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Alteration of a Cyanobacterial Hybrid Vector

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Introduction

The cyanobacteria are prokaryotes capable of performing oxygenic photosynthesis in a manner similarly observed in the chloroplasts of higher plants. Their retention of many important bacterial properties make them amenable to genetic studies. Some of these properties include: single-cell organization, single small genome, rapid generation time, transformation ability, relatively simple production of mutants, and existence of innate plasmids useful as cloning vectors. Thus, cyanobacteria are a simple model system for investigating eukaryotic-type photosynthesis (Stanier and Cohen-Bazire, 1977).

The experimental organism, *Anacystis nidulans* R2, has been well characterized biochemically and biophysically (Olson et al., 1971; Pakrasi, et al., 1985). *A. nidulans* R2 has two indigenous plasmids, an 8 kb plasmid (pUH24) and a 50 kb plasmid (pUH25) (van den Hondel et al., 1979). The smaller cyanobacterial plasmid has successfully been cloned into bacterial plasmids to create hybrid vectors that can be steadily maintained and autonomously replicated in both organisms (Golden and Sherman 1983; Kuhlemier et al. 1981, 1983; Sherman et al. 1984).

In our lab, cyanobacterial hybrid cloning vectors have been constructed for two purposes. They can be used to introduce specifically altered genes into an organism for the purposes of creating photosynthesis mutants which may be analyzed to clarify structural and functional relationships between the polypeptides involved in photosynthesis. Alternatively, they may be used to increase the expression of inserted DNA.

One such vector, pPGV5, constructed by Vann et al., 1986, contains the entire indigenous plasmid, pUH24, of *A. nidulans* ligated to plasmid pLC28 of *Escherichia coli* (Remaut et al., 1981). Plasmid pUH24 is 8.0 kb in size and contains a single *Bam*HI cloning site. Plasmid pLC28 is 2.8 kb and contains the powerful leftward promoter of the bacteriophage lambda, which allows for increased expression of a gene cloned downstream of the promoter. It also contains a gene coding for ampicillin resistance and a single *Bam*H1 cloning site. The hybrid plasmid vector constructed by the ligation of the two *Bam*HI digested plasmids is therefore 10.8 kb and has two *Bam*HI sites (Figure 1).

Plasmid pPGV5 transforms *A. nidulans* efficiently, yielding 2.7 x 10^7 transformants per microgram (Vann et al., 1986). This vector was also used to obtain some increased expression of a 36 kDa polypeptide involved in iron regulation in iron deficient medium (Vann et al. 1986).

Although plasmid pPGV5 showed promise of allowing enhanced expression of cloned genes, these experiments were postponed until the plasmid could be altered to create a more efficient cloning vector. It was altered by removing approximately half of the cyanobacterial sequences to produce a smaller vector capable of more efficient transformations in *E. coli*. The *Bam*HI cloning site most distant from the lambda phage promoter was also removed to create a unique cloning site. The altered cyanobacterial hybrid vector, pTNTV, which is described here is 6.8 kb and contains a single *Bam*HI cloning site conveniently located downstream of the lambda phage promoter.

Materials and Methods

Construction of plasmid pTNTV is depicted in Figure 1. In order to construct this plasmid, two restriction endonuclease digestions were needed: a complete digestion with endonuclease *Xho*I was required to insure that all molecules were cut at

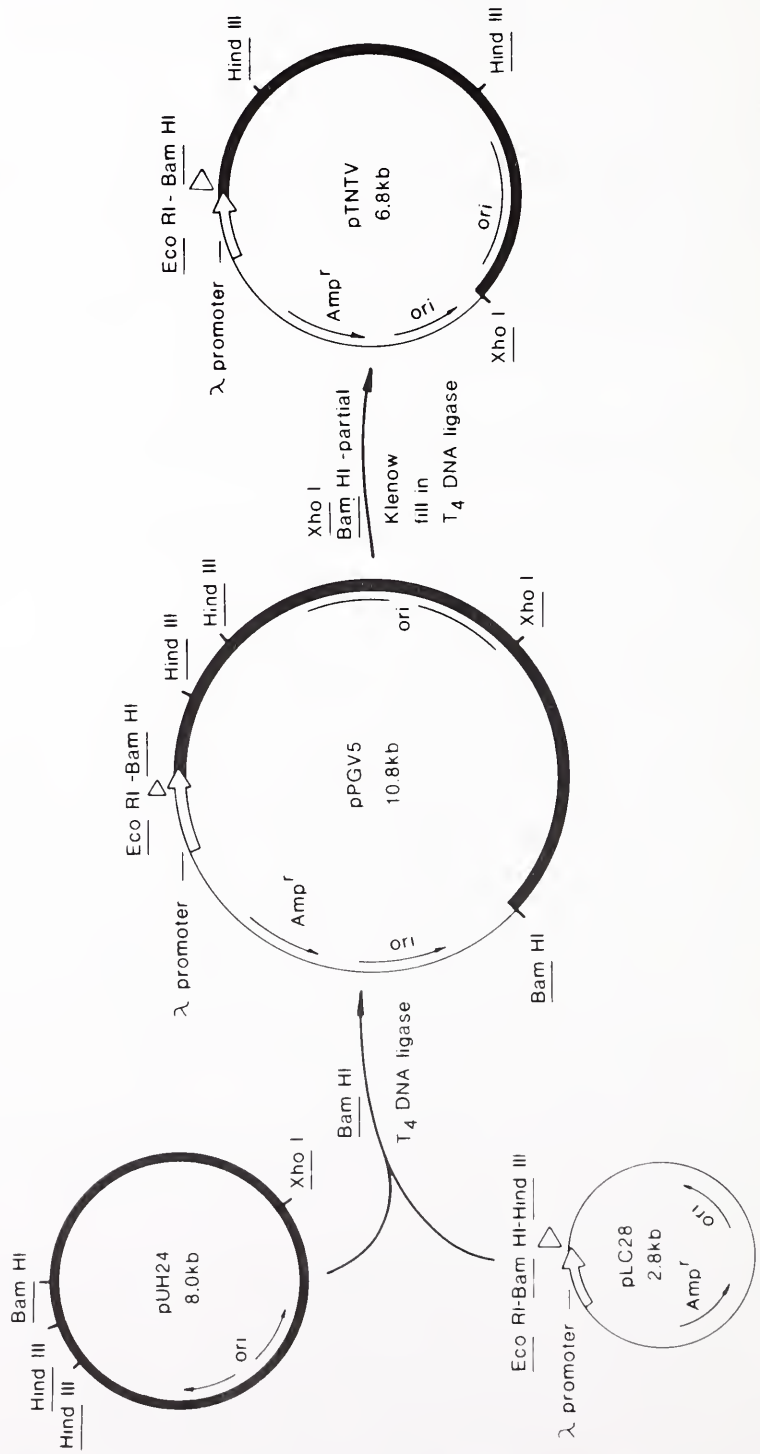

FIGURE 1. Construction of plasmid pTNTV.

this site and a partial digestion with *Bam*HI which would result in some molecules cut only at the *Bam*HI site most distant from the lambda promoter.

First, plasmid PGV5 was first digested with the restriction endonuclease *Xho*I. The digestion was carried out in four separate reaction tubes each containing 3 ug of plasmid DNA in a 15 ul solution of 25 mM Tris-HCL (pH 7.8), 50 mM NaCl, 10 mM $MgCl_2$, 100 ug/ml BSA, 2mM beta-mercaptoethanol, and 5 units of *Xho*I. After incubating at 37 C for 1 hour, the partial digestions with *Bam*HI were performed. The salt concentrations in tubes 1-4 were adjusted to 100 mM NaCl and 5 units of *Bam*HI was added to tube 1. The concentration of *Bam*HI in tube 1 was thus 1.6 units/ug DNA. Serial dilutions of the endonuclease were made by removing 5 ul of the reaction mixture from tube 1 and tube 2. The enzyme concentration in tube 2 had then been diluted to 0.41 units/ug DNA. This fourfold serial dilution was continued through tube 4, transfering 5 ul each time. Digests were incubated 15 minutes at 37 C. The digests were stopped by the addition of phenol. A phenol/chloroform extraction was performed followed by an ethanol precipitation.

Each sample was resuspended in 15 ul of 10 mM Tris-HCL, 1 MM EDTA (pH 8.0) and analyzed by gel electrophores through a 0.7% agarose gel. It was determined that the digestion conditions in tubes 3 and 4 were optimal, yielding a large number of molecules cut twice. These tubes were then pooled and the Klenow fragment from *E. coli* DNA Polymerse I was used to fill-in the restriction enzyme-induced 3' recessed ends. The reaction was carried out using 2 ug of the restriction fragment in a 25 ul solution containing a 2 mM mixture of all four deoxynucleotides, 0.5 M Tris-HCl (pH 7.2), 0.1 M $MgSO_4$, 1 mM dithiothreitol, 100 ug/ml BSA, and 2 units of Klenow. This reaction was incubated at 22 C for 15-30 minutes.

A ligation of the blunt end fragment was then carried out in the same reaction mixture with the addition of 2 units of T4 DNA ligase. After an additional 6 hour incubation at 22 C, the ligation volume was increased to 100 ul with 10 mM Tris, 1 mM EDTA pH 7.6.

Thirty microliters of the ligation mixture was used to transform *E. coli* HB101 using standard techniques (Maniatis 1982). Plasmid DNA was isolated from transformants (Maniatis 1982), and confirmation of plasmid TNTV was obtained via restriction analyses using *Eco* RI, *Hin*dIII and *Bam*HI.

Results and Discussion

The new hybrid cloning vector, pTNTV, was constructed as described above. In Figure 2 it can be seen that this vector is smaller (approximately 6.8 kb) than pPGV5 (10.8 kb) and that pTNTV contains only one *Bam*HI restriction site as compared to pPGV5 which has two. Digestions with *Hin*dIII proved that the new vector retains the cyanobacterial fragment which carries the origin of replication (data not shown). Experiments are in progress to determine if pTNTV will retain the high level of transformation efficiency observed with plasmid pPGV5.

There are several ways that the new vector, pTNTV, can be beneficial. It can be used to explore the effects of enhanced expression of specific wild-type and mutant photosynthesis genes. This is a useful approach for elucidating the possible functions, reactions, and structural relationships of particular proteins involved in the photosynthetic process. The vector may also be used to examine high levels of expression of heterologous genes such as those from higher plants.

Currently we are initiating a project to examine the use of this expression vector as a means to control malaria which is transmitted to humans by the bite of *Anopheles* mosquitoes (dipteran insects). *Bacillus thuringiensis* is a spore-forming bacterium that produces protein inclusions that are lethal to select groups of insects. We have obtained a gene from a strain of *B. thuringiensis* which encodes a dipteran-specific

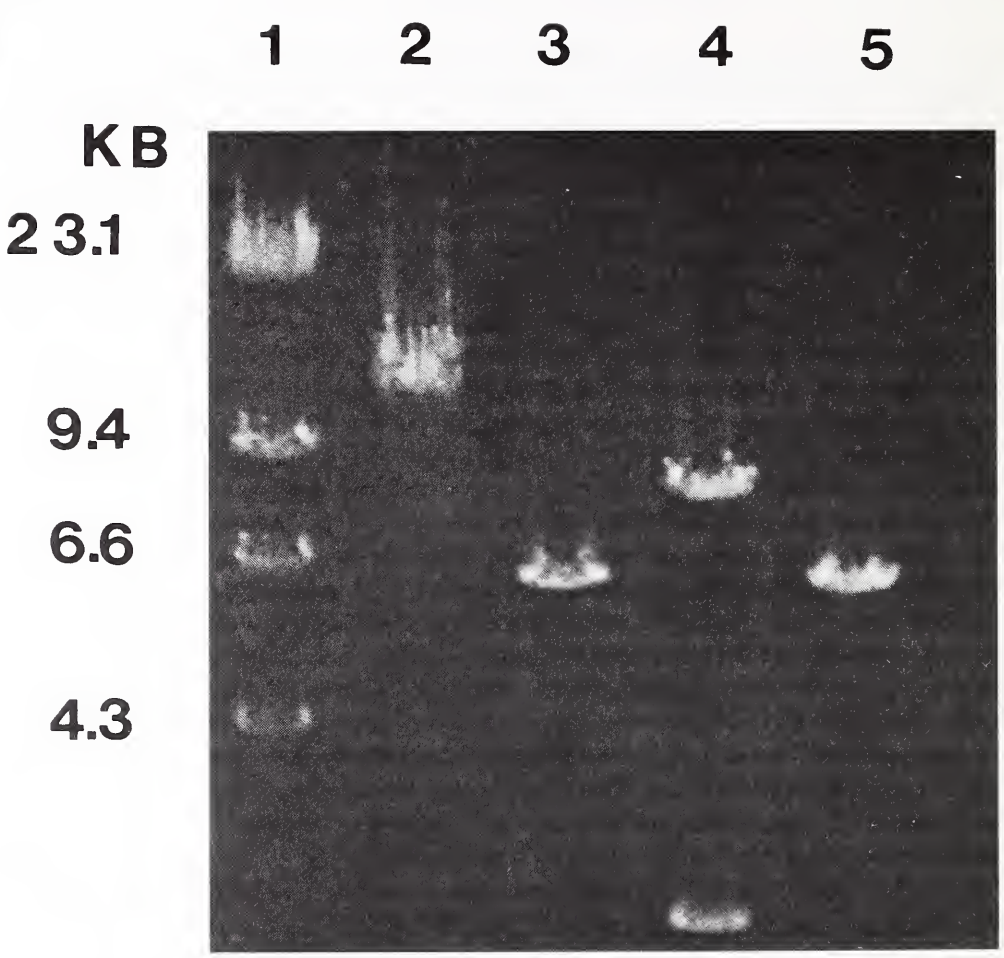

FIGURE 2. Agarose gel electrophoresis demonstrating the construction of plasmid pTNTV. Lane 1 contains the molecular weight markers, bacteriophage lambda digested with endonuclease *Hin*dIII. Lanes 2 and 4 contain plasmid pPGV5 digested with *Eco*RI and *Bam*HI, respectively. Lanes 3 and 5 contain plasmid pTNTV digested with *Eco*RI and *Bam*HI, respectively.

insecticidal protein. We are currently cloning this gene into pTNTV and will then transform *A. nidulans*. If the cyanobacteria survive and a high level of toxin expression is observed, laboratory studies will be performed in which mosquito larvae will be reared with the transformed cyanobacteria. Thus, a more effective method of control of malaria-carrying mosquitos may be obtained.

In conclusion, a new hybrid vector has been constructed which allows for a high level of expression of a gene cloned downstream of the powerful lambda promoter. The vector has two unique cloning sites downstream of the promoter, *Bam*HI and *Eco*RI. The small size of this vector will allow for efficient transformation into *E. coli* or *A. nidulans* R2. Thus, the vector, pTNTV, may prove to be a powerful tool in investigations of the effects of high levels of expression of homologous or heterologous genes in cyanobacteria.

Acknowledgment

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Literature Cited

1. Golden, S. S. and Sherman, L. A., 1983. A hybrid plasmid is a stable cloning vector for the cyanobacterium *Anacystis nidulans* R2. J. Bacteriol. 155: 966-972.
2. Kuhlemeir, C. J., Borrias, W. E., van den Hondel, C. A. M. J. J., Van Arkel, G. A., 1981. Vectors for cloning in cyanobacteria: construction and characterization of two recombinant plasmids capable of transformation to *Escherichia coli* K12 and *Anacystis nidulans* R2. Mol. Gen. Genet. 184: 249-254.
3. Maniatis, T., Fritsch, E. F., Sambrook, J., 1982. Molecular Cloning, a Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor Press, New York.
4. Olson, J. M. and Thornber, J. P., 1979. Photosynthetic Reaction Centers. In Membrane Proteins, Vol 2, Membrane Proteins in Energy Transduction. R. A. Capaldi, ed. pp. 170-340. Marcel Dekker, New York.
5. Pakrasi, H., Reithman, H. C., and Sherman, L. A., 1985. Organization of pigment-proteins in the photosystem II complex of the cyanobacterium, *Anacystis nidulans* R2. Proc. Nat. Acad. Sci. USA 82: 6903-6907.
6. Remaut, E., Stanssens, P., and Fiers, W., 1981. Plasmid vectors for high-efficiency expression controlled by the P_L promoter of coliphage lambda. Gene 15: 81-93.
7. Sherman, L. A., Golden, S. S., and Vann, C., 1984. Transformation and cloning vectors of the cyanobacterium *Anacystis nidulans*. In Biosynthesis of the Photosynthetic Apparatus: Molecular Biology, Development and Regulation. pp. 357-379. Alan R. Liss, Inc.
8. Stanier, R. Y. and Cohen-Bazier, G., 1977. Phototrophic prokaryotes: the cyanobacteria. Annu. Rev. Microbiol. 31: 225-274.
9. van den Hondel, C. A. M. J. J., Keegstra, W., Borrias, W. E. and Van Arkel, G. A., 1971. Homology of plasmids in strains of unicellular cyanobacteria. Plasmid 2: 323-333.
10. Vann, C. N., Playl, L. A., and Sherman, L. A., 1986. The construction and use of hybrid cloning vectors in *Anacystis nidulans* R2. Advances in Photosynthesis Research. Proceedings of the VIIth International Congress on Photosynthesis, Brown University, Providence, RI.