APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1991, p. 385–388
0099-2240/91/020385-04$02.00/0

Vol. 57, No. 2

Improved Vector for Promoter Screening in Lactococci

BOJANA BOJOVIC, GORDANA DJORDJEVIC, AND LJUBISA TOPISIROVIC*

Institute of Molecular Genetics and Genetic Engineering, Vojvode Stepe 283, P.O. Box 794, 11001 Belgrade, Yugoslavia

Received 7 August 1990/Accepted 25 November 1990

Fragments of *Lactococcus lactis* subsp. *lactis* NP45 chromosomal DNA provided promoter activity in *Escherichia coli* when cloned into the promoter probe vector pGKV210. Only 13% of these recombinant plasmids promoted detectable *cat-86* activity when transferred to *L. lactis*, i.e., expressed chloramphenicol resistance. In these promoter-containing versions of pGKV210, the *cat-86* gene specifies chloramphenicol-inducible chloramphenicol acetyltransferase expression. This could be a limiting factor for cloning of promoters with lower activity in *L. lactis*. Therefore, we have constructed a new promoter probe vector, pBV5030, with the mutated version of the *cat-86* gene, which is constitutively expressed when transcriptionally activated by the insertion of a promoter. We found that in *L. lactis* IL1403 the constitutively expressed *cat-86* gene (on a pBV5030 derivative) has four times higher activity than the inducible version of the same gene (on a pGKV210 derivative) when both have the same promoter inserted upstream of the *cat-86* gene. These results suggest that plasmid pBV5030 could be a more efficient vector for the cloning of promoters from lactococci.

Lactic acid bacteria are not pathogenic for humans and animals and therefore could be desirable hosts for cloning and expressing foreign genes. Thus, studying regulation of transcription and translation in lactic acid bacteria is of great importance, since it could facilitate the construction and use of expression vectors.

The analysis of promoter structure and function mainly depends on adequate promoter probe vectors which allow this cloning of various categories of promoters upstream of a promoterless reporter gene. Plasmid pGKV210 is the first one constructed for the selection of promoter-containing fragments in *Lactococcus lactis* (18). It also replicates in *Bacillus subtilis* and *Escherichia coli*. In plasmid pGKV210, the promoterless reporter gene is the inducible *cat-86* gene (7, 14) preceded by a multiple cloning site. Screening of lactococcal promoters with vector pGKV210 has already been done with *L. lactis* subsp. *lactis* and *B. subtilis* as hosts (19). Recently, cloning of promoters from the temperate lactococcal bacteriophage BK5-T into promoter-detecting plasmid pMU1328 has also been reported (12).

Studies of *cat-86* gene expression in *B. subtilis* have shown that inducibility results from the activation of the translation of *cat-86* mRNA by drug-modified ribosomes in a process that has been termed translational attenuation (1). The constitutively expression version of the *cat-86* gene in *B. subtilis* plasmid pPL708ΔAC1 specifies chloramphenicol acetyltransferase (CAT) activity that is four times higher than the activity specified by the inducible version of the gene behind the same promoter (2).

To increase the possibilities of cloning lactococcal promoters of different strengths, we have constructed a new lactococcal promoter probe vector, pBV5030, based on pGKV210 but having a *cat-86* gene which can be constitutively expressed. We present a comparison of activities of the same promoter cloned in front of both versions of the *cat-86* gene (in vectors pGKV210 and pBV5030).

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains, media, and reagents. The bacterial strains and plasmids used in this work are listed in Table 1. Lactococcal strains were cultivated in M17 medium (16) containing glucose (0.5%; GM17). Luria both (LB) was used for cultivating *E. coli*. GM17 and LB broth were solidified with 1.5% agar (Difco) when used as agar plates. Erythromycin (Em), supplied by Boehringer, was added at final concentrations of 2.5 μg/ml for *L. lactis* and 200 μg/ml for *E. coli*. Chloramphenicol (Cm), supplied by Sigma, was added at final concentrations of 5 to 50 μg/ml for *L. lactis* and 20 to 1,000 μg/ml for *E. coli*. Restriction enzymes and T4 DNA ligase, supplied by Pharmacia, were used according to the manufacturer's instructions.

Plasmid, DNA fragments, and chromosomal DNA isolation. Plasmid DNA from *E. coli* was isolated by the alkaline lysis method of Birnboim and Doly (4). Plasmid isolation from lactococci was done by the above-mentioned alkaline method, but with the following modifications: lysis solution was PP buffer (0.04 M ammonium acetate, 0.001 M magnesium acetate, 0.5 M sucrose, pH 7.0) with 4 mg of lysozyme per ml, and instead of NaOH-sodium dodecyl sulfate (SDS) solution we used TE1-SDS solution (0.1 M Tris, 0.01 M EDTA [pH 10.5]–1% [wt/vol] SDS) (20).

After digestion of plasmids pBV502 and pPL703ΔAC1 with *Pst*I and *Hin*dIII restriction enzymes, DNA fragments were separated on 1% agarose (Sigma) gels. DNA fragments were then isolated from the gels by the electroelution method (1 h, 100 V), purified by PCI treatment (phenol, chloroform, and isoamylalcohol in the volume ratio 10:9:1), and resuspended in sterile double-distilled water.

Chromosomal DNA from lactococci was isolated by the method of Gasson (8).

Transformation of cells with ligation mixture. Ligation was carried out by the method of Maniatis et al. (13). The ligation mixture (total, 1 μg of DNA) was incubated at 15°C for 18 h and then purified by PCI treatment and resuspended in 10 μl of sterile double-distilled water.

A logarithmically growing culture of *L. lactis* IL1403 (A_{600} = 0.4; approximately 3×10^8 CFU/ml) was used for electroporation. Cells, washed twice in sterile double-distilled water, were concentrated 50 times in sterile double-distilled

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype	Source or reference
L. lactis subsp. *lactis*		
MG1363	Plasmid-free derivative of NCDO712, Lac$^-$	8
IL1403	Plasmid-free derivative of IL594, Lac$^-$	6
NP45	Nisin-producing strain	11
LGKV210	IL1403 transformant containing plasmid pGKV210	This work
LBV5030	IL1403 transformant containing plasmid pBV5030	This work
LBV502	IL1403 transformant containing plasmid pBV502	This work
LBV503	IL1403 transformant containing plasmid pBV503	This work
E. coli		
MM294	*supE44 hsdR endA1 pro thi*	Laboratory collection
Plasmid		
pPL703ΔAC1	Neo^r, with promoterless constitutively expressible *cat-86* gene	2
pGKV210	Em^r promoter probe vector containing the promoterless *cat-86* gene	18
pBV5030	Em^r promoter probe vector containing the promoterless *cat-86* gene from pPL703ΔAC1	This work
pBV502	Em^r Cm^r pGKV210 derivative with cloned promoter in *Bam*HI site	This work
pBV503	Em^r Cm^r pBV5030 derivative with the same promoter as in pBV502	This work

water. Ligated and purified DNA (prepared as above) was added to 200 μl of this cell suspension and transferred into 2-mm electroporation cuvettes. Electroporation was carried out with a GENE PULSER equipped with the Pulse Controller attachment supplied by Bio-Rad Laboratories. For standard high-efficiency transformation, cell suspensions containing ligated DNA were pulsed at the setting of 25 μF, 2.5 kV and 200 Ω. Immediately after the electric discharge, 1.8 ml of MMP mix (M17; 0.5% glucose, 10 mM $MgCl_2$, 2.5 mM KCl) was added to the cuvette. The whole mixture was incubated for at least 2 h at 30°C with or without induction with sublethal concentrations of chloramphenicol (1 μg/ml). Electroporated cells were plated onto GM17 selective agar plates and incubated at 30°C.

Transformation of competent *E. coli* MM294 cells was performed by a standard procedure (13).

RESULTS

Promoter cloning in plasmid pGKV210. Cloning of promoters from chromosomal DNA of *L. lactis* subsp. *lactis* NP45 was carried out with vector pGKV210. Chromosomal DNA and vector pGKV210, digested with restriction enzymes *Sau*3A and *Bam*HI, respectively, were ligated. The ligation mixture was used to transform *E. coli* MM294. Transformants selected for growth on LB plates containing 100 μg of chloramphenicol per ml (EBV transformants) were screened for resistance to higher levels of chloramphenicol. About 100 transformants resistant to 1 mg of chloramphenicol per ml were chosen for further testing: at least 39 contained plasmids with fragments of different sizes. Plasmids from these 39 EBV transformants were extracted and used to transform *L. lactis* MG1363 (SBV transformants). Selection was made for resistance to erythromycin (2.5 μg/ml).

Testing of the SBV transformants obtained for resistance to chloramphenicol revealed that the cloned promoter-containing fragments could be divided into two classes (Table 2). Chloramphenicol resistance (Cm^r) in *L. lactis* was not as high as that in *E. coli* (Table 2). This result could be explained by the difference in copy number of plasmid pGKV210 in *E. coli* (62 copies) and *L. lactis* (3 copies). These copy numbers were obtained for a plasmid with the same origin of replication (10).

Construction of plasmid pBV5030. Our strategy was to change the regulatory region of the *cat-86* gene responsible for the inducibility by replacing it with the same region from the constitutively expressed *cat-86* gene (Fig. 1). We used plasmid pBV502 (4,770 bp) for this construction. Plasmid pBV502 has a promoter fragment of 370 bp cloned in pGKV210. This promoter is expressed in both *E. coli* MM294 and *L. lactis* MG1363 (Table 2). Plasmid pPL703 ΔAC1 (4,967 bp) was the source of the constitutively expressible *cat-86* gene. This plasmid carris a deletion and duplication within the region involved in the control of

TABLE 2. Maximum chloramphenicol resistance in a plate assay obtained with cloned promoter fragments in pGKV210

Plasmid	Cloned fragment (bp)	Chloramphenicol resistance (μg/ml)	
		E. coli MM294	*L. lactis* MG1363[a]
Class I			
pBV101	740	1,000	10
pBV203	840	1,000	10
pBV301	1,890	1,000	20
pBV502	370	1,000	10
pBV413	690	1,000	10
Class II			
pBV210	1,150	1,000	S
pBV220	850	1,000	S
pBV230	510	1,000	S
pBV120	740	1,000	S
pBV180	550	1,000	S

[a] Transformants obtained after transformation of *L. lactis* subsp. *lactis* MG1363 with purified plasmids isolated from *E. coli* MM294 transformants. S, Sensitive to chloramphenicol (<5 μg/ml). Only 5 of 34 sensitive transformants are shown.

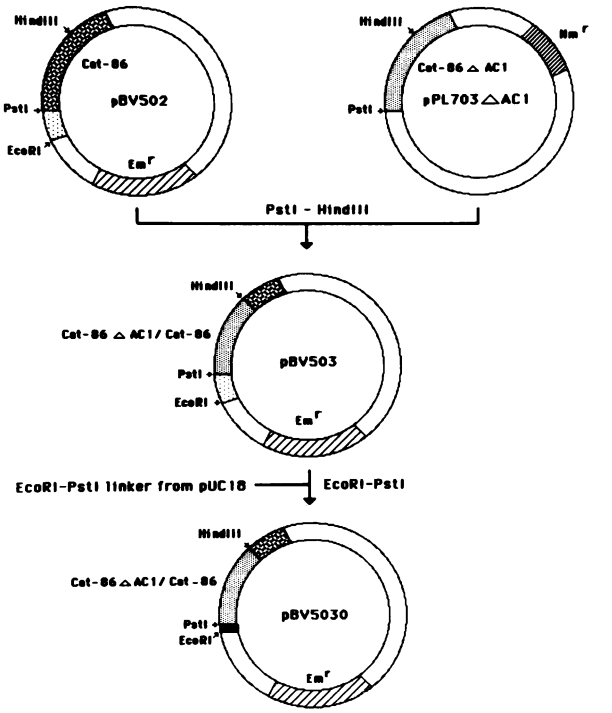

FIG. 1. Construction of plasmid pBV5030. Symbols: ▦, *cat-86* gene; ▦, *cat-86*ΔAC1 gene; ▩ promoter of 370 bp; ■, multiple *Eco*RI-*Pst*I cloning site from pUC18; ▨, erythromycin resistance (Em^r); ▨, neomycin resistance (Nm^r). Only relevant restriction sites are presented.

cat-86 gene inducibility, making it constitutively expressible (2). Plasmids pBV502 and pPL703ΔAC1 were digested with restriction enzymes *Pst*I and *Hin*dIII. The larger fragment of plasmid pBV502 (4,140 bp) and the smaller fragment of plasmid pPL703ΔAC1 (551 bp) were isolated and ligated. The ligation mixture was used to transform *E. coli* MM294. Transformants were directly selected on LB plates containing chloramphenicol (20 μg/ml). Subsequent restriction enzyme analysis of the DNA of one of the transformants proved that we were dealing with the new plasmid, designated pBV503 (Fig. 1). The level of Cm^r in *E. coli* containing plasmids pBV502 and pBV503 was the same (1 mg/ml) (data not shown). The promoter-carrying fragment from plasmid pBV503 was removed by digestion with restriction enzymes *Eco*RI and *Pst*I and replaced by the multiple *Eco*RI-*Pst*I cloning site from pUC18, using the same restriction enzymes. The resulting construct is the new promoter probe vector pBV5030, with a *cat-86* gene that can be constitutively expressed (Fig. 1).

Test of CAT expression in plasmids pBV502 and pBV503. To elucidate the influence of the regulatory region of the *cat-86* gene on the level of Cm^r in lactococci, we transformed *L. lactis* IL1403 with plasmids pBV502, pBV503, pGKV210, and pBV5030, using a selection for resistance to erythromycin (2.5 μg/ml). We tested the expression of the *cat-86* gene in the resulting transformants. The highest level of CAT enzyme synthesis was found in transformant LBV503 (Table 3). This strain has four times more CAT enzyme activity than the transformant LBV502, induced by chloramphenicol. These data are in agreement with the level of Cm^r of transformants LBV502 and LBV503 (Table 3).

TABLE 3. Influence of *cat-86* gene regulation on promoter activity in *L. lactis* subsp. *lactis* IL1403

Transformant/plasmid	CAT activity (nmol min^{-1} mg of $protein^{-1}$)[a]	Chloramphenicol resistance (μg/ml)[b]
LGKV210/pGKV210	0.00	2.7
LBV5030/pBV5030	0.00	3.0
LBV502/pBV502	14.00	14.5
LBV503/pBV503	61.60	54.0

[a] CAT activity was measured at 37°C by the method of Shaw (15). Transformants LBV502 and LBV503 were grown in GM17 medium with chloramphenicol (5 μg/ml). Transformants LGKV210 and LBV5030 were used as controls.

[b] Chloramphenicol concentrations giving 50% inhibition of cell growth.

Shotgun promoter cloning in plasmid pBV5030. To check the possibility of direct shotgun cloning of promoter-containing fragments in lactococci with the promoter probe vector pBV5030, we developed a procedure for electroporation of lactococci with ligation mixtures (see Materials and Methods). A ligation mixture containing plasmid pBV5030 and chromosomal DNA from *L. lactis* NP45, digested with *Bam*HI and *Sau*3A, respectively, was used for electroporation of *L. lactis* IL1403. We obtained 5×10^4 transformants per μg of DNA when selection was for resistance to erythromycin (2.5 μg/ml) and 800 transformants per μg of DNA when directly selecting for resistance to chloramphenicol (5 μg/ml). The resistance to chloramphenicol among transformants varied from 20 to >50 μg/ml (data not shown).

DISCUSSION

There are at least two possible explanations for our results that only 13% of the fragments with promoter activity directly cloned in *E. coli* are functional in *L. lactis*. First, promoters cloned in the heterologous background (*E. coli*) escape any regulation that might exist in the homologous background (*L. lactis*). The second explanation is that not all chromosomal fragments from lactococci that render *E. coli* chloramphenicol resistant represent functional promoters in *L. lactis*. This pseudopromoter activity in *E. coli* could be the result of a high percentage of A+T base pairs in chromosomal DNA of *L. lactis* (9). This idea is supported by the fact that highly A+T-rich chromosomal DNA fragments of *L. plantarum*, larger than 2 kb, cannot be cloned into pBR322 or pUC vectors (3). However, such A+T-rich fragments can be cloned when the vector contains transcription termination signals flanking the multiple cloning site which block a possible pseudopromoter activity (5).

Plasmid pBV502 carries the inducible version of the *cat-86* gene, while plasmid pBV503 carries the constitutive version of the same gene, both controlled by the same promoter. The observation that *L. lactis* containing plasmid pBV503 has four times higher CAT activity than the same strain containing pBV502 (Table 3) is consistent with the already mentioned differences between inducible and constitutive expression of the CAT enzyme in *B. subtilis*. Thus, it is possible that efficient induction of the *cat-86* gene by chloramphenicol in lactococci can be achieved only in the presence of strong promoters. This could also be why we have cloned a small number of promoters functional in lactococci.

Plasmid pGKV210 renders *E. coli* resistant to 90 μg of chloramphenicol per ml in spite of the fact that it carries a promoterless *cat-86* gene (19). Actually, this is because of a highly A+T-rich region upstream of the inducible *cat-86*

gene of pGKV210 which *E. coli* can use as a promoter but *B. subtilis* and *L. lactis* cannot (17). Interestingly, *E. coli* containing plasmid pBV5030 has a lower resistance to chloramphenicol (<20 μg/ml). Thus, a lower concentration of chloramphenicol can be used for selection in promoter-cloning experiments in *E. coli* when plasmid pBV5030 is used as a promoter probe vector instead of pGKV210.

All of these results suggest that plasmid pBV5030 is a more convenient vector for promoter cloning in lactococci. It could possibly facilitate the cloning of promoters having different levels of activities and could be the basis for a more precise determination of functional domains of promoters from lactic acid bacteria.

ACKNOWLEDGMENTS

We are grateful to Jan Kok for providing plasmid pGKV21 and to Paul Lovett for plasmid pPL703ΔAC1. We are also grateful to Jan Kok for critical reading of the manuscript and valuable comments.

This work was supported by the Science Fund of the Republic of Serbia, grant 1.41.5.

REFERENCES

1. **Alexieva, Z., E. J. Duvall, N. P. Ambulos, Jr., U. J. Kim, and P. S. Lovett.** 1988. Chloramphenicol induction of *cat-86* requires ribosome stalling at a specific site in the regulatory leader. Proc. Natl. Acad. Sci. USA **85**:3057–3061.
2. **Ambulos, N. P., S. Mongolsuk, J. D. Kaufman, and P. S. Lovett.** 1985. Chloramphenicol-induced translation of *cat-86* mRNA requires two *cis*-acting regulatory regions. J. Bacteriol. **164**:696–703.
3. **Bates, E. M., and H. J. Gilbert.** 1989. Characterization of a cryptic plasmid from *Lactobacillus plantarum*. Gene **85**:253–258.
4. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1519.
5. **Chen, J. D., and D. A. Morrison.** 1987. Cloning of *Streptococcus pneumoniae* DNA fragments in *Escherichia coli* requires vectors protected by strong transcriptional terminators. Gene **55**: 179–187.
6. **Chopin, A., M. C. Chopin, A. Moillo-Batt, and P. Langella.** 1984. Two plasmid-determined restriction and modification systems in *Streptococcus lactis*. Plasmid **11**:260–263.
7. **Duvall, E. J., D. M. Williams, P. S. Lovett, C. Rudolf, N. Vasantha, and M. Guyer.** 1983. Chloramphenicol-inducible gene in *Bacillus subtilis*. Gene **24**:170–177.
8. **Gasson, M. J.** 1983. Plasmid complements of *Streptococcus lactis* NCDO712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. **154**:1–9.
9. **Kilpper-Balz, R., G. Fischer, and K. N. Schleifer.** 1982. Nucleic acid hybridization of group N and group D streptococci. Curr. Microbiol. **7**:245–250.
10. **Kok, J., J. M. B. M. van der Vossen, and G. Venema.** 1984. Construction of plasmid cloning vectors for lactic acid streptococci which also replicate in *Bacillus subtilis* and *Escherichia coli*. Appl. Environ. Microbiol. **48**:726–731.
11. **Kozak, W., M. Rajchert-Trzpil, and W. T. Dobrzansky.** 1974. The effect of proflavin, ethidium bromide and an elevated temperature on the appearance of nisin-producing strains of *Streptococcus lactis*. J. Gen. Microbiol. **83**:295–302.
12. **Lakshmidevi, G., B. E. Davidson, and A. J. Hillier.** 1990. Molecular characterization of promoters of the *Lactococcus lactis* subsp. *cremoris* temperate bacteriophage BK5-T and identification of a phage gene implicated in the regulation of promoter activity. Appl. Environ. Microbiol. **56**:934–942.
13. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **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.
15. **Shaw, W. V.** 1975. Chloramphenicol acetyltransferase from chloramphenicol-resistant bacteria. Methods Enzymol. **43**:737–755.
16. **Terzaghi, B. E., and W. E. Sandine.** 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. **29**:807–813.
17. **van der Vossen, J.** Personal communication.
18. **van der Vossen, J. M. B. M., J. Kok, and G. Venema.** 1985. Construction of cloning, promoter-screening, and terminator-screening shuttle vectors for *Bacillus subtilis* and *Streptococcus lactis*. Appl. Environ. Microbiol. **50**:540–542.
19. **van der Vossen, J. M. B. M., D. van der Lelie, and G. Venema.** 1987. Isolation and characterization of *Streptococcus cremoris* Wg2-specific promoters. Appl. Environ. Microbiol. **53**:2452–2457.
20. **West, C. A., and P. J. Warner.** 1985. Plasmid profiles and transfer of plasmid-encoded antibiotic resistance in *Lactobacillus plantarum*. Appl. Environ. Microbiol. **50**:1319–1321.