared with 6% for the *met*$^+$ selection. This phenomenon has been observed in similar crosses with other integrated plasmids (Hoch, data not shown). The least frequent in this cross was *met*$^+$, Cm^r, *spo*$^-$, which is the expected quadruple crossover class if the order of markers is *metC3–spo0E11*–pCD4322. The suggested order of markers in this region is shown in Fig. 3. The large deletion, Δ1144, in strain RB1144 that is known to delete the *metC* gene and some of the PBSX prophage (5) also transformed *spoE* strains to *spo*$^+$ at high frequency, indicating that the deletion did not cover the *spo0E* alleles.

Construction of a plasmid to inactivate the transcription unit in plasmid pCD4322. One way to investigate the function of the sigma-28 transcription unit contained within the plasmid pCD4322 insert would be to inactivate this unit by mutation and determine the effect of the mutation on the phenotype of the cell. There are several ways this could be accomplished; we chose to do it by inserting a plasmid within the unit. This was carried out by cloning the middle fragment of the transcription unit, the *Eco*RI-to-*Hin*dIII fragment (Fig. 1), into the vector plasmid pJH101 (Fig. 2) by replacement of the *Eco*RI-to-*Hin*dIII fragment of the vector. Ligation mixtures were transformed into *E. coli* with selection for Amp^r. Tetracycline-sensitive recombinants were isolated and further characterized.

Plasmid pJH101 cannot transform *B. subtilis* for any antibiotic resistance since it lacks a replication origin for this organism (Nguyen et al., manuscript in preparation). If one inserts a region of homology to the *B. subtilis* chromosome in plasmid pJH101, the recombinant plasmid transforms *B. subtilis* strains to Cm^r because the plasmid integrates at its region of homology by a Campbell-type recombination. A recombinant plasmid, pJF501, carrying the middle fragment from the transcription unit, transformed *B. subtilis* 168 to Cm^r at high frequency. Since plasmid pJF501 carries the middle fragment of the transcription unit, upon transformation into the 168 strain the tandem duplication that forms as a result of the recombination event will have both copies of the transcription unit inactivated by the entire plasmid molecule. Several of the Cm^r transformants were characterized to determine the effect of this inactivation.

Figure 4 shows the recombination event generating Cm^r transformants and the expected restriction endonuclease map of this region in the transformants. To verify that the integration event occurred as expected, the structure of the region in the transformants was analyzed by hybridization. Chromosomal DNA from two transformants was double digested with endonucleases *Pst*I and *Hin*cII. The fragments were separated by electrophoresis, transferred to nitrocellulose, and hybridized to plasmid pJF501 labeled by nick translation. The autoradiograph of this experiment is shown in Fig. 5. Chromosomal DNA from strain 168 gave a single labeled band corresponding to 1.4 kb. DNA from strains carrying an integrated plasmid pJF501 lost the

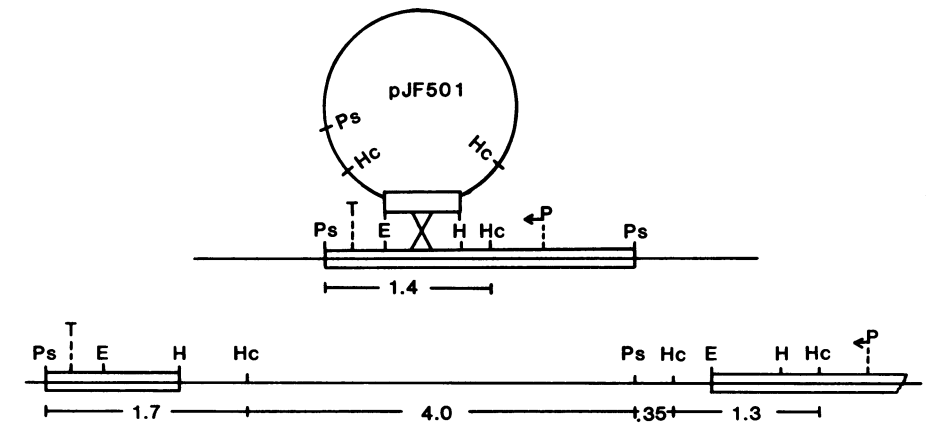

FIG. 4. Scheme for integration of plasmid pJF501 in the *B. subtilis* chromosome and predicted size of the restriction endonuclease fragments generated by this event. Abbreviations: Ps, *Pst*I; E, *Eco*RI; H, *Hin*dIII; Hc, *Hin*cII; P and T, as defined in legend to Fig. 1. Numbers show sizes of fragments in kilobase pairs.

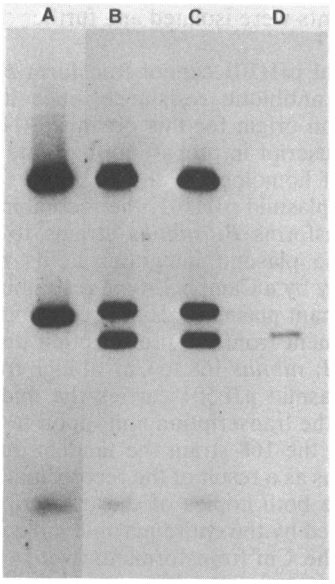

FIG. 5. Electrophoretically separated *Pst*I-*Hin*cII fragments of transformant DNA blotted to nitrocellulose paper and hybridized to labeled plasmid pJF501. (A) Plasmid pJF501 DNA; labeled fragments from top to bottom are 4.0, 1.6, and 0.4 kb. (B, C) DNA from two different transformants; labeled fragments from top to bottom are 4.0, 1.7, 1.3, and 0.4 kb. (D) Chromosomal DNA from strain 168; labeled fragment is 1.45 kb.

1.4-kb fragment, and two new fragments of 1.3 and 1.7 kb appeared in addition to plasmid fragments of 4.0 and 0.35 kb. The sizes of the fragments corresponded to those predicted (Fig. 4).

All of the Cm^r transformants were capable of normal sporulation in rich media, and the yield of spores and rate of sporulation did not differ qualitatively from those of the 168 parental strain. No obvious differences in colony morphology, size, or shape were noted. In addition, none of the transformants gained any auxotrophic requirements.

DISCUSSION

The role of multiple sigma factors in directing transcription from distinct classes of promoters is now firmly established (12). The significance of such sigma factors in the control of cellular development is still in question. At least one of the sigma factors, sigma-37, is responsible for transcription of a gene, the 0.4-kb gene, activated early in sporulation (13). In addition, the transcription of the 0.4-kb gene is under control of the stage 0 sporulation genes (21).

The discovery of a sigma-28-containing RNA polymerase by Wiggs et al. (24) led us to examine whether genes uniquely transcribed by this enzyme were associated with sporulation. The sigma-28-dependent genes cloned by these investigators were analyzed to determine whether they are derived from regions of the chromosome known to contain sporulation genes. Genetic mapping experiments with the integrated plasmid pCD4322 showed that the chromosomal fragment cloned in this plasmid is derived from a region very close to the stage 0 sporulation locus *spo0E*. The order of markers in this region was found to be *metC–spo0E–*::pCD4322–*citL*. The genetic distance between *spo0E* and ::pCD4322 is difficult to determine from experiments of this kind, but it appears that *spo0E* and the sigma-28-dependent gene of plasmid pCD4322 are within a few kilobases of each other on the chromosome. The juxtaposition of these two loci might indicate some common factor in control, although there is no further evidence to support this notion. Another plasmid, pCD4136, isolated by Wiggs et al. (24) and containing a sigma-28-specific gene, was not found to map in the region next to *spo0E* and has not yet been located.

To determine whether the gene in plasmid pCD4322 is required for sporulation, we sought to induce a mutation in it and determine the effect on the cell. Since the ends of the transcript from the gene had been determined by in vitro studies and a convenient middle fragment could be isolated by restriction endonuclease digestion, it was decided to inactivate the gene by inserting a plasmid within it. The internal *Eco*RI-*Hin*dIII fragment of the gene was cloned in plasmid pJH101, which cannot replicate in *B. subtilis* but can insert by a Campbell-like recombination in this organism if a region of homology to the chromosome exists in the plasmid. Transformants analyzed by hybridization were found to have the plasmid integrated as predicted. However, none of the transformants acquired a sporulation or growth defect as a result of this inactivation. In addition, spores from these transformants were as heat- and solvent-resistant as spores from wild-type strains. Such a result does not necessarily indicate that the gene has nothing to do with sporulation. In fact, similar results were obtained when the 0.4-kb gene was inactivated. In this case, however, the frequency of sporulation was decreased, but the spores that formed were normal (20). Mutations in genes for the major structural components of spores, the spore coats, do not lead to a classical sporulation-defective phenotype. Such mutations allow the cell to sporulate normally, but the resultant spores are lysozyme sensitive and are slower to germinate (2, 16). This phenotype or any other subtle change in the spores from the inactivated strains would not be detected without more extensive analysis.

There is one complication in this analysis that

deserves comment. It is unknown whether the transcript that defines the sigma-28-dependent gene on plasmid pCD4322 codes for single or multiple protein products. If the transcript codes for more than one protein product, the strategy used to inactivate by insertion might not have inactivated any of the products.

ACKNOWLEDGMENTS

This research was supported in part by Public Health Service grants GM 19416 and GM 25891 from the National Institute of General Medical Sciences.

Plasmid pCD4322 was kindly provided by Michael Chamberlin and Michael Gilman.

LITERATURE CITED

1. **Anagnostopoulos, C., and J. Spizizen.** 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. **81**:741–746.
2. **Aronson, A. I., and P. C. Fitz-James.** 1975. Properties of *Bacillus cereus* spore coat mutants. J. Bacteriol. **123**:354–365.
3. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
4. **Burgess, R. R., A. A. Travers, J. J. Dunn, and E. K. F. Bautz.** 1969. Factor stimulating transcription by RNA polymerase. Nature (London) **221**:43–46.
5. **Buxton, R. S.** 1980. Selection of *Bacillus subtilis* 168 mutants with deletion of PBSX prophage. J. Gen. Virol. **46**:427–437.
6. **Cohen, S. N., A. Chang, and L. Hsu.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *E. coli* by R-factor DNA. Proc. Natl. Acad. Sci. U.S.A. **69**:2110–2114.
7. **Dagert, M., and S. D. Ehrlich.** 1979. Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. Gene **6**:23–28.
8. **Duffy, J., and E. P. Geiduschek.** 1977. Purification of a positive regulatory subunit from phage SPO1-modified RNA polymerase. Nature (London) **270**:28–32.
9. **Ehrlich, S. D.** 1978. DNA cloning in *Bacillus subtilis*. Proc. Natl. Acad. Sci. U.S.A. **75**:1433–1436.
10. **Fox, T. D., R. Losick, and J. Pero.** 1976. Regulatory gene *28* of bacteriophage SPO1 codes for a phage-induced subunit of RNA polymerase. J. Mol. Biol. **101**:427–433.
11. **Gilman, M. Z., J. L. Wiggs, and M. J. Chamberlin.** 1981. Nucleotide sequences of two *Bacillus subtilis* promoters used by *Bacillus subtilis* sigma-28 RNA polymerase. Nucleic Acids Res. **9**:5991–6000.
12. **Haldenwang, W. G., C. D. B. Banner, J. F. Ollington, R. Losick, J. A. Hoch, M. B. O'Conner, and A. L. Sonenshein.** 1980. Mapping a cloned gene under sporulation control by insertion of a drug resistance marker into the *Bacillus subtilis* chromosome. J. Bacteriol. **142**:90–98.
13. **Haldenwang, W. G., and R. Losick.** 1979. A modified RNA polymerase transcribes a gene under sporulation control in *Bacillus subtilis*. Nature (London) **282**:256–260.
14. **Haldenwang, W. G., and R. Losick.** 1980. Novel RNA polymerase factor from *Bacillus subtilis*. Proc. Natl. Acad. Sci. U.S.A. **77**:7000–7004.
15. **Hanahan, D., and M. Meselson.** 1980. Plasmid screening at high colony density. Gene **10**:63–67.
16. **Jenkinson, H. F.** 1981. Germination and resistance defects in spores of a *Bacillus subtilis* mutant lacking a coat polypeptide. J. Gen. Microbiol. **127**:81–91.
17. **Losick, R., and J. Pero.** 1981. Cascades of sigma factors. Cell **15**:582–584.
18. **McDonnell, M. W., M. N. Simon, and F. W. Studier.** 1977. Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. J. Mol. Biol. **110**:119–146.
19. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick-translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
20. **Rosenbluh, A., C. D. B. Banner, R. Losick, and P. C. Fitz-James.** 1981. Identification of a new developmental locus in *Bacillus subtilis* by construction of a deletion mutation in a cloned gene under sporulation control. J. Bacteriol. **148**:341–351.
21. **Segall, J., and R. Losick.** 1977. Cloned *Bacillus subtilis* DNA containing a gene that is activated early during sporulation. Cell **11**:751–761.
22. **Wahl, G. W., M. Stern, and G. R. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. U.S.A. **76**:3683–3687.
23. **Whiteley, H. R., G. B. Spiegleman, J. M. Lawrie, and W. Hiatt.** 1976. The *in vitro* transcriptional specificities of RNA polymerase isolated from SP82 infected *Bacillus subtilis*, p. 587–600. *In* R. Losick and M. Chamberlin (ed.), RNA polymerase. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Wiggs, J. L., M. Z. Gilman, and M. J. Chamberlin.** 1981. Heterogeneity of RNA polymerase in *B. subtilis*. Evidence for an additional sigma factor in vegetative cells. Proc. Natl. Acad. Sci. U.S.A. **78**:2762–2766.