JOURNAL OF BACTERIOLOGY, Mar. 1984, p. 965–967
0021-9193/84/030965-03$02.00/0

Vol. 157, No. 3

Promoter-Probe Plasmid for *Bacillus subtilis*

CAROLINE E. DONNELLY AND ABRAHAM L. SONENSHEIN*

Department of Molecular Biology and Microbiology, Tufts University Schools of Medicine, Dental Medicine and Veterinary Medicine, and Sackler School of Graduate Biomedical Sciences, Boston, Massachusetts 02111

Received 9 November 1983/Accepted 16 December 1983

We have constructed a promoter-probe expression vector for *Bacillus subtilis*. This plasmid, pCED6, can be used to fuse various DNA sequences to the structural gene of *Escherichia coli* β-galactosidase, permitting analysis of the promoter activity of such sequences. pCED6 replicates and confers drug resistances in both *E. coli* and *B. subtilis*.

Fusion of the regulatory region of a gene of interest to the structural gene for an easily assayed protein has proved to be of great help in understanding the regulation of gene expression (1). We have recently described the construction of such a fusion in which the promoter region of a *Bacillus subtilis* gene was attached to the structural gene for *Escherichia coli* β-galactosidase (4). This promoter region, from the *tms* gene, was shown to direct β-galactosidase expression in both *E. coli* and *B. subtilis*. The plasmid in question, pCED3, has two replicons, pBR322, which allows replication in *E. coli*, and pUB110, which determines replication in *B. subtilis*. The physical map of pCED3 is shown in Fig. 1. The promoter-containing fragment (280-base-pair [bp] *Eco*RI-*Hin*dIII fragment) in this construction provides a transcription initiation site but need not provide any signals for initiation of translation. As shown below, these signals are encoded by *E. coli* DNA from the *trpA* gene and are located just upstream from the β-galactosidase (*lacZ*) sequence.

We have now constructed a promoterless version of this fusion plasmid that allows studies of the activity of known promoter sites and can be used to identify additional promoters. The construction (Fig. 1) required replacing the 1,030-bp *Pst*I-*Hin*dIII fragment of pCED3 with the corresponding restriction fragment (779 bp) from pBR322. It was important to use pCED3 as starting material since it had undergone a spontaneous deletion mutation that removed a sequence from the *lacY-lacA* region of its parent plasmid that caused instability in *B. subtilis* (4). Plasmids pCED3 and pBR322, prepared as described previously (4), were digested with *Pst*I and *Hin*dIII (New England Biolabs) at 37°C in a buffer containing 10 mM Tris-hydrochloride (pH 8), 50 mM NaCl, and 10 mM $MgCl_2$. Complete digestion was verified by electrophoresis in 1% agarose gels. The two treated DNAs were mixed and precipitated by the addition of sodium acetate to 0.4 M and ethanol to 70%. The precipitate was collected by centrifugation, dried, and redissolved in 66 mM Tris-hydrochloride (pH 7.6)–6.6 mM $MgCl_2$–66 μM ATP. After the addition of T4 DNA ligase (Bethesda Research Laboratories), the DNA was incubated at 13°C for 16 h and then used for transformation of competent cells of *E. coli* RV (Δ*lac*) as decribed previously by Cohen et al. (2). Transformed cells were plated on L agar containing 40 μg of 5-bromo-4-chloro-3-indolyl-β-galactoside (X-Gal) per ml, 10 μg of ampicillin per ml, and 5 μg of kanamycin per ml. On this medium, cells containing pCED3 give blue colonies but cells containing pBR322 fail to grow. Among the transformants were some that gave pale-blue colonies. These had the phenotype expected for a plasmid carrying the *lacZ* gene without a reasonably strong promoter. Several pale-blue transformants were purified, and plasmid DNA was extracted from them. They proved to have the *Pst*I-*Hin*dIII fragment of pBR322 inserted into pCED3 in place of the resident fragment. The prototype for these plasmids was designated pCED6. Its physical map is shown in Fig. 1.

B. subtilis BR151 was transformed with pCED6 DNA by the method of Contente and Dubnau (3). Transformants gave white colonies on L agar containing 5 μg of kanamycin per ml and 40 μg of X-Gal per ml. Transformants produced by pCED3 gave blue colonies on the same medium.

Assays of β-galactosidase activity in permeable cells revealed that cells containing pCED6 produced barely detectable levels of the enzyme whether *E. coli* or *B. subtilis* was the host cell (Table 1). These results allowed us to test the notion that insertion of a promoter-containing fragment into pCED6 at a site upstream from *lacZ* would turn on β-galactosidase expression.

First, a *Pst*I-*Hin*dIII fragment containing the *tms* promoter (derived from pLS5ΔRI [4]) was inserted into pCED6. This reconstruction of pCED3 yielded blue colonies as expected. In a second experiment, a 1.9-kilobase *Hin*dIII fragment that contains the promoter site of the *gltA* gene was inserted into the unique *Hin*dIII site of pCED6. Dark-blue colonies were obtained as transformants of *E. coli* RV. The plasmid derived from these transformants, called pRE1, also gave dark-blue colonies when transformed into *B. subtilis* BR151 (R. Emond, C. E. Donnelly, and A. L. Sonenshein, manuscript in preparation). The β-galactosidase activity of permeable cells carrying pRE1 is shown in Table 1.

In addition, a 420-bp *Eco*RI-*Hin*dIII fragment that contains the promoter site for the sporulation gene *spoVG* (7) was inserted into the *Hin*dIII site of pCED6. Transcription of this gene begins about 300-bp upstream from and proceeds toward the *Eco*RI site. The 420-bp fragment, which had been cloned in pBR322 (M. Fine and A. L. Sonenshein, manuscript in preparation), was excised, and its overlapping 5′ ends were filled in by reaction with the Klenow fragment of DNA polymerase (New England Biolabs) in the presence of the deoxynucleoside triphosphates. *Hin*dIII linkers (Bethesda Research Laboratories) were ligated to the blunt-ended fragment, and, after treatment with *Hin*dIII, this DNA was ligated to *Hin*dIII-cleaved pCED6. The β-galactosidase activity of cells carrying plasmids in which the promoter fragment was inserted in the correct orientation relative to *lacZ* (e.g., pCED7) is shown in Table 1. In this case,

* Corresponding author.

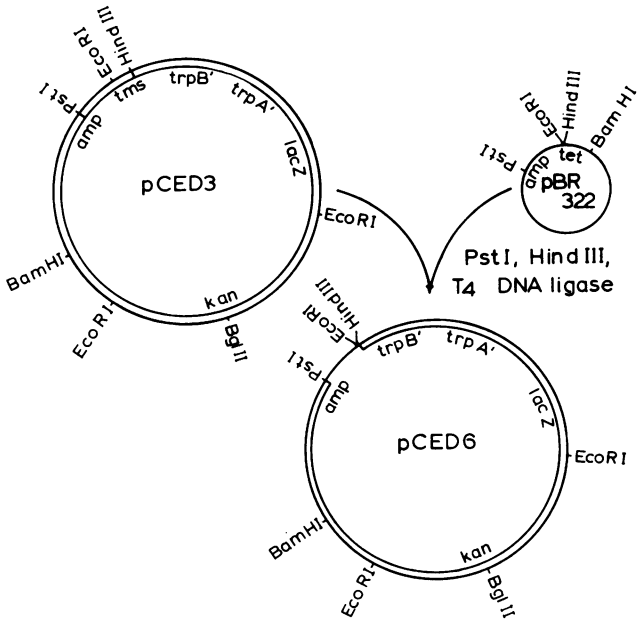

FIG. 1. Construction of pCED6. Plasmids pCED3 and pBR322 were cleaved with restriction endonucleases *Pst*I and *Hin*dIII and ligated together. The ligated DNA was used to transform *E. coli* RV; selection was for transformants that were resistant to ampicillin and kanamycin and unable to express β-galactosidase. The approximate size of pCED6 is 12.5 kilobases.

maximal β-galactosidase activity was observed in cells that had begun to sporulate. A detailed analysis of the dependence of β-galactosidase activity on sporulation in a strain carrying a similar *spoVG-lacZ* fusion has been described by Zuber and Losick (9).

To show that pCED6 could be used to identify additional restriction fragments with promoter activity, we treated chromosomal DNA of wild-type *B. subtilis* with *Hin*dIII and ligated the fragments to *Hin*dIII-treated pCED6. This DNA was used to transform *E.coli* RV to Amp^r. All the Amp^r transformants were pooled and used as a source of plasmid DNA for transformation of *B. subtilis* BR151. (Initial passage through *E. coli* was done to increase the frequency of multimeric plasmids in the DNA preparation.) Of the Kan^r transformants of *B. subtilis* obtained, about 5% formed blue colonies on L agar containing X-Gal. Five blue colonies were picked and purified. Plasmid DNA from each (pLS80 to pLS84) was subjected to restriction analysis, and each strain was tested for β-galactosidase activity during logarithmic growth. The results (Table 1) indicate that a variety of fragments had been inserted in different plasmids. They varied over a 10-fold range in their ability to direct expression of β-galactosidase. One plasmid, pLS81, seemed to contain an insert with particularly potent promoter activity.

An important feature of plasmids derived from pCED6 is that the inserted promoter-containing fragment need not contain translational initiation signals. By sequencing the DNA of the *trpA-lacZ* region used in construction of pCED6, M. Berman (personal communication) has concluded that the natural *lacZ* translation signals are absent and that a hybrid protein containing 59 amino acids of *trpA* and all but the first two amino acids of β-galactosidase is coded for by this sequence. By radioactive labeling and immunoprecipitation of β-galactosidase, we have found that the *lacZ* product coded for by pCED3 in either *E. coli* or *B. subtilis* is the size expected from Berman's data. In Fig. 2, the β-galactosidase coded for by pCED3 in *E. coli* and *B. subtilis* is shown in lanes 5 and 6, respectively. It is identical in size to that produced in *E. coli* when the *lacUV5* promoter is used to drive expression (lane 4) and noticeably larger than the β-galactosidase coded for by F′ *lac* (molecular weight, 116,000, lane 3). If the translation initiation site of the *B. subtilis tms* gene was used by pCED3 to produce β-galactosidase, a fusion polypeptide of 140,000 molecular weight would have been produced. No such polypeptide was seen.

In summary, we have constructed a plasmid that allows the testing of restriction fragments for promoter activity in *B. subtilis* and *E. coli*. The most convenient fragments have *Hin*dIII sites at their ends or a *Pst*I site upstream and a *Hin*dIII site downstream. Since *Hin*dIII "linkers" are readily available, it is practical to consider inserting nearly any type of restriction fragment into the *Hin*dIII site of pCED6. Because pCED6 replicates in both *E. coli* and *B. subtilis*, it is possible to compare activities of particular promoters in the two organisms. An important fact that contributes to this feature of pCED6 is that translation of *lacZ* can initiate at the *trpA* ribosome binding site in either organism. Although this is only one of two instances we know of in which a ribosome binding site of a gram-negative bacterium is used in a gram-positive organism (see reference 10), this allows one to insert promoter-containing restriction fragments without regard to whether they also contain ribosome binding sites or whether they are in proper reading frame with respect to *lacZ*. Furthermore, the intrinsic activity of the β-galactosidase produced is independent of the nature of the promoter fragment since the identical *trpA-lacZ* fusion polypeptide is always produced.

Plasmid pCED6 is formally analogous to other promoter-probe vectors for *B. subtilis*. It differs from pPL603 (8) in that it uses β-galactosidase as the indicator polypeptide rather than an inducible chloramphenicol acetyl transferase

TABLE 1. β-Galactosidase activity coded for by plasmids containing various promoter fragments

Plasmid	Promoter	Size of inserted DNA (kilobases)	β-Galactosidase sp act[a] (U/mg of protein)	
			E. coli RV[b]	*B. subtilis* BR151[c]
None			<0.01	<0.01
pCED6			<0.01	0.11
pCED3	*tms*	0.28	5.21	0.50
pRE1	*gltA*	1.9	7.50	1.00
pCED7	*spoVG*	0.42		2.80[d]
pLS80	Unknown	1.25		0.48
pLS81	Unknown	1.4		4.30
pLS82	Unknown	1.25		0.53
pLS83	Unknown	1.9		1.15
pLS84	Unknown	2.1		0.46

[a] Assayed by the method of Miller (6). One unit of activity produces an increase in absorbance at 420 nm of 1.0 per minute; activity is expressed as units per milligram of protein. Total cellular protein was measured with the Bio-Rad protein assay reagent.

[b] Cells were grown in L broth containing 5 μg of kanamycin per ml and 10 μg of ampicillin per ml and made permeable by CH_3Cl and sodium dodecyl sulfate (4).

[c] Cells were grown in L broth containing 5 μg of kanamycin per ml and were made permeable by toluene treatment (4).

[d] Cells were grown in nutrient sporulation medium containing 5 μg of kanamycin per ml until 2 h after the end of growth and made permeable by toluene treatment.

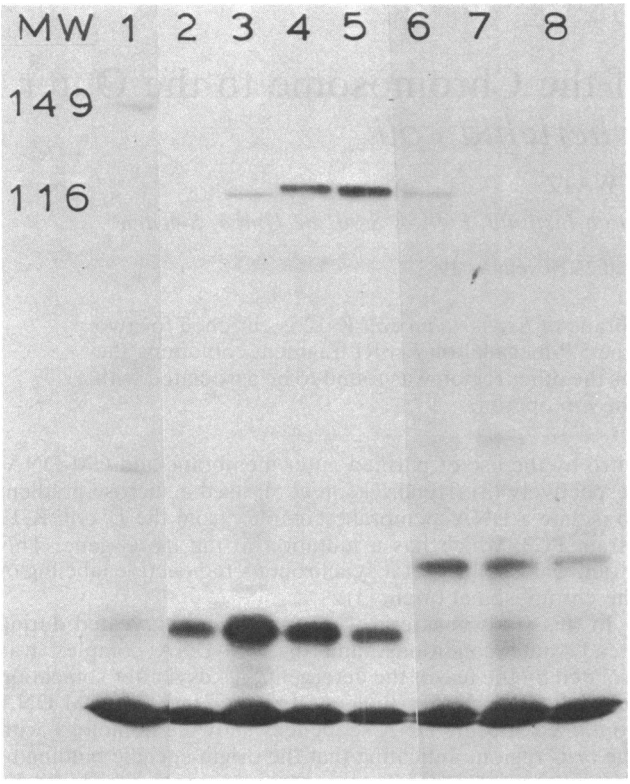

FIG. 2. Gel electrophoresis of β-galactosidase coded for by pCED3. *E. coli* RV and *B. subtilis* BR151 carrying the plasmids indicated below were labeled in methionine-free medium for 15 min with [^{35}S]methionine (1.0553 mCi/mmol; 100 μCi/ml of culture), chilled, washed, and lysed with lysozyme in the presence of DNase I. β-Galactosidase polypeptides were immunoprecipitated by reaction with antibody against *E. coli* β-galactosidase (gifts of A. Wright and R. Huebner) and then with IgSorb (Enzyme Center, Inc.). The precipitate was washed, boiled in dye buffer containing sodium dodecyl sulfate, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lane 1, RV(F′ C521) (this plasmid, obtained from J. Miller and M. Malamy, personal communication, codes for a *pifC-lacZ* fusion polypeptide of 149,000 molecular weight); lane 2, RV(F′ *lac*), uninduced; lane 3, RV(F′ *lac*), induced with isopropylthio-β-D-galactopyranoside; lane 4, pMBO50 (this plasmid, obtained from M. B. O'Connor and M. Malamy, personal communication, has the *lacUV5* promoter upstream from the same *trp-lac* DNA found in pCED6 derivatives); lane 5, RV(pCED3); lane 6, BR151(pCED3); lane 7, BR151(pCED6); lane 8, BR151. Low-molecular-weight proteins appear in all lanes. They are precipitated by antibody to β-galactosidase but are apparently unrelated to the *lacZ* product since they are precipitated from extracts of cells that have no *lacZ* gene. MW, molecular weight × 10^3.

(*cat*) and has a replicon for *E. coli* as well as for *B. subtilis*. It also differs from pGR1 (5), another *cat* plasmid, in that it uses β-galactosidase and does not require the insertion of a ribosome binding site. Plasmid pSK10Δ6 (S. Kamb and J. Pero, personal communication) uses β-galactosidase as an indicator of promoter activity but also requires that the promoter fragment contain a ribosome binding site. The plasmid pTG402 (10) most closely resembles pCED6 in function. It replicates in both *E. coli* and *B. subtilis* and uses as indicator an enzyme whose activity is detectable by a color reaction and whose gene is preceded by a ribosome binding site recognized in *B. subtilis*. The indicator enzyme in this case is catechol 2,3-dioxygenase from *Pseudomonas putida*.

Plasmids such as pCED6 should prove useful in finding new promoter sites and analyzing their function. In particular, transcriptional fusions of the type described here allow one to search for mutations in the promoter region or in chromosomal genes that affect expression.

We thank M. Malamy and A. Wright for their comments on the manuscript, R. Emond for participating in the construction of pRE1, and A. Wright and R. Huebner for gifts of antibody to β-galactosidase.

This work was supported by a Public Health Service grant R01-GM30408 from the National Institutes of Health.

LITERATURE CITED

1. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning on *E. coli*. J. Mol. Biol. **138**:179–207.
2. **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.
3. **Contente, S., and D. Dubnau.** 1979. Characterization of plasmid transformation in *B. subtilis*: kinetic properties and the effects of DNA conformation. Mol. Gen. Genet. **167**:251–258.
4. **Donnelly, C. E., and A. L. Sonenshein.** 1982. Genetic fusion of *E. coli lac* genes to a *B. subtilis* promoter, p. 63–72. *In* A. Ganesan, J. Hoch, and S. Chang (ed.), Molecular cloning and gene regulation in bacilli. Academic Press, Inc., New York.
5. **Goldfarb, D. S., R. H. Doi, and R. L. Rodriguez.** 1981. Expression of Tn9-derived chloramphenicol resistance in *B. subtilis*. Nature (London) **293**:309–311.
6. **Miller, J.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
7. **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.
8. **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**:1163–1165.
9. **Zuber, P., and R. Losick.** 1983. Use of a *lacZ* fusion to study developmental regulation by the *spoO* genes of *Bacillus subtilis*. Cell **53**:275–283.
10. **Zukowski, M. M., D. F. Gaffney, D. Speck, M. Kauffmann, A. Findell, A. Wisecup, and J. P. Le Cocq.** 1983. Chromogenic identification of genetic regulatory signals in *Bacillus subtilis* based on expression of a cloned *Pseudomonas* gene. Proc. Natl. Acad. Sci. U.S.A. **80**:1101–1105.