Reports

Expression of the *Escherichia coli lacZ* Gene on a Plasmid Vector in a Cyanobacterium

Abstract. *A biphasic plasmid vector was used to introduce the* Escherichia coli *K-12* lac *operon into the unicellular cyanobacterium* Agmenellum quadruplicatum *PR-6. The PR-6 transformants expressed* β*-galactosidase at nearly as high a level as did* Escherichia coli *transformants. In order to accomplish this, it was necessary to obtain PR-6 mutants that could be transformed by plasmids with unmodified recognition sites for the endogenous PR-6 restriction endonuclease Aqu I. These mutants were generated by a variation of the ectopic mutagenesis techniques that have been used in other naturally transforming bacteria. The ability to assay the expression of* lacZ *in PR-6 paves the way for the construction of gene fusions with various PR-6 promoters and quantitation of their expression under specific in vivo conditions.*

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The cyanobacteria are the simplest organisms that make use of both photosystems I and II and assimilate inorganic nitrogen in a manner similar to that of higher plants. Their prokaryotic organization makes them a potentially ideal model system in which to study these complex processes at the molecular level. *Agmenellum quadruplicatum* PR-6 (*Synechococcus* sp., PCC7002 and ATCC27264) (*1, 2*) has an efficient, well-characterized, natural DNA uptake system (*3*) and can grow as a facultative photoheterotroph in the presence of glycerol (*2*). We have already isolated and characterized several of the genes encoding components of the light collection apparatus (*4, 5*). Hence, PR-6 is well suited for genetic analysis of the photosynthetic mechanism with plasmid-generated merodiploids. An important aspect of this analysis involves the expression of these PR-6 genes under controlled conditions in which their promoters are fused to the easily assayable *Escherichia coli* β-galactosidase gene. We are now in a position to introduce such fusions into PR-6 on a plasmid vector.

Transformation of PR-6 with biphasic plasmids has been described (*6*). The main obstacle to the general use of biphasic plasmids as cloning vehicles has been the endogenous PR-6 restriction system, Aqu I, an isoschizomer of Ava I (*7*). Since the presence of these restriction sites and the larger monomeric plasmid size have adverse effects on plasmid transformation efficiency in PR-6 (*6*), pAQE12 was constructed as shown in Fig. 1. Modification of the previously described transformation protocol (*6*) (see Table 1) has also improved the efficiency of the procedure several hundredfold. We now obtain an average of 4.6×10^4 transformants per microgram for pAQE12 and 1.3×10^5 transformants per microgram for its dimer, pAQE13; ampicillin resistance (Ap^r) was used for selection. The neomycinphosphotransferase (NPT) II gene from Tn5 (*8*) was cloned into pAQE15 to give pAQE17 as shown in Fig. 1. pAQE17 transformants of PR-6 can be selected at neomycin or kanamycin concentrations of 10 to 1000, μg/ml. With pAQE17, selection for kanamycin resistance (Km^r) gives about a fivefold higher level of PR-6 transformants than does Ap^r selection and also gives more consistent and reproducible results from experiment to experiment. pAQE17 yields 1.3×10^5 transformants per microgram with Km^r selection, whereas its dimer, pAQE18, yields 3.2×10^5 transformants per microgram.

The strategy for cloning the *lac* operon into pAQE17 is outlined in Fig. 2. The final vector construct, pAQE17DL, still contained four Aqu I recognition sites in the *lac* fragment and one in pAQE17 itself. When pAQE17DL was used to transform wild-type PR-6, transformants resistant to both kanamycin and ampicillin could be detected, but they had all deleted most of the *lac* fragment and undergone significant recombinational rearrangements, as had pAQE7 (a derivative of pAQE2 carrying the NPT I gene and six Ava I sites) (*6, 9*).

Elimination of Aqu I restriction activity in the cells appeared to be the most desirable method for circumventing degradation of susceptible plasmids during transformation. We had already observed that mutagenesis by way of ectopic integration during transformation is a highly effective method for generating random PR-6 mutants, as judged by observation of recombinants with altered pigmentation and colony morphology (*10*). This phenomenon was identified in pneumococcus (*11*) and its mechanism has also been studied in that organism (*12–14*). Our strategy is a variation of the procedures reported for pneumococcus. It involves ligating random fragments of total PR-6 DNA, generated by partial

Table 1. Relation of plasmid transformation efficiencies to their number of Ava I or Aqu I sites for PR-6 ectopic recombinants. These were obtained by a plasmid transformation protocol that is a slight variation of the procedure described earlier (*6*). A PR-6 liquid culture was grown to 20 ± 2 percent transmittance (about 4×10^7 cells per milliliter; late log phase), measured at 550 nm, in medium A (*34*). A 0.1 volume of plasmid DNA was added to 0.9 volume of competent cells (at least 1 to 2 μg/ml for maximum levels). This transformation mixture was incubated for 60 to 90 minutes at 39°C with light and CO_2. Dilutions in medium A were plated on the surface of medium A agar plates with 2.5 ml of 0.8 percent agar used per plate. Expression for 40 to 48 hours was carried out at 30° ± 2°C with reduced illumination (plates were covered with a single sheet of typing paper). Platings were challenged with Ap (2 μg/ml) or Km (200 μg/ml) by overlaying them with 2 ml of 0.6 percent agar containing the antibiotic. Transformed colonies appeared after about 4 days of incubation (no paper covering). CFU, colony-forming unit.

Plasmid	Number of Aqu I sites	PR-6 strain	Km^r transformants per CFU
pAQE19	0	A0	9.3×10^{-4}
pAQE19	0	G23	2.0×10^{-3}
pAQE19	0	G38	9.9×10^{-4}
pAQE17	1	A0	9.2×10^{-4}
pAQE17	1	G23	1.1×10^{-3}
pAQE17	1	G38	7.6×10^{-4}
pAQE17L	5	A0	1.2×10^{-5}
pAQE17L	5	G23	5.4×10^{-5}
pAQE17L	5	G38	4.1×10^{-5}
pAQE7	6	A0	$<2.9 \times 10^{-8}$
pAQE7	6	G23	2.6×10^{-6}
pAQE7	6	G38	1.1×10^{-6}

digestion with Sau 3A, to the Tnl Ap^r gene fragment obtained by digestion of pDS1106 (*15*) with Bam HI and Pvu II. This produced DNA molecules that were initially linear and incapable of either ready circularization or autonomous replication. It was hoped that integration of these Ap^r molecules into the PR-6 genome in the region of their homology would cause either insertional (*13, 14*) or deletional (*12*) inactivation of the Aqu I restriction endonuclese gene by any one of a number of possible mechanisms. Similar techniques have been employed for generating specific mutations in *Bacillus subtilis* (*16, 17*), *Saccharomyces cerevisiae* (*18*), and *Dictyostelium discoideum* (*19*), as well as in pneumococcus (*12, 13*). Integration of a drug resistance gene inserted into the host genome in vitro has also been demonstrated in the closely related cyanobacterium *Anacystis nidulans* R2, although it was not used to produce mutations directly (*20*).

A pool of Ap^r recombinants selected in liquid culture (ampicillin, 10 μg/ml) after transformation of wild-type PR-6 with the above ligation mixture was subsequently transformed with Aqu I–sensitive pAQE17DL. Several hundred Km^r transformants were obtained in this manner. Transformed colonies were overlayed with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Sigma) in 0.6 percent agar to 40 μg/ml, and the appearance of blue coloration was sought. Although difficult to discern against the dark green background, some colonies appeared to be slightly blue in color. This was confirmed by exposing replicas of the colonies to chloroform vapor for 30 minutes and then overlaying

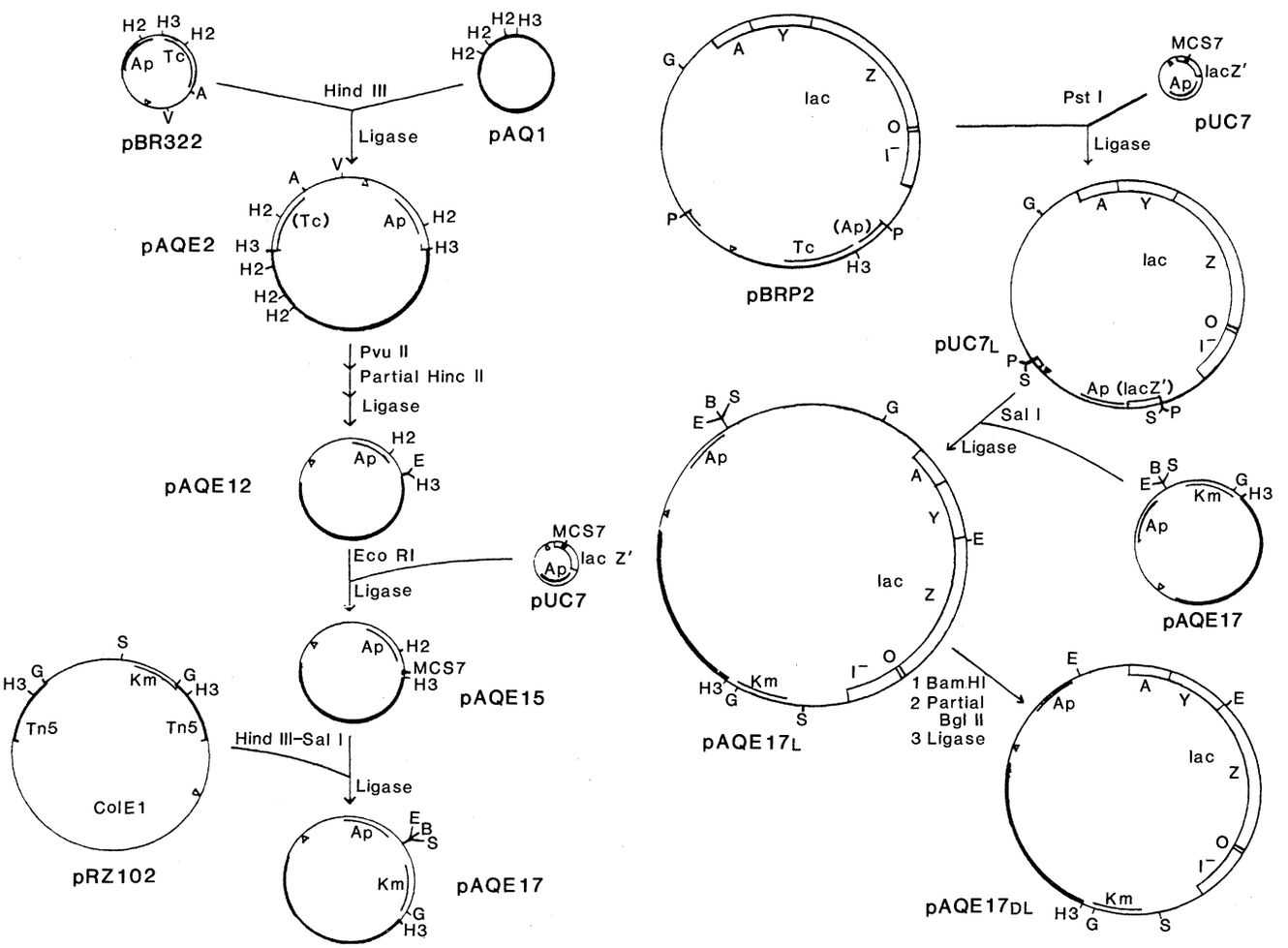

Fig. 1 (left). Development of biphasic plasmid shuttle vectors for use in PR-6. The smallest cryptic PR-6 plasmid, termed pAQ1 (*6*) 4.6 kilobase pairs, was purified and joined with pBR322 (*29*) at their unique Hind III sites in the construction of the first series of biphasic plasmids, represented by pAQE2 (8.9 kb). These plasmids were Tc^s (tetracycline-sensitive) and Ap^r. The Ava I restriction site, sequences of the nonfunctional Tc^r gene, and about 1.0 kb of pAQ1 DNA were eliminated by Pvu II–partial Hinc II digestion and religation to create pAQE12 (5.9 kb). Cloning the 50–base pair restriction site polylinker from pUC7 (*30*), MCS7, into the Eco RI site on pAQE12 effectively provided the new vector pAQE15 (5.9 kb) with at least five unique, available cloning sites (Hind III, Eco RI, Bam HI, Sal I, and Acc I). The NPT II gene, encoding Km^r and NM^r was cloned from pRZ102 (*8*) into pAQE15 as diagramed. The resulting plasmid, pAQE17 (7.3 kb), had at least eight unique, available cloning sites: Hind III, Eco RI, Bam HI, Sal I, Acc I, Ava I, Sma I, and Bgl II. The lysis by alkali procedure (*31*) was used for plasmid isolations from both *E. coli* and PR-6. Restriction enzymes and T4 ligase were obtained from Bethesda Research Laboratories and used under the recommended conditions. Restriction sites: H3, Hind III; H2, Hinc II; A, Ava I or Aqu I; B, Bam HI; E, Eco RI; S, Sal I; G, Bgl II; V, Pvu II; and P, Pst I. Heavier lines indicate pAQ1 sequences, and small triangles show ColE1 replication origins. Fig. 2 (right). Cloning of the *E. coli lac* operon into a PR-6 shuttle vector. The initial Pst I–*lac* clone in pBR322, pBRP2 (15.3 kb), was identical to pBRP1 (*32*) except that it had been converted to *lacI*$^-$ by homogenization in a *lacI3* strain of *E. coli* (KL695) (*33*). The Pst I–*lac* fragment from pBRP2 was cloned into the Pst I site in the center of the symmetrical, multiple-cloning site polylinker MSC7 on pUC7. The fragment could then be cut out with Sal I or Bam HI as well as Pst I, making possible the use of a unique cloning site on pAQE17. After the *lac* operon was cloned into the pAQE17 Sal I site, the resulting plasmid (pAQE17L; 18.3 kb) was subjected to Bam HI–partial Bgl II digestion (followed by religation) to remove about 3 kb of excess, nonessential DNA and reduce the plasmid's size, thereby increasing its potential transformation efficiency. The new plasmid, pAQE17DL (15.3 kb), was prepared in the recombination-proficient Δ(*lac*) *E. coli* strain, RDP244, to obtain a multimeric mixture, further enhancing its potential transformation efficiency (*6*).

them with 0.6 percent agar containing lysozyme at a final concentration of 100 μg/ml and either X-gal at a final concentration of 40 μg/ml or *o*-nitrophenyl-β-D-galactopyranoside (ONPG) (Sigma) at a final concentration of 120 μg/ml. Both of these techniques produced a much more definitive coloration than did intact cells, with the blue color produced from X-gal being more persistent than the yellow from ONPG. Three of these transformants (G23, G25, and G38) actually expressed β-galactosidase activity with this procedure.

Six hundred of the Km^r transformants from the same pool of random ectopic mutants were also tested by colony hybridization (*21*), with 12 showing hybridization to a ^{32}P-labeled M13mp8 probe, which carries an 800–base pair fragment from the *lac* operon (*22*). One of these (D52) was tested for β-galactosidase activity and found to elicit a positive response. Plasmid DNA, extracted (*23*) from this transformant and from two of the Lac^+ transformants selected visually (G25 and G38), contained intact pAQE17DL after transformation back into *E. coli* (*24*). It is possible that some or all of these mutants are siblings, but we have carried two of them through further experimentation for comparison purposes.

The Lac^+ transformants were cured of pAQE17DL by streaking and gridding on nonselective A medium and replica plating for Km^r. The segregants obtained were retransformed to test for enhanced transformation efficiency of Aqu I–sensitive plasmids. A randomly selected Ap^r ectopic recombinant, A0, was used as a control for these experiments, since the presence of the Ap^r fragment in the PR-6 chromosome, homologous with the Ap^r gene on the pBR322-derived vectors, approximately doubled overall transformation efficiency by itself. Kanamycin-sensitive segregants of G23 and G38 were transformed with a series of biphasic plasmids containing up to six Aqu I recognition sites, and their transformation efficiencies were compared to that of A0. The results are shown in Table 1. Both mutants were transformed about five times as frequently with pAQE17L (Fig. 2), as was A0. Using the permeabilization method described above, we detected β-galactosidase activity in 50 percent of the G23 transformants, 73 percent of the G38 transformants, and none of the A0 transformants. Likewise, G38 and G23 were transformed at least 40 times as frequently with the pAQE7 construct, as was A0, and 90 percent of these transformants contained the intact plasmid. Protein extracts (*7*) from G38 and G23 still contained Aqu I endonuclease activity at roughly the same level as that in A0, accounting for the fraction of transformants in which Aqu I had apparently degraded the susceptible plasmids. The percentage of deletions was lower among the pAQE7 transformants, possibly because three of the Aqu I restriction sites are in the NPT I gene (*9*) that was directly selected.

There was very little increase in the transformation frequency of G38 or G23 over that of A0 for plasmids with one (pAQE17) or with no Aqu I recognition sites [pAQE17 with Ava I site removed (pAQE19)] (*25*) (Table 1). Nonetheless, the increased transformation frequencies observed in G38 and G23 appeared to be caused by some change in the Aqu I restriction modification system and not by a generalized transformation enhancement effect. Although it might be argued that these mutants simply exhibit higher transformation efficiencies only with the larger plasmids (pAQE17L and pAQE7), the decrease in relative frequency with which deletions of the larger plasmids were seen in the mutants as opposed to those seen in the control argues against this possibility. Specifically, none of the pAQE17L transformants of A0 were Lac^+, whereas significant fractions of the G23 and G38 transformants were. One explanation for these data relies on the properties of similar recombinants in *B. subtilis* (*16, 17, 26*), pneumococcus (*13, 14*), and *A. nidulans* (*20*). They often have a duplication of the homologous fragment of DNA flanking the nonhomologous inserted fragment. If this also occurs in PR-6, duplication of the entire functional Aqu I methylase gene could have enhanced the cell's ability to protect incoming DNA from the endonuclease without eliminating the restriction activity. This possibility is under investigation.

β-Galactosidase activity in the PR-6 mutants transformed with pAQE17DL was determined by use of a whole cell extract assay developed for *E. coli* (*27*). One enzyme unit equals the amount of enzyme that hydrolyzes 1 nmol of ONPG in 1 minute at 28°C. For this assay, PR-6 was grown in liquid culture to the same density as for transformations in the presence of kanamycin (40 μg/ml). The absorbance of the hydrolyzed ONPG in the PR-6 samples was determined at 420 nm. An identically treated control strain without pAQE17DL, grown to the same density, was used as a blank, since chlorophyll a has a significant absorbance band at this wavelength. In both G23 and G38, pAQE17DL produced 5.0×10^{-7} enzyme units per colony-forming units. The same plasmid produced 8.4×10^{-7} enzyme units per colony-forming unit in *E. coli* KL791 (*28*). The plasmid used carries a *lacI3* mutation, and we have not investigated either *lac* inducibility or possible catabolite repression effects in PR-6.

These ectopic mutants of PR-6 greatly increase our ability to introduce cloned genes into the cyanobacterium by biphasic plasmid transformation. We are also constructing promoterless *lacZ*-fusion plasmid vectors, which will allow us to study the regulation of PR-6 gene expression in response to varying growth conditions. Although we have not yet investigated the possibility of *lac* inducibility in PR-6, the results of this study open up the possibility for expression of other foreign gene products, under control of the *lac* promoter, in this photosynthetic prokaryote.

References and Notes

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35. Supported by NIH grant GM 28609 and by the Agricultural Experiment Station of Pennsylvania State University.

25 March 1985; accepted 19 September 1985