[1] Transposon Mutagenesis of Heterocyst-Forming Filamentous Cyanobacteria

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Introduction

The vegetative cells of certain filamentous cyanobacteria can differentiate morphologically and functionally into one or more of the following: heterocysts, the sites of nitrogen fixation under aerobic conditions; akinetes, also known as spores; and hormogonia, gliding filaments comprised exclusively of small vegetative cells. Photosynthetic oxygen evolution and carbon dioxide fixation are reduced in hormogonia compared to their rates in vegetative cells,[1] and are suppressed completely in heterocysts.[2] Facultatively heterotrophic heterocyst-forming cyanobacteria such as *Nostoc punctiforme* strain ATCC 29133 (PCC 73102; hereinafter referred to as *N. punctiforme*) provide an experimental system for study of the regulation of expression of genes that encode components of the photosynthetic apparatus.

Unicellular and filamentous cyanobacteria establish symbiotic associations with a variety of eukaryotic protists, metazoans, and plants. *N. punctiforme*, for example, was isolated from a symbiotic association with the cycad *Macrozamia* sp.[3] When the eukaryotic partner is heterotrophic, the symbiotic cyanobacterium functions to supply reduced carbon; but when *Nostoc* spp. are in symbiotic association with the bryophyte *Anthoceros punctatus* and other plants, cyanobacterial photosynthetic CO_2 fixation is suppressed and activity of cyanobacterial ribulose-bisphosphate carboxylase/oxygenase is inhibited,[4] presumably facilitating the establishment and stable maintenance of the symbiotic association. Because *N. punctiforme* can grow heterotrophically, mutants of it that are affected in photosynthesis should be isolable. In particular, placement of reporter operons within photosynthetically active genes that are regulated during symbiosis could help to elucidate key features of the symbiotic interaction.

[1] E. L. Campbell and J. C. Meeks, *Appl. Environ. Microbiol.* **55,** 125 (1989).
[2] C. P. Wolk, A. Ernst, and J. Elhai, *in* "The Molecular Biology of Cyanobacteria" (D. A. Bryant, ed.), p. 769. Kluwer Academic Publishers, Dordrecht, 1994.
[3] R. Rippka and M. Herdman, "Pasteur Collection of Cyanobacterial Strains in Axenic Culture, Vol. I: Catalogue of Strains." Institut Pasteur, Paris, 1992.
[4] N. A. Steinberg and J. C. Meeks, *J. Bacteriol.* **171,** 6227 (1989).

As is true also of other diazotrophic cyanobacteria, *N. punctiforme* supplies reduced nitrogen to its symbiotic partner.

Both *Anabaena* (*Nostoc*) sp. strain PCC 7120 (hereinafter referred to as PCC 7120) and *N. punctiforme* grow rapidly and homogeneously in liquid medium, and form visible colonies on plates within 4–7 days after plating. Whereas both strains form heterocysts, only *N. punctiforme* has been found to form akinetes and hormogonial filaments, to grow heterotrophically,[5] and to establish a symbiotic association[6]; however, unlike in PCC 7120,[7,8] mutations of *N. punctiforme* have not been complemented with a cosmid library.[9]

Adjacent cells of filamentous cyanobacteria share an end wall, and therefore are strongly linked, and (at least, on average) each of the cells has multiple chromosomal equivalents. As a consequence, mutant copies of the genome segregate only with difficulty. Selection for antibiotic resistance that results from the presence of a transposon enhances segregation, and antibiotic-resistant colonies that are to be screened for phenotypes of interest are necessarily mutant in some way. Therefore, transposition, relative to other means of mutagenesis, facilitates the isolation of null mutants, and can mark the sites of mutation by the antibiotic resistance gene(s) of the transposon. The conjugal transfer of transposons from *Escherichia coli* has recently been used to generate a variety of mutants that affect heterocyst development[10–14] and symbiotic competence,[6] principally in the cyanobacteria PCC 7120 and *N. punctiforme*. In the two strains mentioned, there is a high probability that the marker and the mutation are coupled. Thus, transposons provide an effective means for identifying genes that correspond to mutant phenotypes. In addition, transposons can stabilize mutations, facilitate mapping[10,15] and—in combination with reporter genes—can directly aid analysis of the transcription of the genes that have been interrupted.

[5] M. L. Summers, J. G. Wallis, E. L. Campbell, and J. C. Meeks, *J. Bacteriol.* **177,** 6184 (1995).
[6] M. F. Cohen, J. G. Wallis, E. L. Campbell, and J. C. Meeks, *Microbiol.* **140,** 3233 (1994).
[7] C. P. Wolk, Y. Cai, L. Cardemil, E. Flores, B. Hohn, M. Murry, G. Schmetterer, B. Schrautemeier, and R. Wilson, *J. Bacteriol.* **170,** 1239 (1988).
[8] W. J. Buikema and R. Haselkorn, *J. Bacteriol.* **173,** 1879 (1991).
[9] J. G. Wallis, Ph.D. Thesis, University of California, Davis (1993).
[10] C. P. Wolk, Y. Cai, and J.-M. Panoff, *Proc. Natl. Acad. Sci. U.S.A.* **88,** 5355 (1991).
[11] A. Ernst, T. Black, Y. Cai, J.-M. Panoff, D. N. Tiwari, and C. P. Wolk, *J. Bacteriol.* **174,** 6025 (1992).
[12] D. Borthakur and R. Haselkorn, *J. Bacteriol.* **171,** 5759 (1989).
[13] T. A. Black, Y. Cai, and C. P. Wolk, *Mol. Microbiol.* **9,** 77 (1993).
[14] E. L. Campbell, K. D. Hagen, M. F. Cohen, M. S. Summers, and J. C. Meeks, *J. Bacteriol.* **178,** 2037 (1996).
[15] T. Kuritz, A. Ernst, T. A. Black, and C. P. Wolk, *Mol. Microbiol.* **8,** 101 (1993).

Choice of a Transposon

Heterocyst-forming cyanobacteria contain insertion sequences (ISs); at least five such sequences have been characterized at the molecular level, including several families of ISs in PCC 7120.[16–19] These IS elements can move within the host genome, perhaps as a consequence of stress; as they transpose, they induce mutations.[18,20] On spontaneous transposition, endogenous IS elements of *Fremyella diplosiphon* (syn. *Calothrix* sp. strain PCC 7601) have insertionally inactivated genes that are involved in synthesis of components of phycobilisomes; electroporation increases their rate of transposition.[21] However, the regulation of the transposition of these IS elements has not been studied and there is no known selection for mutants other than loss of function. No useful, selectable transposon engineered from a cyanobacterial IS has yet been described. We focus here on the use of transposons derived from heterotrophic bacteria.

Transposon Tn*901* (which confers resistance to ampicillin) was transferred by transformation to the unicellular cyanobacterium *Synechococcus* sp. strain PCC 7942 (syn. *Anacystis nidulans* R2),[22] where it transposed spontaneously to an endogenous plasmid. A second transposition produced a methionine auxotroph.[23] The frequency of transposition, and thus of mutagenesis, was very low.[24] Analysis of sites at which the transposon Tn*5* and derivatives of it insert in PCC 7120[12,15] and *N. punctiforme*[6] shows no apparent site or sequence preference. This property makes Tn*5* an appropriate choice as a mutagen.[25] However, transposition of Tn*5* from the same *Synechococcus* 7942 plasmid was even less frequent than transposition of Tn*901*.[24] In contrast, relatively high frequencies of mutation result from transposition of derivatives of wild-type Tn*5*, when delivered by conjugation from *E. coli* to PCC 7120[10] and to *N. punctiforme*[6] in suicide (nonreplicating) plasmids. (Tn*5*, delivered by conjugal transfer of a nonreplicating

[16] I. Bancroft and C. P. Wolk, *J. Bacteriol.* **171,** 5949 (1989).
[17] Y. Cai, *J. Bacteriol.* **173,** 5771 (1991).
[18] J. Alam, J. M. Vrba, Y. Cai, J. A. Martin, L. J. Weislo, and S. E. Curtis, *J. Bacteriol.* **173,** 5778 (1991).
[19] D. Mazel, C. Bernard, R. Schwarz, A. M. Castets, J. Houmard, and N. Tandeau de Marsac, *Mol. Microbiol.* **5,** 2165 (1991).
[20] Y. Cai and C. P. Wolk, *J. Bacteriol.* **172,** 3138 (1990).
[21] B. U. Bruns, W. R. Briggs, and A. R. Grossman, *J. Bacteriol.* **171,** 901 (1989).
[22] C. A. M. J. J. van den Hondel, S. Verbeek, A. van der Ende, P. J. Weisbeek, W. E. Borrias, and G. A. van Arkel, *Proc. Natl. Acad. Sci. U.S.A.* **77,** 1570 (1980).
[23] N. Tandeau de Marsac, W. E. Borrias, C. J. Kuhlemeier, A. M. Castets, G. A. van Arkel, and C. A. M. J. J. van den Hondel, *Gene* **20,** 111 (1982).
[24] C. J. Kuhlemeier and G. A. van Arkel, *Methods Enzymol.* **153,** 199 (1987).
[25] D. E. Berg, *in* "Mobile DNA" (D. E. Berg and M. M. Howe, eds.), p. 185. American Society for Microbiology, Washington, D.C., 1989.

TABLE I
TRANSPOSONS DERIVED FROM TN*5* CITED IN THIS CHAPTER[a]

Transposon	Size (bp)	Resistance markers	Promoter for antibiotic operon	Factor-independent terminator	Reporter gene	*oriV*	Reference
Tn*5*-764	4145	Km/Nm	*psbA*	Present	None	p15A	29, this chapter
Tn*5*-765	6572	Km/Nm	*psbA*	Present	*V.f. luxAB*	p15A	29, this chapter
Tn*5*-770	4948	CmEm	Native	Present	None	p15A	This chapter
Tn*5*-771	7375	CmEm	Native	Present	*V.f. luxAB*	p15A	This chapter
Tn*5*-800	5000	CmEm	Native	None	None	pMB1	This chapter
Tn*5*-801	7427	CmEm	Native	None	*V.f. luxAB*	pMB1	This chapter
Tn*5*-1058	5395	Km/NmBmSm	*psbA*	Present	None	p15A	10
Tn*5*-1062	7832	Km/NmBmSm	*psbA*	Present	*V.h. luxAB*	p15A	This chapter
Tn*5*-1063	7834	Km/NmBmSm	*psbA*	Present	*V.f. luxAB*	p15A	10
Tn*5*-1065	8117	Km/NmBmSm	*psbA*	Present	T7 gene 1	p15A	30
Tn*5*-1087b	5331	CmEm	Native	None	None	pMB1	11
Tn*5*-1088a,b	7251	CmEmSmSp	Native	None	None	pMB1	11
Tn*5*-1140	8910	CmEm	Native	None	*lacZ*	pMB1	This chapter

[a] For reasons presented in Ref. 31, we do not recommend, for routine reporting, further derivatives described therein that contain entire *lux* operons as a reporter. The geneology of the transposons listed above is as follows: Tn*5*-1058→Tn*5*-764[→(Tn*5*-765, Tn*5*-770→Tn*5*-771)], Tn*5*-1062, Tn*5*-1063, Tn*5*-1065, Tn*5*-1087b[→Tn*5*-800→(Tn*5*-801, Tn*5*-1140)], Tn*5*-1088a,b.

plasmid, has also been reported to have integrated into the genomes of a marine filamentous *Pseudanabaena* sp. and two unicellular cyanobacteria[26]; unfortunately, it was not verified in those cases that Tn*5* had actually transposed; see below.) As in *E. coli* and other bacteria examined,[25] a 9-bp direct repeat of target sequences is generated on insertion, although 8-bp repeats are occasionally generated.[27,28]

We recommend the use of Tn*5*-based transposon Tn*5*-1058 or three groups of derivatives of it (Table I). These transposons have several advantages relative to wild-type Tn*5*. First, an *oriV* from the medium-copy-number plasmid p15A (the same *oriV* that functions in pACYC177[32]) is engineered into many of these derivatives to facilitate the recovery in *E. coli* of the transposon together with flanking genomic sequences from

[26] K. Sode, M. Tatara, H. Takeyama, J. G. Burgess, and T. Matsunaga, *Appl. Microbiol. Biotechnol.* **37,** 369 (1992).
[27] F. Fernández-Piñas, F. Leganés, and C. P. Wolk, *J. Bacteriol.* **176,** 5277 (1994).
[28] Y. Cai and A. N. Glazer (1996).
[29] Y. Cai and C. P. Wolk, *J. Bacteriol.* **179,** 258 (1997).
[30] C. P. Wolk, J. Elhai, T. Kuritz, and D. Holland, *Mol. Microbiol.* **7,** 441 (1993).
[31] F. Fernández-Piñas and C. P. Wolk, *Gene* **150,** 169 (1994).
[32] A. C. Y. Chang and S. N. Cohen, *J. Bacteriol.* **134,** 1141 (1978).

resultant mutants.[10] Second, relative to wild-type Tn*5*, these derivatives have an enhanced frequency of transposition. Furthermore, in those that retain the Tn*5* determinants for resistance to the antibiotics neomycin (Nm) and kanamycin (Km), bleomycin (Bm), and streptomycin (Sm), the resistances have been enhanced by replacement of the wild-type promoter with the *psbA* promoter from the chloroplast genome of the higher plant, *Amaranthus hybridus*. The sequence of this promoter approaches the consensus sequence of the eubacterial σ^{70}-family of promoters that yield a high level of transcription in vegetative cells of unicellular and filamentous cyanobacteria.[33] In most of those same derivatives, a *rho*-independent terminator was inserted downstream from the highly expressed antibiotic resistance operon to minimize antisense interference with the expression of the transposase gene. Streptomycin resistance in cells of *E. coli*[34] and PCC 7120[10] was further enhanced by introduction of a 6-bp deletion in the 3′ portion of the Sm^r gene (in Tn*5*-1058, Tn*5*-1063, and certain others).

In one group of derivatives of Tn*5*-1058 (e.g., Tn*5*-770), only the antibiotic resistance genes have been replaced. Use of these transposons may be advantageous, compared with transposons that confer resistance to Nm, when the cyanobacterium to be mutagenized shows high natural resistance to Nm. (Our results also suggest that strains that bear Tn*5*-1058 may derive metabolic nitrogen when exposed to high levels of Nm.[35]) Plasmid pRL764SX[29] (Fig. 1), bearing Tn*5*-764, facilitates the generation of derivatives of Tn*5* to fit specific needs. In this plasmid, unique restriction sites flank the *oriV* (origin of replication in *E. coli*), the *oriT* (origin of conjugal transfer), and the antibiotic resistance segments, allowing facile replacement of these components.

Those in a second group of derivatives of Tn*5*-1058 (see Table I) bear a promoterless reporter gene or operon (e.g., *luxAB* from *Vibrio fischeri* (Tn*5*-765, Tn*5*-771, Tn*5*-1063], or *Vibrio harveyi* [Tn*5*-1062], *lacZ* [Tn*5*-1140], T7 gene 1 [Tn*5*-1065]) very close to the L end of the transposon, enabling formation of transcriptional (not translational) fusions on transposition into the genome. Mutant strains of PCC 7120[2,10,29] bearing the *luxAB*-containing transposons can show environmentally responsive production of light. In Tn*5*-1065 the promoterless gene 1 of coliphage T7 gene 1, encoding RNA polymerase, functions as an amplifier in a reporting cascade.[30] The gene 1 product, induced from a transcriptional fusion, activates transcription of a T7-specific promoter controlling, for example, *luxAB*. Expression of the reporter gene can be enhanced relative to direct promoter

[33] S. E. Curtis and J. A. Martin, this chapter, Ref. 2, p. 613.
[34] P. Mazodier, O. Genilloud, E. Giraud, and F. Gasser, *Mol. Gen. Genet.* **204,** 404 (1986).
[35] Y. Cai (1996).

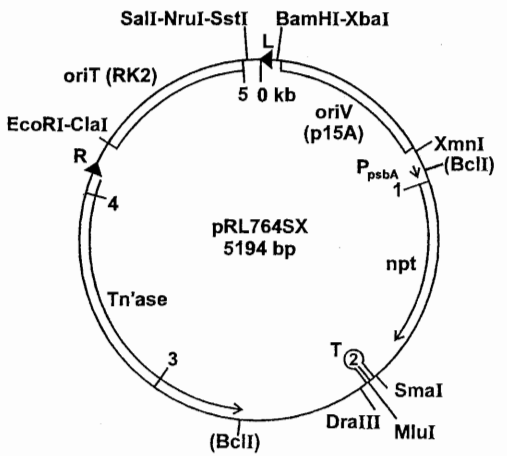

FIG. 1. Simplified schematic of pRL764SX bearing transposon Tn*5*-764. *oriV*(p15A), origin of replication (from plasmid p15A); P_{psbA}, promoter of the *Amaranthus hybridus* gene that encodes the 32-kDa protein of photosystem II; *npt*, gene that encodes neomycin phosphotransferase, which confers resistance to neomycin and kanamycin; T, *rho*-independent terminator of *lpp* gene; Tn'ase, gene (with modified promoter incorporated[36]) that encodes the transposase of transposon Tn*5*; *oriT*(RK2), transfer origin of broad-host-range plasmid RK2; R and L: right and left ends, respectively, of Tn*5*. Unique enzyme sites are shown that permit modular replacement of the *oriV* and the antibiotic-resistance determinant. (The *Xba*I site shown is not unique, but is the only *Xba*I site that can be cut when the plasmid is taken from a *dam*$^+$ strain of *E. coli*.)

reporter fusions.[30] However, high activity of T7 gene 1 appears toxic to PCC 7120 (perhaps its product recognizes some sequence in the genome), so that this system is effective only for genes that are weakly transcribed during growth.

The Tn*5* derivatives (Table I) bear an RK2 *oriT* region on their respective plasmids to allow for conjugal transfer from *E. coli*.[10] They can be introduced into PCC 7120 and *N. punctiforme* by conjugation with *E. coli* and by electroporation, but not by simple transformation. In a final group of derivatives of Tn*5*-1058, a group that includes Tn*5*-800, Tn*5*-801 and Tn*5*-1087b, only the transposase-encoding IS*50*R and the *oriT* are retained as a *Bam*HI-*Bcl*I cassette, easily combined with many other plasmids that provide an *oriV* and a selectable marker.

An advantage of the series of Tn*5* derivatives mentioned that is apparently not shared by wild-type Tn*5*,[37,38] at least in PCC 7120, is the stabilization of mutations. This difference may be attributable to the greater promotion of the antibiotic-resistance operon in the derivative transposons, but may also be attributable in part to greater excision of the wild-type transposon. Excision is stimulated by the presence of extensive inverted repeats at the termini of a transposon.[25] In the derivative transposons of Table I, the lengthy (ca. 1.5-kb) repeat represented by IS*50*L in wild-type Tn*5* has been reduced to less than 0.1 kb. In none of the numerous PCC 7120 mutants studied so far has the Tn*5*-derived transposon been shown to have moved from its original point of insertion.[28]

[36] J. C. P. Yin, M. P. Krebs, and W. S. Reznikoff, *J. Mol. Biol.* **199,** 35 (1988).
[37] J. Liang, L. Scappino, and R. Haselkorn, *Proc. Natl. Acad. Sci. U.S.A.* **89,** 5655 (1992).
[38] J. Liang, L. Scappino, and R. Haselkorn, *J. Bacteriol.* **175,** 1697 (1993).

Further Considerations

The cyanobacterium to be transposon-mutagenized must be in some way transformable, so that the transposon can enter it, the transposon DNA must escape restriction and degradation in the recipient, and the transposase gene and its products must function in the recipient. Preliminary results indicate that *F. diplosiphon,* which is the preferred organism for studies of complementary chromatic adaptation, is susceptible to mutagenesis with certain derivatives of Tn*5*-1058 that lack sites for the restriction endonuclease *Sph*I.[39] All of ca. 70 exconjugants of *Anabaena variabilis* strain ATCC 29413 that had been mated with *E. coli* bearing Tn*5*-1088a and Tn*5*-1088b were both spectinomycin^r and Em^r, suggesting that the strain may be susceptible to transposon mutagenesis.[40] Two of seven analyzed derivatives of *Nostoc ellipsosporum* that had received pRL1063a (bearing Tn*5*-1063) were products of transposition.[41] In the other five, the following series of events appears to have taken place. Native insertion sequence IS*891*N (whose sequence differs by one bp from the corrected sequence of IS*891* [GenBank accession no. M30792]) transposed into the DNA of incoming plasmid pRL1063a. The resulting plasmid then integrated into the genome via homologous recombination of its copy of IS*891*N with a corresponding element in the genome of *N. ellipsosporum.*[41] Exconjugants that result from such recombinational events (or from the very unlikely possibility that the transposon-bearing plasmid can replicate in the cyanobacterium) rather than from transposition can be identified by the continued presence of the *oriT* region of the plasmid.

PCC 7120 contains at least three type II restriction endonucleases, isoschizomers of *Ava*I, *Ava*II and *Ava*III,[42] that initiate degradation of DNA that has unmethylated sites for those nucleases. Chloramphenicol^r

[39] J. Cobley, personal communication (1996).
[40] C. P. Wolk (1996).
[41] F. Leganés, F. Fernández-Piñas, and C. P. Wolk, *Mol. Microbiol.* **12,** 679 (1994).
[42] T. Thiel, this chapter, Ref. 2, p. 581.

(Cm^r) helper plasmids pRL528 and pRL623, derivatives of pDS4101,[43] carry genes that code for *Ava*I and *Eco*47II methylases that modify sequences recognized by *Ava*I and *Ava*II, respectively; pRL623 also carries the gene that encodes the *Eco*T22I methylase, which confers resistance to *Ava*III. Use of pRL528 or pRL623 in *E. coli* to premethylate the plasmid carrying the transposon is essential for efficient transfer to, and transposition in, PCC 7120.[10,44,45] Plasmids pRL1045 and pRL1124, used with Cm^r transposons such as Tn*5*-1087b, are Km^r derivatives of pACYC177[32] that bear the same methylase genes as pRL528 and pRL623, respectively.[45] No type II restriction endonuclease activity has been detected in *N. punctiforme,* and no beneficial result was observed on premodification of the transposon-bearing plasmid with pRL528. Although we routinely transfer replicating plasmids to *N. punctiforme* by electroporation,[5,14] attempted transfer of Tn*5*-1063 by electroporation of pRL1063a has yielded no transposition events.[46]

Protocol for Mutagenesis

Conjugation

The triparental mating procedures used to transposon-mutagenize *N. punctiforme*[6] differ from those devised for PCC 7120,[10,12,44,47] illustrating the possible need to optimize conditions when developing a transposition protocol for yet another strain. The triparental system consists of a (cyanobacterial) recipient strain, an *E. coli* donor conjugal strain carrying RP4 (a broad host range conjugal plasmid) or a closely related plasmid,[44] and an *E. coli* donor cargo strain carrying the plasmid to be transferred (in this case the plasmid bearing the transposon) and (if necessary for mobilization and/or modification) a helper plasmid. Unlike the *oriT* site on many pMB1-derived cargo plasmids (pMB1 is a progenitor of pBR322), the RK2 *oriT* in the plasmids carrying the Tn*5*-1058 derivatives requires only the trans-acting factors supplied by the broad host range conjugal plasmid; thus, helper plasmids such as pDS4101 and pRL528 are not needed to mobilize those transposons. However, as mentioned earlier, experiments using PCC 7120 supplement the cargo plasmid in *E. coli* with a methylating plasmid

[43] F. Finnegan and D. Sherratt, *Mol. Gen. Genet.* **185,** 344 (1982).
[44] J. Elhai and C. P. Wolk, *Methods Enzymol.* **167,** 747 (1988).
[45] J. Elhai, A. Vepritskiy, A. M. Muro-Pastor, E. Flores, and C. P. Wolk, *J. Bacteriol.* **179,** 1998 (1997).
[46] M. F. Cohen, Ph.D. Thesis, University of California, Davis (1996).
[47] T. Thiel and C. P. Wolk, *Methods Enzymol.* **153,** 232 (1987).

to methylate *Ava*I, *Ava*II, and *Ava*III sites on the DNA of the transposon-bearing cargo plasmid prior to transfer. Transfer of RK2 *oriT*-containing plasmids to *N. punctiforme*[6] is enhanced by use of conjugal plasmid pRK2013,[48] whose copy number is greater than that of RK2.

The *E. coli* donor strains are grown in Luria–Bertani (LB) liquid medium with selecting antibiotics(s) (generally 25 μg/ml Km for the conjugal strain carrying pRK2013; 50 μg/ml of Ap and/or Km for the strain carrying RP4; and 50 μg/ml Km plus 25 μg/ml Cm for the strain carrying a Km^r cargo plasmid such as pRL1058 [bearing Tn*5*-1058] and a Cm^r helper such as pRL528, or a Cm^r cargo plasmid such as pRL1087b and a Km^r helper such as pRL1045). The cells are harvested by centrifugation at 2000*g* for 10 min, washed in LB medium lacking antibiotics, and resuspended to the desired cell density (see below).

The recipient cyanobacterium is prepared similarly for both mating procedures. To reduce the chance of spontaneous mutation to a Fox^- phenotype (inability to fix N_2 in the presence of oxygen),[11] cultures of both strains are maintained on N_2, and then subcultured 1 : 200 (v/v) to medium that contains nitrate (PCC 7120) or ammonium (*N. punctiforme;* 2.5 m*M*, buffered with 5 m*M* 3-[*N*-morpholino]propanesulfonic acid [MOPS]) for growth of the culture to be transposon mutagenized. Liquid cultures of less than 4 μg/ml of chlorophyll *a* (Chl *a*) of both cyanobacteria (such relatively dilute cultures are strongly recommended) are subjected to mild cavitation in a sonic bath[49] to break up clumps of filaments (*N. punctiforme)* to facilitate spread-plating and, by fragmenting the filaments to a length of 3 to 10 cells, to help ensure that there will be only one transposition event per colony-forming unit (cfu; because a viable filament is a cfu, the fewer cells per filament the lower the probability of multiple transpositions per cfu). The fragmented filaments are washed once by centrifugation at 1000 *g* for 5 min, resupended in growth medium (for *N. punctiforme,* supplemented with MOPS-buffered NH_4Cl) and incubated under photoautotrophic growth conditions for 6–10 hr. It may be possible to increase the frequency of transposition into certain genes by inducing their transcription shortly before mating,[25,35] e.g., by removal of combined nitrogen for heterocyst-formation and nitrogen-fixation genes, or by a shift to light of different quality and/or quantity for light harvesting-related genes.

Conjugal contact is established by spreading a mixture of cells of the conjugal and the cargo/helper *E. coli* donor strains and the recipient cyanobacterium on detergent-free membrane filters (Millipore HATF or Nucle-

[48] D. H. Figurski and D. R. Helinski, *Proc. Natl. Acad. Sci. U.S.A.* **76,** 1648 (1979).
[49] C. P. Wolk and E. Wojciuch, *Arch. Mikrobiol.* **91,** 91 (1973).

pore REC-85) that lie atop cyanobacterial minimal medium (BG11[50] or AA[51] with or without combined nitrogen) solidified with agar (1% Difco Bacto agar purified by the method of Braun and Wood[52]) and supplemented with 5% (PCC 7120) or 0.5% (*N. punctiforme*) (v/v) separately autoclaved LB medium. The density of the parental strains spread on the filters is an important difference between the two mating procedures. For a single plate mating with PCC 7120, the conjugal and donor strains of *E. coli* are grown overnight, diluted 1 : 40, and 2.5–3 hr later, cells from a 10-ml portion of each culture are harvested by centrifugation, separately washed, concentrated, combined with the other *E. coli* strain, and mated with PCC 7120 cells containing a total of 15 μg of Chl *a* per filter (1 μg Chl *a* corresponds to approximately 3×10^6 cells of PCC 7120[44]). The cells are spread by placing the mixture of cells atop five 1-mm glass beads (Sigma Chemical Co., St. Louis, MO; acid-washed, thoroughly rinsed with distilled water, and autoclaved) resting on the mating filter, then shaking vigorously in the plane of the filter.

Such a high density of *E. coli* cells is detrimental to the viability of *N. punctiforme*[6]; therefore, approximately 20-fold fewer *E. coli* cells are used. Roughly equal amounts of the conjugal and cargo donor cells are harvested, washed, and combined, and the mixture is resuspended to an OD_{600} of 9 to 10. A 0.5-ml aliquot of this mixture of *E. coli* donor cells is then mixed in a microcentrifuge tube with a 0.5-ml suspension of *N. punctiforme* cells containing 50 μg Chl *a* (1 μg of Chl *a* corresponds to about 5.7×10^6 cells of *N. punctiforme*[6]). The mixture is then centrifuged at 4000*g* for 30 sec, all but about 150 μl decanted, and the remainder gently mixed using a wide-bore pipette tip (the mixture should be somewhat pasty) and spread on the filters. A 30-ml culture of each of the *E. coli* donor strains at OD_{600} of about 1 is sufficient for 12 plate matings of *N. punctiforme.* The higher numbers of cyanobacterial cells used in the *N. punctiforme* mating procedure compensates partially for the lower number of *E. coli* donor cells, but the yield of transpositions is nonetheless about five-fold lower than from a typical PCC 7120 mating.

To allow time for plasmid transfer and for expression of transposon-encoded antibiotic resistance, the mating filters are incubated for at least 48 hr (for *N. punctiforme*, not more than 4 days) in the light at 30° (PCC 7120) or 28° (*N. punctiforme*) prior to exposure to antibiotics. During this period, noticeable growth should occur. *N. punctiforme* has a higher mating

[50] R. Rippka, J. Deruelles, J. B. Waterbury, M. Herdman, and R. Y. Stanier, *J. Gen. Microbiol.* **111,** 1 (1979).
[51] M. B. Allen and D. I. Arnon, *Plant Physiol.* **30,** 366 (1955).
[52] A. C. Braun and H. N. Wood, *Proc. Natl. Acad. Sci. U.S.A.* **48,** 1776 (1962).

efficiency and grows more rapidly on plates when air is supplemented with 1% (v/v) CO_2. CO_2 supplementation has not been tried for plate growth of PCC 7120.

Selection for Colonies in which Transposition Has Occurred

The filters are then transferred to medium supplemented with one or more antibiotics. Cyanobacteria vary in their natural sensitivity to different antibiotics. Neomycin is often more selective than kanamycin in filamentous strains. For PCC 7120, selection after mating is carried out with either Nm at 400 μg/ml, Sm at 5 μg/ml, or a combination of Nm at 25 μg/ml plus Sm at 1 μg/ml when Tn*5*-1063 or similar Nm^rSm^r transposons are used[10]; for *N. punctiforme* a combination of Nm at 10 μg/ml plus Sm at 1 μg/ml is used.[6] At low levels of antibiotics exconjugant strains in which the antibiotic resistance genes are driven by the very strong P_{psbA} promoter from *A. hybridus* become antibiotic resistant due to the presence of transferred, albeit nonreplicating (but not degraded) copies of the transposon-bearing plasmid. One of us (YC) recommends transfer of the filters to fresh selective medium when biliproteins from lysed cells have diffused into, and colored, the agar medium beneath the filters. Transfer of the mating filters to fresh selective medium tends to accelerate the clearing of the background and the growth of exconjugant colonies. Exconjugants appear as dark green colonies atop a white or pale yellow background of dead cells about 1–3 weeks after the start of antibiotic selection. At this point the colonies on the filters can be screened for mutant phenotypes. For example, to screen for mutants defective in nitrogen fixation, filters are transferred to a medium without combined nitrogen (subsequent transfer to the same medium may be desirable) and colonies that develop yellowing edges within 7 days are further characterized. To prevent growth of wild-type cells, the filters should be transferred to fresh Nm-containing agar medium at regular intervals or the Nm concentration increased to, e.g., 100–400 μg/ml.

Purifying Transposition-Derived Colonies

A colony formed on a mating filter can be streaked on selective agar medium, or can be inoculated directly into an antibiotic-supplemented minimal liquid medium. Culturing and subculturing is an effective means of ridding the exconjugants of contaminating *E. coli* cells.[44] Cultures are tested for retention of viable *E. coli* by putting a drop of culture on an LB agar plate and incubating overnight at 37°. An axenic cyanobacterial culture will show no growth on the LB plate. Once an axenic culture is obtained, Southern analysis is used to indicate whether the transposon has inserted into the chromosome of the exconjugant by transposition.

Segregation of the Mutant and Wild-Type Chromosomes

Many cyanobacteria, including PCC 7120 and *N. punctiforme*, are polyploid; e.g., on average a cell of PCC 7120 has been calculated to contain more than 10 copies of the chromosome (see Ref. 20). Complete segregation of a mutant genotype is normally desirable, although for certain mutations it may not be attainable. Continuous subculturing in liquid medium with sufficiently strong antibiotic selection, coupled with occasional mild cavitation in a sonic bath to fragment the filaments[49] facilitates the process, but plating following fragmentation is essential to ensure segregation from all wild-type copies of the genome. Southern analysis of genomic DNA with an appropriate probe is used to establish whether the wild-type chromosome is present or absent (see below).

Difficulty in obtaining completely segregated mutants could indicate inadequate antibiotic selection or that the mutated gene is important to cellular survival under given growth conditions. While the former difficulty is easily addressed by increasing the concentration of antibiotic used, the latter may require procedures beyond the scope of this article. Change of growth conditions and nutrient supply may be tried. Several transposon-generated mutants of PCC 7120 have been obtained in which the insertions are within genes essential for growth, such as the *rpl*15 gene coding for the 50S ribosomal protein L32. A common characteristic of these mutants is their inconsistency in growth. When cells of such a mutant are inoculated from a selective agar medium to a selective liquid medium, survival and growth of the inoculum in the liquid medium is unpredictable; once a liquid culture is obtained, streaking on a selective agar medium often gives spotty, instead of homogeneous, cell growth in areas of predicted high cell density.[28]

Reconstructing the Mutation

In two mutants of PCC 7120 that were tagged with wild-type Tn*5*,[37,38] the mutations were not linked to the site of insertion of the transposon. In *N. ellipsosporum*[41] and *Anabaena* sp. strain M-131[16] and perhaps in PCC 7120,[53] conjugation may stimulate mutagenic transposition of IS elements. Although there is no evidence for such a response in *N. punctiforme*, it is essential to reconstruct the mutation with the transposon or with an interposon in wild-type cells to determine whether the mutant phenotype is a direct result of the specific insertion and not the consequence of a secondary mutation. While regeneration of the mutation can be done with a simple

[53] C. C. Bauer, personal communication cited in Ref. 37.

interposon such as an antibiotic-resistance cassette, such a construction may generate a novel phenotype.[54]

To reconstruct the mutation, the transposon with flanking cyanobacterial genomic DNA must first be recovered from the mutant. The phenol-chloroform glass-bead method for isolation of genomic DNA,[20] applicable to virtually all bacteria including those cyanobacteria that we have tried, is suitable for this purpose. If one wants to avoid handling phenol and obtain genomic DNA of high molecular mass, multistep lysis–nucleic acid precipitation protocols are also available.[6,55]

Genomic DNA prepared from the mutant is digested with a restriction endonuclease that does not cut within the transposon, but cuts the cyanobacterial genomic DNA frequently[10]; the digested DNA is diluted and then ligated to circularize the genomic fragments. For Tn*5*-1058 or Tn*5*-1063, candidate enzymes include *Cla*I, *Eco*RI, *Eco*RV, *Pvu*I, *Sca*I, and *Spe*I. Successful transformation of *E. coli* should occur only with those circularized fragments that contain the p15A *oriV* carried by the Tn*5* derivatives. We recommend transformation or electroporation using *E. coli* strains DH10B or DH5α-MCR (which lack host-specific and methylation-dependent restriction systems[56]). The presence of methylation-dependent restriction systems in numerous other strains of *E. coli*, such as the popular DH5α, can greatly reduce the transformation frequency of isolated DNA because many cyanobacteria have variously methylated DNA.[57] The Tn*5* derivatives mentioned earlier that have an *oriV* from p15A provide relatively stable maintenance of the transposon-derived plasmids in *E. coli*. If the cloned DNA appears at all unstable, the host *E. coli* should be grown at 30° and promptly stored at −80° rather than repeatedly subcultured.

Provided that the length of DNA flanking each side of the transposon suffices for homologous recombination (if not, the transposon should be excised from the cyanobacterial chromosome with a different restriction endonuclease), the recovered plasmid is prepared for interposon mutagenesis of the wild-type strain, usually using *sacB*-mediated positive selection for double recombinants.[6,13,20] The minimum length of homologous flanking DNA with which we have tested and observed a recombination event in *N. punctiforme* is 700 bp, whereas a 200-bp length suffices in PCC 7120 for a second crossover. First, a frameshift mutation is introduced into the transposase gene by filling in the *Not*I site (if unique in the plasmid).[6] This

[54] T. A. Black and C. P. Wolk, *J. Bacteriol.* **176,** 2282 (1994).
[55] C. Franche and T. Damerval, *Methods Enzymol.* **167,** 803 (1988).
[56] S. G. Grant, J. Jesse, F. R. Bloom, and D. Hanahan, *Proc. Natl. Acad. Sci. U.S.A.* **87,** 4645 (1990).
[57] R. N. Padhy, F. G. Hottat, M. M. Coene, and P. P. Hoet, *J. Bacteriol.* **170,** 1934 (1988).

step can often be omitted in PCC 7120 reconstructions because homologous recombination often occurs at a much higher frequency than transposition. Second, a positive selection cassette, such as those that contain *sacB*, is placed between the two outer ends of the flanking DNA. The *sacB* gene product confers sensitivity to sucrose in many organisms (references cited in Ref. [20]). A *sacB*-containing vector, pRL1075 (Cm^rEm^r), has been especially designed for ligation to the recovered transposon-derived plasmids,[13] and Sm^rSp^r variants (pRL1130a[29] [Fig. 2] and pRL1130b) made for use with Cm^rEm^r transposons such as Tn*5*-1087b. In addition to the *sacB* gene from *Bacillus subtilis* and an RK2 *oriT*, pRL1075 and pRL1130a and b contain symmetrical polylinkers that flank a pMB1 *oriV*; this *oriV* is replaced by the linearized excised chromosomal fragment which supplies replication functions from its p15A *oriV*.

We routinely verify sucrose sensitivity (Suc^s) conferred by the resulting plasmid in *E. coli* prior to transfer of the plasmid into the wild-type cyanobacterium. The plasmid is mobilized from an *E. coli* strain to wild-type *N. punctiforme* by pRK2013 or to PCC 7120 by RP4, by a standard triparental

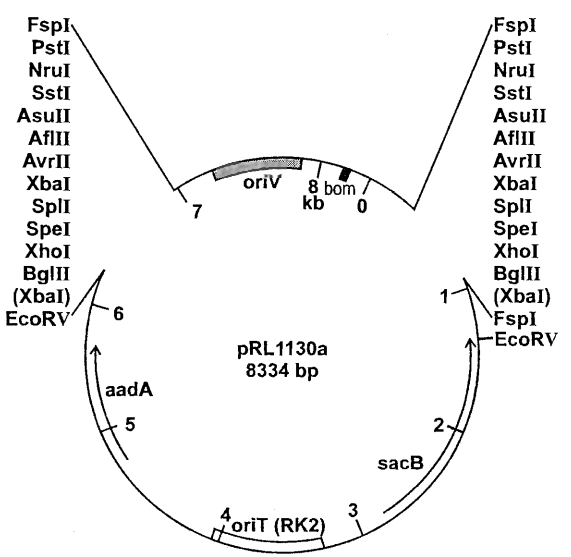

FIG. 2. Simplified schematic of *sacB*-containing positive selection vector pRL1130a. *oriV* and *oriT*(RK2) as in Fig. 1; *bom*, basis of mobilization (for pRL1130a, not normally used); *sacB*, gene that confers sensitivity to the host strain to sucrose; *aadA*, gene that confers resistance to streptomycin and spectinomycin. For reconstructions of transposon-induced mutations, transposon- (and thereby *oriV*-)containing plasmids excised from the chromosome are inserted between the inverted polylinkers of pRL1130a, replacing the *oriV*- and *bom*-containing segment of that plasmid.

mating with (PCC 7120) or without (*N. punctiforme*) a methylation-helper plasmid. Plasmids generated from pRL1075 that bear Tn*5*-1063 (or a derivative of it in which the transposase has been inactivated) are $Bm^rCm^rEm^rNm^rSm^r$ and Suc^s. Single recombinants are typically maintained on solid medium with Em at 15 to 20 μg/ml (*N. punctiforme*) or 10 μg/ml (PCC 7120) or grown in liquid medium with 5 μg/ml Em and 10 μg/ml Nm (*N. punctiforme*) or with Em and Nm both at 2 μg/ml (PCC 7120). To accumulate double recombinants, a single-recombinant colony is inoculated into liquid medium with Nm (10 or 50 μg/ml for *N. punctiforme* or PCC 7120, respectively), but without Em, and incubated under growth conditions for several weeks.[6,20] Cells from such a culture are then used for positive selection for double recombinants (Suc^r). Filaments are first subjected to brief, mild cavitation, washed, and plated, usually in several cell densities, onto cyanobacterial growth medium supplemented with 5% (w/v) sucrose and Nm at 10 μg/ml (*N. punctiforme*)[6] or 200 μg/ml (PCC 7120).[20] (When the *Eco*RV site immediately 3′ from *sacB* of pRL1075 has been used for cloning, 10% [w/v] sucrose can be more effective than 5%.) Use of higher light intensity tends to accelerate the killing of single-recombinant background cells that bear a functional copy of *sacB*, and the formation of sucrose-resistant colonies. One should not assume that all sucrose-resistant colonies are double recombinants, because inactivation of *sacB* in single recombinants by point mutation or by insertion of cyanobacterial IS elements is not uncommon.[20,35] These "pseudo-double recombinants" can be easily identified in this case by simply checking for their ability to grow on Em-containing medium, unless they have accumulated a rare second mutation in the Em^r gene. True double recombinants have lost the Em^r-, *sacB*-containing fragment from pRL1075, and so are Em^s. Nonetheless, Southern analysis to confirm the reconstruction is highly recommended. Further studies of the mutated genomic DNA are warranted if the double recombinant strain bearing the reconstruction exhibits the same phenotype as the original transposon-insertion mutant. If the original mutant phenotype is interesting but evidently not caused by the transposon, it may be cloned by complementation from cosmid libraries (PCC 7120).

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