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Construction of a new vector for the expression of foreign genes in *Zymomonas mobilis*

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SUMMARY

Broad host range plasmids have previously been shown to be suitable as vectors to introduce antibiotic resistance genes into *Z. mobilis*. However, attempts to use these vectors to carry other genes with enteric promoters and controlling elements have resulted in limited success due to poor expression. Thus we have constructed a promoter cloning vector in a modified pBR327 and used this vector to isolate 12 promoters from *Z. mobilis* which express various levels of β-galactosidase in *Escherichia coli*. Four of these were then subcloned into pCVD 305 for introduction into *Z. mobilis*. All expressed β-galactosidase in *Z. mobilis* with activities of 100 to 1800 Miller units. One of these retained a *Bam*HI site into which new genes can be readily inserted immediately downstream from the *Z. mobilis* promoter. Genetic traits carried by pCVD 305 were initially unstable but spontaneous variants were produced during sub-culture in which the plasmid was resistant to curing at elevated temperature. One of these variants was examined in some detail. The increased stability of this variant appears to result from an alteration in the plasmid rather than a chromosomal mutation or from chromosomal integration.

INTRODUCTION

Zymomonas mobilis is a gram-negative bacterium with considerable potential for the commercial production of fuel ethanol [12]. However, this organism is extremely limited in substrate range and natural isolates are capable of fermenting only glucose, fructose, sucrose and sorbitol [16]. Previous studies in our laboratory have attempted to increase this substrate range by introducing an enteric transposon encoding the β-galactosidase and lactose permease genes [3] using plasmid pGC91.14, a derivative of RPI [4]. Neither the lactose permease or β-galactosidase were expressed at high levels in strains of *Z. mobilis* carrying this plasmid although activities were easily detectable. A seond problem was also noted, i.e., the instability of this plasmid during growth at temperatures above 30°C.

In this study, we have investigated the use of *Z. mobilis/E. coli* gene fusions for the efficient expression of β-galactosidase in *Z. mobilis*. Several of these expressed high levels of β-galactosidase activ-

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ity in *Z. mobilis* strain CP4, although none expressed lactose permease.

MATERIALS AND METHODS

Strains, plasmids and growth conditions. The organisms and plasmids used in this study are listed in Table 1. *Z. mobilis* strain CP4 was grown at 30°C in complex medium (YGP) containing 20 g/l glucose as described by Skotnicki et al. [15] unless stated otherwise. Strains of *E. coli* were grown on Luria broth [8] lacking added carbohydrate. MacConkey agar containing lactose (2 g/l) was used to screen for lactose utilization in *E. coli*. YGP medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) was used to screen for β-galactosidase production by *Z. mobilis*. Tetracyline was used for the selection of both *E. coli* and *Z. mobilis* transformants at a concentration of 12.5 μg/ml. Ampicillin was used in *E. coli* selections at a final concentration of 100 μg/ml.

DNA preparation and analysis. Plasmid DNA was isolated from strains of *E. coli* using the method of Birnboim and Doly [1] and purified on cesium chloride/ethidium bromide gradients as described by Maniatis et al. [10].

Chromosomal DNA was isolated from *Z. mobilis* strain CP4 as follows. A 1-g cell pellet from an exponential culture was washed with 10 mM EDTA, 25 mM Tris-HCl (pH 8) and resuspended in 10 ml of this buffer containing 10% glycerol. Solid lysozyme (10 mg) was added and the cells were allowed to digest overnight at 37°C. Protease K was added to a final concentration of 200 μg/ml and the incubation was continued for 12 h at 50°C. 1 ml of 10% *N*-laurylsarkosinate was added and the incubation was continued for an additional 12 h at 50°C. 20 ml of 95% ethanol were added and the high-molecular-weight DNA was spooled out on a glass rod. This was rinsed in two tubes of 70% ethanol, briefly air dried, and redissolved in 4 ml of 1 mM EDTA, 10 mM Tris-HCl (pH 8). Cesium chloride was added to a density of 1.57 g/ml. The DNA preparation was spun for 14 h at 200 000 × *g* in a VTi65 rotor and the viscous DNA fraction which banded near the center was collected. After dialysis, this procedure yielded 1.5–3 mg of DNA with a

Table 1

Plasmids and strains used in this study

Organism and/or plasmid	Relevant properties	Source
Z. mobilis strain CP4	wild type	A. Ben-Basset, Cetus Corp., Berkeley, CA
E. coli strain HB101	*recA, layY*	K.T. Shanmugam, Univ. Florida Gainesville, FL
E. coli strain S17-1	*rec.A,* mobilizing strain	R. Simon, Bielefeld, F.R.G.
Plasmid pGE 172	λ plac Mu fusion with derivative of pBR327 amp	G.M, Weinstock, Univ. of Texas, Houston, TX.
Plasmid pCVD 305	derivative of pRK290 with polylinker fragment fragment, tet	this paper
Plasmid pLOI 100	deletion of pGE 172	this paper
Plasmid pLOI 101	deletion of pLOI 100	this paper
Plasmid pLOI 102	*lacZ* fusion	this paper
Plasmid pLOI 103	*lacZ* fusion	this paper
Plasmid pLOI 104	*lacZ* fusion	this paper
Plasmid pLOI 105	*lacZ* fusion	this paper
Plasmid pLOI 106	*lacZ* fusion	this paper
Plasmid pLOI 107	*lacZ* fusion	this paper

260:280 nm ratio of 1.8 and consisted of high-molecular-weight DNA as judged by agarose gel electrophoresis.

Partial digests of *Z. mobilis* DNA were prepared with restriction enzyme *Sau*3A essentially as described by Silhavy et al. [13]. The fractions containing fragments with predominant sizes of 1–4 kilobases were used as a source of *Z. mobilis* promoters for the construction of fusion genes without further purification. Restriction enzymes were used in the buffers supplied by the manufacturer or their equivalents. Restriction fragments were analyzed by agarose gel electrophoresis using Tris-borate buffer as described by Maniatis et al. [10]. Ligations were performed essentially as described by Darzins and Chakrabarty [7] at 15°C.

Plasmid mini-screens were performed on *E. coli* transformants using the modification of the Birnboim and Doly [1] procedure described by Silhavy et al. [13]. This procedure did not work well with *Z. mobilis* strain CP4. Plasmid DNA was isolated from *Z. mobilis* using the alkaline lysis procedure described by Crosa and Falkow [5].

Transformation and conjugation. E. coli strains were transformed using the calcium chloride procedure of Mandel and Higa [9]. Plasmids were mobilized from *E. coli* strain S17-1 into *Z. mobilis* strain CP4 using the centrifugation/filter disc procedure described by Simon et al. [14].

Enzyme assays. Assays for β-galactosidase and lactose permease were conducted according to the procedure desribed by Miller [11].

RESULTS AND DISCUSSION

Construction of β-galactosidase gene fusion vector

We have constructed a vector for the cloning of a *Z. mobilis* promoter along with the translational start and amino-terminal end to make fusion proteins with β-galactosidase. This vector was made by deleting most of λ-DNA from the plasmid, pGE 172 [2], using the *Sma*1 sites (Fig. 1). This vector contains a deletion derivative of pBR327, the lactose permease gene and a large carboxy-terminal fragment of β-galactosidase. Both genes for lactose utilization were included so that they could be transferred together into *Z. mobilis* during subsequent manipulations under the control of a single *Z. mobilis* promoter. A *Bam*H1 site is located im-

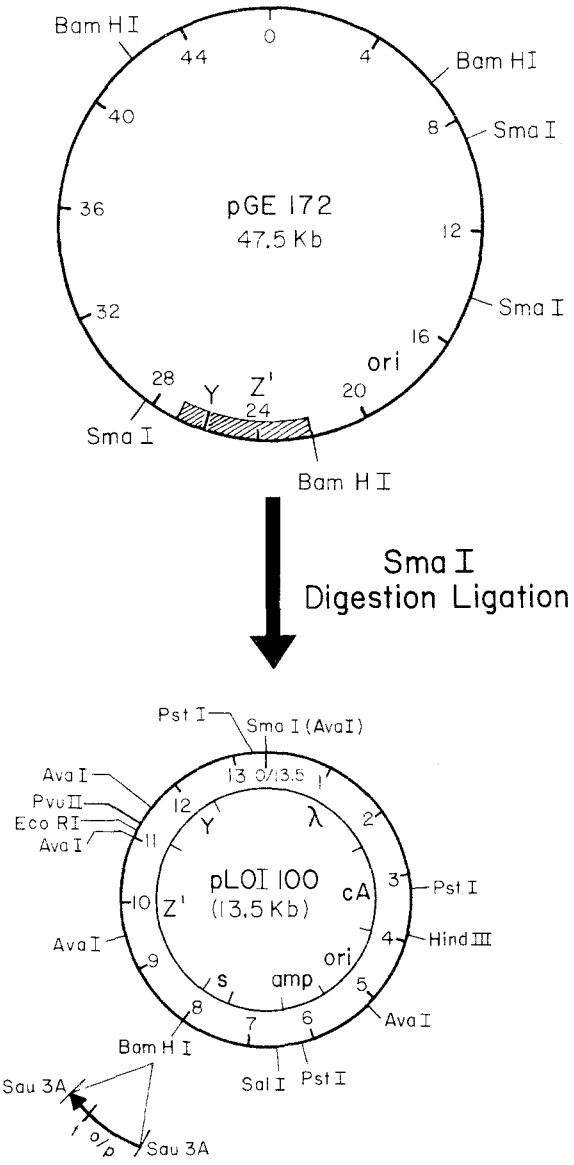

Fig. 1. Construction of pLOI 100. The plasmid pLOI 100 contains a *Bam*H1 site immediately upstream from the truncated *lacZ* gene into which fragments of *Z. mobilis* DNA were inserted. The addition of an operator/promoter, ribosomal-binding sequence, translational start, and amino-terminal peptide are required for the production of a functional β-galactosidase fusion protein. The abbreviations cA and s refer to parts of the plasmid Mu genome.

Table 2

Expression of *Z. mobilis*/*lacZ* gene fusions and lactose permease in *E. coli*

Plasmid	Enzyme activity (Miller units)	
	β-galactosidase	lactose permease
pLOI 100	<2	<2
pLOI 102	3300	510
pLOI 103	1010	200
pLOI 104	370	230
pLOI 105	140	75
pLOI 106	105	70
pLOI 107	30	25

mediately upstream from the truncated β-galactosidase gene (*lacZ*) and serves as a site for the introduction of foreign gene fragments to construct fusion proteins in vitro. To produce a functional β-galactosidase gene, the DNA fragment inserted must contain an operator/promoter site, ribosomal binding site, translational start, and an amino-terminal portion of a protein ligated in the proper reading frame.

Cloning and physical properties of Z. mobilis gene fragments forming functional fusion proteins

Approximately 200 μg of a *Sau*3A partial digest of *Z. mobilis* DNA was ligated with 2 μg of vector DNA and transformed into HB101. Ampicillin-resistant recombinants were selected on lactose-MacConkey medium. Approximately 4% of the colonies were red, indicating lactose utilization. These varied somewhat in intensity of color and 18 were purified and screened for activity. Activities varied from 30 Miller units of β-galactosidase to over 3000 Miller units and six clones were selected for further study which encompassed this array of activities (Table 2). These were assigned plasmid numbers in the order of decreasing β-galactosidase activity with pLOI 102 being the most active (three times the level of an induced wild-type *E. coli*) and pLOI 107 being the least active. The lactose permease was expressed in all cases, although the ratio of activities between the permease and the cleavage enzyme did not remain constant. This may be due in part to differences in the stability or activity of the β-galactosidase fusion proteins. The relative levels of both enzymes are consistent the same comparative pattern of promoter strengths in *E. coli* with the possible exception of pLOI 104, which appears equal or stronger than pLOI 105 based upon the level of lactose permease produced.

The approximate sizes of the *Z. mobilis* inserts and some of their restriction sites are listed in Table 3. The three most active promoters in *E. coli* were the smallest fragments, 400–500 base pairs. The weaker promoter fragments were 1.4–2.4 kilobases. Each *Z. mobilis* fragment had its own unique set of restriction enzyme sites, indicating that all were different. Plasmid pLOI 102 was particularly interesting because the original *Bam*H1 site was regenerated immediately adjacent to the truncated β-galactosidase gene. A deletion derivative of this plas-

Table 3

Physical properties of the *Z. mobilis* DNA fragments forming β-galactosidase fusion genes

n.d., not determined.

Plasmid	Insert size (kilobases)	Restriction sites in insert					
		*Bam*H1	*Hin*dIII	*Eco*R1	*Pst*1	*Ava*1	*Sma*1
pLOI 102	0.4	1	1	0	0	0	0
pLOI 103	0.5	0	0	0	0	0	0
pLOI 104	0.5	n.d.	0	0	0	1	0
pLOI 105	2.3	0	0	1	0	0	0
pLOI 106	2.4	1	2	1	0	1	0
pLOI 107	1.4	1	0	1	0	1	0

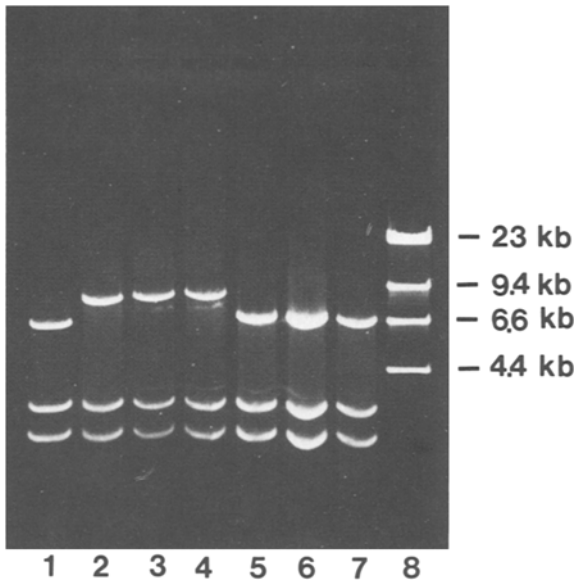

Fig. 2. Restriction digestion of plasmids containing β-galactosidase gene fusions with *Pst*1. Lane 1, pLOI 100; lanes 2–7, pLOI 107–pLOI 102, respectively; lane 8, λ*Hin*dIII digest as size marker.

mid was constructed by removing the 3.2 kilobase fragment (primarily λDNA). This plasmid is potentially useful for the introduction of genes into the *Bam*H1 site under the control of a *Z. mobilis* promoter.

All of the fusion genes constructed lacked new Pst 1 sites, facilitating the removal of a single large fragment which included the fusion gene and the lactose permease gene for subsequent sub-cloning (Fig. 2). The *Pst*1 fragments from four of these were ligated into pCVD 305, a modified pRK290 containing the *Hae*II polylinker fragment from pUC 8 (Ref. 6 and J.B. Kaper, unpublished results). Tetracycline-resistant recombinants of HB101 were selected on lactose-MacConkey agar. Approximately 10% of these were deep red, indicating the presence of the lactose genes. Correct construction was confirmed by plasmid size and digestion with *Pst*-1.

The pCVD 305 plasmids containing the lactose genes were subsequently transformed into strain *E. coli* S17-1 and conjugated into *Z. mobilis*. *Z. mobilis* recombinants were selected for tetracyline resistance on YGP plates and on plates which contained lactose instead of glucose. No recombinants were obtained on the plates lacking glucose. All expressed β-galactosidase activity on X-gal plates.

During growth on X-gal plates, all colonies became sectored even in the presence of tetracycline, indicating extreme instability of these plasmids in *Z. mobilis*. No such instability was observed in *E. coli* containing the same plasmids. Fig. 3 illustrates the degree of instability of pRK290, of pCVD 305 and of pCVD 305 carrying the lactose genes from pLOI 105. All were equally unstable and over 95% of the population lost these plasmids after 10 generations in the absence of tetracycline selection. The growth of these plasmid-containing cells was retarded. Spontaneous tetracycline-resistant mutants grew more rapidly on plates and in broth during subculturing, although clones with the plasmid and β-galactosidase activity could be easily recovered.

During subculturing and plating on X-gal plates, intensely blue colonies appeared infrequently with the plasmids containing inserts from pLOI 105 and

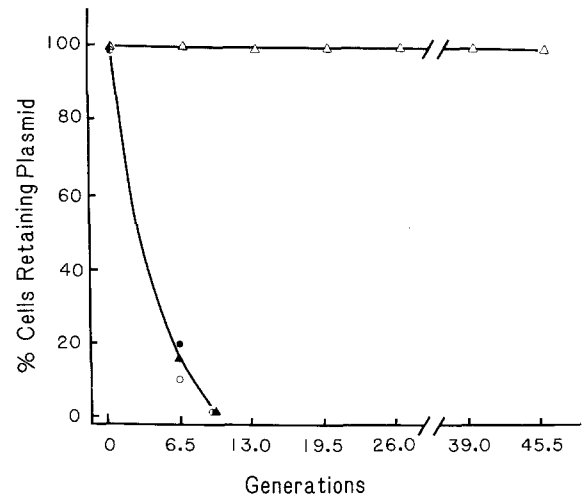

Fig. 3. Stability of plasmids in *Z. mobilis* in the absence of antibiotic selection. Serial transfers were made using 1:100 dilutions at 24-h intervals; samples were diluted and plated. 50 colonies were then screened from each culture to determine the persistance of plasmid markers. Cultures containing pRK290 and pCVD 305 were screened for tetracyline resistance. Cultures containing pCVD 305 in which the lactose genes had been inserted were scored on X-gal and tetracycline plates. In all cases, tetracycline resistance and β-galactosidase activity were lost together. ●, pRK290; ○, original pCVD 305; ▲, pCVD 305 containing the lactose genes from pLOI 105; △, stabilized derivative of pCVD 305 containing the lactose genes from pLOI.

pLOI 106. These spontaneous derivatives were found to be very stable in *Z. mobilis* during subculture at 30°C (Fig. 3). Although these still did not grow on lactose, β-galactosidase activity and tetracycline resistance were stably maintained during subculturing at 37°C (not shown) or 30°C in the absence of antibiotic selective pressure. To determine whether this stabilization had resulted from plasmid changes or from chromosomal changes, plasmid DNA was re-isolated from several of these stable clones, transformed into *E. coli* strain S17-1 and conjugated back into *Z. mobilis*. In all cases, the plasmids were stably maintained in the resulting *Z. mobilis* recombinants, indicating that some change had occurred in the plasmids during subculturing in *Z. mobilis*. This change does not appear to have involved extensive rearrangement, since no changes in the sizes of the 10 *Ava*1 restriction fragments could be detected in comparison with the original plasmid.

Expression of lactose genes in Z. mobilis

The activities of the *Z. mobilis*/*E. coli* β-galactosidase fusion proteins and the lactose permease were determined in four original *Z. mobilis* recombinants (Table 4). Although these had been grown in the presence of tetracycline, only approximately 1/3 of the cells actually contained the lactose plasmid at the time of assay. However, β-galactosidase activity was expressed by all transformants. Considerably higher activities were observed in the stabilized derivatives containing fusion genes from pLOI 105 and pLOI 106, 1870 Miller units and 250 Miller units, respectively. It is interesting to note that these two plasmids were the two most poorly expressed in *E. coli*, indicating that there are differences in the recognition sequences for transcription, ribosomal binding, and/or translational start between *Z. mobilis* and *E. coli*.

None of the recombinant strains of *Z. mobilis* grew on medium in which lactose replaced glucose. This was presumably due to the lack of sufficient lactose permease activity (Table 4). Repeated attempts to isolate lactose fermenting *Z. mobilis* cultures using enrichments with mixtures of lactose and glucose, with and without mutagenesis were unsuccessful. However, the recombinants could be grown on lactose if glucose-grown cells which had been inactivated with chloroform were added as an enzyme source. Limited growth was also observed on medium containing lactose and 2% dimethylsulfoxide, a cell permeant.

Table 4

Expression of *Z. mobilis*/*lacZ* gene fusions and lactose permease in *Z. mobilis*

Values in parenthesis indicate the activities present in the stable derivatives of the original transformants.

Plasmid	Enzyme activity (Miller units)	
	β-galactosidase	lactose permease
pLOI 100	0	0
pLOI 102	20	0
pLOI 103	100	0
pLOI 105	140 (1870)	<2
pLOI 106	45 (290)	<2

CONCLUSIONS

Our results demonstrate the feasibility of employing gene fusion technology for the expression of foreign genes in industrially important microorganisms. In these studies we have cloned several fragments of *Z. mobilis* DNA which serve as efficient promoters for gene expression in *E. coli* and in *Z. mobilis*. Further, we have shown the S17-1-mobilizing strain of *E. coli* which contains an integrated RP4 can be used to mobilize smaller plasmids into *Z. mobilis*. Problems still remain, however, such as the inherent instability of the plasmid vector, pCVD 305, in *Z. mobilis*. This instability was not altered by the construction of β-galactosidase fusion genes. In two cases, it was possible to isolate spontaneous plasmid derivatives which were stably maintained in *Z. mobilis*. Such derivatives may be of value for future construction of more stable vectors for these industrially important microorganisms.

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REFERENCES

1 Birnboim, H.C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acid Res. 7: 1513–1523.

2 Bremer, E., T.J. Silhavy, J.M. Weisemann and G.M. Weinstock. 1984. λ plac Mu: A transposable derivative of bacteriophage lambda for creating lac Z fusions in a single step. J. Bacteriol. 158: 1084–1093.

3 Carey, V.C., S.K. Walia and L.O. Ingram. 1983. Expression of a lactose transposon (Tn951) in *Zymomonas mobilis*. Appl. Environ. Microbiol. 46: 1163–1168.

4 Cornelis, G., D. Ghosal and H. Saedler. 1979. Multiple integration sites for the lactose transposon Tn951 on plasmid RP1 and establishment of a coordinate system for Tn951. Mol. Gen. Genet. 168: 61–67.

5 Crosa, J.H. and S. Falkow. 1981. Plasmids. In: Manual of Methods for General Bacteriology, American Society for Microbiology, Washington, DC. (Gerhardt, P., R.G.E. Murray, R.N. Costilow, E.W. Nester, W.A. Wood, N.R. Kreig and G.B. Phillips, eds.), pp. 266–282.

6 Datta, A.R., J.B. Kaper and A.M. MacQuillan. 1984. Shuttle cloning vectors for the marine bacterium *Vibrio parahaemolyticus*. J. Bacteriol. 160: 808–811.

7 Darzins, A. and A.M. Chakrabarty. 1984. Cloning of genes controlling alginate biosynthesis from a mucoid cystic fibrosis isolate of *Pseudomonas aeruginosa*. J. Bacteriol. 159: 9–18.

8 Luria, S.E. and M. Delbruck. 1943. Mutations of bacteria from virus sensitivity to virus resistance. Genetics 28: 491–511.

9 Mandel, M. and A. Higa. 1970. Calcium dependent bacteriophage DNA infection. J. Mol. Biol. 53: 159–162.

10 Maniatis, T., E.F. Fritsch and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. 545 p. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

11 Miller, J.H. 1972. Experiments in molecular genetics, 466 p. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

12 Rogers, P.L., K.L. Lee, M.L. Skotnicki and D.E. Tribe. 1982. Ethanol production by *Zymomonas mobilis*. Adv. Biochem. Eng. 23: 37–84.

13 Silhavy, T.J., M.L. Berman and L.W. Enquist. 1984. Experiments with Gene Fusions, 301 p. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

14 Simon, R., U. Priefer and A. Puhler. 1983. A broad host range mobilization system for in vivo genetic engineering: Transposon mutagenesis in gram negative bacteria. Biotechnology 1: 784–791.

15 Skotnicki, M.L., K.L. Lee, D.E. Tribe and P.L. Rogers. 1981. Comparison of ethanol production by different *Zymomonas* strains. Appl. Environ. Microbiol. 41: 889–893.

16 Swings, J. and J. Deley. 1977. The biology of *Zymomonas*. Bacteriol. Rev. 41, 1–46.