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MOLECULAR GENETIC APPROACHES TOWARDS THE UNDERSTANDING OF HETEROCYST DIFFERENTIATION AND PATTERN FORMATION IN THE CYANOBACTERIUM *Anabaena* SP.

presented by

Yuping Cai

has been accepted towards fulfillment
of the requirements for

the Ph.D. degree in Botany & Plant Pathology

Major professor

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MOLECULAR GENETIC APPROACHES TOWARDS THE UNDERSTANDING OF HETEROCYST DIFFERENTIATION AND PATTERN FORMATION IN THE CYANOBACTERIUM *Anabaena* SP.

By

Yuping Cai

A DISSERTATION

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ABSTRACT

MOLECULAR GENETIC APPROACHES TOWARDS THE UNDERSTANDING OF HETEROCYST DIFFERENTIATION AND PATTERN FORMATION IN THE CYANOBACTERIUM *Anabaena* SP.

By

Yuping Cai

Certain cyanobacteria (blue-green algae) are the only free-living prokaryotes capable of both oxygenic (higher-plant type) photosynthesis and aerobic nitrogen fixation. *Anabaena* spp. are of developmental interest because, when deprived of fixed nitrogen, they differentiate to form N_2-fixing cells called heterocysts in a patterned array along the cyanobacterial filaments.

In order to facilitate analysis of the development of *Anabaena* spp., a positive selection system for gene replacement was developed. The conditionally lethal gene *sacB* from *Bacillus subtilis* is inserted into the vector portion of a suicide plasmid bearing a mutant version of a chromosomal gene. Cells in which such a plasmid has integrated into the chromosome through single recombination are plated on solid medium containing 5% sucrose, which is toxic to cells bearing *sacB*. A small fraction of the cells becomes sucrose-resistant. Most of the cells in that fraction have undergone a second recombinational event

in which the *sacB*-containing vector has been lost, and the wild-type form of the chromosomal gene has been replaced by the mutant form. Three versatile plasmids were constructed to facilitate the use of this technique.

The conditionally lethal nature of *sacB* was also used to entrap insertion sequences (ISs) from *Anabaena* sp. strain PCC 7120. Analysis of spontaneous sucrose-resistant colonies derived from cells bearing the *sacB*-containing, replicating plasmid pRL250 revealed insertion of at least six different ISs in *sacB*. IS*892*, most frequently observed to transpose in this study, was sequenced and further characterized.

To help elucidate the early responses to nitrogen-stepdown which are significant for the onset of heterocyst differentiation and pattern formation, a derivative of transposon Tn*5* that incorporates the promoterless bacterial luciferase genes, *luxAB*, as a reporter of transcriptional activity was used to isolate nitrogen-responsive mutants. Two mutants, TLN2 and TLN6, showed increased luminescence within 1 or 4 h, respectively, when cultures were shifted from NO_3^- to N_2 as a nitrogen source. Using a T7-based transcription-amplifying/reporting system, *tln6*-directed luciferase activity at a single-cell level was observed by microscopic photon-counting and was found distributed unevenly along the filament at 7 h after nitrogen-stepdown. Mutations in TLN2 and TLN6 were characterized and localized in the chromosome. The fusion of each gene to *luxAB* was regenerated in the wild-type chromosome by gene replacement using *sacB*, and the resulting mutants were subjected to secondary mutagenesis by a Tn*5* derivative lacking *luxAB*. Four secondary mutants were isolated that showed altered *tln6::luxAB* expression as well as defective differentiation of heterocysts.

To

my Mother

ACKNOWLEDGMENTS

I thank my advisor C. Peter Wolk for nurturing my professional growth. His strict scientific attitude, creative imagination and astute advice have made my graduate career a very rewarding one, both scientifically and ethically. He also jointly performed some of the experiments described in chapter 4. I thank Lee McIntosh, Harold Sadoff and Loren Snyder for serving in my guidance committee and for their thoughtful suggestions.

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I thank past and present members of the Wolk lab for all the stimulating discussions and helpful suggestions. Specifically, I thank Jeff Elhai for making, and teaching me to use, the computer program permitting superposition of luminescent and bright-field images and the computer program for processing bright-field and photon-counting images of single filaments, Doron Holland for helpful advice on DNA sequencing, Elaine Oren for her extensive work on the

mapping of *tln2*::Tn*5*-1063 and *tln6*::Tn*5*-1063 in the chromosome by pulsed-field gel electrophoresis, and Anneliese Ernst for her unpublished results of the nitrogenase assay of the *nifD* mutant *Anabaena* PS263. I greatly appreciate the generosity of members of this lab whose many unpublished constructs were used during the course of this study. I would also like to thank members of the DOE Plant Research Laboratory for making PRL a wonderful place to work in.

Special thanks go to Lily and Sunny who make a warm home to look forward to after everyday's work. Finally I thank my best friend "CAT", whose long-lasting friendship has greatly enriched my life.

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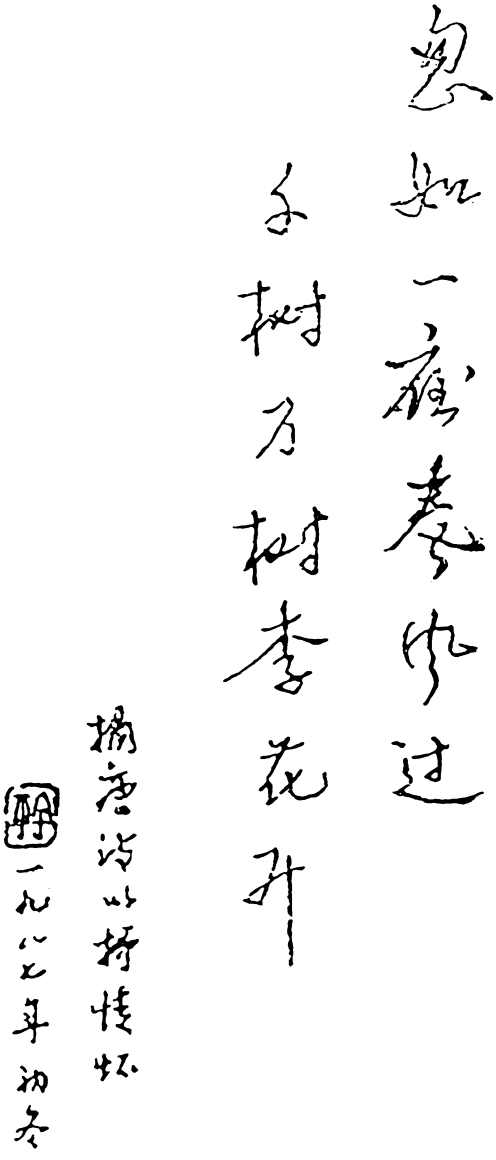

This one-thousand-plus-year old Chinese poem expressed the poet's pleasant surprise by the stunning morning spectacle created by the first snow of the Winter overnight.

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LIST OF ABBREVIATIONS AND SYMBOLS

::	Novel joint of DNA
( )	Designates carriage of a replicating plasmid
+	In possession of a functional gene* or a phenotype**
−	Lack of a functional gene* or a phenotype**
▲	Deletion, when used in the description of genotypes
AA	Allen and Arnon medium (see Appendix A)
AAD	Aminoglycoside adenylyltransferase
ANT	Aminoglycoside nucleotidyltransferase
Ap	Ampicillin
APH	Aminoglycoside phosphotransferase
ATP	Adenosine 5'-triphosphate
bp	Basepair(s)
BLOS	A family of 5.6-kb gene cassettes that contains a ***b**om* (*oriT*) region, the genes *luxAB*, an ***o**riV*, and a **S**m^r/Sp^r determinant
Bom	Basis of mobilization (*oriT*, see Appendix E)
CAT	Chloramphenicol acetyltransferase
Cm	Chloramphenicol

EDTA	Ethylenedinitrilotetraacetic acid
Em	Erythromycin
Gm	Gentamicin
GOGAT	Glutamine:oxoglutarate aminotransferase (glutamate synthase)
GS	Glutamine synthetase
IPTG	Isopropyl-thiogalactoside
IS	Insertion sequence
Kb	Kilobasepair(s)
Kd	Kilodalton(s)
Km	Kanamycin
LB	Luria-Bertani medium
MCS	Multiple cloning sites
MDRS	Methylation-dependent restriction systems
MSX	L-methionine-D,L-sulfoximine
N	Fixed nitrogen (NO_3^- or NH_4^+)
NMT	An rRNA N^6-amino adenine N-methyltransferase
Nm	Neomycin
Nx	Nalidixic acid
ORF	Open reading frame
^{r}	Resistant
^{s}	Sensitive

SDS	Sodium dodecyl sulfate, also called sodium lauryl sulfate
SEM	Standard deviation of the mean
Sm	Streptomycin
Sp	Spectinomycin
Suc	5% sucrose
Tc	Tetracycline
TES	N-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid
Tn	Transposon
Xgal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

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* Names of genes are presented in italic letters.

** Names of phenotypes are presented with their first letters capitalized.

Chapter 1

GENERAL INTRODUCTION

INTRODUCTION

Cyanobacteria, formerly called blue-green algae, constitute a highly diverse group of prokaryotes that pose, as well as provide organisms to answer, a variety of biological and evolutionary questions. Cyanobacteria constitute the major group of prokaryotes that perform oxygenic (higher-plant type) photosynthesis, and many species are capable of nitrogen fixation (Stanier, 1977; Stanier and Cohen-Bazire, 1977). The recently discovered and characterized Prochlorales are another group of photosynthetic prokaryotes that have chlorophyll *a*, but unlike cyanobacteria, have chlorophyll *b* rather than phycobilins (Lewin, 1976; Burger-Wiersma, 1986). According to the endosymbiont hypothesis (Margulis, 1981), cyanobacteria and the Prochlorales are considered the progenitors of chloroplasts of eukaryotic plants based on their morphological, biochemical, and genetic similarities (Whitton et al., 1971; Morden and Golden, 1989; Kuhsel et al., 1990; Xu et al., 1990) .

Cyanobacteria are comprised of unicellular and filamentous species. The ca. 300 strains axenically cultured in the Pasteur collection have been divided into five sections based on comparative studies of their structure and development (Rippka et al., 1979; in this dissertation I refer to this system as the 1979 Rippka

Assignment). The cyanobacterial strain that was almost exclusively used throughout this study, *Anabaena* sp. strain PCC 7120, was originally named *Nostoc muscorum*. But in the 1979 Rippka Assignments this strain was placed in the genus *Anabaena*, rather than *Nostoc*, under Section IV. Although the name *Nostoc* was recently recommended again for this strain (Rippka, 1988), the name *Anabaena* has been used in the majority of publications of studies of this strain and is therefore used throughout this presentation.

Since the discovery of genetic transformability of the cyanobacterium *Anacystis nidulans* in 1970 (Shestakov and Khuyen, 1970), several strains (Porter, 1986) of unicellular cyanobacteria (all in Section I of the 1979 Rippka Assignment), including *Synechocystis* sp. strain PCC 6803 that is capable of light-activated heterotrophic growth (Anderson and McIntosh, 1991), have been extensively used in molecular genetic studies of the structural and functional aspects of oxygenic photosynthesis (Bryant and Tandeau de Marsac, 1988; McFadden and Small, 1988). The heterocystous filamentous cyanobacteria (Section IV and V of the 1979 Rippka Assignment), on the other hand, were studied extensively long before the availability of molecular genetic means because they, unlike almost all other prokaryotes, are capable of cellular differentiation to form heterocysts and akinetes (also called spores; Nichols and Adams, 1982), as well as of aerobic nitrogen fixation.

When grown in media with a sufficient amount of fixed nitrogen such as nitrate or ammonium, the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 forms filaments that are comprised of only vegetative cells. However, when deprived of a source of fixed nitrogen, this and other *Anabaena* spp. differentiate to form thick-walled cells called heterocysts at semi-regular intervals along the

filaments (Wolk, 1982). In such nitrogen-fixing filaments, vegetative cells and heterocysts perform different but interdependent metabolic functions. Vegetative cells use light as an energy source to split water (producing reductant and O_2) and fix CO_2, and thus to maintain growth. Heterocysts fix nitrogen and export the fixed nitrogen to the vegetative cells. Part of the reductant produced in vegetative cells is transported to the heterocysts and used for reduction of dinitrogen. Heterocysts, terminally differentiated cells, have unique morphological and biochemical characteristics suited for nitrogen fixation. They form a thick envelope that is very little permeable to O_2 (Murry and Wolk, 1989) and have a much reduced connection with neighboring vegetative cells (Wilcox et al., 1973). The activity of photosystem II, which produces O_2, is absent, and heterocysts devote photosystem I to cyclic photophosphorylation to produce ATP needed for nitrogen fixation (Haselkorn, 1978). Heterocysts are thought also to respire a large fraction of the reductant that they receive from vegetative cells, in order to maintain a very low internal pO_2 (Murry and Wolk, 1989), and thus to preserve the activity of the inherently O_2-sensitive, nitrogen-fixing enzyme, nitrogenase (Wolk, 1982).

In *Anabaena variabilis* about 15 to 25% of the genome is devoted to the production of RNA transcripts that are heterocyst-specific under aerobic conditions (Lynn et al., 1986). In the process of heterocyst differentiation, genes of various functions are differentially turned on or off (Elhai and Wolk, 1990). Mutants that are defective in various aspects of heterocyst differentiation have been isolated and complemented (Wolk et al., 1988; Buikema and Haselkorn, 1991a), and some of the mutated genes studied in detail (Holland and Wolk, 1990; Buikema and Haselkorn, 1991b). In addition, DNA is rearranged at a late

stage of heterocyst differentiation (Golden et al., 1985; Haselkorn, 1989). The development of the endospore of the well studied Gram-positive bacterium, *Bacillus subtilis*, is governed by a regulatory transcriptional cascade in which the expression of certain genes is dependent on prior activity of other genes (Stragier and Losick, 1990). However, very few of the *Anabaena* genes involved in the heterocyst differentiation process have been characterized. The regulation of the expression of these genes and possible regulatory relationships among them remain virtually unknown.

Because of its morphological simplicity, *Anabaena* sp. can be used as a model organism for the elucidation of the detailed biochemical mechanisms that control the formation of multicellular patterns (Dworkin, 1985). The mechanisms that govern the initiation of the pattern of heterocyst spacing in *Anabaena* sp. may differ from the mechanism(s) that preserves that pattern because once the spatial pattern of heterocysts is formed, it may be maintained by the gradient of nitrogenous compound(s) exported from heterocysts. The initial establishment of the heterocyst pattern is, however, independent of nitrogen fixation because mutants unable to fix nitrogen can form normally spaced heterocysts, and patterned heterocyst formation can be induced under growth conditions free of N_2. Two distinct, but experimentally distinguishable mechanisms have been proposed to explain the initial formation of the pattern of spaced heterocysts (Wolk, 1989). One mechanism proposed (which I denote **the volunteer model**) is that the first cells to sense nitrogen-stepdown differentiate to form heterocysts, and simultaneously produce the heterocyst pattern by exporting a differentiation-inhibiting substance to adjacent vegetative cells. The second mechanism proposed (which I call **the scavenger model**), is that the first cells to sense N-

stepdown activate a nitrogen-scavenging system that drains available nitrogen from adjacent cells, thus permitting the scavenging cells to continue growing vegetatively. Subsequent similar action of neighboring cells puts the cell that senses nitrogen deprivation the last farthest away from the cells that sense N-stepdown first, and leads to the formation of a heterocyst by this cell. The two proposed mechanisms can be tested experimentally if one can identify an *Anabaena* gene that responds rapidly to removal of fixed nitrogen, and can follow the developmental fate of the cells in which that gene is first activated after N-stepdown. According to the volunteer model, this cell will differentiate into a heterocyst, whereas according to the scavenger model, this cell will be distant from the heterocysts that are formed.

GOAL OF THIS STUDY

In 1984, the Wolk laboratory successfully transferred foreign DNA from *Escherichia coli* to *Anabaena* sp. by conjugation (Wolk et al., 1984). This achievement marked the start of manipulatory molecular genetic studies of the filamentous cyanobacteria. However, it was difficult to introduce a site-directed mutation into the genome of *Anabaena* sp. because when homologous DNA sequences within suicide plasmids were transferred to *Anabaena* sp. by conjugation, single-crossover events (integration recombinations) occur far more frequently than do double-crossover events (replacement recombinations). In order to facilitate analysis of the development of *Anabaena* sp., I therefore sought a means to select for double recombinants. The application of a conditionally lethal gene, the *sacB* from *B. subtilis*, led to the technique of positive selection for double recombinants in *Anabaena* spp. The conditionally lethal nature of

sacB was also used to entrap insertion sequences in cells of *Anabaena* spp., leading to the discovery of at least six different insertion sequences in *Anabaena* sp. strain PCC 7120, and to an insight into dynamic changes occurring in the genome of that strain. In the later part of my studies, two additional powerful techniques were made available by work of this laboratory: use of bacterial luciferase as a reporter to report transcriptional activity at a single-cell level (Elhai and Wolk, 1990) and the efficient transposition of Tn*5* derivatives that can produce fusions of the bacterial luciferase genes, *luxAB*, with the genome of *Anabaena* sp. (Wolk et al., 1991). Two genes, *tln2* and *tln6*, that respond rapidly to removal of fixed nitrogen were identified. I have also tentatively identified mutations that affect heterocyst differentiation as well as the expression of *tln6*. Such mutations are likely to be in the very early genes that sense nitrogen-starvation and are involved in the initiation of patterned formation of heterocysts.

Chapter 2

DEVELOPMENT OF A SYSTEM OF POSITIVE SELECTION FOR GENE REPLACEMENT IN THE FILAMENTOUS CYANOBACTERIUM *Anabaena* SP. [1]

SUMMARY

Use of the conditionally lethal gene *sacB* provides a simple, effective, positive selection for double recombinants in *Anabaena* sp. strain PCC 7120, a filamentous cyanobacterium. This gene, which encodes the secretory levansucrase of *Bacillus subtilis*, is inserted into the vector portion of a suicide plasmid bearing a mutant version of a chromosomal gene. Cells of colonies in which such a plasmid has integrated into the chromosome of *Anabaena* sp. through single recombination are plated on solid medium containing 5% sucrose. Under this condition, the presence of the *sacB* gene is lethal. A small fraction of the cells from initially sucrose-sensitive colonies becomes sucrose-resistant; the majority of these sucrose-resistant derivatives has undergone a second recombinational event in which the *sacB*-containing vector has been lost, and the

[1] Most of the content of this chapter has been published in the Journal of Bacteriology (**Cai and Wolk**, 1990).

wild type form of the chromosomal gene has been replaced by the mutant form. By the use of this technique several selected wild-type genes in the chromosome of *Anabaena* sp. strain PCC 7120 have been mutated, and replaced by corresponding mutant genes. This *sacB*-mediated positive selection system was further improved by the construction of three plasmids, pRL271, pRL277 and pRL278. By using one of these plasmids, the introduction of a specific mutation into the chromosome of *Anabaena* sp. can be as easy as one subcloning plus one conjugal transfer, provided that a marked, mutated fragment of DNA from *Anabaena* sp. is readily available.

INTRODUCTION

Molecular genetic studies of cyanobacteria have been greatly facilitated by advances in techniques for genetic transfer (Wolk et al., 1984; Porter, 1986). However, site-directed modification of the chromosome, a powerful tool for study of gene function, has been applied nearly exclusively to unicellular cyanobacteria (Porter, 1986) because of the ease with which double recombinants can be isolated from these organisms.

Isolation of double recombinants from filamentous cyanobacteria such as *Anabaena* species has been difficult for two reasons. First, when homologous DNA sequences within suicide plasmids are transferred to *Anabaena* sp. by conjugation, single-crossover events (integration recombinations) occur far more frequently than double-crossover events (replacement recombinations) (Golden and Wiest, 1988; this work). Second, because cells of *Anabaena* sp. have multiple genomic equivalents as calculated from the genetic complexity (Herdman et al., 1979b; Bancroft et al., 1989) and the amount of DNA per cell (Craig et al.,

1969), and are linked, isolation of recessive mutants requires both segregation of mutant and wild-type forms of the genome and physical disjunction of adjacent cells of a filament bearing the two genomic forms (Currier et al., 1977).

Golden and Wiest (1988) first introduced an insertional mutation into the *xisA* gene in the chromosome of *Anabaena* sp., by screening for double-recombinant derivatives of selected single recombinants. Their screening experiment made use of a 17-kb, homologous DNA fragment, with a Sm^r/Sp^r cassette (the Ω cassette; Prentki and Krisch, 1984) inserted near the middle to inactivate the gene of interest (an antibiotic resistant gene used in this manner has been referred to as an "inactivation cassette" [Golden, 1988]). Quantitative work with *Escherichia coli* has shown that the rate of recombination decreases greatly as the length of a homologous sequence decreases (Shen and Huang, 1986). Recombination in *Anabaena* sp. may have a similar size-dependence. In *Anabaena* sp. strain PCC 7120, exhaustive screens have failed to isolate double recombinants when the size of the homologous region was below 4 kb (J. Elhai, personal communication; this work). For this reason, we sought to isolate double recombinants by positive selection for loss of the vector portions of plasmids that had integrated into the chromosome. Such positive selection can be achieved by inclusion of a conditionally lethal gene within the vector portion of the plasmid.

The conditionally lethal gene, *sacB*, has been used to isolate double recombinants in the Gram negative bacterium, *Erwinia chrysanthemi* (Ried and Collmer, 1987). This gene, from the unicellular Gram-positive bacterium, *Bacillus subtilis*, encodes levansucrase (sucrose:2,6-ß-D-fructan 6-ß-D-fructosyltransferase; E.C. 2.4.1.10), a 50-kd secretory protein, production of which is induced by sucrose (Gay et al., 1983 and 1985). The gene has been cloned

(Gay et. al, 1983) and sequenced (Steinmetz et al., 1985), and its expression has been well studied (Aymerich et al., 1986 and Klier et al., 1987). Expression of the gene is lethal to such Gram negative bacteria as *E. coli*, *Agrobacterium tumefaciens*, *Rhizobium meliloti* and *E. chrysanthemi* in the presence of 5% sucrose in solid medium (Gay et al., 1983 and 1985; Ried and Collmer, 1987). Growth is inhibited and cells lyse within as little as 1 h after induction of *sacB* expression by sucrose (Gay et al., 1983). The mechanism of lethality of the *sacB* product to a variety of Gram-negative bacteria is not well understood. Lethality may be due to transfructosylation from sucrose to various metabolically important acceptors (Gay et al., 1983), or to accumulation of unsecreted protein in the cell membranes because of inadequate cleavage and export (Beckwith and Silhavy, 1983). Because cyanobacteria have the peripheral structure characteristic of Gram-negative bacteria, I tested two strains of *Anabaena* sp., and found both of them susceptible to sucrose when bearing the *sacB* gene. I further found that the conditional lethality of *sacB* in *Anabaena* sp. enables direct selection for double recombinants on sucrose-containing solid medium.

MATERIALS AND METHODS

Media and growth conditions. The bacterial strains used are listed in Table A.2 of Appendix B. Axenic strains of *Anabaena* sp. were grown photoauxotrophically at 30°C in AA-based media (Allen and Arnon, 1955; see Appendix A) in air under cool white fluorescent lighting of ca. 60 $\mu E\ m^{-2}\ s^{-1}$, agitated on a rotary shaker when liquid medium was used. *E. coli* was grown, usually at 37°C, in LB (Luria-Bertani)-based media (Maniatis et al., 1982). Antibiotic concentrations used for selection are listed in Appendix F. A 50%

aqueous solution of sucrose, sterilized by filtration though a filter of pore size 0.22 μm (type GA) or 0.45 μm (type HA) (Millipore Corp., Bedford, MA), was added to autoclaved LB agar or AA+NO_3^- agar medium to a final concentration of 5% sucrose.

Isolation and manipulation of DNA. Restriction enzymes and modifying enzymes used were mostly from Bethesda Research Laboratories (BRL) of Life Technologies, Inc. (Gaithersburg, MD), New England Biolabs, Inc. (Beverly, MA), United States Biochemicals Corp. (Cleveland, OH), and Boehringer Mannheim Corp. (Indianapolis, IN), and were used essentially according to the recommendations of the suppliers.

Plasmids used are listed in Table A.3 of Appendix C. Cloning procedures followed standard methods (Maniatis et al., 1982). Plasmid mini-preps from *E. coli* was performed essentially as published (Holmes and Quigley, 1981).

Total DNA was isolated from *Anabaena* sp. by the following modification of a published technique (Golden et al., 1985; D. Holland, personal communication). Cells of *Anabaena* sp. in the mid- or late-log phase of growth were harvested from 25 to 50 ml of liquid culture and resuspended in a final volume of 400 μl in a 1.5-ml microfuge tube with 10 mM Tris·Cl, 0.1 mM EDTA (pH 7.5). Then, 150 μl of sterile glass beads (Sigma Chemical Co. catalog No. G-9143), 20 μl of 10% Sodium docecyl sulfate (SDS; Boehringer Mannheim Biochemicals, Indianopolis, IN), and 450 μl of a 1:1 (v/v) mixture of phenol and chloroform were added. The mixture was subjected to a cycle of vigorous vortexing for 1 min followed by cooling on ice for 1 min, for a total of 4 to 6 times. The resulting suspension was centrifuged at 15,000 x *g* for 15 min, and the clear

supernatant solution was transferred to a new microfuge tube, phenol- and then chloroform-extracted, and its DNA ethanol-precipitated.

DNA probes were labelled with [α-^{32}P]-deoxyadenosine 5'-triphosphate ([α-^{32}P]dATP, as ca. 13.0 μM triethylammonium salt at a concentration of ca. 10.0 mCi per ml) purchased from New England Nuclear (Boston, MA), or Amersham Corp. (Arlington Heights, IL), using a random primer labeling kit purchased from BRL. Southern analyses were performed as follows: DNA digested with appropriate restriction enzyme(s) was first separated by electropheresis in a 0.7% agarose gel (Ultrapure agarose from BRL), then transferred (Davis et al., 1986) to a nitrocellulose membrane (Immobilon-NC Transfer Membranes, pore size: 0.45 μM, type HA, Millipore Corp.) and hybridized by a standard procedure (Maniatis et al., 1982) with the following specifics: hybridizations were always carried out at 65° C, followed by one 5-min wash and then two 30-min washes, all with 0.5 X SSC (formula for the SSC solution is found in Maniatis et al., 1982) and 0.1% SDS at 65° C (high stringency washes) or room temperature (low stringency washes).

Genetic transfer and selection. Preparation of competent cells and transformation of *E. coli* were carried out according to a published procedure (Hanahan, 1985). Plasmid DNA was introduced into cells of *Anabaena* sp. by conjugation from *E. coli*, with pRL528 as helper plasmid, following standard procedures (Wolk et al., 1984; Elhai and Wolk, 1988b).

To isolate double recombinants of *Anabaena* sp., initial exconjugants were suspended in 50 ml of AA/8+NO_3^- medium, shaken under growth conditions for 4 to 6 h, subjected to cavitation in a sonic cleaning bath for 5 to 10 min, and washed twice with the same medium. About 10^6 to 10^7 cells were then plated

on AA+NO_3^- agar containing Sm, Sp, and 5% sucrose. I refer to this procedure as direct plating. In some experiments, exconjugants were also subcultured in 50 ml of AA/8+NO_3^-+Sm+Sp liquid medium, with cavitation of the culture for 5 min at 4- to 7-da intervals, for 1 month. The cells were then washed once and plated on solid medium of AA+NO_3^-+Sm+Sp+Suc at 10^6, 10^5, and 10^4 cells/plate. Cell density was estimated from methanolic extracts of cells (Mackinney, 1941) by assuming a content of 0.4 pg of chlorophyll *a* per cell.

RESULTS

Construction of plasmids pRL250, pRL256, and pRL263 for the initial experiments. Plasmid pRL57 (Elhai and Wolk, 1988a), a positive selection shuttle cloning vector, was constructed by ligation of the pDUI-containing *Cla* I-*Nde* I fragment of pRL1 (Wolk et al., 1984) with the *nptII*-containing *Nde* I-*Asu* II fragment of pRL44 (Table A.2; for a list of genes described in this study, see Appendix E). Plasmid pRL250 (Fig. 2.1a) was constructed by cloning the 3881-bp *Bam*H I fragment containing the *sacB-nptI* cartridge from pUM24 (Ried and Collmer, 1987) between the two *Bam*H I sites of pRL57.

The suicide (integration) plasmid pRL256 (Fig. 2.1b) was constructed as follows. Sm^r/Sp^r cassette C.S4 (Bancroft and Wolk, 1989), bounded by *Sma* I sites, from plasmid pRL171PSm (Table A.2) was inserted into the *Nru* I site of the 3.5-kb, partial-*Sau*3A I fragment of *Anabaena* DNA in plasmid pRL52, inactivating the *hetA* gene which is required for complementation of the Fox^- (Fixation of nitrogen in the presence of oxygen; nomenclature and list of phenotypes described in this study are presented in Appendices D and F) mutant EF116 (Wolk et al., 1988; Holland and Wolk, 1990). The resulting plasmid, pRL61, was

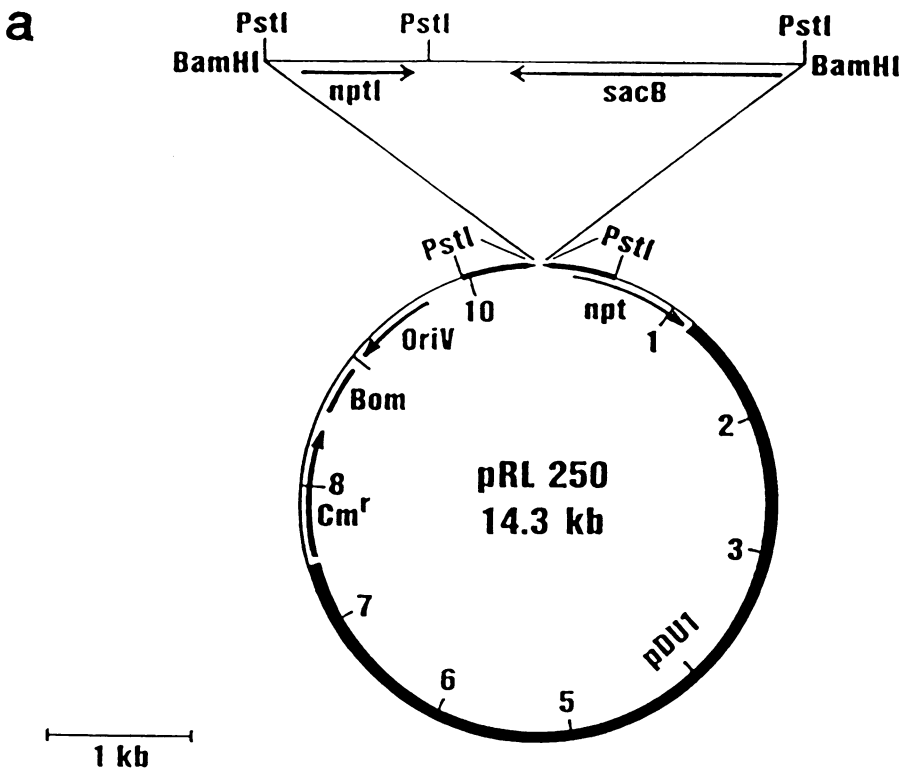
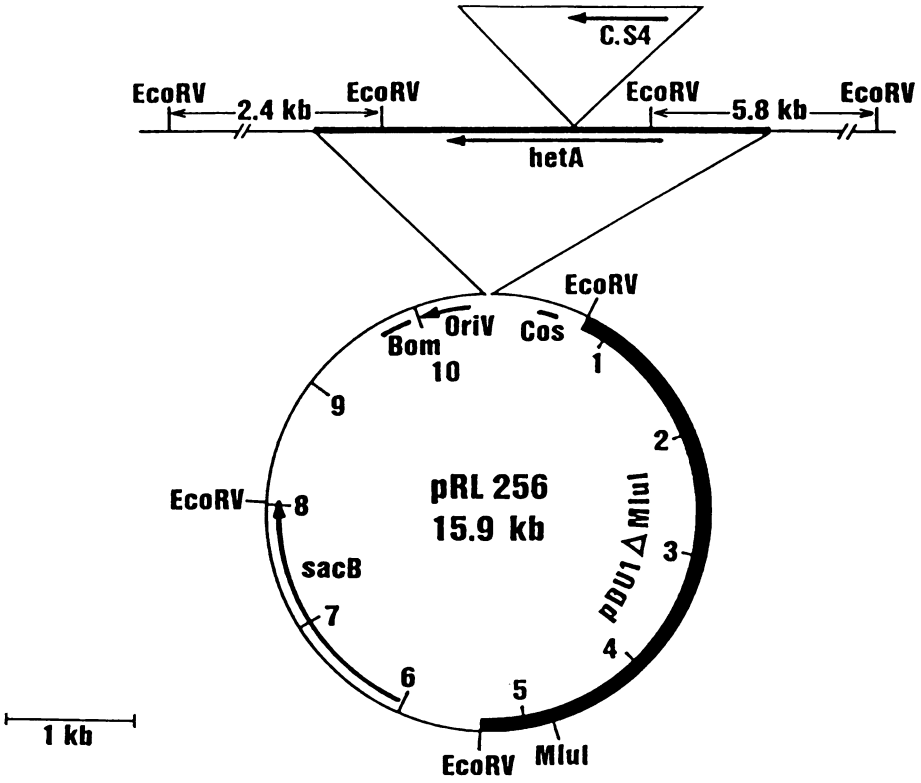


Figure 2.1. Essential features of plasmids (a) pRL250, (b) pRL256, and (c) pRL263. The horizontal line in B and C. represents a portion of the chromosome of *Anabaena* sp. strain PCC 7120; the bold region represents the portion subcloned into the corresponding plasmids. For information on the *hetA* gene, see Holland and Wolk, 1990.

b


C.S4
EcoRV
2.4 kb
EcoRV
EcoRV
5.8 kb
EcoRV
hetA
EcoRV
OriV
Cos
Bom
10
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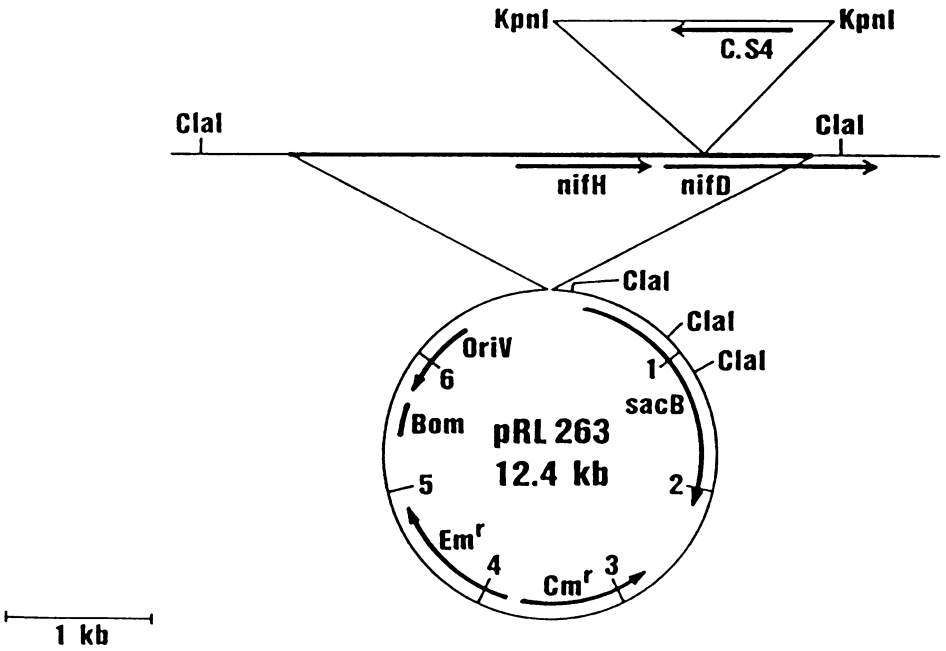
9
2
EcoRV
8
pRL 256
15.9 kb
pDU1 ΔMlul
3
sacB
7
4
6
5
1 kb
EcoRV
Mlul

c


KpnI
KpnI
C.S4
ClaI
ClaI
nifH
nifD
ClaI
ClaI
ClaI
OriV
1
6
sacB
Bom
pRL 263
12.4 kb
5
2
Em^r
4
Cm^r
3
1 kb

then stripped of its ability to replicate autonomously in *Anabaena* sp. by deleting a 1.7-kb *Mlu* I fragment from the pDU1 portion (Schmetterer and Wolk, 1988), yielding plasmid pRL61M. Finally, the 2597-bp *Bam*H I-*sacB-Pst* I fragment from plasmid pUCD800 (Gay et al., 1985) was inserted between the *Bgl* II and *Pst* I sites in the *nptII* region of pRL61M.

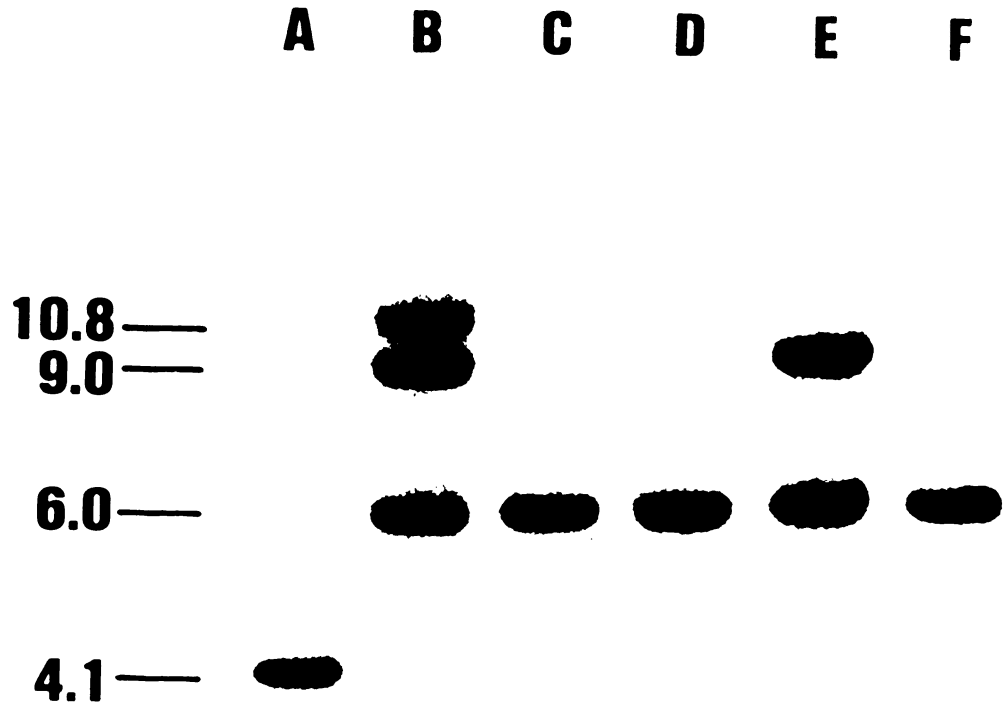
The suicide plasmid pRL263 (Fig. 2.1c) was constructed as follows. The *cat* gene of plasmid pRL517b (Table 2.2), excised with *Nhe* I and *Asu* II, was replaced by the 3851-bp *Xba* I-*sacB-nptI-Acc* I fragment from pUM24. The *nptI* gene was then cut out with *Pst* I and replaced by cassette C.CE1 (Cm^r and Em^r; Elhai and Wolk, 1988) from *Nsi* I to *Hind* III supplemented with a 42-bp *Hind* III-*Pst* I fragment from plasmid πvx (Seed, 1983).

Effect of *sacB* expression on cells of *Anabaena* spp. The two strains of *Anabaena* sp. that were tested, PCC 7120 and M-131, grow as well, and often better, on media containing 5% sucrose than on media lacking sucrose. When bearing plasmid pRL250 that contains the *sacB* gene (Fig. 2.1a), both strains, *Anabaena* sp. strains PCC 7120(pRL250) and M131(pRL250), become extremely sensitive to 5% sucrose in solid medium. Following plating of ca. 10^7 cells of newly derived exconjugants, filaments decomposed and cells bleached within 12 h, and few (see chapter 3) or no colonies subsequently arose. A similar phenotype was observed when the *sacB* gene was present as a single copy in the chromosome of *Anabaena* sp. strain PCC 7120 (see below, strains PCC 7120::pRL256 and PCC 7120::pRL263). Thus, despite the complexity of the regulatory region of the *sacB* gene from *B. subtilis* (Steinmetz et al., 1985), that gene appears to be well expressed in *Anabaena* spp.

Site-directed inactivation of the *nifD* gene in the chromosome of *Anabaena* sp. strain PCC 7120. Although plasmid pRL263 (Fig. 2.1c) cannot replicate in *Anabaena* sp. strain PCC 7120, the cyanobacterium can acquire resistance to Sm/Sp by homologous recombination with cloned *Anabaena* DNA that flanks the drug-resistance cassette. Single recombinants would be predicted to exhibit the phenotype Sm^r/Sp^r Em^r Suc^s Fox^+ and double recombinants to exhibit the phenotype Sm^r/Sp^r Em^s Suc^r Nif^- (therefore Fox^-). Following mating, exconjugants arose at a frequency of ca. 10^{-5} of cells plated. All 200 Sm^r/Sp^r exconjugants that I tested were Fox^+, and were therefore provisionally considered single recombinants and denoted PCC 7120::pRL263. Another 200 colonies derived from a two-month-old culture of a single-recombinant colony (see Fig. 2.3, lane C) were screened for Fox^- phenotype arising as a result of double recombination; that phenotype was not observed.

I selected for double recombinants by plating exconjugants on AA+NO_3^- solid medium containing Sm, Sp and 5% sucrose. Positively selected sucrose-resistant colonies (denoted PS263) appeared at a frequency of ca. 10^{-5} in experiments using direct plating, and approximately 10^{-4} when initial exconjugants were grown for one month in liquid medium before cavitation and plating. Of the 20 PS263 colonies resulting from direct plating, all but one, PS263-42, had an Sm^r/Sp^r Em^s Suc^r Fox^- phenotype, suggestive of double recombination. Replacement of the wild-type *nifD* gene by the *nifD*::C.S4 derivative as a result of double recombination was confirmed by Southern analysis (Fig. 2.2). PS263-42 had an Sm^r/Sp^r Em^r Suc^r Fox^+ phenotype and showed a pattern of hybridization predicted for a single recombinant (Fig. 2.2). Presumably, the *sacB* gene had been inactivated, rather than lost as in authentic double recombinants.

Figure 2.2. Southern analysis of DNA from *Anabaena* PS263 colonies. Markers indicate sizes of DNA (in kilobases). Total DNA from wild-type PCC 7120 (lane A), single-recombinant PCC 7120::pRL263 (lane B), double recombinants PS263-1, -2, and -50, respectively (lanes C, D, and F), and pseudo-double recombinant PS263-42 (lane E) was digested with *Cla* I and probed with linearized plasmid pRL393 (see table A.3). The origins of the bands observed can be deduced from Fig. 2.1c. The 4.1-kb band in the wild type containing the *nifH* gene and part of the *nifD* gene is replaced by a 6.0-kb band in all other colonies, in which the 1.9-kb cassette C.S4 is inserted within the *nifD* gene. Single recombinant PCC 7120::pRL263 (lane B) and pseudo-double recombinant PS263-42 (lane E) have a 9.0-kb band generated from the junction to the vector, and lane B contains also a 10.8-kb band which resulted from duplication of the whole plasmid. In the double recombinants (lanes C, D, and F), the 6.0-kb band which contains the *nifD* gene with the C.S4 insert is present, but the 9.0-kb band to which the vector contributes has been lost.

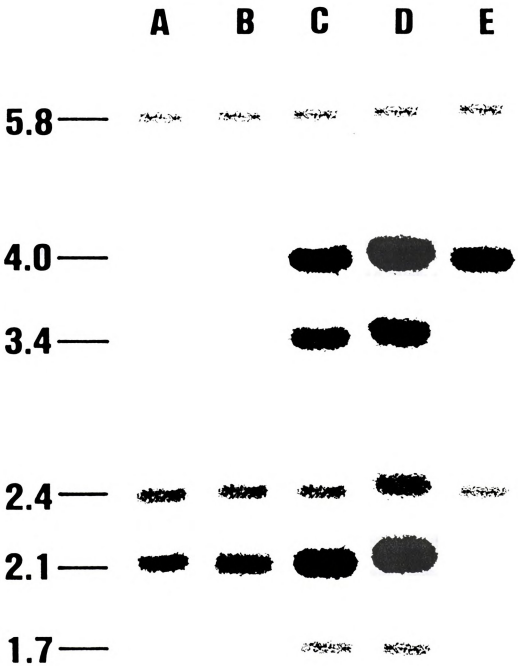

A
B
C
D
E
F
10.8
9.0
6.0
4.1

I refer to such strains as "pseudo-double recombinants." Possible mechanisms for their occurrence are discussed below. Thirty PS263 colonies isolated after extra cycles of growth in liquid, and cavitation, were examined, and 28 proved to have the phenotype of a double recombinant. Two, PS263-53 and PS263-54, resembled PS263-42 in phenotype.

Anabaena strain PS263-1, a representive, authentic double recombinant, is indistinguishable from wild-type *Anabaena* sp. strain PCC 7120 in both growth rate and morphology when grown in medium containing fixed nitrogen. When transferred to medium free of fixed nitrogen, in which it cannot grow, *Anabaena* PS263-1 develops heterocysts that are not distinguishable, by light microscopy, from those of the wild type strain. On the average, heterocysts are separated by fewer vegetative cells in the mutant than in the wild type strain (data not shown), presumably because the mutant is more deficient in nitrogen. The Nif$^-$ phenotype of this strain was confirmed by the negative result of acetylene-reduction assay of nitrogenase activity under aerobic and anaerobic conditions (A. Ernst, personal communication).

Site-directed inactivation of the *hetA* gene in the chromosome of *Anabaena* sp. PCC 7120. The *hetA* gene is a differentially regulated gene that affects the biosynthesis of the polysaccharide layer of the heterocyst envelope. A *hetA* mutant, EF116, generated by UV irradiation, can be complemented by a 3.5-kb sequence of chromosomal DNA that harbors the *hetA* gene; complementation is abolished when a drug cassette is inserted into the *Nru* I site within *hetA* (Wolk et al., 1988; Holland and Wolk, 1990). Using *sacB*-mediated positive selection and the suicide plasmid pRL256 (Fig. 2.1b), the wild-type *hetA* gene in the chromosome of PCC 7120 was replaced, through double recombination, by a

Figure 2.3. Southern analysis of DNA from *Anabaena* PS256 colonies. Markers indicate sizes of DNA (in kilobases). Total DNA was digested with *Eco*R V and probed with linearized plasmid pRL351 (see table A.3). The bands of 2.4, 2.1 and 5.8 kb correspond to the three *Eco*RV segments in the horizontal line of Fig. 2.1b. The bands of 3.4 and 1.7 kb, which appear only in single and pseudo-double recombinants, arise from the vector-insert junctions on the left and right sides of Fig. 2.1b, respectively. Wild-type strain PCC 7120 (lane A) and mutant EF116 (lane B) have bands of equal size, indicating that there is no significant variation in the *hetA* region of the mutant chromosome. In double recombinant PS256-17 (lane E), the 2.1-kb band is replaced by a 4.0-kb band, reflecting the insertion of C.S4 into the *hetA* gene. Pseudo-double recombinant PS256-5 (lane D) has the same pattern of hybridization as single recombinant PCC7120::pRL256 (lane C).

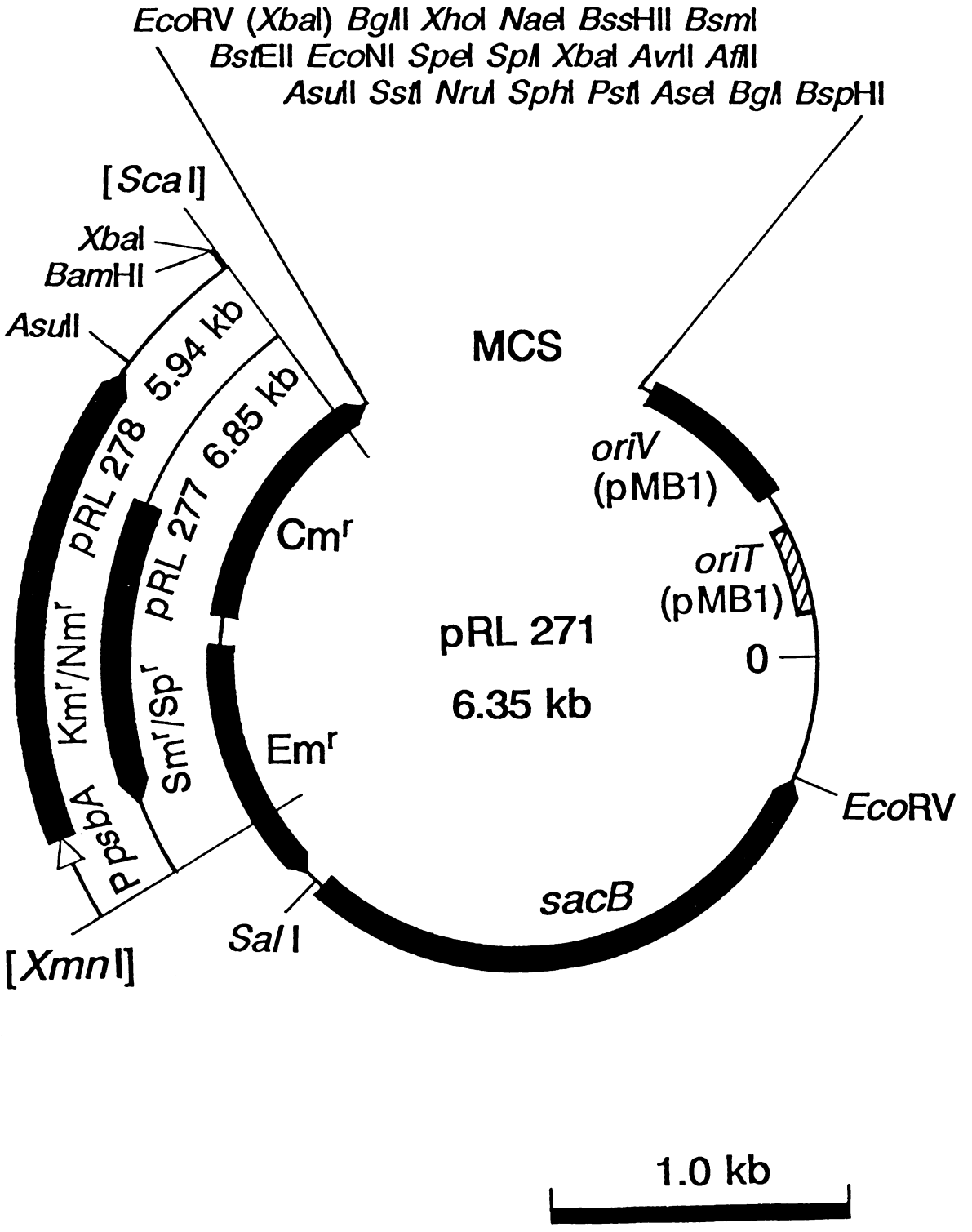

A
B
C
D
E
5.8
4.0
3.4
2.4
2.1
1.7

mutant *hetA* gene in which the Sm^r/Sp^r cassette C.S4 had been inserted into its *Nru* I site. Resulting *hetA* mutants, represented by PS256-17, are Sm^r/Sp^r Fox^-. Pseudo-double recombinants, represented by PS256-5, were identified by their phenotype (Sm^r/Sp^rFox^+) and by Southern analysis (Fig. 2.3). Single and double recombinants arose in these experiments with frequencies slightly lower than those observed in the *nifD*-inactivation experiments described above.

In medium free of fixed nitrogen the mutant strain PS256-17 develops cells that have the shape and spacing of heterocysts but that show no deposition of heterocyst envelope polysaccharide. In contrast, heterocyst envelope polysaccharide is deposited irregularly in mutant EF116 (Wolk et al., 1988). The difference may be attributable to the type of mutation present in *hetA* of the two mutants.

Construction of plasmids pRL271, pRL277, and pRL278, and facilitated application of the *sacB*-mediated positive selection system for gene replacement. A set of plasmids was designed to simplify the introduction of a mutated DNA fragment from *Anabaena* sp. into a *sacB*-containing suicide plasmid. Plasmid pRL271 (Fig. 2.4) has the following composition: bp 1 (*Eco*47 III, destroyed) to bp 2369 (*Sal* I) contains the gene *sacB* from pUM24; following the *Sal* I site is the sequence 5' CTGCA 3' followed by the Em^r gene (bp 1995-2896 of plasmid pE194; Horinouchi and Weisblum, 1982), then followed by the sequence CGAATTCA (contains an *Eco*R I site), then the 954-bp Cm^r gene from bp 4357 (*Asu* II, destroyed) to bp 5310 (*Asu* II, destroyed) of plasmid pBR325 (Balbás et al., 1986), followed by the *Eco*R V-*Bgl* II fragment of the pIC20H polylinker (Marsh et al., 1984). Thereafter comes a 527-bp *Xho* I-*Sst* I polylinker fragment derived from bp 1943 to 1572 and bp 1443 to 1289 of plasmid pJRD184 (Heusterspreute

Figure 2.4. Essential features of plasmids pRL271, pRL277, and pRL278. See text for composition of the plasmids. The 970-bp region of multiple cloning sites (MCS) between *Eco*R V (excluded) and *Bsp*H I (included) has sites for 20 different, hexanucleotide-specificity restriction endonucleases that cut nowhere else in the plasmids with the following exceptions: in pRL277, *Bsm* I also cuts within the Cm^r determinant; in pRL277, *Nae* I, *Bss*H II, *Bst*E II, *Ase* I, *Bgl* I, and *Bsp*H I also cut the Sm^r/Sp^r determinant; and in pRL278, *Nae* I, *Bss*H II, *Sph* I, and *Pst* I also cut the Km^r/Nm^r determinant. The *Xba* I site in parenthesis is *dam*-methylated. Three sites (not shown), *Eag* I, *Xmn* I, and *Xcm* I, upstream from the *aadA* gene (Sm^r/Sp^r) are unique in plasmid pRL277 and therefore can be used also as cloning sites. There is a *Sph* I site between those three addtional cloning sites and the MCS region, which draws caution to using *Sph* I in the subcloning because digestion by this enzyme removes most of the cloning sites from pRL277. Three sites, *Asu* II, *Bam*H I, and *Xba* I, downstream from the *nptII* gene (Km^r/Nm^r) in pRL278 may also be used as cloning sites. The *Xmn* I and *Sca* I sites in pRL277 and pRL278 are destroyed, and the antibiotic resistance cassettes between these two sites are not drawn to scale.


EcoRV (XbaI) BglII XhoI NaeI BssHII BsmI
BstEII EcoNI SpeI SphI XbaI AvrII AflII
AsuII SstI NruI SphI PstI AseI BglI BspHI
[ScaI]
XbaI
BamHI
AsuII
5.94 kb
6.85 kb
pRL 278
pRL 277
Km^r/Nm^r
Sm^r/Sp^r
PpsbA
[XmnI]
Cm^r
Em^r
SalI
MCS
oriV
(pMB1)
oriT
(pMB1)
pRL 271
6.35 kb
0
EcoRV
sacB
1.0 kb

et al., 1985; bp 1571 to 1444 was deleted by treatment with T4 DNA polymerase of the *Bgl* II and *Pst* I ends, and subsequent ligation), followed by the *Nru* I to *Pst* I fragment of the polylinker from pIC20H. Finally, the *oriV* (pMB1)- and *oriT* (pMB1)-containing portion of the plasmid is derived from the *Pst* I to *Pvu* II (destroyed) fragment of pBR322 (Balbás et al., 1986). The *Xmn* I and *Sca* I sites (both blunt) in the 3' portion of the Em^r and Cm^r genes, respectively, permit convenient replacement of antibiotic resistance cassettes to generate derivatives of pRL271. A Sm^r/Sp^r derivative (pRL277) was made by ligation of the *Dra* I-bounded *aadA*-containing Ω cassette (from pHP45Ω; Prentki and Krisch, 1984), and a Km^r/Nm^r derivative (pRL278) was made by ligation of the *nptII*-containing cassette C.K3 (Elhai and Wolk, 1988a; the *Xba* I ends were made blunt by filling-in reactions using the Klenow fragment), with the larger *Xmn* I-*Sca* I fragment of pRL271 (Fig. 2.4).

These three plasmids are identical except for their antibiotic resistance genes that permit selection in both *E. coli* and *Anabaena* spp. The region of multiple cloning sites (MCS) has 20 possible sites that are compatible with the ends generated by many restriction endonucleases. The relative simplicity of using one of these plasmids to achieve gene replacement in *Anabaena* sp. was illustrated by the inactivation, in cooperation with D. Holland, of the gene *conA*.

Positioned 3' from *hetA* is a single-copy gene (partially characterized, and denoted ORF2 in Holland and Wolk, 1990) to which I refer as *conA* because it is **con**stitutively expressed during growth with NO_3^- and during heterocyst differentiation. Because the *hetA* mutant PS256-17 (see above) could not be complemented by cosmids that complement mutant EF116 (D. Holland, unpublished results), we speculated that the function of *conA* might be needed

for the function of *hetA*. The Sm^r/Sp^r Ω fragment (Prentki and Krisch, 1988), provided with *Xba* I ends by passage through plasmid pRL453 (Elhai and Wolk, 1988a), was inserted into the *Xba* I site in the 5' region of *conA*, disrupting the predicted ORF. A 5-kb *Pvu* II fragment containing this insertionally mutated *conA* and the entire *hetA* was then cloned into the unique *Nru* I site of pRL271. The resulting plasmid, pRL743, was transferred into *Anabaena* sp. strain PCC 7120 by conjugation. Sucrose-resistant exconjugants (denoted PS743) derived from a sucrose-sensitive single recombinant (*Anabaena* sp. strain PCC 7120::pRL743) were analyzed by Southern analysis. Five of the six colonies examined were double recombinants in which the mutated *conA* gene had replaced the wild-type gene (data not shown). None of the five mutants differed phenotypically from the wild type, suggesting that the product of *conA* is dispensable under the growth conditions employed.

Plasmid pRL270, a precursor of pRL271 which lacks the polylinker fragment from pJRD184, was similarly used to inactivate the gene (*prcA*) encoding a calcium-dependent protease in the genomes of *Anabaena* spp. strains PCC 7120 and ATCC 29413 FD (Maldener et al., 1991).

DISCUSSION

Utilization of the conditional lethalitity of *sacB* permits positive selection for double recombinants in the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 and presumably in any other cyanobacterial strains that show similar susceptibility to sucrose when bearing the *sacB* gene. Using this technique with *Anabaena* sp. strain PCC 7120, I inserted a Sm^r/Sp^r cassette (C.S4) into the *nifD* gene in the chromosome to create a *nifD* mutant PS263-1, and into the *hetA*

gene to create a *hetA* mutant PS256-17 that is defective in heterocyst formation and Fox$^-$.

This technique has significant advantages relative to screening. Double recombinants can be obtained within less than one month following conjugation. As in the experiment using plasmid pRL263, pseudo-double recombinants can be easily distinguished by testing for the antibiotic resistance (Em^r) conferred by the vector. Experimental data available so far (J.-M. Panoff, personal communication) indicate that as little as 0.2 kb of homologous DNA bordering the inactivation cassette is sufficient for isolating double recombinants. By contrast, it is very difficult, if at all possible, to isolate double recombinants by screening when there is nearly as small an amount of bordering, homologous DNA. Because the size of homologous DNA required is small, it is relatively easy to find an unique site at which to insert an inactivation cassette into the gene of interest. Moreover, use of *sacB* should allow an unmarked mutation to be introduced into the chromosome (Ried and Collmer, 1987).

In the history of molecular genetic studies of *Anabaena* sp. strain PCC 7120, many genes such as the nitrogenase genes *nifHDK* (Rice et al., 1982), the ribulose-1,5-bisphosphate carboxylase genes *rbcLS* (Curtis and Haselkorn, 1983), and the ATP synthase (*atp*) genes (Curtis, 1988) were identified by heterologous hybridization and subsequent nucleotide sequencing. Although valuable information has been obtained from studies of such gene homologs and their presumed promoters, the true identity and function of those proposed genes (e.g., the second copy of *nifH*; Rice et al., 1982) and promoters (e.g., the series of *glnA* promoters; Tumer et al., 1985) remain theoretical without mutational investigation. Some of the presumptions such as the initially defined

nifD open reading frame (Lammers and Haselkorn, 1983), and the proposed *nifK* promoter and its sequence *per se* (Mazur and Chui, 1982) have later proved incorrect (Golden et al., 1985, and Haselkorn, 1986). Development of the *sacB*-mediated positive selection system provides an easy means of achieving gene replacement and should facilitate the verification of proposed genetic constituents. The insertional mutation introduced into the *nifD* gene in this study provided the first confirmation of the function of a gene of *Anabaena* sp. strain PCC 7120 that had been proposed on the basis of heterologous hybridization and sequencing. Inactivation of *conA* provided the first experimental demonstration that a constitutive gene in *Anabaena* sp. could be mutated without causing any obvious change of the phenotype of the organism (except for the antibiotic resistance introduced into the mutant). Accordingly, a similar experiment can determine whether insertional mutation of any chosen constitutive gene is lethal to *Anabaena* sp.

One problem in the use of the *sacB* system is the production of pseudo-double recombinants as a result of inactivation of the *sacB* gene. Spontaneous mutations of *sacB* in cells of *Anabaena* sp., including point mutations, deletions and the insertion of IS elements (see chapter 3), can certainly contribute to the appearance of pseudo-double recombinants. My results upon plating cells of *Anabaena* strain PCC 7120(pRL250) in the presence of 5% sucrose suggest that spontaneous mutations of *sacB* in cells of *Anabaena* sp. accumulate with time of cell culture.

Nonetheless, Inactivation of the *sacB* gene in *E. coli* cells prior to conjugation is probably the major cause of the appearance of pseudo-double recombinants. I have observed that an overnight liquid culture inoculated with

a single small sucrose-sensitive colony of *E. coli* strain DH5(pUM24) gave rise to large and small sucrose-resistant colonies at a total frequency of 10^{-3}. Restriction analysis of plasmid pUM24 from these sucrose-resistant colonies suggested that in 1% of the total, and mostly in large colonies, the plasmid had experienced insertion of transposable elements. IS*2*- and IS*10*-like elements, tentatively identified by size and by restriction analysis (Ghosal et al., 1979; Halling et al., 1982; Galas and Chandler, 1989), were found in the *sacB* gene in this test. If *sacB*-bearing cells of *E. coli* are subcultured repeatedly prior to conjugation, the percentage of pseudo-double recombinants among sucrose-resistant colonies of *Anabaena* sp. can exceed 50%. However, if care is taken in choosing sucrose-sensitive colonies for inoculation of plasmid-donating cultures of *E. coli* and in their length of culture, the percentage of pseudo-double recombinants can usually be contorlled to be under 5%. Similarly, although a longer period of culturing single-recombinant colonies increases the absolute number of double recombinants, such subculture of single recombinants has also been observed to increase the percentage of pseudo-double recombinants among sucrose-resistant colonies, presumably due to an accumulation of spontaneous mutations of the *sacB* gene in the cells of *Anabaena* sp.

Surprisingly, *Anabaena* sp. strians PCC 7120::pRL256 and PCC 7120::pRL263, both of which have a functional copy of *sacB* in the chromosome, grow well in the liquid medium AA/8+NO_3^-+5% sucrose, so that culture in sucrose-containing liquid medium does not enrich double recombinants. I cannot account for this difference in results between solid and liquid media. Similarly, UV-irradiation of single recombinants in liquid (with the intent of increasing the frequency of a second recombinational event prior to selection on

sucrose plates) only increases the percentage of pseudo-double recombinants, possibly by increasing the mutation rate of *sacB* in the cells of *Anabaena* sp.

Construction of the suicide plasmids pRL256 and pRL263 for the initial experiments of gene replacement was case-specific and the two contructs were not useful for introduction of other mutated genes into the chromosome. Construction of plasmids pRL271, pRL277 and pRL 278 facilitated such application by providing a set of plasmids, with a variety of antibiotic resistance markers, that are much more adaptable to other cloned fragments. As described in this chapter and in chapter 4, by using one of these plasmids or their derivatives, introduction of a specific mutation into the chromosome of *Anabaena* sp. can be as easy as one subcloning plus one conjugal transfer, followed by selection, provided that a marked, mutated DNA fragment from *Anabaena* sp. is readily available. During the past two years the three plasmids, and their immediate progenitors, have been distributed to 19 laboratories worldwide to use for this and similar purposes in cyanobacteria or other Gram-negative bacteria.

Chapter 3

USE OF A CONDITIONALLY LETHAL GENE TO ENTRAP INSERTION SEQUENCES IN *Anabaena* SP. STRAIN PCC 7120 AND CHARACTERIZATION OF THE FAMILY OF THE INSERTION SEQUENCE IS*892* [1]

SUMMARY

The conditionally lethal nature of the *sacB* gene was used to entrap insertion sequences from *Anabaena* sp. strain PCC 7120. Selected, spontaneously sucrose-resistant colonies derived from cells bearing the *sacB*-containing, autonomously replicating plasmid pRL250 were analyzed. Inactivation of *sacB* proved to be largely due to insertions into the gene by a variety of insertion sequences in cells of *Anabaena* sp. At least six different, presumed insertion sequences were found in this study.

IS*892*, the insertion sequence most frequently observed in this study, has been further characterized. It is 1675 basepairs (bp) in size with 24-bp near-

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[1] Most of the data presented in this chapter have been published in the Journal of Bacteriology (**Cai and Wolk, 1990**; **Cai, 1991**).

perfect inverted terminal repeats, and has two open reading frames (ORFs) that could code for proteins of 233 and 137 amino acids, respectively. Upon insertion into target sites, which are usually A/T rich, this IS generates an 8-bp directly repeated target duplication. A 32-bp sequence in the region between ORF1 and ORF2 is similar to the sequence of the inverted termini. Similar inverted repeats are found within each of those three segments, and the sequences of these repeats bear some similarity to the 11-bp direct repeats flanking the 11-kb insertion interrupting the *nifD* gene of this strain (Golden et al., 1985). A sequence similar to that of a binding site for the *E. coli* integration host factor (IHF) is found about 120 bp from the L end of IS*892*. Partial nucleotide sequences of active IS elements IS*892*N and IS*892*T, members of the IS*892* family from the same strain of *Anabaena* sp., were shown to be very similar, but not identical, to the sequence of IS*892*.

INTRODUCTION

Insertion sequences (IS) are transposable DNA-elements that are generally smaller than transposons and normally bear only genes related to transposition (for a review, see Galas and Chandler, 1989). Because IS elements lack selectable markers, direct genetic selection for transposition of these elements is generally not possible. However, IS elements can be detected indirectly as a consequence of their transposition into, and inactivation of, a marker gene or an operon (Malamy, 1970; Fiandt et al., 1972; Gay et al., 1985). IS elements have been isolated from a variety of prokaryotes. Some of the elements have been sequenced and studied in detail. Most IS elements are 0.8 to 2.5 kb in size and have near-perfect inverted terminal repeats ranging from 8 to 41 bp. Almost all

bacterial IS elements characterized to date generate directly repeated duplications of their target DNA sequences upon insertion, presumably as a result of staggered cutting of target DNA. Many of these elements generate a duplication of a fixed number of base pairs, ranging from 2 to 13 bp, as a characteristic of the element (Galas and Chandler, 1989).

Cyanobacteria differ physiologically and phylogenetically (Woese, 1987) from other eubacteria. Two active IS elements have been isolated and sequenced from cyanobacteria: IS*701* from *Calothrix* sp. strain PCC 7601 (Mazel et al., 1988) and IS*891* from *Anabaena* sp. strain M-131 (Bancroft and Wolk, 1989). IS*701* appears to be a typical IS element with inverted terminal repeats and the generation of target duplications (Galas and Chandler, 1989). By contrast, IS*891* lacks inverted terminal repeats and fails to generate a target duplication upon insertion. In addition, genetic elements, collectively denoted the *mys* family, from the cyanobacterium *Anabaena* sp. strain PCC 7120 were suggested to be insertion sequences on the basis of the structural similarity of their nucleotide sequence to that of typical IS elements (Alam and Curtis, 1985).

The gene *sacB* is conditionally lethal to some Gram-negative bacteria and has been used to entrap insertion sequences in some of those bacteria (Gay et al., 1985). The cyanobacterium *Anabaena* sp., when bearing that gene, was shown to be sensitive to sucrose (see chapter 2). In this chapter, I shall discuss the discovery, by the use of *sacB*, of a variety of insertion sequences in cells of *Anabaena* sp. strain PCC 7120, and a detailed study of one of the insertion sequences, IS*892*, and related elements in this strain of *Anabaena*.

MATERIALS AND METHODS

Bacterial strains, growth media, cultural conditions, most molecular biological techniques used in this study, and chemical suppliers have been described in chapter 2. A nitrocellulose filter that was to be rehybridized for further Southern analysis was first stripped of radioactivity by immersion for 10 min in 1 liter of boiling 5 mM EDTA (pH 8.0).

Isolation of plasmid DNA from *Anabaena* spp. Plasmid DNA was extracted from *Anabaena* sp. strains PCC 7120 and M-131 by a boiling procedure modified from Holmes and Quigley (1981): cells from a 50-ml liquid culture in early stationary phase were harvested, washed with 1.0 ml of H_2O, and mixed with 700 μl of STET solution (2% sucrose, 5% Triton X-100, 50 mM EDTA [pH 8.0], 10 mM Tris·HCl [pH 8.0]) plus 50 μl of 10 mg lysozyme per ml of H_2O. After 5 min at room temperature, the suspension was heated in boiling water for 40 s. The mixture was then centrifuged at 21,000 x *g* for 20 min at 4°C, and the pellet removed. The supernatant solution was extracted with phenol and then with chloroform. DNA was precipitated by isopropanol at -70°C and resuspended in 50 μl of $T_{1/10}E$ solution (10 mM Tris·HCl, 0.1 mM EDTA [pH 8.0]).

Determination and analysis of DNA sequence. DNA sequence was determined by using synthetic DNA primers and ordered deletions of fragments subcloned into vectors pUC118 and pUC119 (Vieira and Messing, 1987). The sequencing strategy used is shown in Fig. 3.1. Deletions were made by a combination of timed digestion by exonuclease III and treatment with mung bean nuclease according to a protocol provided by Stratagene (La Jolla, CA). DNA oligonucleotide primers were synthesized and purified with equipment and reagents supplied by Applied Biosystems, Inc. (Foster City, CA). Double-

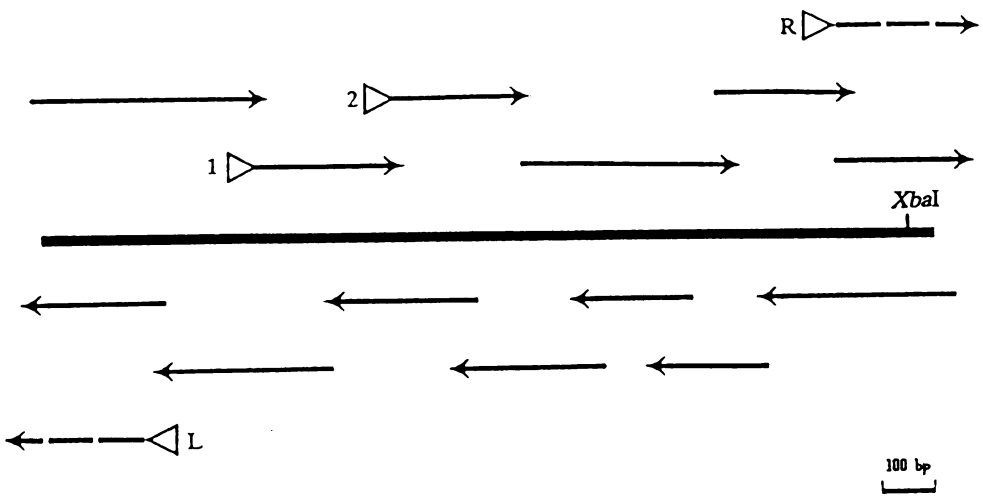


Figure 3.1. The sequencing strategy used for determination of the nucleotide sequence of IS*892*. The *Xba* I site is shown for orientation. All arrows point from 5' to 3'. An open triangle indicates a synthetic DNA primer. Primers L and R were used mainly for sequencing of insertion target sites. The sequences of the synthetic primers were:

1. 5' TGCTTATATAGGAGC 3'
2. 5' TTGCTTATCAGGAGA 3'

L. 5' GCTGTAGTTCTACTAC 3'

R. 5' TGCCTGTGCCATCGC 3'

-stranded DNA and the chain termination technique using dideoxynucleotides (Sanger et al., 1977) were used in DNA sequencing, utilizing Sequenase version 2.0 from United States Biochemical Corp., and adenosine 5'-α-[^{35}S]thiotriphosphate ("^{35}S-ATP", as ca. 1.0 mg per ml triethylammonium salt at a concentration of 10 mCi per ml) from Amersham Corp. Electrolyte gradient polyacrylamide sequencing gels were prepared and run as described previously (Sheen and Seed, 1988). The gels were fixed in a solution of 5% methanol plus 5% acetic acid (Biggin et al., 1983) and dried at 80°C *in vacuo* before autoradiography. Both strands of the nucleotide sequence presented in Fig. 3.3 were sequenced. Nucleotide and amino acid sequences were analyzed with the assistance of the software Editbase (Purdue Research Foundation and USDA/ARS), HIBIO DNASIS, and HIBIO PROSIS (Hitachi America Ltd., San Bruno, CA).

Nucleotide sequence accession number. The nucleotide sequence of IS*892* shown in Fig. 3.3 has been deposited in GenBank under accession number **M64297**.

RESULTS AND DISCUSSION

Spontaneous mutations in *Anabaena* sp. strain PCC 7120. Plasmid pRL250 (See Fig. 2.1a of chapter 2) bears the gene *sacB* and a clone of the cyanobacterial plasmid pDU1 that confers autonomous replication in *Anabaena* spp. (Wolk et al., 1984; Schmetterer and Wolk, 1988). *Anabaena* sp. strain PCC 7120 bearing this plasmid cannot grow on sucrose-containing solid medium because of the presence of a functional *sacB* gene (see Chapter 2). One such colony of *Anabaena* strain PCC 7120(pRL250) was subcultured continuously for

2 months in liquid medium AA/8 plus NO_3^-, and about 10^7 cells were then plated on solid medium AA plus NO_3^-, neomycin, and 5% sucrose. Approximately 300 colonies were recovered after 10 days. The pRL250-like plasmids isolated from twenty-two of these colonies (denoted PS250-N, where N=1, 2..., 22; see Appendix B) were analyzed by Southern hybridization (Fig. 3.2a). In 15 of these plasmids, the 2.6-kb *sacB*-containing *Pst* I fragment of pRL250 was replaced by a larger *Pst* I fragment, while other *Pst* I fragments of the plasmid were unchanged. In the remaining seven cases the 2.6-kb fragment showed no visible change in size, or appeared to have been deleted entirely. In the strains that showed no visible change in the 2.6-kb fragment the *sacB* gene may have been inactivated by a point mutation or a small deletion, thus accounting for the viability of the strains on sucrose-containing medium.

The 15 variant plasmids of pRL250 that showed an increase in size of the 2.6-kb fragment were recovered by transformation of *Escherichia coli* strain HB101 or DH5α with total or plasmid DNA extracted from the corresponding *Anabaena* colonies (the *E. coli* strain HB101 is better suited than strain DH5α for recovery of *Anabaena* DNA: see Appendix B), and are listed in Table A.3 of Appendix C. Although colony PS250-1 appeared (see Fig. 3.2a) to contain a second variant bearing a 1.7-kb insertion that hybridizes to IS*892* (see Fig. 3.2b), this variant was not recovered. On the basis of limited data from restriction mapping and Southern analysis, the 15 presumptive IS elements that entered these 15 plasmids have been tentatively grouped and named (Table 3.1).

Plasmid pRL272, a variant of pRL250 recovered from colony PS250-3, was restriction-mapped. The data revealed a 1.7-kb insertion in the 58-bp region from *Eco*R I to *Pvu* II of *sacB* (Cai and Wolk, 1990). This insertion element, denoted

Figure 3.2. Southern analysis of DNA from colonies of *Anabaena* PS250-N (N=1, 2, ..., 22). Total DNA from PS250-1 to PS250-22 (lanes 1 to 22) and from PCC 7120(pRL250) (lane 23) and DNA of plasmid pRL250 (lane 24) were digested with *Pst* I. (a) Insertion of IS elements into *sacB* of pRL250. The blotted filter was probed with labelled plasmid pRL250. The band of 2.6 kb corresponds to the *Pst* I fragment that bears the entire *sacB* gene, while the bands of 9.5, 1.2, and 0.5 kb are from the rest of plasmid pRL250 (Fig. 2.1a). (b) Detection of IS*892*-related elements. The same filter, stripped of radioactivity from the above hybridization, was reprobed with the internal *Dra* I-*Eco*R V fragment from IS*892* (see Fig. 3.3). The unnumbered lanes on the left in both panels provide size markers of DNA (in kilobases).

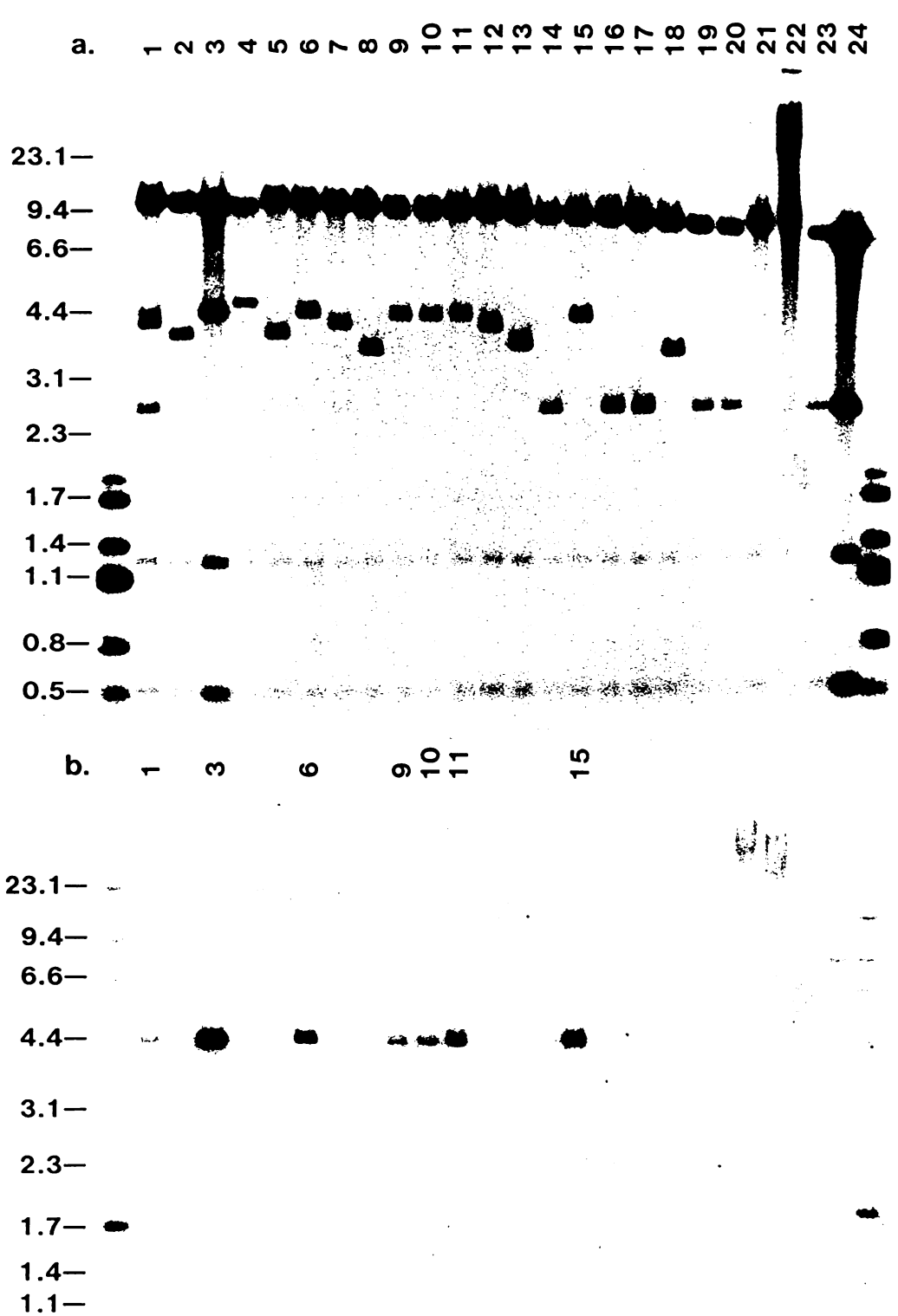

a.
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
23.1—
9.4—
6.6—
4.4—
3.1—
2.3—
1.7—
1.4—
1.1—
0.8—
0.5—
b.
1 3 6 9 10 11 15
23.1—
9.4—
6.6—
4.4—
3.1—
2.3—
1.7—
1.4—
1.1—
0.8—
0.5—

Table 3.1. Insertion sequences found in *Anabaena* sp. strain PCC 7120

IS element	Size (kb)	Source of insertion	Comment
IS*892*	1.7 kb	PS250-3	Also PS250-6, -9, -10, -11 and -15; see text
IS*893*	1.2 kb	PS250-2	Possibly also PS250-13
IS*894*	1.9 kb	PS250-4	
IS*895*	1.2 kb	PS250-5	A *mys* element; see text
IS*897*	1.5 kb	PS250-7	Possibly also PS250-1 and PS250-12
IS*898*	1.0 kb	PS250-8	Possibly also PS250-18

IS*892*, and other hybridizing elements discovered in this experiment were further characterized (see below).

The 0.8-kb *Hin*d III-*Eco*R I fragment from plasmid pAn625 (a gift of S. E. Curtis, North Carolina State University) that contains most of the presumed insertion element *mysA* (Alam and Curtis, 1985), hybridized strongly to the IS element (denoted IS*895*) that had inserted into the *sacB* gene of pRL250 in colony PS250-5 (data not shown), suggesting extensive homology between the two DNA elements. The IS*895*-containing plasmid pRL745 was therefore sent to S. E. Curtis for further characterization. A detailed study of the family of IS*895* from *Anabaena* sp. strain PCC 7120 has been published (Alam et al., 1991).

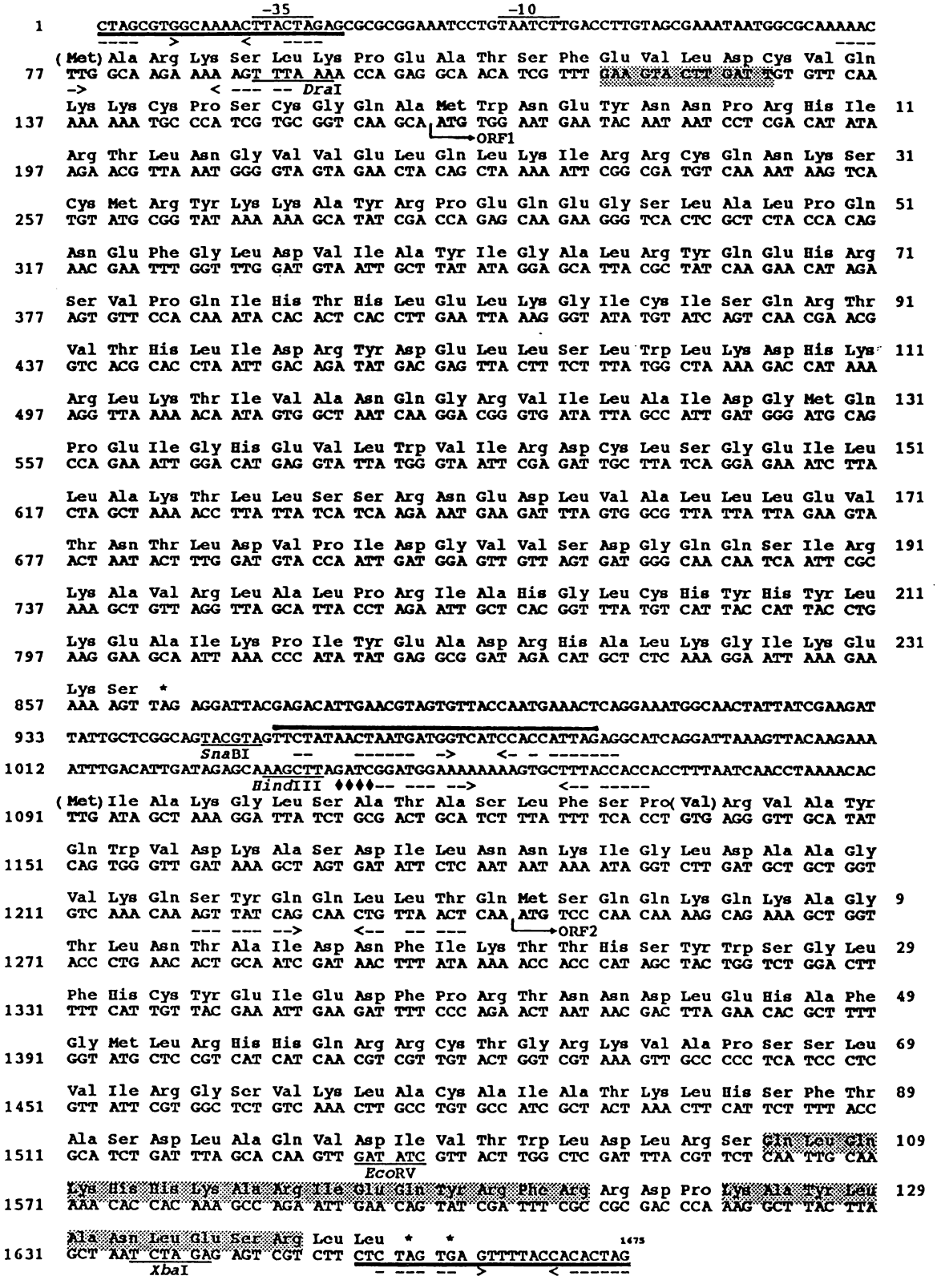
The transposable element IS*891*, isolated from the closely related *Anabaena* sp. strain M-131, hybridized to the genome of *Anabaena* sp. strain

PCC 7120 (Bancroft and Wolk, 1989), but failed to hybridize to the 15 active insertion sequences discovered in this experiment.

General features of IS*892*. IS*892* (Fig. 3.3) is 1,675 bp in length and has 24-bp near-perfect (21 out of 24 bp; Fig. 3.6) inverted terminal repeats that show no significant sequence similarity to termini of other known bacterial IS elements (Galas and Chandler, 1989). Two open reading frames (ORFs) are present in tandem on the same DNA strand. When the first methionine residue is taken as the translational initiation codon, the two ORFs, ORF1 and ORF2, are predicted to code for proteins of 233 and 137 amino acids, respectively. Possible alternative start codons TTG (Zhang et al., 1989; Tsinoremas et al., 1991) and GTG (Reddy et al., 1988) could extend ORF1 to 262 amino acids and ORF2 to 188 or 173 amino acids (Fig. 3.3). As has been observed in a number of cyanobacterial genes (Tandeau de Marsac and Houmard, 1987) and the other three sequenced cyanobacterial IS elements (Bancroft and Wolk, 1989; Alam et al., 1991; Tandeau de Marsac, personal communication), neither ORF of IS*892*, in defined or extended version, is preceded by a typical ribosome binding sequence. The complementary strand does not contain complete ORFs of greater than 82 codons with a reasonably positioned initiation codon. An incomplete reading frame, initiated at the first methionine codon at bp 357 to 355, extends for 118 codons and out of the left (L) end of IS*892* without a stop codon.

An *E. coli*-type promoter (Mulligan et al., 1984) is present 5' to ORF1 (Fig. 3.3). The -35 region of that presumed promoter, 5' TTACTA 3', lies within the L-end terminal repeat sequence. A -35 region of an *E. coli*-like promoter, pointing outward, can be found in the inverted termini: 5' TTGCCA 3' at the L end and 5' TTACCA 3' at the right (R) end. Such outward-pointing half promoters have been

Figure 3.3. Complete nucleotide sequence of the transposable element IS*892* from the cyanobacterium *Anabaena* sp. strain PCC 7120. The noncoding strand of the sequence is presented in 5' (left [L] end) to 3' (right [R] end) direction. Numbering of the nucleotide sequence and of the amino-acid sequences of ORF1 and ORF2 are presented, respectively, to the left and to the right of the sequence. The deduced amino acid sequences of ORF1 and ORF2 and their possible 5' extensions are displayed above the DNA sequence, with presumed translational initiation sites printed in boldface. Possible alternative start codons are indicated in parentheses. Also shown are the -35 and -10 regions of a presumed promoter for ORF1. The inverted termini are double underlined. Potential stem-loop structures are indicated by pairs of counterpointing arrows under the nucleotide sequence. Restriction sites mentioned in the text are underlined, and the site for *dam* methylation is marked with filled diamonds. The M sequence, from bp 953 to 984, which is similar to sequences of the terminal repeats (see Fig. 3.6), is highlighted by a bar above the sequence. The shaded DNA sequence around bp 125 is a possible binding site for the *E. coli* integration host factor (IHF, see text), and the shaded amino acid sequences at the carboxyl end of ORF2 may be a potential helix-turn-helix DNA binding structure.


-35
-10
DraI
ORF1
ORF2
SnaBI
HindIII
EcoRV
XbaI

found at the ends of many IS elements, and their implication has been discussed previously (Galas and Chandler, 1989).

The sequence 5' GAAGTACTTGATT 3', from bp 116 to 128 (Fig. 3.3), matches well with the consensus sequence 5' TAAnTnnTTGATT 3' (Goodrich et al., 1990) of binding sites for the *E. coli* integration host factor (IHF). In *E. coli*, the histonelike protein IHF (Drlica and Rouviere-Yaniv, 1987; Friedman, 1988) has been shown to participate in the transposition of IS*1* and IS*10*, which have IHF binding sites at or near their end sequences (Gamas et al., 1987; Morisato and Kleckner, 1987). Possible IHF binding sites have been found in IS elements of various origins (Galas and Chandler, 1989). The presence of a putative IHF-binding site near one end of IS*892*, in IS*701* (Galas and Chandler, 1989), and in IS*895* (Alam et al., 1991) makes it tempting to speculate that an IHF-like protein, although not yet observed in cyanobacteria, could be involved in the transposition of their IS elements.

Several strains of cyanobacteria, including *Anabaena* sp. strain PCC 7120, exhibit *dam* methylation of their DNA (Padhy et al., 1988). Sites for *dam* methylation found at the ends of IS*10*, IS*50*, and IS*903* have been reported to influence transposition of these IS elements in *E. coli* (Roberts et al., 1985; Dodson and Berg, 1989). The sole site for *dam* methylation found in IS*892* is located at the end of a stem-loop structure 5' to ORF2 (the N sequence, see Fig. 3.3 and discussion below). Whether that site influences the activity of IS*892* is, however, unknown.

The two proteins predicted by ORF1 and ORF2 in IS*892*, 26.8 and 15.8 kDa in molecular mass, respectively, are likely cytosolic proteins because each has an overall hydropathy index of -0.1 with no peaks over +/-- 1.0 (window size:

19 amino-acid residues; von Heijne, 1987). Although calculated isoelectric points are close to 7.0, both proteins have a moderately high content of basic amino acids (arginine and lysine residues account for ca. 15% of the amino acids in each protein), consistent with possible interactions of the proteins with DNA (Galas and Chandler, 1989). Prediction of secondary structure by the Garnier-Robson method (von Heijne, 1987) suggested that a helix-turn-helix conformation, a structural motif found repeatedly in prokaryotic DNA binding proteins (Pabo and Sauer, 1984), could form at the carboxyl end of ORF2 (Fig. 3.3).

The G+C contents and codon usage of the genome of *Anabaena* sp. strain PCC 7120 and of IS*892* were compared (Table 3.2 and 3.3). IS*892* has a markedly lower G+C content than that of the genome, and its codon usage differs extensively from that of abundantly expressed chromosomal genes: of the 18 amino acids that have multiple synonymous codons, only five amino acids (Pro, Gln, Ala, Val, and Glu) are represented by similar codon preferences. The altered codon usage in IS*892* seems consistent with its lower G+C content: almost all changed codon preferences favor codons ending with A or U. Alternatively, codon usage, often not correlated with the G+C content of an organism (Ikemura, 1985), may be attributable to the presumed low expressivity of the genes of IS*892* (Gouy and Gautier, 1982).

Computer-assisted sequence comparisons (Lipman and Pearson, 1985; von Heijne, 1987) between IS*892* and other cyanobacterial IS elements IS*701* (Tandeau de Marsac, personal communication), IS*891*, and IS*895* failed to identify regions of significant similarity of nucleic acid or protein sequence. A search covering both the GenBank and the EMBL data bases also failed to

Table 3.2. Comparison of G+C contents of IS*892*, the chromosome, and the *nifD* element of *Anabaena* sp. strain PCC 7120 [a]

G+C content	*Anabaena* sp. strain PCC 7120	*nifD* element	IS*892*
Overall	42.5	38.7	38.7
Of ORFs	47.9	40.5	39.7

[a] Data on overall G+C content of the strain is as previously published (Herdman et al., 1979a), and that of the *nifD* element is calculated from published sequence data (Lammers et al., 1986, 1990). The G+C content of the ORFs of this strain of *Anabaena* is calculated from eight abundantly expressed chromosomal genes (Tandeau de Marsac and Houmard, 1987), that of the *nifD* element is calculated from five proposed ORFs in the sequenced region (Lammers et al., 1986, 1990), and that of IS*892* is calculated from its two ORFs.

recognize a known transposable element that shares significant sequence similarity with IS*892*.

A family of IS*892*-related insertion sequences. The internal *Dra* I-*Eco*R V fragment of IS*892* (Fig. 3.3) was used to re-probe the filter used in Fig. 3.2a. Six bands of the same size as the band from PS250-3 showed strong hybridization (Fig. 3.2b), suggesting that the IS elements from these seven colonies are homologous. Plasmids were recovered from these colonies (except for colony PS250-1, see above), and the L-end portion of their insertions partially sequenced. The partial sequences (ca. 400 bp) of the insertions from PS250-6, -11, and -15 were identical to the corresponding sequence of IS*892*. However,

Table 3.3. Comparison of codon usage of IS*892*, the chromosome, and the *nifD* element of *Anabaena* sp. strain PCC 7120 [a]

Amino acid	Gene codon	Codon usage frequency of: *Anabaena* sp. strain PCC 7120	*nifD* element	IS892	Amino acid	Gene codon	Codon usage frequency of: *Anabaena* sp. strain PCC 7120	*nifD* element	IS892
Arg	CGA	1.5	13.7	20.0	Ile	AUA	1.4	31.8	29.6
	CGC	29.4	28.8	13.3		AUC	69.5	23.5	18.5
	CGG	6.6	11.0	10.0		AUU	29.1	44.7	51.9
	CGU	51.5	20.5	23.3					
	AGA	9.6	20.5	26.7	Lys	AAA	54.7	80.5	79.2
	AGG	1.5	5.5	6.7		AAG	45.3	19.5	20.8
Leu	CUA	11.1	17.9	14.9	Asn	AAC	91.7	32.3	28.6
	CUC	14.6	10.6	12.8		AAU	8.3	67.7	71.4
	CUG	19.4	13.0	4.2					
	CUU	4.4	9.7	12.8	Gln	CAA	80.4	74.0	73.7
	UUA	16.6	30.9	48.9		CAG	19.6	26.0	26.3
	UUG	40.0	17.9	6.4					
					Ilis	CAC	91.0	33.3	36.8
Ser	UCA	6.8	21.0	33.3		CAU	9.0	66.7	63.2
	UCC	28.4	13.6	9.5					
	UCG	0.0	6.2	0.0	Glu	GAA	80.4	68.0	76.2
	UCU	39.8	23.4	28.6		GAG	19.6	32.0	23.8
	AGC	21.6	14.8	4.8					
	AGU	3.4	21.0	23.8	Asp	GAC	55.4	34.2	27.8
						GAU	44.6	65.8	72.2
Thr	ACA	25.6	37.8	5.9					
	ACC	59.5	28.4	29.4	Tyr	UAC	80.2	38.1	46.2
	ACG	2.4	9.5	17.6		UAU	19.8	61.9	53.8
	ACU	12.5	24.3	47.1					
					Cys	UGC	68.2	45.4	12.5
Pro	CCA	26.4	27.6	54.5		UGU	31.8	54.6	87.5
	CCC	23.6	25.9	27.3					
	CCG	0.0	3.4	0.0	Phe	UUC	73.6	25.0	0.0
	CCU	50.0	43.1	18.2		UUU	26.4	75.0	100.0
Ala	GCA	26.8	33.3	26.9	Met	AUG			
	GCC	9.0	21.2	19.2					
	GCG	7.1	7.4	7.7	Trp	UGG			
	GCU	57.1	37.1	46.1					
					Stop	UAA	66.7	40.0	0.0
Gly	GGA	7.0	35.6	38.9		UAG	22.2	20.0	66.7
	GGC	17.1	27.1	5.5		UGA	11.1	40.0	33.3
	GGG	2.3	8.5	22.2					
	GGU	73.6	28.8	33.3					
Val	GUA	46.8	25.4	35.0					
	GUC	6.4	20.0	10.0					
	GUG	6.4	20.0	15.0					
	GUU	40.4	34.6	40.0					

[a] Codon usage frequency is presented as a percentage of the total usage of corresponding sets of synonymous codons. Methionine and tryptophan are not compared because a single codon corresponds to each of those amino acids. See footnote *a* of Table 3.2 for references on ORFs used in the calculation.

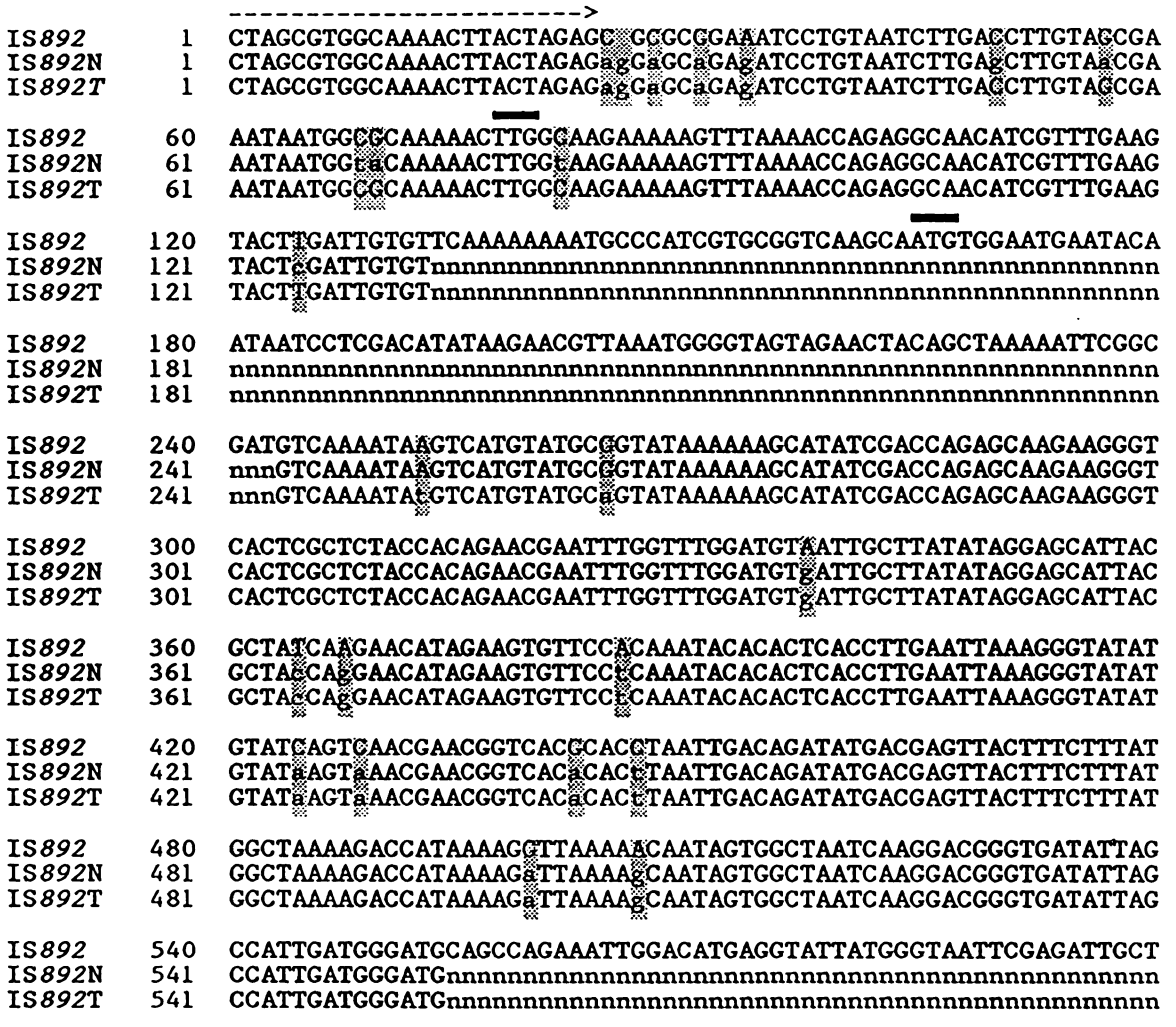


Figure 3.4. Comparison of partial nucleotide sequences of three members of the IS*892* family from the cyanobacterium *Anabaena* sp. strain PCC 7120. Their L-end terminal repeats are highlighted by an arrow above the sequence. Basepairs different from those of IS*892* are in lower case and the changed regions shaded. Stretches of n's represent regions not sequenced. Both possible start codons of ORF1 are marked with a bar on top.

sequences of the IS elements from PS250-9 and PS250-10 (denoted IS*892*N and IS*892*T, respectively) differed slightly from that of IS*892* as well as from each other. Compared with IS*892*, the incompletely sequenced insertion sequences IS*892*N and IS*892*T have a 1-bp insertion following the L-end terminus and have at least 20 and 16 base pair changes (transition/transversion ≈ 2:1), respectively (Fig. 3.4). There are 8 base pair differences between the sequenced regions of IS*892*N and IS*892*T. Most of the changes within ORF1 affect the second or the third base of a codon and do not result in any amino-acid replacement. A few changes affect the first base of a codon and generate conservative amino acid replacements (data not shown). None of the basepair changes leads to the disruption of ORF1 (even in the extended version), supporting the idea that this ORF encodes a functional protein.

Target sequence and specificity. The junctions produced by insertion of members of the IS*892* family into the *sacB* gene were sequenced. All such insertions were shown to lie within the ORF of *sacB* (Steinmetz et al., 1985). The data showed that IS*892* makes directly repeated 8-bp target duplications (Table 3.4). All three members of the IS*892* family inserted into A/T rich target sites. The sequence AAAT(a/t) appeared in all the target sites for IS*892* and IS*892*T in this experiment. The site 5' AAAATATC 3' appears to be particularly favorable because at least two independent insertions by IS*892*, in colonies PS250-3 and PS250-11, targeted this site. These two insertions recognized the same site but inserted in opposite orientations, indicating that the orientation of a target site does not necessarily dictate the orientation of insertion.

Table 3.4 Target duplications produced by insertion of members of the IS*892* family in *Anabaena* sp. strain PCC 7120 [a]

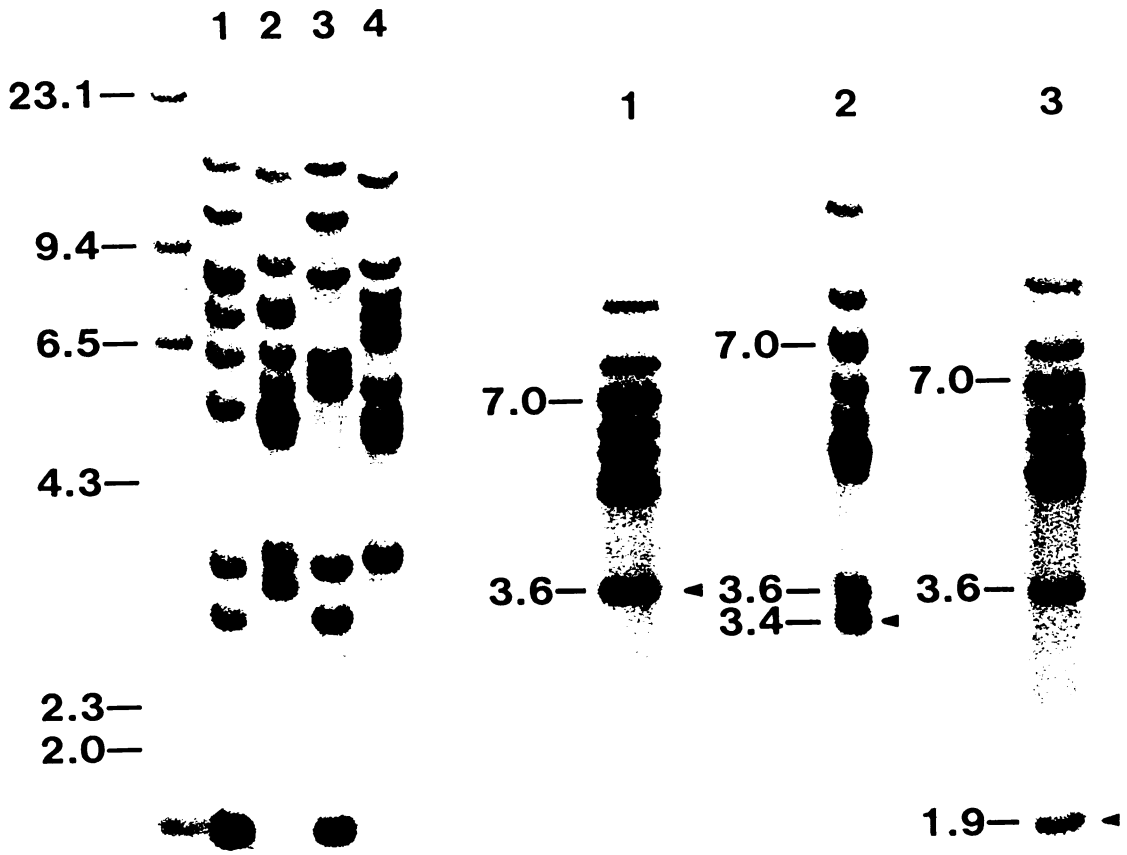
Source of insertion	IS element	Target duplication
PS250-3	IS*892*	5' ^{684} AAAATATC ^{691} 3'
PS250-15	IS*892*	5' ^{684} AAAATATC ^{691} 3'
PS250-11	IS*892*	5' ^{691*} GATATTTT ^{684*} 3'
PS250-6	IS*892*	5' ^{1508*} TTTTAAAG ^{1501*} 3'
PS250-9	IS*892*N	5' ^{1468*} GTTAGATG ^{1461*} 3'
PS250-10	IS*892*T	5' ^{1231} CAAATACT ^{1238} 3'

[a] Target sequences in the *sacB* gene are presented from 5' to 3' where insertion immediately follows in the same orientation as shown in figure 3.3. Basepair numbering is after the published *sacB* sequence (Steinmetz et al., 1985), and numbers with an asterisk indicate sequence of the complementary strand. The R-end junctions of IS*892*N and IS*892*T were not satisfactorily sequenced, so that 8-bp duplications are partially assumed.

The changing genome of *Anabaena* sp. strain PCC 7120 and the IS*892* family. The presence of active insertion sequences may strongly influence the structure and stability of the genome by transposition, and by acting as substrates for homologous recombination (Grindley and Reed, 1985; Galas and Chandler, 1989). When three batches of total DNA, extracted at 1-year intervals from serially subcultured wild-type *Anabaena* sp. strain PCC 7120 (always in liquid medium, with or without fixed nitrogen source; inoculation interval: ca. 2.5

weeks), were digested with *Xba* I or *Eco*R V and probed with the *Dra* I-*Eco*R V fragment of IS*892*, three similar, but nonidentical patterns of hybridization were observed (Fig. 3.5b) A more dramatically different banding pattern was observed in the genome of a culture recovered from an 8-year old frozen sample (data not shown). The changes may reflect the activity of the IS*892* family, although DNA rearrangement not related to IS*892* remains a possibility. By contrast, IS*895* did not show any changed pattern of hybridization to total DNA of several cultures of wild-type *Anabaena* sp. strain PCC 7120, including one with which the hybridization pattern of IS*892* had changed (S. E. Curtis, personal communication; personal observation). Given the comparison with IS*895*, and the fact that they were most frequently observed among insertions into the *sacB* gene, members of the IS*892* family appear to transpose actively. *Anabaena* species have multiple copies of the chromosome per cell (see Chapter 2) and is filamentous. It is unknown whether random chance or some unidentified selective pressure led to conversion of all copies of the chromosome to the new configuration in a relatively short period of time. It has been suggested that some cyanobacterial strains lost some of their properties, such as the production of gas vacuoles, in the history of pure culture (Rippka, 1988). Similarly, among the filamentous *Anabaena* species, strain PCC 7118 has lost its ability to form mature heterocysts (Elhai and Wolk, 1990), strain M-131 has lost its capability of heterocyst differentiation (C. P. Wolk, personal communication), and strain PCC 7120 is thought to have lost its ability to produce hormogonia (Rippka, 1988). The activity of transposable DNA elements, as visualized in cells of *Anabaena* sp. strain PCC 7120, may be one of the mutational forces that led to loss of cellular characteristics.

Figure 3.5. Southern hybridization of IS*892* to total DNA of *Anabaena* spp. (a) Copy number of IS*892*-like elements in the genomes of *Anabaena* sp. strains PCC 7120 and M-131. Total DNAs from strain PCC 7120 (lanes 1 and 2) and strain M-131 (lanes 3 and 4) were digested with *Eco*R V (lanes 1 and 3) or *Xba* I (lanes 2 and 4) and probed with the radioactively labelled internal *Dra* I-*Eco*R V fragment from IS*892*. The unnumbered lane on the left indicates sizes of DNA (in kilobases). (b) DNA rearrangement in the genome of *Anabaena* sp. strain PCC 7120. Total DNA extracted from a serial subculture of this strain of *Anabaena* sp. at the beginning of the years 1989 (lane 1), 1990 (lane 2) and 1991 (lane 3) were digested with *Xba* I and hybridized with the same probe used in panel A. One of two 3.6-kb bands in the earliest batch of DNA was replaced by a 3.4- or 1.9-kb band in subsequent DNA samples (arrowheads).



a.

b.

Southern analysis of *Eco*R V- or *Xba* I-digested total DNA from four cultures (one shown in Fig. 3.5a) of wild type *Anabaena* sp. strain PCC 7120 showed that there were at least nine copies of members of IS*892* family in the genome (two cultures showed ten distinct bands). It was not determined which hybridizing band corresponded to a particular member of the IS*892* family or whether all hybridizing copies were capable of transposition. No data are available to indicate whether members of the IS*892* family transpose in a conservative or replicative manner. The same probe was also used to probe total DNA of *E. coli* HB101 (pRL528) and DH5α, transient hosts of plasmid pRL250 during conjugation and transformation. Under the standard, high-stringency conditions employed, no hybridization was observed, thus excluding the possibility that IS*892* had been derived from cells of *E. coli*.

Unique nucleotide structure of IS*892*. The sequence comprising the two stem-loop structures in the region between ORF1 and ORF2 show some similarity to those of the terminal repeats. When properly aligned, a 32-bp sequence, which I denote the M sequence (bp 953 to 984, in which the first stem-loop is formed [Fig. 3.3]), could be viewed as an imperfect direct repeat of the R-end terminal sequence (and therefore as an imperfect inverted repeat of the L end). Discounting the 6 bases that introduce gaps in the alignment, 20 of 26 bases in the M sequence are identical, in order, to 20 of the 26 bases at the R-end (Fig. 3.6). Downstream from the M sequence is another stem-loop structure (denoted the N sequence) which is immediately preceded by the *dam* methylation site (Fig. 3.3). The sequence of one arm of this stem-loop, 5'TTTACCAC 3' (bp 1060 to 1067), is exactly repeated in the R-end sequence. With such unique sequence structure, IS*892* could form, in addition to a normal " racket frame " structure

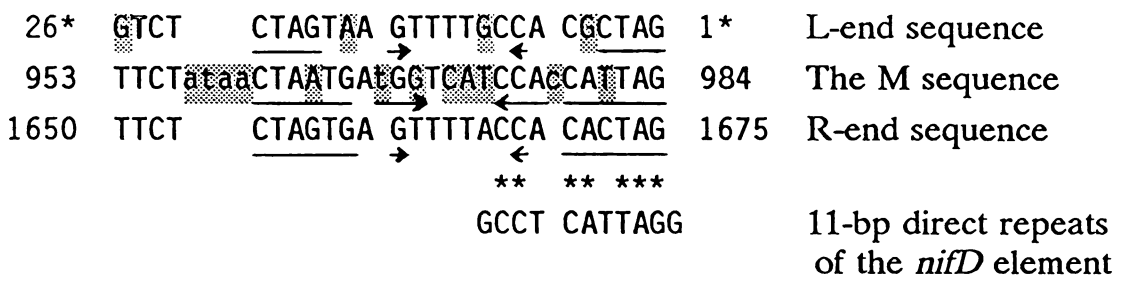


Figure 3.6. Alignment and comparison of the M sequence to the L-end and R-end sequences of IS*892*, and of those three sequences to the 11-bp directly repeated sequence flanking the 11-kb insertion (the *nifD* element) interrupting the *nifD* gene in the chromosome of the same strain of *Anabaena* sp. All sequences are presented in 5' to 3' order. Inverted repeats in the three sequences are marked with arrows underneath the sequences. The strand complementary to the L-end sequence in Fig. 3.3 is shown (numbered from large to small, with an asterisk), and base 25 (T) is for IS*892*N and IS*892*T only. The six bases in the M sequence that introduce gaps in the alignment are presented in lower case. Non-matching bases are shaded. Matching bases between the 11-bp repeat of the *nifD* element and the other three sequences are indicated with asterisks.

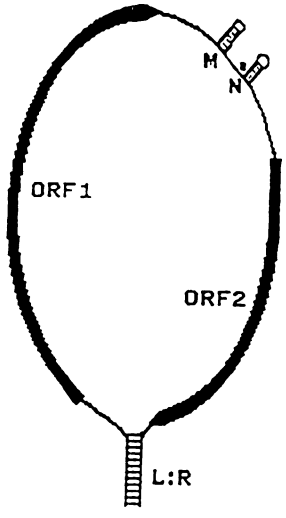
(Sakaguchi, 1990), an alternative secondary structure that contains a mini IS*892* element bearing only ORF1 (Fig. 3.7).

Alternatively, the M and R-end sequences could be viewed as direct repeats containing intrinsic inverted repeats. Such a structure surrounding ORF2 is reminiscent of the *aadA* and *sat* genes in Tn*7* and related Tn*1825*, which were suggested to have inserted into the transposons via integrase-mediated site-specific recombinations (Fling et al., 1985; Sundström et al., 1991).

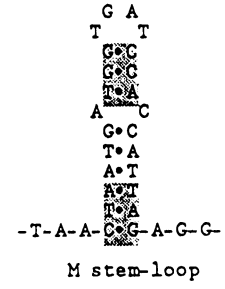
To observe whether the postulated mini IS*892* transposes, or whether ORF2 is removable from IS*892*, the 0.85-kb *Dra* I-*Sna*B I fragment (L end to M) and the 0.5-kb *Hin*d III-*Eco*R V fragment (M to R end) from IS*892* (see Fig. 3.3) were individually used to probe *Eco*R V- or *Xba* I-digested total DNA. Patterns of hybridization by both probes were identical to that by the *Dra* I-*Eco*R V fragment containing both ORF1 and ORF2 (Fig. 3.5a), suggesting that transposition of the complete IS*892* is the predominant event. A computer search of both ORFs of IS*892* failed to identify a structural motif similar to the one that is conserved in the integrase family of site-specific recombinases (Argos et al., 1986).

Resemblance of IS*892* and the *nifD* element. The *nifD* element is an 11-kb sequence interrupting the *nifD* gene in the chromosome of vegetative cells of the same strain of *Anabaena* sp., and it is excised from the chromosome by site-specific recombination between its 11-bp directly repeated border sequences in a late stage of heterocyst differentiation, whereupon a functional *nifD* gene is created (Golden et al., 1985; Lammers et al., 1986; Golden and Wiest, 1988). Six or 7 bases of the sequence of that 11-bp recombination site were found to be identical to corresponding bases (no gaps introduced in the alignment) in the

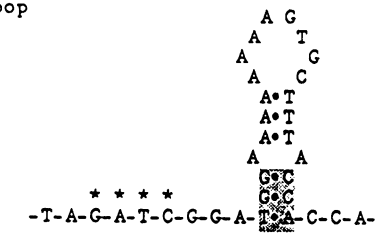
Figure 3.7. Possible nucleotide secondary structures that could be formed by IS*892*. Uper panel: a normal "racket frame" structure in which the inverted termini pair to form quadruple-stranded DNA juxtaposed by mediation of proteins such as transposase and IHF (Sakaguchi, 1990). Also notice two stem-loop structures (M and N) separating ORF1 and ORF2. Lower panel: an alternative double-loop secondary structure in which the L end and the M sequences pair to form a possible mini IS*892* element that bears only ORF1. Pairing of the N and the R-end sequences puts ORF2 in a smaller loop (when this smaller loop is not formed the R-end sequence can by itself form a stable stem-loop structure). Detailed basepairing is shown to the right of the drawings. One or both of the pairing motifs, 5' TAG 3' and 5' CCA 3' (shaded), are shared by all stem structures.



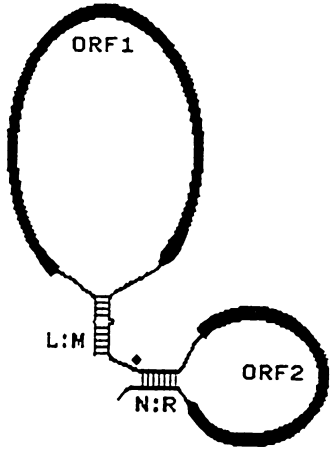
L•R stem-loop

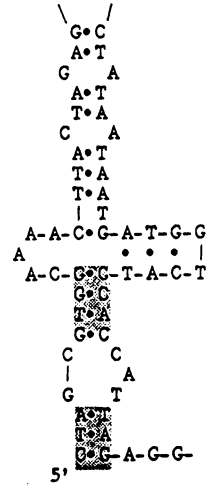


M stem-loop

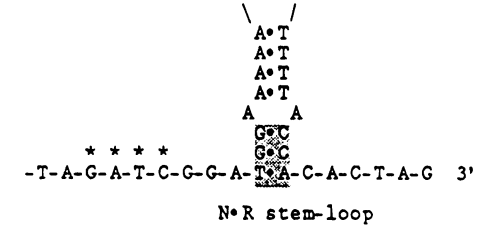


N stem-loop

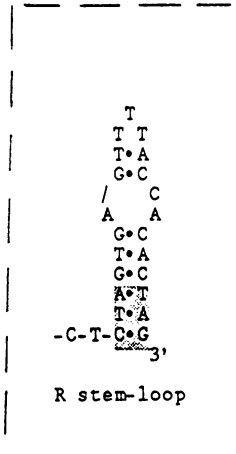




L•M stem-loop



N•R stem-loop



R stem-loop

sequences comprising the intrinsic repeats in the termini of IS*892* and in the M sequence (Fig. 3.6). The *nifD* element also has essentially the same G+C content and codon usage as IS*892* (Table 3.2 and 3.3). However, no significant sequence similarity was found between IS*892* and the *nifD* element, except for their termini.

Distribution of IS*892*. IS*892* from *Anabaena* sp. strain PCC 7120 hybridized to genomic DNA from several other strains of *Anabaena* spp. and *Nostoc* spp., which are filamentous (in Section IV of the 1979 Rippka Assignment [Rippka et al., 1979]), including *Anabaena* sp. strain M-131 (lanes 3 and 4 of Fig. 3.5a), *Anabaena* sp. strain PCC 7118, *Nostoc* sp. strain Mac, *Nostoc ellipsosporum*, and *Nostoc* sp. strain ATCC 29150 (none of the hybridizations give identical banding patterns; T. Thiel, personal communication), but did not hybridize to genomes of the heterocystous filamentous strain *Anabaena variabilis* ATCC 29413, the nonheterocystous filamentous strain *Pseudanabaena* sp. (in Section III of the 1979 Rippka Assigment), and the unicellular strains *Synechocystis* sp. strain PCC 6803 and *Synechococcus* sp. strain PCC 7002 (both are in Section I of the 1979 Rippka Assigment) (T. Thiel, personal communication). I attempted to entrap an active IS*892*-like transposable element from *Anabaena* sp. strain M-131, which is also sensitive to sucrose when bearing pRL250 (see Chapter 2), but failed (unpublished results). It is possible that the IS*892*-hybridizing elements in *Anabaena* sp. strain M-131 transpose relatively infrequently or no longer at all.

Chapter 4

STUDY OF GENES THAT RESPOND EARLY TO NITROGEN-STEPDOWN AND OF OTHERS THAT REGULATE THEIR EXPRESSION [1]

SUMMARY

Filamentous cyanobacteria of the genus *Anabaena* are of developmental interest because, when deprived of fixed nitrogen, they show patterned differentiation of N_2-fixing cells called heterocysts. To help elucidate the early responses of these organisms to nitrogen-stepdown, mutants were isolated that responded rapidly to removal of fixed nitrogen. This was accomplished using a derivative of transposon Tn*5* that incorporates the promoterless bacterial luciferase genes, *luxAB*, as a reporter of gene transcription. Two mutant strains, TLN2 and TLN6, showed increased luminescence within 1 or 4 hr, respectively. Using a coliphage T7-based transcription-amplifying system, *tln6*-directed luciferase activity at single-cell level was observed by microscopic photon-

[1] Part of the contents of this chapter has been published in the Proceedings of the National Academy of Sciences, U.S.A. (**Wolk, Cai, and Panoff, 1991**).

counting and was found distributed unevenly along the filament at the earliest time point of observation, 7 hr after nitrogen-stepdown.

Mutations in strains TLN2 and TLN6 were further characterized and localized in the chromosome. The fusion of each mutant gene to *luxAB* was regenerated in the wild-type chromosome using *sacB*-mediated selection for gene replacement, and the resulting mutants were subjected to secondary mutagenesis by a Tn*5* derivative that lacks *luxAB*. Four secondary mutants were isolated which showed altered *tln6::luxAB* expression and were defective in heterocyst differentiation.

INTRODUCTION

The cyanobacterium *Anabaena* sp. responds to starvation of fixed nitrogen by initiating the differentiation of N_2-fixing cells called heterocysts at semi-regular intervals along its filaments (Wolk, 1982). Genetic studies in recent years have been directed toward analysis of 1) the process of heterocyst differentiation and 2) the mechanism of heterocyst pattern formation.

The availability of molecular tools has made *Anabaena* sp. a model prokaryote for study of multicellular development and cellular differentiation. *Bacillus subtilis*, a gram-positive, sporulating bacterium, and *Myxococcus xanthus*, a gram-negative bacterium which shows colonial morphogenesis, are other such models that have been extensively studied in these regards (Gottesman, 1984; Dworkin, 1985; Kroos et. al., 1986). Studies on *B. subtilis* suggest that a phospho-transfer pathway (Burbulys et al., 1991) is involved in the initiation of sporulation, and that during endospore formation, gene expression is controlled by a cascade of sigma (σ) factors (Stragier and Losick, 1990).

Expression of many developmentally regulated genes in *Myxococcus* sp. has been shown to be dependent on a sequence of intercellular interactions (Kroos and Kaiser, 1987). In *Anabaena* sp., mutants lacking or defective in heterocyst differentiation have been isolated by UV or chemical mutagenesis (Wolk et al., 1988; Buikema and Haselkorn, 1991a). Some of these mutants have been complemented and the complementing genes studied in detail (Holland and Wolk, 1990; Buikema and Haselkorn, 1991b). In addition, rearrangements of DNA at a late stage of heterocyst differentiation have also been reported (Golden et al., 1985; Haselkorn, 1989). Nonetheless, few genes that are important specifically for the development of *Anabaena* sp. have been characterized. The regulation of the expression of these genes and possible regulatory relationships between them, are virtually unknown. The differentiation process in *Anabaena* sp. is accompanied by the compartmentalization of gene expression, with certain genes (e.g., the *rbcLS* genes) being transcribed only in vegetative cells and others (e.g., the *nifHDK* genes) only in heterocysts (Elhai and Wolk, 1990). The possible role of RNA polymerase modification by alternative σ factors has begun to be examined. At least two genes for σ factors have been identified tentatively on the basis of their hybridization to the gene (*sigA*) encoding the "housekeeping" σ factor of *Anabaena* sp. strain PCC 7120 (Brahamsha and Haselkorn, 1991). One of the proposed genes, *sigB*, was observed to be transcribed only under nitrogen-fixing conditions. Insertional inactivation of the gene, however, did not appear to affect heterocyst differentiation or nitrogen fixation (Brahamsha, 1991; B. Brahamsha, personal communication).

Anabaena sp. provides an opportunity to elucidate, in a morphologically simple organism, the detailed biochemical mechanisms that govern the formation

of multicellular patterns. The pattern of spaced heterocysts has been suggested to arise *de novo* by two very distinct, but experimentally distinguishable mechanisms (Wolk, 1989).

According to the first mechanism, sometimes called the "altruistic" model, and which I call **the volunteer model**, the first cells that sense nitrogen-stepdown differentiate to form heterocysts voluntarily, and produce and maintain the heterocyst-distribution pattern by exporting a differentiation-inhibiting substance to adjacent vegetative cells. It should be noted that in this model the inhibitory substance that ultimately governs pattern formation is distributed simultaneously with the onset of heterocyst differentiation by the few early volunteer proheterocysts. All genes induced by -N condition are directly involved in the differentiation *program* although they are not necessarily involved in either differentiation or pattern formation.

According to the second mechanism, sometimes called the "selfish" model, and which I call **the scavenger model**, the first cells that sense N-stepdown activate a system that scavenges nitrogen from adjacent cells; consequent deprivation of the latter cells of nitrogen leads them to activate a nitrogen-scavenging system that drains nitrogen from their distal, neighboring cells; and so on along a filament. The last cell of a group of contiguous cells that senses nitrogen deprivation, unable to tap any source of nitrogen other than N_2, is stimulated to differentiate to form a heterocyst. The scavenging stage that occurs between vegetative growth and the onset of heterocyst differentiation is a distinct feature of this model. Mutation of genes involved in scavenging may not prevent the formation of heterocysts although the pattern of heterocysts would be expected to be altered.

Because the two models lead to different predictions about possible spatial relationships between the cells that differentiate and the cells that first sense N-deprivation, the models might be tested experimentally as follows. The promoter of a gene that is activated very early after N-stepdown should be fused to a promoterless reporter operon, e. g., the luciferase genes *luxAB*, and expression observed at a single-cell level. According to the volunteer model, expression would be observed first in the cells that will eventually become heterocysts. In contrast, according to the scavenger model, that expression would be initiated in cells that are distant from the sites of eventual heterocyst formation (Wolk, 1989).

The wild-type transposon Tn*5* (Berg, 1989) has been shown to transpose in *Anabaena* sp. strain PCC 7120 (Borthakur and Haselkorn, 1989), and derivatives of Tn*5*, including those that contain reporter genes, have been constructed for use in Gram-negative eubacteria (Boivin et al., 1988; Simon et al., 1989; de Lorenzo et al., 1990). The genes (*luxAB*) for the light-emitting protein complex, luciferase, have been shown to permit localization of transcription from specific promoters to individual cells of a filament (Elhai and Wolk, 1990). A derivative of Tn*5* that incorporates the *Vibrio fischeri luxAB* as a transcriptional reporter was constructed and used to mutagenize wild-type *Anabaena* sp. strain PCC 7120. *Anabaena* genes that are activated rapidly in response to removal of fixed nitrogen were thereby identifed. One of these genes, *tln6*, increases its expression within 4 hr after removal of nitrate from the medium. Expression of *tln6::luxAB* along the filament was also examined.

Restriction with enzymes for which sites are present in the transposon and in few copies in the genome (Bancroft and Wolk, 1989) allowed mapping of the

site of insertion with a resolution of ca. 10 kb, by pulsed-field gel electrophoresis. The direction of the transcription reported by the transposon was determined by hybridization to blots of the pulsed-field gels. Genomic DNA contiguous to the insertions was recovered by transfer to *E. coli* by electroporation. The *tln6::luxAB* fusion was then regenerated by introduction into the wild-type genome of a *luxAB*-bearing cassette that bears no homology to transposon Tn*5*. The resulting mutant was subjected to secondary transposon mutagenesis to identify genes that control both the expression of *tln6* and eventual heterocyst differentiation. Such genes may be useful in elucidating the mechanisms underlying heterocyst differentiation and pattern formation. They may include the genes that, by sensing N-starvation, lead to initiation of patterned formation of heterocysts.

MATERIALS AND METHODS

Bacterial strains and plasmids used are listed in Table A.2 and Table A.3 of Appendixes B and C, respectively. *Anabaena* sp. strain PCC 7120 and its transposition- or gene replacement-generated mutants were grown with AA-based media (Allen and Arnon, 1955) supplemented with various concentrations of neomycin (Nm), streptomycin (Sm), and/or spectinomycin (Sp), as appropriate. The concentrations of antibiotics used are presented in Appendix F. Media used are described in Appendix A. L-methionine-D,L-sulfoximine (MSX), an inhibitor of glutamine synthetase (GS), was used at a concentration of 1.0 mM.

Plasmids bearing transposons were transferred from *E. coli* to *Anabaena* sp. by triparental matings (Wolk et al., 1984) which were performed on filters (Type REC-85 pre-sterilized filters, Nuclepore Corp., Pleasanton, CA) atop solidified AA plus nitrate media (with or without 5% LB). About 1×10^7 to 5×10^7

cells of *Anabaena* sp. were plated on a 100-mm Petri dish. Cell counts were estimated from methanolic extracts of cells (Mackinney, 1941) by assuming a content of 0.4 pg of chlorophyll *a* per cell. Nitrogen-stepdown of transposition-generated colonies was initiated by moving the filter onto solid medium free of fixed nitrogen. Colonies of interest were purified as described (Wolk et al., 1984).

Luminescence of colonies on a Petri dish was monitored (Maly et al., 1988) with the Hamamatsu Photonic System model C1966-20 (Photonic Microscopy, Inc., Oak Brook, IL) coupled to a Nikon 35-mm f 2.0 macro-lens. Several µl of n-decanal ($C_{10}H_{20}O$; catalog # D-7384, Sigma Chemical Co., St. Louis, MO.), spread inside the top of a glass Petri dish, provided substrate for luciferase. The top of a plastic disposable Petri dish was used in the initial experiments but this procedure was later discontinued because the aldehyde slowly etched, and reduced the transparency of, the plastic. Exposure of cells to aldehyde was kept to no more than a few min., usually less than 2 min. After imaging of luminescence, the Petri dishes were blown with sterile air until free of an odor of aldehyde, and were provided with new covers. Microscopy and localization of luciferase activity of individual cells along the filament were performed as described previously (Elhai and Wolk, 1990)

Mutants with a particular temporal response of *luxAB* activity to N-stepdown were first identified on mating filters. Such mutants were then picked and streaked onto NO_3^--containing agar medium. Cells from these streaks were resuspended in AA/8 + NO_3^- liquid medium for 6 to 16 hr under growth conditions and then 4 or 6 replicative spots were made from 2 or 3 µl of each of the suspensions on a filter atop NO_3^--containing agar medium. After 1 to 2 days of cell growth, half of the filter was transferred to agar medium free of fixed N for

a period of time before the other half was transferred to the same petri dish and imaged under the Photonic camera. Many N-responsive mutants initially picked from mating filters were re-examined this way. More precise measurements of induced luxAB activity were performed by the **time-course induction assay of artificial colonies**: 2-μl portions of a culture of a mutant of interest, growing exponentially or nearly so ($O.D._{665}$ of a methanolic extract of a liquid culture ≤ 0.2) in medium AA/8 + nitrate + appropriate antibiotic(s), were spotted onto cut pieces of filter atop solidified AA medium containing nitrate or ammonium plus appropriate antibiotic(s) to form homogeneous and nearly identical artificial colonies. About 2 days thereafter, and at various times, pieces of filter bearing artificial colonies were transferred to solidified basal AA medium plus antibiotic(s) before imaging of luminescence from all spots (see Fig. 4.10).

Most molecular biological techniques were standard, as described in chapters 2 and 3. DNA of high molecular weight was prepared, digested with appropriate restriction endonucleases, and subjected to pulsed-field gel electrophoresis, and Southern analysis of the gels performed, as described previously (Bancroft et al., 1989) except that digested samples were not treated with Proteinase K. In order to recover genomic sequences contiguous with the transposon, DNA extracted from *Anabaena* sp. (using the glass-bead method, see chapter 2) was digested with appropriate restriction enzymes, self-ligated, and transferred into *E. coli* HB101 by electroporation (Dower and Ragsdale, 1988). DNA so extracted was also used for most of the Southern analyses. Introduction of a specific mutation into the *Anabaena* genome was achieved using the *sacB*-mediated gene replacement procedure described in chapter 2.

RESULTS

Construction of Tn*5*-derived transposons and of the BLOS cassettes. The promoter-probe transposon, Tn*5*-1063 (Fig. 4.1), was constructed by C. P. Wolk (Wolk et al., 1991). This Tn*5* derivative retains, from Tn*5 lac* (Kroos and Kaiser, 1984; Kroos et al., 1986), 53 bp of the outside end of IS50L that enable transposition, and that allow outside promoter activity to control transcription of the reporter genes *luxAB* from *V. fischeri* (via pRL488; see Elhai and Wolk, 1990) that are placed immediately downstream. The 53-bp fragment introduces stop codons in all three reading frames, preventing the formation of fusion proteins with the reporter luciferase. The transposon contains a p15A origin of replication (*oriV*) from the plasmid pACYC184 (Chang and Cohen, 1978; Rose, 1988) that functions in *E. coli* (but not in *Anabaena* sp.), and therefore enables DNA contiguous with the transposon to be recovered from the *Anabaena* genome by excision, circularization, and transfer to *E. coli*. Excision is possible using enzymes, including *Cla* I, *Dra* I, *Eco*R I, *Eco*R V, *Sca* I, and *Spe* I, that cut the genome frequently but do not cut Tn*5*-1063. In addition, *Eco*47 III and *Mlu* I may be used to clone *Anabaena* DNA lying to the L end of the transposon, and *Xba* I to clone *Anabaena* DNA lying to the R end of the transposon (see Fig. 4.1).

Wild-type *Anabaena* sp. is sensitive to Sm, Nm or Bm at 1, 25, or 0.2 μg/ml when (ca. 5×10^7) cells containing 15 μg of chlorophyll *a* are plated on agar medium in a 100-mm Petri dish. *Anabaena* sp. bearing wild-type Tn*5* was reported to be resistant to Nm at 30 μg/ml (Borthakur and Haselkorn, 1989) but is sensitive to 1 μg Sm/ml (our observation). In Tn*5*-1063 the wild-type Tn*5* promoter of the antibiotic resistance operon is replaced with the *psbA* promoter from *Amaranthus hybridus* (Hirschberg and McIntosh, 1983; L. McIntosh,

personal communication), and the wild-type Sm^r gene is replaced with the Sm^r gene from plasmid pPM111*. A 6-bp deletion in the 3' region of the Sm^r gene in pPM111* results in greatly enhanced resistance to Sm in *E. coli* (Mazodier et al., 1986). The presence of Tn*5*-1063 confers greatly enhanced resistance to Bm, Nm, and Sm upon *Anabaena* sp. Strains of *Anabaena* sp. bearing Tn*5*-1063 can be selected on 400-500 μg Nm/ml, 1-4 μg/ml Bm, or 5-20 μg/ml Sm. Selection after mating was carried out with either 400 μg Nm/ml or 10 μg Sm/ml. At 400 μg Nm/ml, inoculation with more than ca. 3.8×10^7 cells (i.e., containing >15 μg of chlorophyll *a*) of *Anabaena* sp. per mating filter can lead to confluent growth. The lawn on the mating filter can then be cleared, and true transposition-derived colonies permitted to grow, by transferring the filter to 20 μg Sm/ml or 4 μg Bm/ml. Experiments (Fig. 4.2) in which ca. 3,000 transposon-derived colonies were generated per filter were performed with mating filters treated with 20 μg Sm/ml after overgrowth of *Anabaena* sp. on initial Nm selection.

Two additional modifications were incorporated into Tn*5*-1063 with the aim of increasing the frequency of transposition relative to wild-type Tn*5*. Addition of a rho-independent transcriptional terminator from the *lpp* gene of *E. coli* (T_{lpp}) (from pJDC406; Coleman et al., 1985) near the 3' end of the Sm^r gene may reduce antisense transcription of the transposase gene from the strong *psbA* promoter. The 5' GATC 3' sequences in the promoter region of the transposase, which when *dam*-methylated reduce transposition of wild-type Tn*5* (Yin et al., 1988, Dodson and Berg, 1989) in *E. coli*, were eliminated by replacement with sequences from pRZ1107 (Yin et al., 1988) in order that the frequency of transposition of the transposon not be reduced by the *dam* methylation of *Anabaena* sp. (Padhy et al., 1988).

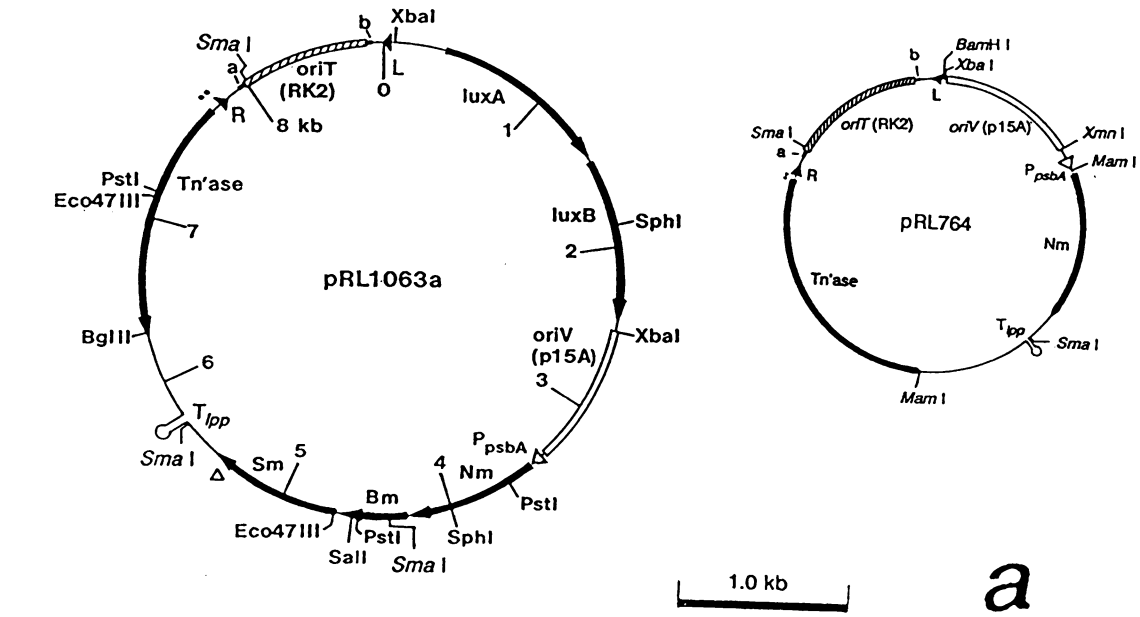
A 0.7-kb fragment containing an RK2 origin of transfer (*oriT*) from plasmid pAT187 (Trieu-Cuot et al., 1987), bordered by polylinkers and short sequences from ColE1 and pBR322, connects the ends of Tn*5*-1063 to form plasmid pRL1063a. This *oriT* permits efficient mobilization of the plasmid from *E. coli* to *Anabaena* sp. Plasmid pRL1063a lacks sites for *Ava* III and *Avr* II, Type-II restriction enzymes derived from strains of *Anabaena* sp. (Tandeau de Marsac and Houmard, 1987), and so would not be restricted by those enzymes upon transfer into those strains.

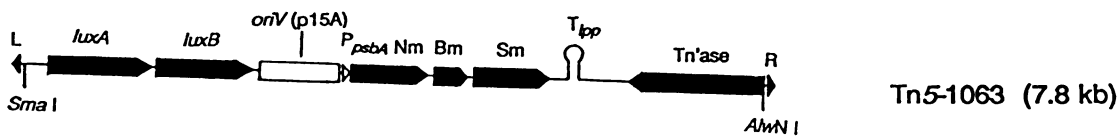
The Tn*5* derivative Tn*5*-764 is present in plasmids pRL764 (Fig. 4.1) and pRL764SX. Plasmid pRL764 was derived from plasmid pRL1058 (the *luxAB*-less progenitor of pRL1063a) by deletion of the 1,250-bp *Sma* I fragment that contains a large portion of the Bm^r gene and the entire Sm^r gene. Plasmid pRL764SX was derived from pRL764 by deletion of the short fragment from *Sma* I (at the transposase-proximal end of *oriT*) to *Eco*R V (in polylinker a). In addition to retaining useful features of pRL1058, except for the Bm^r and Sm^r determinants, pRL764SX allows easy replacement of the *oriV*-containing fragment (*Bam*H I or *Xba* I to *Xmn* I) and the antibiotic resistance cassette (*Xmn* I or partial *Mam* I to *Sma* I) with desired alternatives.

The transposition frequencies of Tn*5*-1063 (7.83 kb) and Tn*5*-764 (4.15 kb) in *Anabaena* sp. strain PCC 7120 are ca. 1×10^{-5} to 4×10^{-5}, and 3×10^{-5} to 9×10^{-5} per cell, respectively. The slightly higher frequency of transposition of Tn*5*-764 may be attributable to its smaller size, which is closer in size to the wild-type Tn*5* (5.8 kb; Berg, 1989) than is Tn*5*-1063.

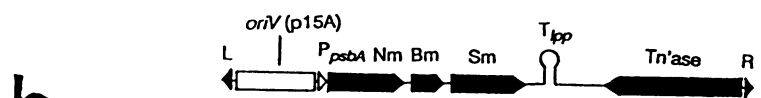
Two other Tn*5* derivatives, Tn*5*-800 and Tn*5*-1087b, were also used in the experiments described in this chapter. Plasmid pRL800, bearing Tn*5*-800, is a

Figure 4.1. Essential features of (a) plasmids pRL1063a and pRL764; (b) transposons Tn*5*-1063, Tn*5*-1058 and Tn*5*-764; and (c) the BLOS cassettes. Details of the structure of plasmid pRL1063a are presented in Wolk et al. (1991). Plasmid pRL1058, bearing Tn*5*-1058, is the precursor of pRL1063a (bearing Tn*5*-1063) and pRL764 (bearing Tn*5*-764). The genes *luxAB* from *Vibrio fischeri* in the form of a *Bam*H I fragment from pRL488 (Elhai and Wolk, 1990), provided with *Xba* I ends by passage through pRL498 (Elhai and Wolk, 1988a), was inserted into the *Xba* I site of pRL1058, giving pRL1063a. Plasmid pRL764 was made from pRL1058 by deleting the 1.25-kb *Sma* I fragment that includes a large portion of the Bm^r gene and the entire Sm^r gene. Only restriction sites within the transposon and mentioned in the text are shown. Polylinker a is, clockwise, *Eco*R I - *Cla* I - *Eco*R V - (*Xba* I) - *Bgl* II - *Xho* I - *Sst* I - *Nru* I - *Hin*d III - *Sph* I - *Pst* I. Polylinker b is *Sal* I - *Pst* I - *Sph* I - *Hin*d III - *Nru* I - *Sst* I - *Xho* I - *Bgl* II - (*Xba* I). The *Xba* I sites in parentheses are methylated by dam^+ strains of *E. coli*. The left (L) and right (R) ends of the transposons are indicated (◂, ▸). An open triangle at the end of the Sm^r gene depicts the 6-bp deletion, and the two dots at the promoter region of the transposase gene represent the two mutated 5' GATC 3' sequences (see text). Components of the BLOS cassettes are described in text. The 54-bp *Eco*R V-*Eco*47 III fragment is deleted in cassette BLOS2; in addition to that 54-bp deletion, the *Bam*H I site (not shown) 3' from *luxAB* is also eliminated in cassette BLOS3 (see text).

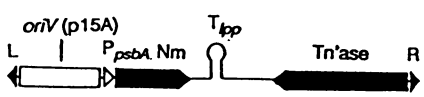




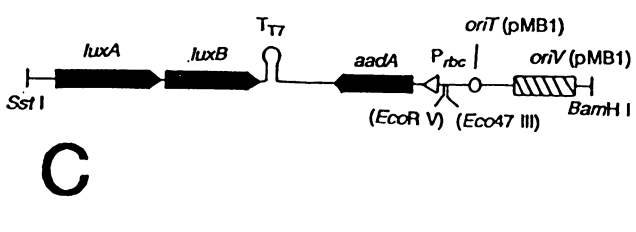
Tn*5*-1063 (7.8 kb)



Tn*5*-1058 (5.4 kb)



Tn*5*-764 (4.2 kb)



BLOS (5.6 kb)

derivative of pRL1058. In pRL800, the sequence of pRL1058 from bp 65 (*Xba* I at the L end, destroyed) to bp 3871 (*Bcl* I, destroyed) is replaced by the following sequence (in the same direction): first, 36 bp from bp 1584 (*Asu* II, destroyed) to bp 1620 (*Sna*B I, destroyed) of plasmid pJRD184 (Heusterspreute et al., 1985), then an A, then the 956-bp Cm^r determinant from bp 4357 (*Asu* II, destroyed) to bp 5312 (*Asu* II, destroyed) of plasmid pBR325 (Balbás et al., 1986), then bp 3292 (*Eco*R I) to bp 2378 (*Sal* I, destroyed) and bp 6115 (*Nde* I, destroyed) to bp 4616 (*Avr* II, destroyed) from plasmid pRL271 (chapter 2), then 5'AG 3'. This sequence was joined to bp 3871 of pRL1058 by ligation of a *Bam*H I-generated 5' overhang to a *Bcl* I-generated 5' overhang. The termination codon (TGA) of the transposase gene, falling within the *Bcl* I site, was not altered in the manipulation. Overall, the *Bam*H I-*Xba* I-*oriV* (p15A)-Nm^r-Bm^r-Sm^r-*Bcl* I fragment in Tn*5*-1058 is replaced by a *Bam*H I-*Xba* I-Cm^r-Em^r-*oriV* (pMB1)-*Bam*H I (destroyed) fragment in Tn*5*-800. The transposon Tn*5*-1087b (C. P. Wolk, unpublished) is very similar to Tn*5*-800. In Tn*5*-800 the *cat* and *erm* genes (comprising the Cm^r Em^r cassette C.CE3) point in the same direction, while in Tn*5*-1087b (which contains cassette C.CE2) the *cat* gene points in the opposite direction. Because the *cat* gene of Tn*5*-1087b is not followed by a transcriptional termination signal, the *cat* transcript may read out of the L end, or read-through from outside of the L end may produce an anti-*cat* transcript, after transposition. Both Tn*5*-800 and Tn*5*-1087b transpose in cells of *Anabaena* sp. strain PCC 7120 at a frequency close to that of Tn*5*-764.

The BLOS cassettes (Fig. 4.1) were designed specifically for replacement of transposon Tn*5*-1063 in recovered *Anabaena* DNA fragments into which the transposon had inserted. Cassette BLOS1 has the following components: bp 1

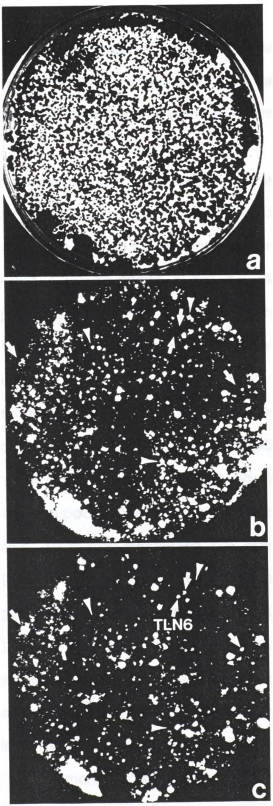
to 2415 are *Sst* I-*luxAB*-*Bam*H I from pRL488 (Elhai and Wolk, 1990), bp 2416 t
2560 are *Bam*H I-T_{T7}-*Bgl* II (destoyed) from pET3 (Rosenberg et al., 1987), b
2561-2567 are *Bam*H I (destroyed)-*Dra* I (destroyed) from pRL25 (Wolk et a
1988), the following 1724 basepairs are a trimmed Sm^r/Sp^r cassette C.S
(Bancroft and Wolk, 1989) which now retains the portion from bp 1,463 (*Stu*
destroyed) to bp 5 (*Ava* I, destroyed) of the *aadA* sequence (Fling et al., 198
and bp -410 to bp -160 (*Nla* III; in the article by Bancroft and Wolk [1989] it wa
incorrectly reported as -240; C. P. Wolk, personal communication) that contair
the promoter for *rbcLS* (P_{rbc}) of *Anacystis nidulans* (Shinozaki and Sugiur
1985). Next (this ligation generated an *Eco*R V site), bp 4292-4374 are bp 44
to 525 (*Sau*96 I) of pBR322, and then bp 4375-5658 are *Sau*96 I (bp 1951)-*ori*
oriV-*Dra* I (bp 3234, destroyed) from pBR322. Finally, a *Sma* I (destroyed
*Bam*HI linker at bp 5659-5666 completes cassette BLOS1. The *Eco*R V si
upstream from P_{rbc} in BLOS1 was eliminated by cutting with *Eco*R V and *Eco*
III, and religating, thus deleting 54 bp and forming BLOS2. The ends of th
BLOS cassettes (BLOS1 is present in pRL739, pRL739B, and pRL739S; BLOS
is present in pRL759 and pRL759B) are connected by different polylinke
depending on the plasmids in which they reside. The polylinker in pRL739 ar
pRL759 is (from the *luxAB*-distal end) *Bam*H I - *Xba* I - *Sal* I - *Pst* I - *Sph*
*Hin*d III - *Nru* I - *Sst* I - *Xho* I - *Bgl* II - (*Xba* I) - *Kpn* I - *Sst* I (*luxAB*-proximal enc
in pRL739B and pRL759B is *Bam*H I - *Sma* I - *Kpn* I - *Sst* I; and in pRL739S
*Bam*H I - *Xba* I - *Sal* I - *Pst* I - *Sph* I - *Hin*d III - *Nru* I - *Sst* I. The *Xba* I site
parenthesis is methylated by *dam*$^+$ strains of *E. coli*. The *Bam*H I site 3' fro
luxAB in BLOS2 in pRL759 was eliminated by a filling-in reaction using T4 DI

polymerase, producing cassette BLOS3 in plasmid pRL759D (T. Black and C. P. Wolk, unpublished results).

As described in the text below, a BLOS cassette is used both as a reporter of transcription and as an inactivation cassette (see chapter 2). A mutation in a DNA fragment of *Anabaena* sp. containing a BLOS cassette was introduced into the wild-type chromosome by the *sacB*-mediated positive selection for double recombinants (chapter 2); in the two particular manipulations described in this chapter, the *sacB*-containing plasmid pRL278R was used. Plasmid pRL278R was derived from plasmid pRL278 (Fig. 2.4) by digestion of pRL278 with *Eco*R V and religation. The desired product of this ligation, pRL278R, had the 2.5-kb *Eco*R V-*oriV*-*oriT*-*Eco*R V fragment inverted as compared to pRL278. The *sacB*-C.K3 (Km^r/Nm^r) fragment of plasmid pRL278R can be excised by digestion with *Asu* II (which produces *Cla* I-compatible 5' overhangs), *Bam*H I plus *Bgl* II, *Eco*R V, or *Xba* I.

Use of Tn*5*-1063 with luciferase as a reporter to identify genes that respond rapidly to nitrogen-stepdown, and study of the induction of those genes. When viewed through the Photonic System, a mating filter (on solid medium containing NO_3^-) with hundreds or thousands of Tn*5*-1063 transposition-derived colonies (Fig. 4.2a) shows many sources of light (Fig. 4.2b). The luminescent intensity of the colonies is re-observed after transfer of the colony-bearing filter to agar medium free of fixed nitrogen for a period of time (Fig. 4.2c). Comparison of images of luminescence observed before and after the shift of medium identifies light sources that respond to nitrogen-stepdown. By superimposing an image of luminescence and a corresponding analog (bright-field)

Figure 4.2. Screening of transposon-mutagenized colonies of *Anabaena* sp. strain PCC 7120 to identify mutants that respond to removal of fixed nitrogen from the medium by increase or decrease of luminescence. (a) Photograph of a filter bearing thousands of colonies derived from transposition of Tn*5*-1063, and luminescent (photon-counting) images of the filter prior to N-deprivation (b) and after 6.5 hr of deprivation of fixed N (c). Colonies indicated with arrows, including the colony from which mutant TLN6 was derived, showed increased luminescence in response to N-deprivation. Colonies indicated by arrowheads showed reduced luminescence.


a
b
TLN6
c

image, specific colonies of interest can be readily identified and picked from the mating filter.

Filters containing several thousand colonies derived from transposition of Tn*5*-1063 were used in the initial experiments. Two mutants, TLN2 and TLN6, that responded rapidly to N-stepdown by increase of luminescence were chosen for intensive study.

The genes fused with *luxAB* in mutant *Anabaena* strains TLN2 and TLN6 were denoted *tln2* and *tln6*, respectively. Artificial colonies of purified mutants TLN2 and TLN6 showed increased luminescence within 1 or 4 hr, respectively, after N-stepdown (Fig. 4.3). As shown in Fig. 4.3, induction of *tln2* and *tln6* appeared to be specific to the removal of nitrate from the medium rather than to the reduced ionic strength in the nitrate-free medium from which nitrate had simply been omitted.

In cyanobacteria, nitrate is reduced to nitrite and then to ammonium by ferredoxin-dependent nitrate reductase and nitrite reductase (Manzano et al., 1976). Ammonium, the end product of the nitrate-reduction pathway, is funneled to the cellular amino-acid pool by the glutamine synthetase/glutamate synthase (GS/GOGAT) system. Glutamine synthetase (GS, encoded by the *glnA* gene; Tumer et al., 1985), which catalyzes the ATP-dependent combination of ammonium and glutamate to form glutamine, is inhibited by L-methionine-D,L-sulfoximine (MSX) (Meeks et al., 1977; see Fig. 4.4a). Ammonium has also been observed to inhibit cellular uptake of nitrate (Flores et al., 1980) and the activity of nitrate reductase (Herrero et al., 1981) in cyanobacteria, but appears to exert such inhibitory effects via ammonium-derived nitrogenous compounds (possibly glutamine), rather than ammonium *per se*, because MSX blocks those inhibitions.

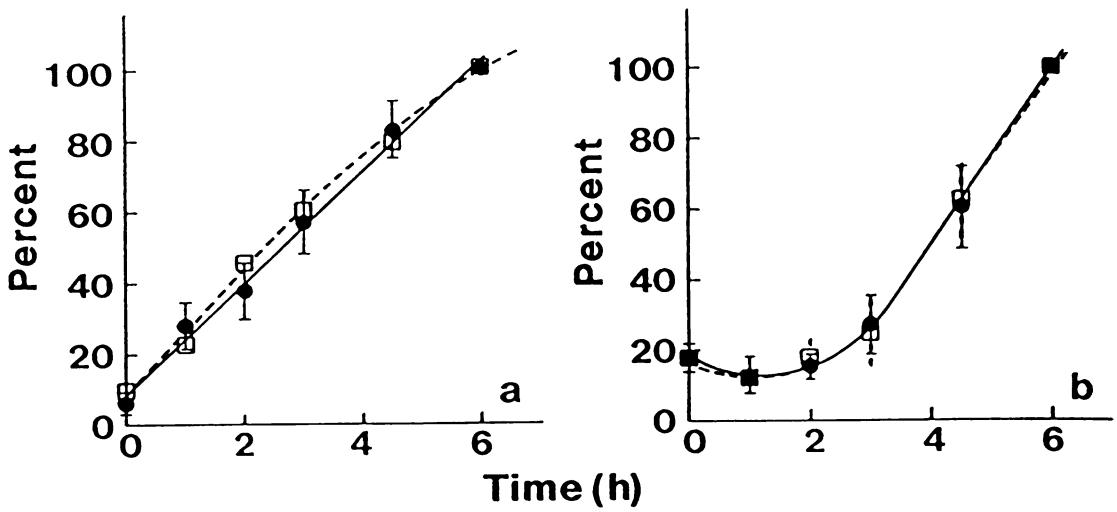
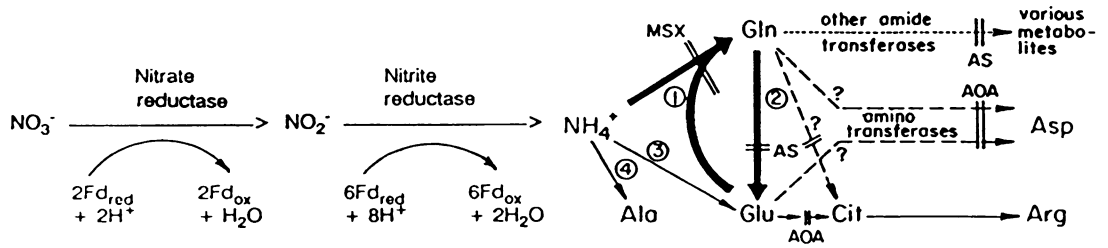


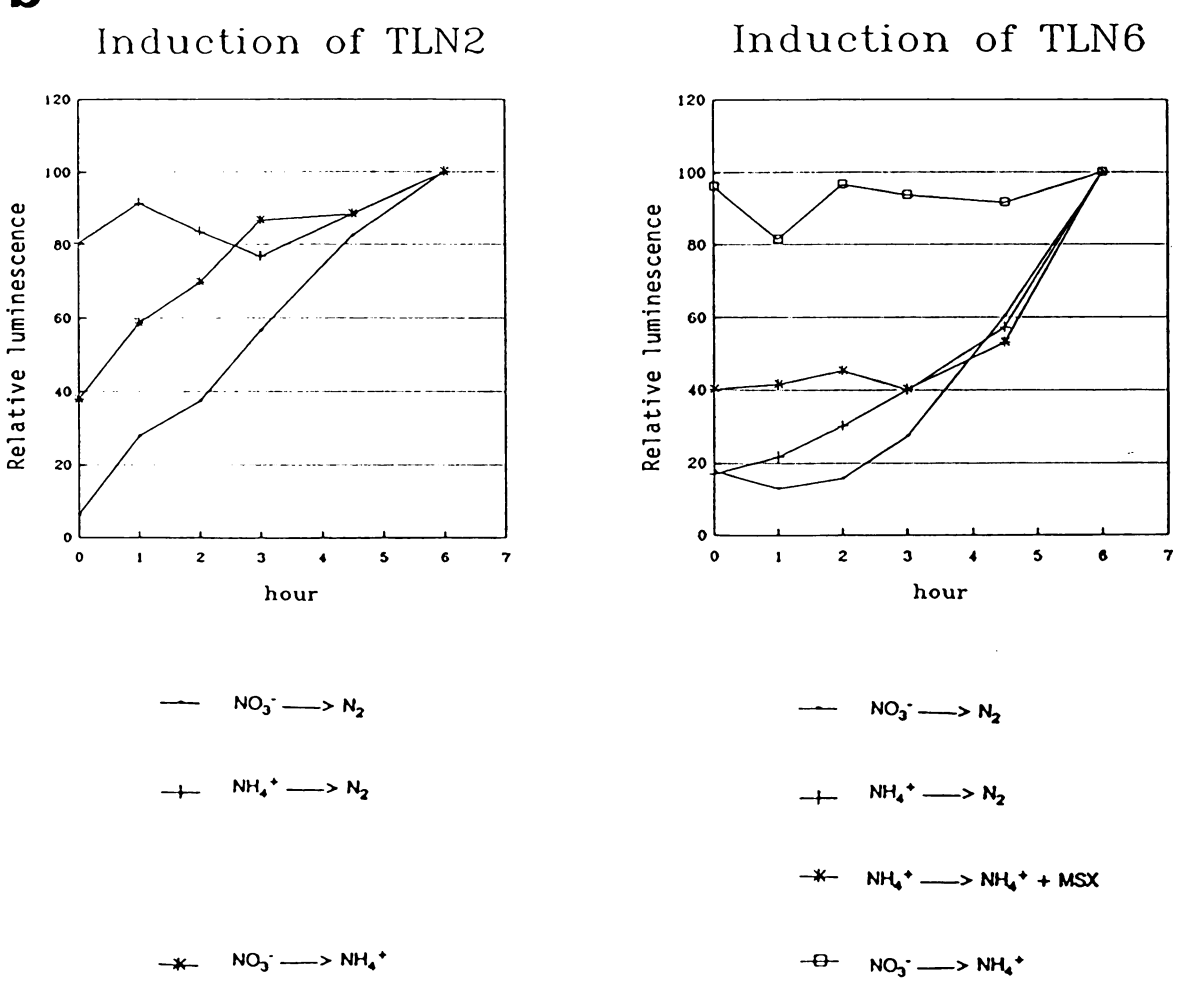
Figure 4.3. Response of mutants TLN2 (a) and TLN6 (b) to deprivation of fixed nitrogen (NO_3^-). N-stepdown was initiated by moving cells in artificial colonies (see Materials and Methods) from agar medium AA + 10 mM Na/KNO_3 + Sm (10 μg/ml) to NO_3^--free agar medium AA + Sm (10 μg/ml) without (●) or, as a control having unaltered ionic strength, with (□) 10 mM Na/KCl. Relative luminescent intensities were measured. Each value is the mean of measurements from three independent experiments, ± SEM. Mean luminescence did not change significantly when cells were transferred to fresh NO_3^--containing medium (data not shown).

Figure 4.4. Utilization of nitrate and of ammonium by cyanobacteria and response of mutants TLN2 and TLN6 to removal of nitrate or of ammonium from the growth media. (a) Scheme of metabolic pathways in the utilization of nitrate and of ammonium in cyanobacteria (adopted from Meeks et al., 1977, and Tsinoremas et al., 1991). (b) Response of mutants TLN2 and TLN6 to removal of nitrate or of ammonium from the growth media. Experimental procedures are identical to those described in Fig. 4.3; growth media are AA + Sm (10 μg/ml), with or without sources of fixed nitrogen (see Appendix A) as indicated. Relative luminescent intensities were measured. Each value is the mean of measurements from three independent experiments. SEM, not shown, is smaller than 15% of the corresponding mean value for most of the measurements.

a


Nitrate reductase
Nitrite reductase
NO3-
NO2-
NH4+
2Fdred + 2H+
2Fdox + H2O
6Fdred + 8H+
6Fdox + 2H2O
MSX
Gln
other amide transferases
various metabo-lites
AS
AOA
amino transferases
Asp
Ala
Glu
Cit
Arg

b


Induction of TLN2
Induction of TLN6
Relative luminescence
hour
NO3- ——> N2
NH4+ ——> N2
NO3- ——> NH4+
NH4+ ——> NH4+ + MSX

Observations from alternative N-stepdown experiments (Fig. 4.4b) suggested that *tln2* and *tln6* respond differently to the removal of various nitrogenous compounds. The gene *tln2* appeared to respond to NO_3^- concentration *per se*, rather than to an ammonium-derived metabolic pool because *tln2::luxAB* 1) was rapidly induced in response to the shift from NO_3^- to -N, 2) showed similar induction in response to the shift from NO_3^- to NH_4^+, and 3) remained highly expressed when mutant TLN2 was grown with ammonium as nitrogen source, and did not respond to the removal of ammonium from the medium (Fig. 4.4b). Gene *tln6*, on the other hand, appeared to sense the concentration (or ratio) of ammonium-derived metabolites because *tln6::luxAB* 1) was not induced when shifted from NO_3^- to NH_4^+ (remains at the low, constitutive level of expression), 2) responded with an induction similar to the one shown in Fig. 4.3 (but the lag period of ca. 3.5 hr was replaced by a slow but continuous increase) when mutant TLN6 was transferred from NH_4^+ to -N, and 3) showed induction when shifted from NH_4^+ to NH_4^+ + MSX (Fig. 4.4b).

Both *Anabaena* mutants, TLN2 and TLN6, appear to differentiate normally, forming N_2-fixing heterocysts, and are similar to the wild type in regard to growth rate and morphology.

Genetic characterization of *tln2* and *tln6*, and regeneration of their *luxAB* fusions in the wild-type chromosome. Southern analysis of total DNA from mutants TLN2 and TLN6, digested with the enzymes *Eco*R I, *Eco*R V, and *Cla* I, none of which cuts inside the transposon, showed only single bands of hybridization, suggesting that the genome of each mutant bears only one copy of the transposon Tn*5*-1063 (Fig. 4.5). Because pRL1063 has no known homology with the genome of *Anabaena* sp. strain PCC 7120, integration of the

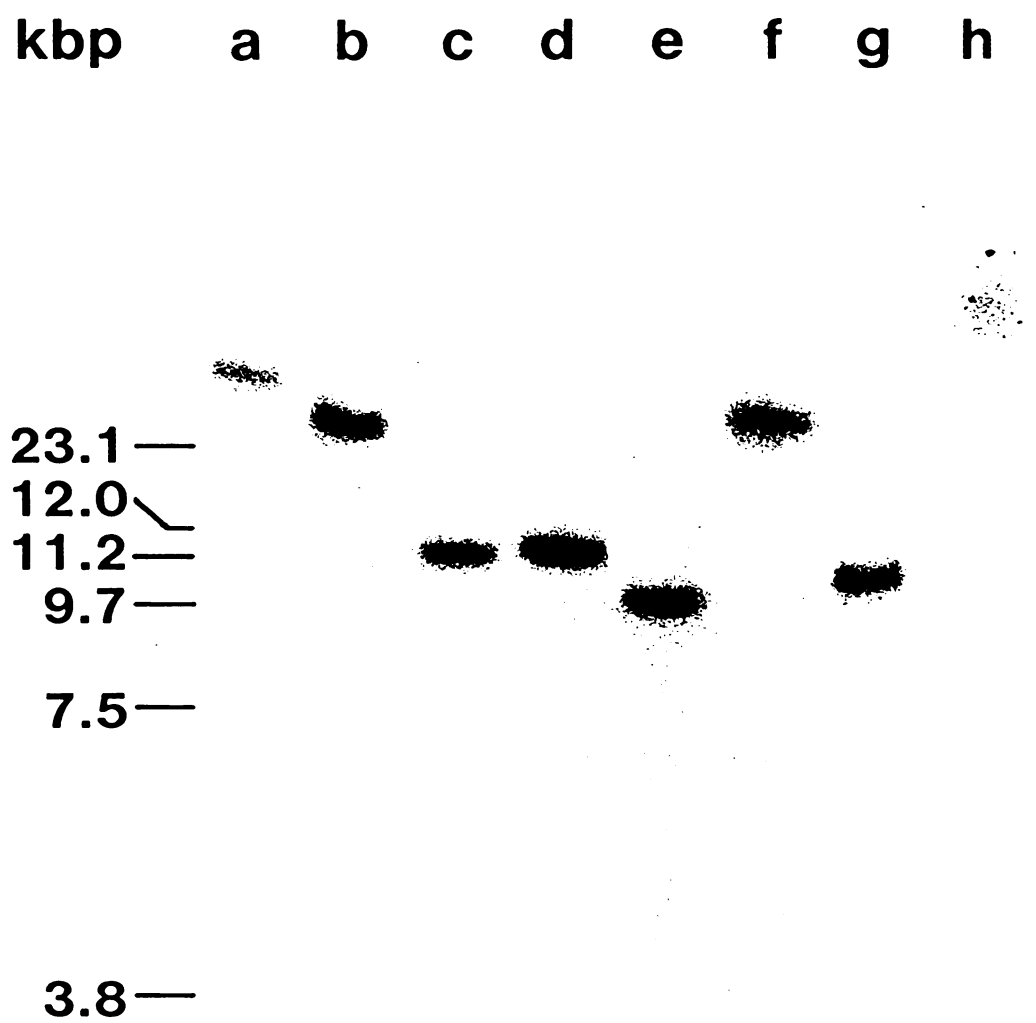


Figure 4.5. Southern analysis of total DNA isolated from mutants TLN2 and TLN6 of *Anabaena* sp. Samples of total DNA isolated from mutants TLN2 (lanes a, c, e, and g) and TLN6 (lanes b, d, f, and h) were digested with the restriction endonucleases *Eco*R I (lanes a and b), *Eco*R V (lanes c and d), *Cla* I (lanes e and f), or *Bgl* II (lanes g and h), subjected to electrophoresis, blotted, and probed with the two larger *Bgl* II fragments of pRL1063a that span Tn*5*-1063 (Fig. 4.1) and that were ^{32}P-labelled. Generation of bands produced by digestion with *Cla* I or *Eco*R V is illustrated in Fig. 4.6.

plasmid into the chromosome would be by illegitimate recombination. Plasmid pRL1063a is comprised of three *Bgl* II fragments of sizes 6.4, 1.6, and 0.8 kb; illegitimate recombination would give rise to least one band of one of the three sizes. However, *Bgl* II-digested total DNA from TLN2 and TLN6 showed no hybridizing band of size below 7 kb (Fig. 4.5). Therefore, the transposon cannot have integrated into the chromosome simply by recombination.

The *Cla* I and *Eco*R V fragments containing Tn*5*-1063 and contiguous *Anabaena* DNA in the genome of TLN2 and TLN6 (Fig. 4.6) were recovered by digestion of DNA with *Cla* I or *Eco*R V, circularization by ligation, and transfer to *E. coli* by electroporation. Sizes of all recovered fragments were consistent with sizes of hybridizing bands shown in Fig. 4.5. The recovered 9.6-kb *Cla* I fragment (pRL731) from TLN2 has 0.8 kb of contiguous chromosomal DNA upstream from the L end of the transposon and *luxAB*, and 1.0 kb adjacent to the R end of the transposon. The 11-kb *Eco*R V fragment (pRL732) has 0.5 kb contiguous DNA at the L end and 2.7 kb at the R end. The recovered 29-kb *Cla* I fragment (pRL733) from TLN6 has 1.2 kb adjacent to the L end and *luxAB*, and ca. 20 kb at the R end of Tn*5*-1063. The 11.1-kb *Eco*R V fragment (pRL734) has 0.6 kb at the L end and 2.7 kb at the R end (Fig. 4.6).

The locations of insertion in the chromosomes of mutants TLN2 and TLN6 were determined by pulsed-field gel electrophoresis and hybridization in cooperation with E. Oren and C. P. Wolk (Fig. 4.7). The banding pattern of digestion with *Sal* I (Fig. 4.7, lanes 2 and 5) and hybridization (lanes 3 and 4) with a ^{32}P-labelled fragment containing the *luxAB* genes localized the transposon in TLN2 and TLN6 within fragments SalB and SalJ, ca. 170 and 80 kb from the nearest *Sal* I sites, respectively. Hybridization with DNA from IS*50* R (contains the

Figure 4.6. Diagram of a portion of the chromosomes of *Anabaena* mutants (a) TLN2 and PS760 and (b) TLN6 and PS763. The four longer lines reprent a portion of the chromosome. Also indicated are the origins of plasmids pRL731 and pRL732 (from mutant TLN2), and pRL733 and pRL734 (from mutant TLN6) that contain Tn*5*-1063 and contiguous *Anabaena* DNA. L and R indicate the left (luciferase) and the right (transposase) ends of the transposon Tn*5*-1063, respectively. In mutant PS760 most of the transposon Tn*5*-1063 is replaced by the cassette BLOS1; in mutant PS763 most of Tn*5*-1063 is replaced by BLOS2 (see text). The presumed promoter (not shown) of *tln2* (in TLN2 and PS760) or of *tln6* (in TLN6 and PS763) is upstream from the L end of Tn*5*-1063, directing expression of the *luxAB* genes.

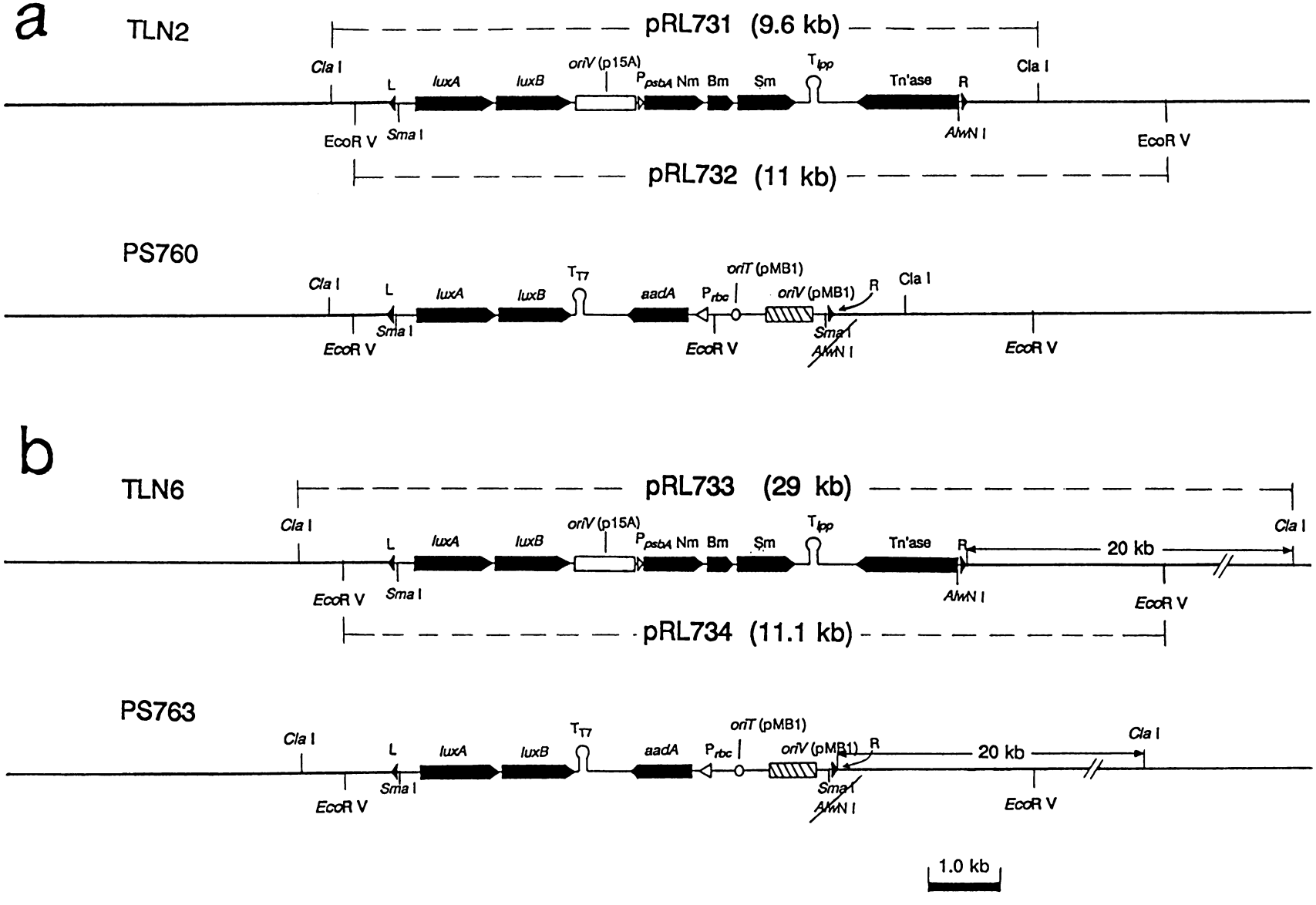
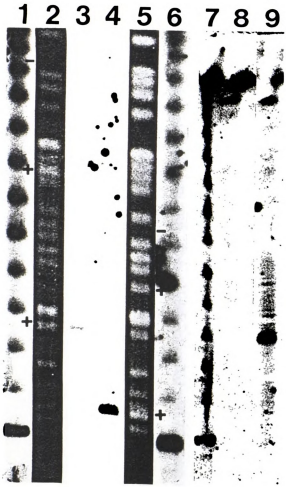
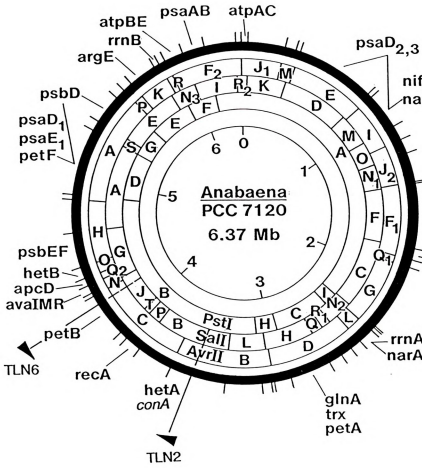

a
TLN2
pRL731 (9.6 kb)
Cla I
L
luxA
luxB
oriV (p15A)
P psbA
Nm
Bm
Sm
T lpp
Tn'ase
R
Cla I
EcoR V
Sma I
AlwN I
EcoR V
pRL732 (11 kb)
PS760
Cla I
L
luxA
luxB
T T7
aadA
P rbc
oriT (pMB1)
oriV (pMB1)
R
Cla I
Sma I
EcoR V
EcoR V
Sma I
AlwN I
EcoR V
b
TLN6
pRL733 (29 kb)
Cla I
L
luxA
luxB
oriV (p15A)
P psbA
Nm
Bm
Sm
T lpp
Tn'ase
R
20 kb
Cla I
EcoR V
Sma I
AlwN I
EcoR V
pRL734 (11.1 kb)
PS763
Cla I
L
luxA
luxB
T T7
aadA
P rbc
oriT (pMB1)
oriV (pMB1)
R
20 kb
Cla I
Sma I
EcoR V
Sma I
AlwN I
EcoR V
1.0 kb

Figure 4.7. Localization of *tln2*::Tn*5*-1063 and *tln6*::Tn*5*-1063 in the chromosomes of mutants TLN2 and TLN6, respectively, by pulsed-field gel electrophoresis and Southern hybridization. Genomic DNAs from mutant strains TLN2 (lanes 2,3, and 9) and TLN6 (lanes 4 and 5) and wild-type *Anabaena* sp. strain PCC 7120 (lane 8) were cut with *Sal* I (lanes 2-5) or with *Avr* II plus *Sph* I (lanes 8 and 9). Lanes 1, 6, and 7 are concatamers of coliphage λ DNA. Lanes 2 and 5 are from an ethidium bromide-stained pulsed-field gel, and lanes 1, 3, 4, and 6 are an autoradiograph of a Southern blot of those lanes and of λ ladders, hybridized with *luxAB* plus λ DNA. In mutants TLN2 and TLN6, new *Sal* I restriction fragments (+) replace, respectively, fragments SalB and SalJ (—; compare Bancroft et al., 1989). A separate hybridization (lanes 7 to 9) with the transposase gene plus λ DNA shows cross-reaction at 190 kb. The location and orientation of transposon Tn*5*-1063 in mutants TLN2 and TLN6 are indicated by arrowheads, with *luxAB* at the wide end of the arrowhead, in the map (to the right) of the chromosome of *Anabaena* sp. strain PCC 7120 (adapted from Bancroft et al., 1989). The locations of other genes mentioned in this dissertation, such as *atp*, *conA*, *hetA*, *hetB*, *glnA*, *nif*, *prcA*, and *rbcLS*, are also shown in the map.


1 2 3 4 5 6 7 8 9


Anabaena
PCC 7120
6.37 Mb
atpBE
rrnB
argE
psaAB
atpAC
psaD2,3
nifH2
narC
psbD
psaD1
psaE1
petF
prcA
leuB6
nifK
rbcLS
rpoBC1C2
psbEF
hetB
apcD
avaIMR
petB
TLN6
recA
hetA
conA
TLN2
glnA
trx
petA
rrnA
narA
PstI
SalI
AvrII

R end and the entire transposase gene of Tn*5*) after restriction with a combination of *Avr* II plus *Sph* I (lane9) localized the transposon in the chromosome of mutant TLN2 to a position of 3.5 Mb on the map, with *luxAB* pointing clockwise. This site of insertion was further confirmed by restriction with *Pst* I, and hybridization (data not shown). TLN6 was tentatively localized at 4.2 Mb, with *luxAB* oriented counterclockwise, by a weak hybridization of the AvrN fragment with the 80-kb *Sal* I fragment generated by insertion of Tn*5*-1063 (data not shown).

In order to perform second-round mutagenesis with Tn*5*-derived transposons to identify mutations that alter the expression of *tln6* (see below), the *tln6::luxAB* fusion was regenerated in the wild-type chromosome as follows, using the *sacB*-mediated gene replacement technique described in chapter 2. Plasmid pRL734 (Fig. 4.6) contains the *tln6::luxAB* fusion, and lacks *Alw*N I and *Sma* I sites outside of Tn*5*-1063. Most of Tn*5*-1063 was removed from this plasmid by digestion with *Alw*N I and *Sma* I, the 3' overhang of the *Alw*N I end was made blunt by treatment with Klenow fragment, and the transposon was replaced with the cassette BLOS2 by ligation to *Sma* I-opened plasmid pRL759B. A transformant (denoted pRL762) in which *tln6::luxAB* (oriented as before) was regenerated, was selected for further use. Plasmid pRL762, opened with *Eco*R V, was ligated to the 3.4-kb *sacB*- and C.K3-containing *Eco*R V fragment of pRL278R to give plasmid pRL763. The suicide plasmid pRL763 was transferred to the cells of wild-type *Anabaena* sp. strain PCC 7120 by conjugation to produce a sucrose-sensitive single recombinant *Anabaena* sp. strain PCC 7120::pRL763. A sucrose-resistant double recombinant, *Anabaena* PS763, was derived from the single recombinant, and its structure confirmed by Southern analysis. The

*Eco*R V-hybridizing fragment in the genome of PS763 is 8.9 kb (lane 1 of Fig. 4.11), 2.2 kb smaller than that of TLN6 (lane d of Fig. 4.5), reflecting the size difference (illustrated in Fig. 4.6) between Tn*5*-1063 (7.8 kb) and the BLOS2 cassette (5.62 kb). The temporal pattern of induction of *luxAB* in mutant PS763 was virtually identical to that observed with TLN6 (see Fig. 4.10). Using a similar method and *Asu* II-digested pRL278R, Tn*5*-1063 in plasmid pRL731 was replaced with the cassette BLOS1 (from plasmid pRL739) and the *tln2::luxAB* fusion was regenerated in the wild-type chromosome to give mutant strain *Anabaena* PS760 (illustrated in Fig. 4.6) whose temporal pattern of induction of *luxAB* is indistinguishable from that of TLN2 (data not shown).

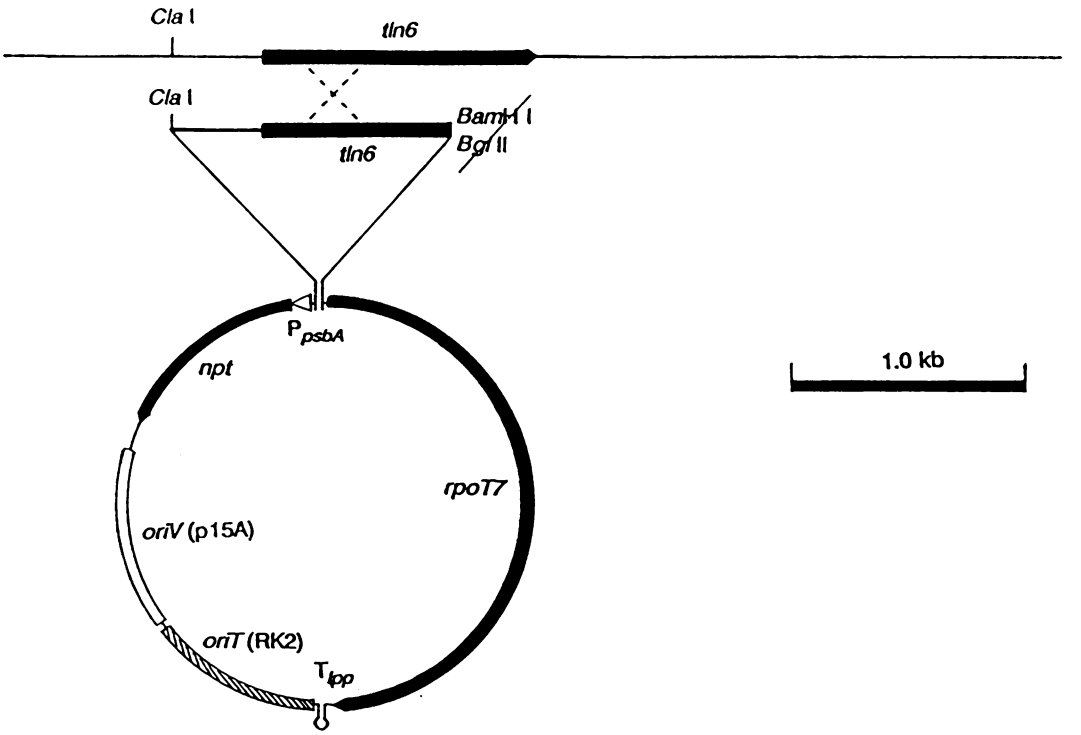
Observation, at a single-cell level, of expression of *tln6* along the filament, using a T7 RNA polymerase-based transcription-amplifying reporter system. Genes *tln2* and *tln6* respond to removal of fixed nitrogen rapidly, and so the *tln2::luxAB* and *tln6::luxAB* fusions provide an opportunity to examine whether genes activated shortly after N-stepdown show patterned expression along a filament. Unfortunately, both genes were expressed so weakly that with neither of the *luxAB* fusions imaged at a single-cell level was I able to observe light at a level above the electronic background seen with our photon-recording system. I therefore made use of a binary vector system for the amplification of transcriptional reporting that had been constructed by C. P. Wolk (unpublished).

The rifampicin (Rif)-resistant RNA polymerase of phage T7 has been widely used to over-express genes cloned downstream from the T7 ϕ10 promoter (Tabor and Richardson, 1985; Rosenberg et al., 1987). The binary vector system for transcriptional amplification constructed for use in *Anabaena* sp. consists of a replicating plasmid and an integrating plasmid. The replicating plasmid

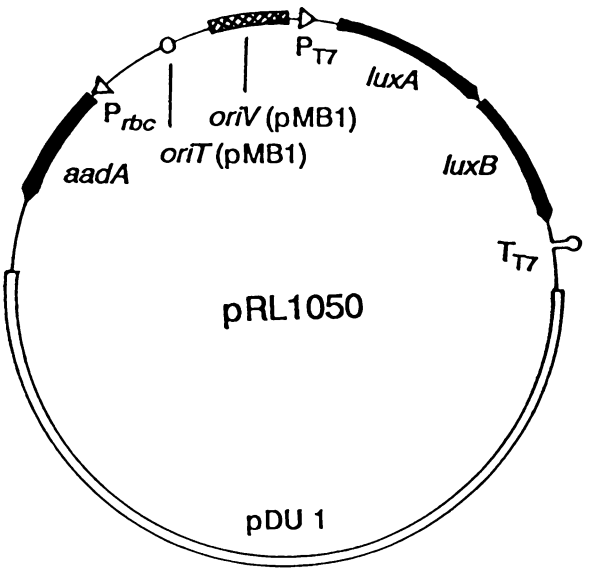
pRL1050 (Fig. 4.8) contains 1) the reporter operon *luxAB* (as in Tn*5*-1063 and the BLOS cassettes, from *Vibrio fisheri*) that is under the control of the T7 ϕ10 promoter (P_{T7}), a promoter that is highly specific for the RNA polymerase encoded by the phage T7 ϕ1 gene (*rpoT7*), and is terminated with a T7 terminator; 2) the cyanobacterial plasmid pDU1 that permits replication in *Anabaena* spp. (Wolk et al., 1984; Schmetterer and Wolk, 1988); 3) the pMB1-derived *oriV* and *oriT* from pBR322 that permit replication in, and mobilization from, *E. coli* (Balbás et al., 1986); and 4) the Sm^r/Sp^r cassette C.S4 (Bancroft and Wolk, 1989). The precursor of the final integrating plasmid is plasmid pRL1081 which was designed to contain no sequence homologous to pRL1050. This suicide plasmid was constructed from 1) the *rpoT7* gene, terminated with the bidirectional transcriptional terminator T_{lpp} from plasmid pJDC406; 2) multiple cloning sites immediately upstream of rpoT7 for insertion of a gene of interest; 3) a p15A-derived *oriV* from pACYC184 for replication in *E. coli* and an RK2-derived *oriT* from pAT187; and 4) the Km^r/Nm^r cassette C.K3 (see Appendix F), all on a scaffold of the polylinker from pJRD184 (Heusterspreute et al., 1985).

The 0.76-kb and 1.2-kb *Cla* I-*Bam*H I fragments upstream from the L end of Tn*5*-1063 in plasmids pRL731 and pRL733, respectively (Fig. 4.6; the *Bam*H I site is to the left of the *Sma* I site next to the L end of Tn*5*-1063), were cloned between the *Cla* I and *Bgl* II sites in the multiple cloning region of pRL1081, giving integrating plasmids pRL772 and pRL776, respectively. Both plasmids were individually introduced into *Anabaena* sp. strain PCC 7120(pRL1050) by conjugation. Selected exconjugants bore *tln2::rpoT7* (in *Anabaena* strain TAL2) and *tln6::rpoT7* (in *Anabaena* strain TAL6) fusions in the chromosome by single homologous recombination. In strain TAL2 or TAL6, promoter activity of *tln2* or

Figure 4.8. Scheme of amplified reporting of transcription of *tln6* in *Anabaena* sp. strain TAL6 by the T7-based binary vector system. The 1.2-kb *Cla* I-*Bam*H I fragment overlapping the L end of Tn*5*-1063 in pRL733 (see text) was inserted into plasmid pRL1081 between *Cla* I and *Bgl* II to give plasmid pRL776. Integration of pRL776 into the chromosome (top horizontal line) by single homologous recombination puts the phage T7 ϕ1 gene (*rpoT7*) under the control of the *tln6* promoter. The product of gene *rpoT7*, the phage T7 RNA polymerase, binds specifically to the phage ϕ10 promoter P_{T7} and transcribes the *luxAB* genes. No information is available on the size of *tln6*, so that the drawing of that gene in the figure is hypothetical.


Cla I
tln6
Cla I
BamH I
Bgl II
tln6
P psbA
npt
rpoT7
oriV (p15A)
oriT (RK2)
T lpp
1.0 kb

pRL776


P T7
luxA
P rbc
oriV (pMB1)
oriT (pMB1)
aadA
luxB
T T7
pRL1050
pDU 1

1.0 kb

tln6 directs production of the T7 RNA polymerase which in turn binds specifically to P_{T7} and strongly expresses *luxAB* (Fig. 4.8). Luminescence of TAL2 and TAL6 is much greater than that of TLN2 and TLN6 under both non-inducing and inducing conditions (data not shown). The control strain, *Anabaena* sp. strain PCC 7120(pRL1050), showed very little luminescence, suggesting that there is minimal read-through promotion into the luciferase genes (C. P. Wolk, unpublished; personal observation).

The distribution of luminescence was examined along filaments of strain *Anabaena* TAL6 (*tln6::rpoT7* + P_{T7}::*luxAB*) (but not yet along filaments of TAL2) by photon-counting microscopy. At the earliest time point after N-stepdown that was studied, 7 hr, virtually all cells along the filament showed luminescence. A few cells, often spaced well apart, showed much lower luminescence, close to the background level (Fig. 4.9a, arrows). At 21 hr after N-stepdown, (nearly) mature heterocysts could be seen in the filaments, suggesting that heterocysts differentiate at a normal rate in this strain of *Anabaena* sp. At this time point, heterocysts showed virtually no luminescence; possibly insufficient O_2 was present to support luminescence. Although most cells other than heterocysts showed luminescence, a few cells luminesced only very weakly (Fig. 4.9b, arrows). These observations are based on two independent experiments; additional experiments are needed to assess the reproducibility of the observations.

Figure 4.9. Observation of *tln6*-directed luciferase activity along the filament of *Anabaena* strain TAL6 at 7 hr (a) and 21 hr (b) after nitrogen-stepdown. Vegetative cells are outlined in green and heterocysts in red. There is variation in the degree of luminescence among vegetative cells, and those that show *markedly* lower luminescence than do others are indicated by arrows. The gap (+) in the upper filament in (a) is the result of a cell that had burst before photon-recording (see Discussion).

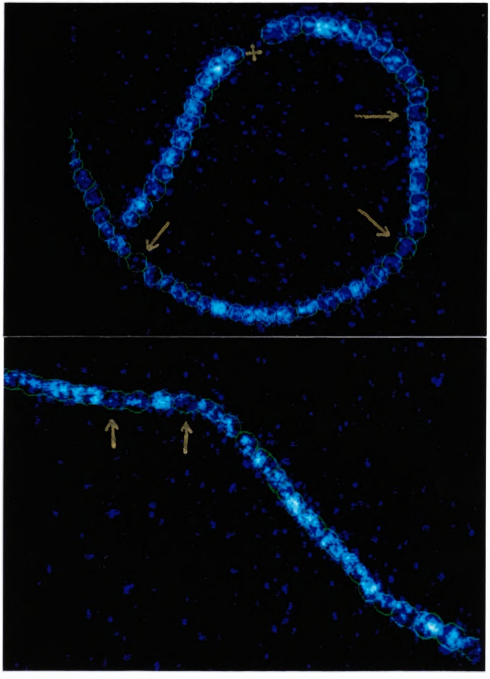


Figure 4.9a

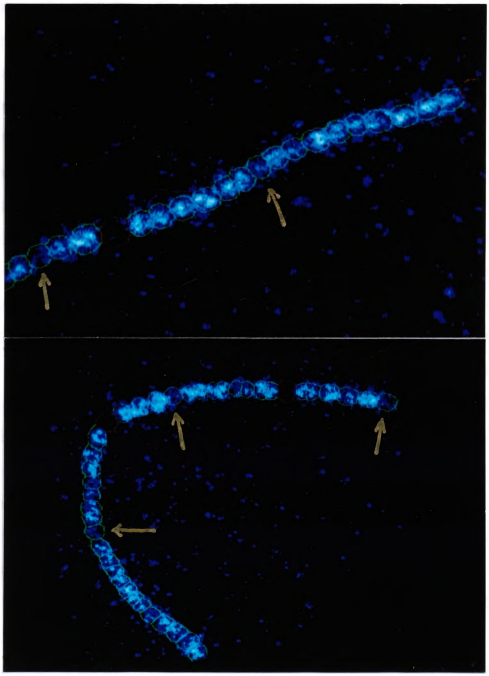


Figure 4.9b

Secondary transposon-mutagenesis to identify genes that regulate both the expression of *tln6* and the differentiation of heterocysts. * I tried, by random transposon mutagenesis of strains that contain the *tln6::luxAB* fusion, to identify regulatory genes whose products control both the expression of *tln6* and the differentiation of heterocysts. In the initial experiments, transposons Tn*5*-1087b (C. P. Wolk, unpublished) and Tn*5*-800 were used to mutagenize mutant *Anabaena* TLN6 (*tln6*::Tn*5*-1063). Although both transposons appeared to transpose frequently in TLN6, Southern analysis (data not shown) of two such secondary mutants (denoted TTL62 and TTL68) showed that the transposon (Tn*5*-1087b) had integrated into the chromosome not by transposition, but through single homologous recombination of plasmid pRL1087b with the resident Tn*5*-1063 within the transposase sequence. Because Tn*5*-1063 in TLN6 and other Tn*5* derivatives share the 1.43-kb sequence from IS*50*R that contains the whole transposase and *Anabaena* sp. strain PCC 7120 is not a *recA* strain (a defect in the *recA* gene results in the virtual elimination of homologous recombination of DNA in *E. coli*; Radding, 1985), using TLN6 as target to perform subsequent transposon-mutagenesis by other Tn*5* derivatives might frequently lead to production of recombinants rather than, or in addition to, transposon-mutants. Such experiments were therefore terminated. Although *recA* genes have been found in the cyanobacteria *Anabaena* variabilis ATCC 29413 (Owttrim and Coleman, 1989), *Synechococcus* sp. strain PCC 7002 (Murphy et al., 1990), and *Anabaena* sp. strain PCC 7120 (J. R. Coleman, personal communication),

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* Attempts at secondary mutagenesis of a strain with a *tln2::luxAB* fusion were unproductive, and are therefore not reported here.

a complete *recA* mutation appears to be lethal to *Synechococcus* sp. strain PCC 7002 (Murphy et al., 1990). Therefore, use of a *recA* strain of *Anabaena* sp. strain PCC 7120 may not be the solution to this problem of unwanted homologous recombination.

Mutant strain *Anabaena* PS763 (*tln6*::BLOS2) retains the *tln6::luxAB* fusion and less than 120 bp (a maximum of 67 bp at the L end and 52 bp at the R end) of sequence homologous to other *luxAB*-lacking Tn*5* derivatives that we have constructed. Use of *Anabaena* PS763 as target to perform secondary transposon-mutagenesis should dramatically reduce the probability of homologous recombination of the incoming Tn*5*-derivatives with the chromosome, as well as possibly increase their transposition frequency because, unlike TLN6, cells of *Anabaena* PS763 do not contain the Tn*5* transposase gene which encodes for both the transposase and an inhibitor of transposition (Berg, 1989).

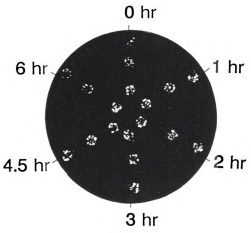
The transposons Tn*5*-764 and Tn*5*-1058 (Fig. 4.1) were used to mutagenize cells of *Anabaena* PS763. Thousands of secondary mutants on mating filters were screened for those whose *tln6::luxAB* expression had been altered. Theoretically one can screen for those that show low luminescence under -N (induced) condition but it proved impractical because looking for such colonies was like fishing out a dead bulb in a field of "a thousand points of light". Nonetheless, screening under +N (non-inducing) condition identified several mutants (denoted TTL631, TTL632, TTL633, and TTL635) that showed strong luminescence constitutively (data not shown). However, these mutants were capable of normal heterocyst differentiation and nitrogen fixation, and therefore were not further studied.

In order to isolate mutants that are altered both in the expression of *tln6::luxAB* and in heterocyst formation, a different strategy was employed. I first screened for secondary mutants that were unable to fix dinitrogen, and then screened such mutants for possible alterations in the induction of *tln6::luxAB*. Fox$^-$ mutants are easily identified because they become and stay yellow after a mating filter bearing many small, transposition-generated colonies is transferred from +N to -N conditions. More than a hundred secondary Fox$^-$ mutants were recovered in this way. Four of them (denoted TTL615, TTL616, TTL619, and TTL620) were found to be altered in the induction of *tln6::luxAB* in response to N-stepdown: mutants TTL615 and TTL620 stay at the non-induced level and do not appear to respond to N-stepdown, while mutants TTL616 and TTL619 constitutively express *tln6::luxAB* at a high level (Fig. 4.10). Southern analysis showed that all four secondary insertions by Tn*5*-764 or Tn*5*-1058 were independent, and apparently not closely linked to the original mutation of *tln6*::BLOS2 (Fig. 4.11).

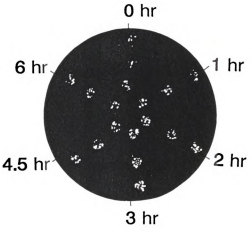
Preliminary examinations by bright-field microscopy revealed a distinct phenotype for each mutant. When grown in the presence of NO_3^-, mutant TTL615 had short, fragmented filaments, whereas the other three mutants had a normal morphology. The four mutants responded in differing ways to N-stepdown. Mutant TTL615 became even more fragmented, and produced, with delayed timing, only immature heterocysts that tended to have widened junctions with vegetative cells. Mutant TTL616 appeared to differentiate normally. Mutant TTL619 tended to break into short filaments and produced heterocysts that were "empty" and thin-walled. Finally, mutant TTL620 became yellow very slowly (suggesting that the cells were not degrading phycocyanin in response to N-

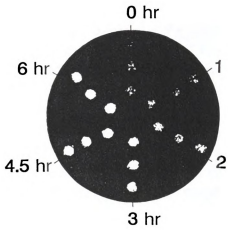
Figure 4.10. Altered expression of *tln6::luxAB* in secondary mutants TTL615, TTL616, TTL619, and TTL620, as compared to that of the primary mutant PS763 (TLN6). See Materials and Methods for experimental procedure. Nitrogen-stepdown of the secondary mutants was initiated by, at various times, transferring filters bearing artificial colonies from AA + 10 mM Na/KNO_3 + Nm (200 μg/ml) + Sp (5 μg/ml) agar medium to AA + Nm (20 μg/ml) + Sp (5 μg/ml) agar medium. Mutants TLN6 and PS763 (shown) were induced by a transfer from AA + 10 mM Na/KNO_3 + Sm (2.5 μg/ml) to AA + Sm (2.5 μg/ml) and showed virtually identical expression of *tln6::luxAB*. In the -N medium the concentration of Nm is reduced from 200 μg/ml to 20 μg/ml to avoid use by the cell of Nm, a nitrogenous compound, as a source of fixed nitrogen (see Appendix F).

TTL615

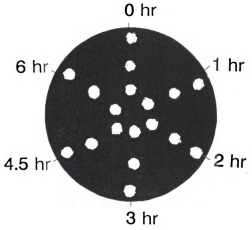


TTL620

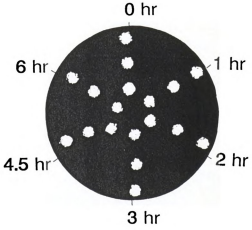




TLN6/PS763

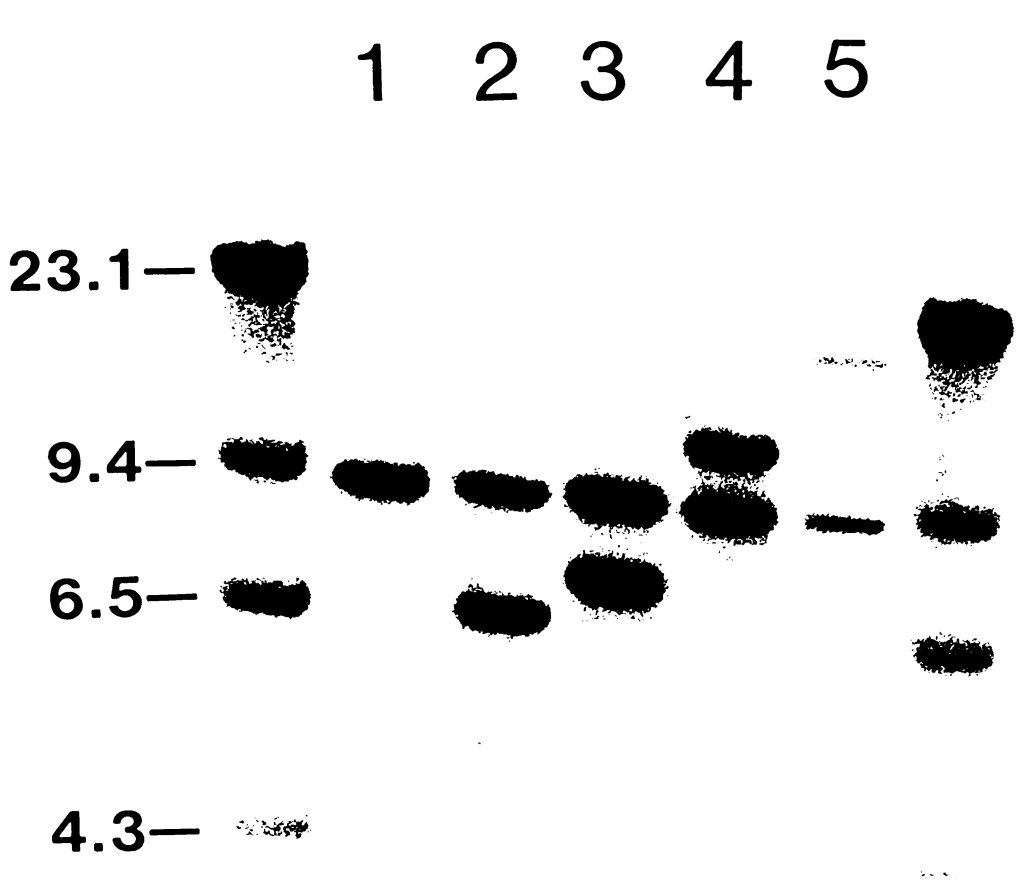


TTL619



TTL616

Figure 4.11. Southern analysis of secondary mutants produced by transposition of Tn*5*-764 or of Tn*5*-1058 in mutant *Anabaena* PS763. Total DNA isolated from strain PS763 (lane 1) and secondary mutants TTL615 (lane 2), TTL616 (lane 3), TTL619 (lane 4), and TTL620 (lane 5) were digested by *Eco*R V and hybridized with a ^{32}P-labelled probe made from the *luxAB*-containing *Bam*H I fragment from pRL1022a (see Appendix C) and from the entire plasmid pRL764 that had been linearized by digestion with *Bam*H I. The 8.9-kb hybridizing band in lanes 1 to 5 represents the *Eco*R V fragment that contains the primary *tln6::luxAB* mutation with the cassette BLOS2 (Fig. 4.6). Additional single bands suggest independent insertion of Tn*5*-764 (lanes 4 and 5) or Tn*5*-1058 (lanes 2 and 3) in the genome of *Anabaena* PS763. Plasmids pRL764 and pRL1058 (bearing transposon Tn*5*-764 and Tn*5*-1058, respectively) cannot have integrated, intact, into the genome in these four secondary mutants because such recombination would have produced two, instead of one, hybridizing bands in addition to the 8.9-kb band. The unnumbered lanes on both sides provide DNA size markers (in kilobases).


1 2 3 4 5
23.1—
9.4—
6.5—
4.3—
2.3—
2.0—

starvation; Bradley and Carr, 1976) and did not appear to initiate heterocyst differentiation (one observation of a culture 5 days after removal of fixed nitrogen from the medium showed some cells that could possibly be considered proheterocysts). I have not yet determined whether 1) the morphogenetic aspects of the phenotype of these mutants and 2) the effects on expression of *tln6::luxAB*, are consequences of insertion of the transposon that has rendered them Nm^r.

Introduction, by gene replacement, of a known secondary mutation to study its possible regulatory relationships with genes *tln2* and *tln6*. Possible regulatory relationships between *tln2*, *tln6*, and other known genes were also explored using the *sacB*-mediated gene replacement technique described in chapter 2. Mutation in the gene *hetR* blocks heterocyst differentiation (Buikema and Haselkorn, 1991a and b). A *hetR* ::Tn*5*-1058 insertional mutant (α41) was independently obtained by C. P. Wolk in our lab, and the gene was shown to be induced within 3.5 hr after N-stepdown (T. Black and C. P. Wolk, unpublished results). The 9.8-kb *Eco*R V fragment bearing Tn*5*-1058 plus 4.4 kb of contiguous *Anabaena* DNA was recovered (as pRL880) from mutant α41, and ligated to the *Eco*R V fragment that contains *sacB* and the C.CE2 cassette (Cm^r and Em^r) from plasmid pRL1075, a derivative of pRL271 (see Appendix C), to give positive selection plasmid pRL908 (T. Black and C. P. Wolk, unpublished results). The *hetR* ::Tn*5*-1058 mutation was then introduced into mutants PS760 and PS763 by conjugation using pRL908. Suc^r Fox^- colonies *Anabaena* PS908L2 and PS908L6 from Suc^s Fox^+ single-recombinants PS760::pRL908 and PS763::pRL908, respectively, showed inductions of *tln2::luxAB* and *tln6::luxAB* similar to those of PS760 and PS763, respectively, suggesting that the *hetR*

mutation does not affect expression of either *tln2* or *tln6*. Nonetheless, whereas strain *Anabaena* PS908L6 is morphologically normal when grown with NO_3^-, series of partially plasmolyzed cells are frequently seen in strain PS908L2 grown with NO_3^--containing media. The reasons for the expression of such a phenotype remain to be investigated.

DISCUSSION

Mutagenesis using transposon Tn*5*-1058 and its derivatives in *Anabaena* sp. exhibits distinct advantages over chemical- and UV-mutagenesis. First, many colonies representing presumptive transpositions are obtained, and each such colony is a genuine mutant. Second, because we have yet to find evidence of multiple insertions, the great majority of mutants probably bear only a single lesion resulting from transposition. Third, because each mutated gene is linked to the transposon, which contains an origin of replication (*oriV*) functional in *E. coli*, at least part of the gene is easily cloned in *E. coli* for further study. Fourth, transcriptional activity of the mutated gene can be monitored by a reporter gene in the transposon (e. g., Tn*5*-1063) if insertion has been in the appropriate orientation. Fifth, the site of mutation and the orientation of the mutated gene in the chromosome can be mapped by pulsed-field gel electrophoresis and Southern analysis. In addition, by introducing the mutated gene, fused to a reporter, into the wild-type chromosome, secondary mutagenesis can be used to identify trans-acting regulatory genes.

Fusions to *lacZ*, encoding ß-galactosidase, have been invaluable in the elucidation of bacterial regulatory networks (Gottesman, 1984; Silhavy and Beckwith, 1985), and is similarly applicable to cyanobacteria (Elhai and Wolk,

1990; Scanlan et al., 1990). Nonetheless, use of luciferase as a reporter has several advantages relative to use of ß-galactosidase. Its product, light, is rapidly dissipated; and because luciferase appears to turn over in cells of *Anabaena* sp. with a half-life ≤ 2.6 hr (Wolk et al., 1991), repeated measurements can assess decreases, as well as increases, in promoter activity (Fig. 4.2). Luciferase activity, easily assayed, is not obscured by the pigmentation of cyanobacterial cells. The assay, subject only to instrumental background, provides sufficient sensitivity and spatial resolution to permit single-cell measurements *in vivo* as shown by Elhai and Wolk (1990) and further illustrated in this chapter.

In the experiment of Fig. 4.2, the photonic detector was operated at < 1% of its maximum sensitivity, to prevent overloading by bright colonies. This probably accounts for the relatively low ratio of light sources to colonies in Fig. 4.2. In experiments lacking sources of "intense" light, sources of weaker luminescence can be studied with greatly increased instrumental sensitivity. A high density of colonies on mating filters (Fig. 4.2) enhances the probability that rare types of mutants will be represented on a given number of filters, but increases the difficulty of identifying and isolating a particular colony, and prolongs the experimentation because stronger selection is needed following overgrowth. We therefore now perform conjugation to cells containing, per filter, ≤ 15 µg of chlorophyll *a*.

The reaction catalyzed by bacterial luciferases can be summarized as follows (Meighen, 1991):

$$FMNH_2 + RCHO + O_2 \longrightarrow FMN + H_2O + RCOOH + h\nu\,(490\text{ nm})$$

The substrate provided in our experiments was n-decanal because it was shown to cross the cell membrane much more readily than do longer-chain aldehydes

(Meighen, 1991). However, assay of luciferase with n-decanal appeared to be toxic to cells of *Anabaena* sp. The toxicity may be due to one or more of the following reasons: 1) the hydrophobic aldehyde substrate accumulates in the cell membranes; 2) the reaction catalyzed by luciferase consumes a large amount of cellular $FMNH_2$; 3) the weakly acidic fatty acid produced by the oxidation of aldehyde is a protonophore which can directly dissipate the proton gradient, a core element of Mitchell's chemiosmotic hypothesis (Mitchell, 1961), and thus inhibit bioenergetic work (Krulwich et al., 1990). Therefore, I exposed *Anabaena* cells to aldehyde for a minimal possible length of time in assays for luciferase activitry. Colonies on Petri dishes were usually viable after being subjected to a luciferase assay carried out as described in the Materials and Methods section. *Microscopic* observation of luciferase activity, however, poses the following problem. To initiate the luciferase assay, n-decanal was added to a suspension of cells of *Anabaena* sp. to a final concentration of 0.01% (v/v; Elhai and Wolk, 1990), and was often left in contact with the cells for a period of more than 5 min. in the dark for photon counting. This procedure appeared to be very stressful to the *Anabaena* cells, and frequently led to cells bursting (Fig. 4.9a). A possibly better condition of assay, sustained endogenous production of aldehyde by cells of *Anabaena* sp., is being sought but has yet to be achieved (C. P. Wolk, personal communication).

The gene *tln6* increases its expression 4 hr after N-stepdown (Fig. 4.3), and by 7 hr (Fig. 4.9a), it has apparently been expressed in most, if not all, cells of the filament. The product of this gene could be, among other possibilities, part of a nitrogen-uptake/scavenging system that is stimulated by a lowered level of an ammonium-derived nitrogenous compound. Cells that showed much reduced

tln6 expression in the filament N-starved for 21 hr were often either heterocysts or presumptive proheterocysts, the latter appearing usually several cells away from the heterocysts (Fig. 4.9b). Perhaps, therefore, the dim cells in the 7-hr, N-starved filaments (Fig. 4.9a) were also presumptive heterocysts. I have not yet tried to follow the fate of individual cells that respond rapidly to N-stepdown to see whether these cells differentiate into heterocysts (as predicted by the volunteer model) or prove distant from eventual heterocysts (as predicted by the scavenger model). Because gene *tln6* responds relatively slowly to N-stepdown compared with *hetR* (T. Black and C. P. Wolk, unpublished observations), *tln6* seems inadequate for use to distinguish the two proposed models of pattern formation experimentally.

I have found several secondary mutations that alter the expression of *tln6*. Four of these mutations, in TTL615, TTL616, TTL619, and especially TTL620, appear also to affect heterocyst differentiation. This work thus represents a step toward determining whether heterocyst differentiation is " the culmination of a process which begins as a non-developmental response to nitrogen deprivation" (Wolk, 1982), or whether that differentiation is elicited independently, for example by more stringent N-deprivation.

As results in chapter 3 suggest, *Anabaena* sp. strain PCC 7120 may have a high rate of spontaneous mutation. When a wild-type culture maintained in nitrate-containing medium was used in transposition mutagenesis and subsequently screened for Fox^- mutant colonies, the frequency of such colonies was usually around 10% to 15% (personal observation), compared to below 5% when a culture maintained under nitrogen-fixing conditions was used. To reduce the interference of spontaneous Fox^- mutants, *Anabaena* cells used for mating

in later experiments were maintained on solid or liquid media without fixed nitrogen, but then grown to moderate cell density in nitrate-containing liquid medium for mating (to achieve high growth rate and to reduce clumping of filaments). In the strain *Anabaena* PS763 the BLOS cassette that provides *luxAB* reporting of transcription of gene *tln6* is bordered by the 53-bp L- and 52-bp R-end sequences of Tn*5* (Fig. 4.6), and is potentially mobile when a transposase is provided in *cis* (de Lorenzo et al., 1990). When a Tn*5* derivative is introduced into the cells in secondary transposon mutagenesis, Tn*5*-BLOS could transpose to a new location in the genome and possibly put *luxAB* under the control of a different promoter or no promoter (although Tn*5* transposes conservatively, the original *tln6::luxAB* might or might not be lost after transposition of Tn*5*-BLOS; Berg, 1989). Results of Southern analysis have essentially ruled out transposition of Tn*5*-BLOS. However, the Fox$^-$ phenotype in the four secondary mutants could still possibly be the result of independent spontaneous mutations. Therefore, reintroduction of an isolated secondary mutation into the genome of *Anabaena* PS763 to observe whether the same phenotype recurs will be an essential step in the characterization of each of the four secondary mutations.

APPENDICES

APPENDIX A

GROWTH MEDIA FOR *Anabaena* SPP.

AA-based media used in this study were made by following a modification of the formula described by Allen and Arnon (1955). The composition and preparation of the basal medium are listed in Table A.1

Agar was purchase from Difco Laboratories (Detroet, MI) or Sigma Chemical Co. (St. Louis, MO). Solidified media were prepared by two different procedures: 1) by addition of agar, purified as described (Braun and Wood, 1962), to the final solution to a final concentration of 1% prior to autoclaving or, 2) by autoclaving separately, 2% agar (Difco) and a 2X concentrated final solution from which the P_i stock is omitted, and by then combining the agar, the double-strength solution and sterile P_i stock solution 1:1:0.0125 (v/v). I prefer agar plates made by the second procedure because they seem to be more consistent between different batches.

Nitrate, 5 mM KNO_3 plus 5 mM $NaNO_3$, was generally used as fixed nitrogen source where so indicated. A concentrated stock solution was added, 1:100 (v/v), before autoclaving. When ammonium was used to replace nitrate as fixed nitrogen source, 200 mM NH_4Cl solution (sterile filtered) was added to

autoclaved agar medium to a final concentration of 2 mM. Ten mM N-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES, pH 7.5) was added to such ammonium medium to maintain neutral pH.

The ingredients are diluted 8-fold (except for nitrate which, if present, is diluted only 2-fold) in all AA-based liquid media.

Table A.1. Composition and preparation of the modified basal AA medium for *Anabaena* spp.

Final solution (1,000 ml)	
ddH_2O	969.0 ml
AA-P stock solution	25.0 ml
P_i stock solution	6.25 ml

AA-P stock solution (1,000 ml)	
$MgSO_4$ stock solution	250 ml
$CaCl_2$ stock solution	250 ml
NaCl stock solution	250 ml
Microelement stock solution	250 ml

Microelement stock solution (1,000 ml)	
$MnCl_2 \cdot 4H_2O$	288.0 mg
MoO_3	28.8 mg
$ZnSO_4 \cdot 7H_2O$	35.2 mg
$CuSO_4 \cdot 5H_2O$	12.6 mg
H_3BO_3	457.6 mg
$CoCl_2 \cdot 6H_2O$	6.4 mg
Fe-EDTA stock solution	128.0 ml

Fe-EDTA stock solution (1,000 ml)	
KOH	9.12 g
$Na_2EDTA \cdot 2H_2O$	35.79 g
$FeSO_4 \cdot 7H_2O$ (or $FeCl_2$)	24.01 g (17.19 g)

Other simple stock solutions	
P_i stock solution (1,000 ml)	57.38 g of K_2HPO_4
$MgSO_4$ stock solution (1,000 ml)	40.98 g of $MgSO_4 \cdot 7H_2O$
$CaCl_2$ stock solution (1,000 ml)	12.18 g of $CaCl_2 \cdot 2H_2O$
NaCl stock solution (1,000 ml)	38.68 g of NaCl
Na/KNO_3 stock solution (1,000 ml)	42.51 g of $NaNO_3$ (0.5 mole) + 50.56 g of KNO_3 (0.5 mole)

APPENDIX B

BACTERIAL STRAINS DISCUSSED IN THIS STUDY

TABLE A.2. Bacterial strains discussed in this study

Strains	Marker [1]	Other relevant properties [2]	Note
Cyanobacteria			
Anabaena sp. strain PCC 7120 and its derivatives			
PCC 7120	—	Fox$^+$ Het$^+$; contains at least six different insertion sequences including IS*892* and IS*895*	3, 4
α41	Bm^r Nm^r Sm^r	*hetR* ::Tn*5*-1058; Hdi$^-$ Fix$^-$	5, 6
EF114	—	Fox$^-$, Hgl$^-$	7
EF116	—	*hetA*; Fox$^-$ Hep$^-$	7, 8
M8	Bm^r Nm^r Sm^r	Hen$^-$ Hep$^+$ Hgl$^+$; PCC 7120::Tn*5*-1063; defective in connection between heterocyst envenlope and the cell membrane	8
N10	Bm^r Nm^r Sm^r	Fox$^+$, Pat$^-$; PCC 7120::Tn*5*-1063; has much reduced heterocyst frequency	9
P9	Bm^r Nm^r Sm^r	Fix$^-$ Het$^+$; PCC 7120::Tn*5*-1037	10
PCC 7120(pRL250)	Nm^r	Suc^s	
PCC 7120(pRL1050)	Sm^r/Sp^r	P_{T7}-*luxAB* in pDUI-base plasmid	5
PCC 7120::pRL256	Sm^r/Sp^r	Suc^s Fox$^+$	
PCC 7120::pRL263	Em^r Sm^r/Sp^r	Suc^s Fox$^+$	

Table A.2 (*continued*)

Strains	Marker	Other relevant properties	Note
PCC 7120::pRL743	Em^r Sm^r/Sp^r	Suc^s Fox^+	
PCC 7120::pRL760	Sm^r/Sp^r	Suc^s	
PCC 7120::pRL763	Sm^r/Sp^r	Suc^s	
PS250-1	Nm^r	Suc^r variant of PCC 7120(pRL250), with a 1.5-kb insertion in *sacB*	
PS250-2	Nm^r	Suc^r variant of PCC 7120(pRL250); *sacB*::IS*893*	
PS250-3	Nm^r	Suc^r variant of PCC 7120(pRL250); *sacB*::IS*892*	
PS250-4	Nm^r	Suc^r variant of PCC 7120(pRL250); *sacB*::IS*894*	
PS250-5	Nm^r	Suc^r variant of PCC 7120(pRL250); *sacB*::IS*895*	
PS250-6	Nm^r	Suc^r variant of PCC 7120(pRL250); *sacB*::IS*892*	
PS250-7	Nm^r	Suc^r variant of PCC 7120(pRL250); *sacB*::IS*897*	
PS250-8	Nm^r	Suc^r variant of PCC 7120(pRL250); *sacB*::IS*898*	
PS250-9	Nm^r	Suc^r variant of PCC 7120(pRL250); *sacB*::IS*892*N	
PS250-10	Nm^r	Suc^r variant of PCC 7120(pRL250); *sacB*::IS*892*T	
PS250-11	Nm^r	Suc^r variant of PCC 7120(pRL250); *sacB*::IS*892*	
PS250-12	Nm^r	Suc^r variant of PCC 7120(pRL250), with a 1.5-kb insertion in *sacB*	
PS250-13	Nm^r	Suc^r variant of PCC 7120(pRL250), with a 1.2-kb insertion in *sacB*	
PS250-14	Nm^r	Suc^r variant of PCC 7120(pRL250)	
PS250-15	Nm^r	Suc^r variant of PCC 7120(pRL250); *sacB*::IS*892*	
PS250-16	Nm^r	Suc^r variant of PCC 7120(pRL250)	
PS250-17	Nm^r	Suc^r variant of PCC 7120(pRL250)	
PS250-18	Nm^r	Suc^r variant of PCC 7120(pRL250), with a 1.0-kb insertion in *sacB*	

Table A.2 (*continued*)

Strains	Marker	Other relevant properties
PS250-19	Nm^r	Suc^r variant of PCC 7120(pRL250)
PS250-20	Nm^r	Suc^r variant of PCC 7120(pRL250)
PS250-21	Nm^r	Suc^r variant of PCC 7120(pRL250); ▲*sacB*
PS250-22	Nm^r	Suc^r variant of PCC 7120(pRL250); ▲*sacB*
PS256-5	Sm^r/Sp^r	Suc^r variant of PCC 7120::pRL256; Fox^+ Hep^+
PS256-17	Sm^r/Sp^r	*hetA*::C.S4; Fox^- Hep^- Suc^r
PS263-1 (also -2,-50)	Sm^r/Sp^r	*nifD*::C.S4; Het^+ Nif^- Suc^r Em^s
PS263-42 (also -53,-54)	Em^r Sm^r/Sp^r	Suc^r variant of PCC 7120::pRL263; Fox^+
PS743	Sm^r/Sp^r	*conA*::C.S3; Fox^+; Suc^r
PS760	Sm^r/Sp^r	*tln2::luxAB* (BLOS1); Fox^+ Suc^r
PS760::pRL908	Em^r Nm^r Sm^r/Sp^r	Suc^s
PS763	Sm^r/Sp^r	*tln6::luxAB* (BLOS2); Fox^+ Suc^r
PS763::pRL908	Em^r Nm^r Sm^r/Sp^r	Suc^s
PS908L2	Nm^r Sm^r/Sp^r	*tln2::luxAB* plus *hetR* ::Tn*5*-1058; Hdi^- Suc^r
PS908L6	Nm^r Sm^r/Sp^r	*tln6::luxAB* plus *hetR* ::Tn*5*-1058; Hdi^- Suc^r
TAL2	Nm^r Sm^r/Sp^r	*tln2::rpoT7* plus pRL1050
TAL6	Nm^r Sm^r/Sp^r	*tln6::rpoT7* plus pRL1050
TLN2	Bm^r Nm^r Sm^r	*tln2::luxAB* (Tn*5*-1063); Fox^+
TLN6	Bm^r Nm^r Sm^r	*tln6::luxAB* (Tn*5*-1063); Fox^+
TTL62	Em^r Sm^r/Sp^r	PS763::Tn*5*-1087b; Fox^+
TTL68	Em^r Sm^r/Sp^r	PS763::Tn*5*-1087b; Fox^+
TTL615	Nm^r Sm^r/Sp^r	PS763::Tn*5*-1058; Fox^-
TTL616	Nm^r Sm^r/Sp^r	PS763::Tn*5*-1058; Fox^-
TTL619	Nm^r Sm^r/Sp^r	PS763::Tn*5*-764; Fox^-
TTL620	Nm^r Sm^r/Sp^r	PS763::Tn*5*-764; Fox^- (Het^- ?)
TTL631	Nm^r Sm^r/Sp^r	PS763::Tn*5*-764; Fox^+
TTL632	Nm^r Sm^r/Sp^r	PS763::Tn*5*-764; Fox^+
TTL633	Nm^r Sm^r/Sp^r	PS763::Tn*5*-764; Fox^+
TTL635	Nm^r Sm^r/Sp^r	PS763::Tn*5*-764; Fox^+

Table A.2 (*continued*)

Strains	Marker	Other relevant properties	Note
WJB216	—	hetR; Fix$^-$ Hdi$^-$	11
Y34	Cm^r Em^r	Fix$^-$; PCC 7120::Tn*5*-5-1087a	12
Y46	Cm^r Em^r	Fix$^-$; PCC 7120::Tn*5*-5-1087a	12
Anabaena sp. strain M-131		Het$^-$; contains IS*891* and homologs of IS*892* and IS*895*	4, 13, 14
Anabaena sp. strain PCC 7118		Fix$^+$, Hdi$^+$, Het$^-$; contains homologs of IS*892* and IS*895*	14, 15, 16
Anabaena variabilis ATCC 29413		Het$^+$; capable of facultitive heterotrophic growth; contains no IS*892* homolog	16, 17
FD		A mutantg that is a better strain for genetic transfer	17, 18
Anacystis nidulans		Also called *Synechocossus* sp.; Unicellular; capable of genetic transformation; donor of P*rbc* of C.S4	13, 19, 20
Calothrix sp. strain PCC 7601		Filamentous; contains IS*701*	21
Nostoc ellipsosporum		Filamentous; contains homologs of IS*892*	16
Nostoc sp. strain Mac		Filamentous; contains homologs of IS*892*	16
Nostoc sp. strain ATCC 29150		Filamentous; contains homologs of IS*892*	16
Synechococcus sp. strain PCC 7002		Unicellular; capable of genetic transformation; contains no IS*892* homolog	16, 22

Table A.2 (*continued*)

Strains	Marker	Other relevant properties	Note
Synechocystis sp. strain PCC 6803		Unicellular; capable of genetic transformation and light-activated hetrotrophic growth; contains no IS*892* homolog	16, 22, 23
Non-cyanobacteria			
Bacillus subtilis		Unicellular Gram-positive bacterium capable of endospore development; source of *sacB*	24, 25
Escherichia coli		A Gram-negative enteric bacterium	
DH5α	Nx^r	ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *endA1 gyrA96 recA1 hsdR17*(r_K^-, m_K^+)	26, 27
DH5α(pUM24)	Km^r Nx^r	Suc^s	
HB101	Sm^r	*mcrBC mrr hsdS20*(r_B^-, m_B^-) *recA rpsL20*	27, 28
HB101(pRL528)	Cm^r Sm^r	Transient host of conjugal plasmids	
Myxococcus xanthus		A Gram-negative soil bacterium capable of multicellular fruiting body formation	24

Note to Table A.2:

1. Resistance-conferring genes and usage of antibiotics are listed in Appendix F.

2. Nomenclature and list of phenotypes are presented in Appendix D, list of genes is in Appendix E. Name of a gene listed in this column indicates mutation in the gene (a genotype).
3. Wolk et al., 1984.
4. Cai, 1991.
5. C. P. Wolk, unpublished.
6. T. Black and C. P. Wolk, unpublished results.
7. Wolk et al., 1988.
8. A. L. Ernst and C. P. Wolk, unpublished results.
9. D. N. Tiwari and C. P. Wolk, unpublished data.
10. A. L. Ernst, J.-M. Panoff, and C. P. Wolk, unpublished results.
11. Buikema and Haselkorn, 1991a and 1991b.
12. A. L. Ernst, Y. Cai, and C. P. Wolk, unpublished results.
13. Bancroft and Wolk, 1989.
14. Alam et al., 1991.
15. Elhai and Wolk, 1990.
16. T. Thiel, personal communication.
17. Currier and Wolk, 1979.
18. Maldener et al., 1991.
19. Golden et al., 1989.
20. Shinozaki and Sugiura, 1985.
21. Mazel et al., 1988.
22. Porter, 1986.
23. Anderson and McIntosh, 1991.
24. Dworkin, 1985.
25. Gay et al., 1983.
26. Grant et al., 1990.
27. The *E. coli* strain DH5α was used in most of the transformation experiments using DNA isolated from *E. coli* in the couse of this study because this strain gives high frequency of transformation and mini-prep DNA of good quality, partially due to its *endA1* mutation which eliminates the production of endonuclease 1 (1991 BRL catalog, life technologies, Inc., Gaithersburg, MD). However, this strain retains all three of its methylation-dependent restriction systems (MDRS): McrA, McrBC, and Mrr (see *mcrA*, *mcrBC*, and *mrr* in Appendix E), and therefore exhibits strong restriction on DNAs methylated in a foreign pattern (Grant et al., 1990). In fact, in my experience transformation of competent DH5α with DNA isolated from *Anabaena* sp. strain PCC 7120 consistently gave very poor results, which led to my switch to strain HB101. The *E. coli* strain HB101

shows much less restriction on foreign methylated DNA, including DNA isolated from *Anabaena* sp. strain PCC 7120, because both of its McrBC and Mrr systems have been rendered nonfunctional (Raleigh et al., 1988).

28. Raleigh et al., 1988.

APPENDIX C

PLASMIDS USED IN THIS STUDY

Table A.3. Plasmids used in this study

Plasmids	Marker [1]	Other relevant features [2]	Note
pACYC184	Cm^r Tc^r	Source of *oriV* (p15A)	3
pAn625	Ap^r Tc^r	IS*895*C (*mysA*), *psbA*1	4, 5
pAT187	Km^r	Source of *oriT* (RK2)	6
pBR322	Ap^r Tc^r	Cloning vector, source of pMB1 *oriV* and *oriT*	7
pBR325	Ap^r Cm^r Tc^r	Source of Cm^r determinant	7
pDU1	—	Confers capacity to replicate in *Anabaena* spp.	8, 9
pE194	Em^r	Source of Em^r determinant	10
pET3	Ap^r	Doner of P_{T7} and T_{T7}	11
pIC20H	Ap^r	Cloning vector; *lacIZ'*	12
pJDC406	Ap^r	Source of bidirectional transcriptional terminator T_{lpp}	13
pJRD184	Ap^r Tc^r	Restriction site bank	14
pPM111*	Ap^r	Source of modified Sm^r gene (*str**)	15
pRL1	Cm^r	Shuttle vector	8
pRL44	Km^r/Nm^r Sm^r/Sp^r	S.K3+L.HEH2+C.S3	16
pRL52	Km^r/Nm^r	*hetA* in pRL25C	17, 18
pRL57	Cm^r Km^r/Nm^r and Sm^r/Sp^r	S.K5+L.HEH2+C.S3; positive selection shuttle cloning vector	2
pRL61	Km^r/Nm^r Sm^r/Sp^r	*hetA*::C.S4 derivative of PRL52	16

Table A.3 (*continued*)

Plasmids	Marker	Other relevant features	Note
pRL61M	Km^r/Nm^r Sm^r/Sp^r	*Mlu* I deletion of pRL61; a suicide plasmid	
pRL171PSm	Ap^r Sm^r/Sp^r	S.A1+L.HEH1+C.S4	16, 19
pRL250	Cm^r Km^r/Nm^r	pRL57-based shuttle vector, contains *sacB*	
pRL256	Sm^r/Sp^r	*hetA*::C.S4, *sacB*	
pRL263	Cm^r Em^r Sm^r/Sp^r	*nifD* ::C.S4, *sacB*	
pRL270	Cm^r Em^r	*sacB*; precursor of pRL271	
pRL271	Cm^r Em^r	*sacB*-containing cloning vector; C.CE2	
pRL272	Cm^r Km^r/Nm^r	*sacB*::IS*892*; a variant of pRL250 isolated from *Anabaena* PS250-3	
pRL274	Cm^r Km^r/Nm^r	*sacB*::IS*892*N; a variant of pRL250 isolated from *Anabaena* PS250-9	
pRL277	Sm^r/Sp^r	*sacB*-containing cloning vector; C.K3	
pRL278	Km^r/Nm^r	*sacB*-containing cloning vector; C.S4	
pRL278R	Km^r/Nm^r	A derivative of pRL278, *sacB*+C.K3 can be cut out by *Asu* II, *Bam*H I plus *Bgl* II, *Eco*R V, and *Xba* I,	
pRL351	Ap^r	*hetA* in pUC8	18, 20
pRL393	Km^r/Nm^r	*nifH* and part of *nifD* in pRL19B	2, 21
pRL453	Ap^r Sm^r	pUC18/19 containing L.EHE1 and C.S1	2
pRL488	Km^r/Nm^r	Donor of *luxAB* from *Vibrio fischeri*	22
pRL498	Km^r/Nm^r	S.K3+L.HEH1	2
pRL517b	Cm^r Sm^r/Sp^r	*nifH* and part of *nifD* from pAn154; C.S4 inserted in *Kpn* I of *nifD*	19, 23
pRL528	Cm^r	Helper plasmid for conjugal transfer; contains M.*Ava* I and M.*Eco*47 II methylases	24
pRL731	Bm^r Km^r/Nm^r Sm^r	*tln2*::Tn*5*-1063; *Cla* I-recovered fragment from mutant TLN2	25
pRL732	Bm^r Km^r/Nm^r Sm^r	*tln2*::Tn*5*-1063; *Eco*R V-recovered fragment from mutant TLN2	25
pRL733	Bm^r Km^r/Nm^r Sm^r	*tln6*::Tn*5*-1063; *Cla* I-recovered fragment from mutant TLN6	25

Table A.3 (*continued*)

Plasmids	Marker	Other relevant features	Note
pRL734	Bm^r Km^r/Nm^r Sm^r	*tln6*::Tn*5*-1063; *Eco*R V-recovered fragment from mutant TLN6	25
pRL739	Sm^r/Sp^r	Bears cassette BLOS1, unique polylinker; *luxAB*	
pRL739B	Sm^r/Sp^r	Bears cassette BLOS1, unique polylinker; *luxAB*	
pRL739S	Sm^r/Sp^r	Bears cassette BLOS1, unique polylinker; *luxAB*	
pRL741	Cm^r Km^r/Nm^r	A variant of pRL250 isolated from *Anabaena* PS250-1, with a 1.5-kb insertion in *sacB*	
pRL742	Cm^r Km^r/Nm^r	*sacB*::IS*893*; a variant of pRL250 isolated from *Anabaena* PS250-2	
pRL743	Cm^r Em^r Sm^r/Sp^r	*conA*::C.S3, contains *sacB*	
pRL744	Cm^r Km^r/Nm^r	*sacB*::IS*894*; a variant of pRL250 isolated from *Anabaena* PS250-4	
pRL745	Cm^r Km^r/Nm^r	*sacB*::IS*895*; a variant of pRL250 isolated from *Anabaena* PS250-5	
pRL746	Cm^r Km^r/Nm^r	*sacB*::IS*892*; a variant of pRL250 isolated from *Anabaena* PS250-6	
pRL747	Cm^r Km^r/Nm^r	*sacB*::IS*897*; a variant of pRL250 isolated from *Anabaena* PS250-7	
pRL748	Cm^r Km^r/Nm^r	*sacB*::IS*898*; a variant of pRL250 isolated from *Anabaena* PS250-8	
pRL750	Cm^r Km^r/Nm^r	*sacB*::IS*892*T; a variant of pRL250 isolated from *Anabaena* PS250-10	
pRL751	Cm^r Km^r/Nm^r	*sacB*::IS*892*; a variant of pRL250 isolated from *Anabaena* PS250-11	
pRL752	Cm^r Km^r/Nm^r	A variant of pRL250 isolated from *Anabaena* PS250-12, with a 1.5-kb insertion in *sacB*	
pRL753	Cm^r Km^r/Nm^r	A variant of pRL250 isolated from *Anabaena* PS250-13, with a 1.2-kb insertion in *sacB*	
pRL755	Cm^r Km^r/Nm^r	*sacB*::IS*892*; a variant of pRL250 isolated from *Anabaena* PS250-15	

Table A.3 (*continued*)

Plasmids	Marker	Other relevant features	Note
pRL758	Cm^r Km^r/Nm^r	A variant of pRL250 isolated from *Anabaena* PS250-18, with a 1.0-kb insertion in *sacB*	
pRL759	Sm^r/Sp^r	Bears cassette BLOS2, unique polylinker; *luxAB*	
pRL759B	Sm^r/Sp^r	Bears cassette BLOS2, unique polylinker; *luxAB*	
pRL759D	Sm^r/Sp^r	Bears cassette BLOS3 which lacks internal BamH I sites; polylinker same as in pRL759; *luxAB*	26
pRL760	Km^r/Nm^r Sm^r/Sp^r	*tln2*::BLOS1, *sacB*	
pRL762	Ap^r Sm^r/Sp^r	*tln6*::BLOS2	
pRL763	Km^r/Nm^r Sm^r/Sp^r	*tln6*::BLOS2, *sacB*	
pRL764	Km^r/Nm^r	Bears Tn*5*-764; P_{psbA}-*npt*	
pRL764SX	Km^r/Nm^r	Bears Tn*5*-764; P_{psbA}-*npt*; unique *Sma* I site	
pRL772	Km^r/Nm^r	*tln2::rpoT7*; C.K3	
pRL776	Km^r/Nm^r	*tln6::rpoT7*; C.K3	
pRL800	Cm^r Em^r	Bears Tn*5*-800; C.CE3	
pRL880	Bm^r Km^r/Nm^r Sm^r	*hetR* ::Tn*5*-1058; *Eco*R V-recovered fragment from mutant α41	25, 26
pRL908	Cm^r Em^r Km^r/Nm^r and Sm^r	*hetR* ::Tn*5*-1058, *sacB*	25, 26
pRL1022a	Km^r/Nm^r	*luxAB* (*Bam*H I)/L.HEH1	16
pRL1050	Sm^r/Sp^r	P_{T7}-*luxAB*, P_{rbc}-*aadA*; pDU1-based replicating plasmid	16
pRL1058	Bm^r Km^r/Nm^r Sm^r	Bears Tn*5*-1058; P_{psbA}-*npt-ble-str**	25, 27
pRL1063a	Bm^r Km^r/Nm^r Sm^r	Bears Tn*5*-1063; *luxAB*, P_{psbA}-*npt-ble-str**	23, 27
pRL1075	Cm^r Em^r	A derivative of pRL271, *sacB*+C.CE2 in an inverted polylinker	16
pRL1081	Km^r/Nm^r	A suicide plasmid containing promoterless *rpoT7*; C.K3	16
pRL1087b	Cm^r Em^r	Bears Tn*5*-1087b; C.CE2	16
pRZ1107	Gm^r	The two *dam* methylation sites in the promoter of Tn*5* are mutated	28

Table A.3 (*continued*)

Plasmids	Marker	Other relevant features	Note
pUC118	Ap^r	*lacIZ'*; A cloning vector that carries the intergenic region (IG) from phage M13 for production of single-stranded plasmid DNA.	29
pUC119	Ap^r	Same as pUC118 but the polylinker is in the opposite orientation	29
pUCD800	Km^r	Contains *sacB*	30
pUM24	Ap^r Km^r/Nm^r	Contains the *sacB-nptI* cartridge	31
πvx	—	pBR322-derived vector for screening of recombinants	32

Note to Table A.3:

1. See Appendix F for information on resistance-conferring genes and dosage of corresponding antibiotics.
2. See Elhai and Wolk (1988a) for nomenclature of polylinkers and antibiotic resistance cassettes.
3. Chang and Cohen, 1978, and Rose, 1988.
4. S. E. Curtis, personal communication
5. Alam et al., 1991.
6. Trieu-Cuot et al., 1987
7. Balbás et al., 1986, and references therein.
8. Wolk et al., 1984.
9. Schmetterer and Wolk, 1988.
10. Horinouchi and Weisblum, 1982.
11. Rosenberg et al., 1987
12. Marsh et al., 1984
13. Coleman et al., 1985.
14. Heusterspreute et al., 1985.
15. Mazodier et al., 1986.
16. C. P. Wolk, unpublished.
17. Wolk et al., 1988.
18. Holland and Wolk, 1990.
19. Composition of the Sm^r/Sp^r cassette C.S4 is described in Bancroft and Wolk (1989).

20. D. Holland and C. P. Wolk, unpublished.
21. G. Schmetterer, unpublished.
22. Elhai and Wolk, 1990
23. J. Elhai and C. P. Wolk, unpublished; for information on plasmid pAN154 and the *nifHD* genes, see Rice et al., 1982.
24. Elhai and Wolk, 1988b.
25. In *E. coli* the Sm^r gene in Tn*5*-1058 and Tn*5*-1063 confers only weak resistance to Sm, see Appendix F.
26. T. Black and C. P. Wolk, unpublished.
27. Wolk et al., 1991.
28. Yin et al., 1988.
29. Vieira and Messing, 1987
30. Gay et al., 1985
31. Ried and Collmer, 1987
32. Seed, 1983.

APPENDIX D

NOMENCLATURE AND LIST OF PHENOTYPES [1]

There are many different *Anabaena* mutants with phenotypes that are related to nitrogen fixation and heterocyst differentiation. Names of phenotypes used to describe such mutants have been inadequate and often unclear. A series of new names for different phenotypes is proposed and used in this presentation. Names and definitions of the phenotypes, and representative mutants are listed below. Possible inclusion/exclusion relationships of the phenotypes (except for Suc^{s}) are illustrated in Fig. A.1. I would like to point out the restrictions of this listing: 1) this listing, although somewhat extensive, is by no means intended to be encyclopedic. With the accumulation of our knowledge this listing will surely be expanded and improved; 2) only one form of differentiation, the differentiation of heterocysts, is considered because our knowledge on the other, the differentiation of akinetes, is very limited; 3) concerning nitrogen fixation, only that of the conventional (molybdenum-dependent) nitrogenase is considered because the alternative (vanadium-dependent) nitrogenase present in some *Anabaena* spp. (Kentemich et al., 1988;

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[1] The nomenclature incorporates information from personal communications with A. L. Ernst, C. P. Wolk, and T. Black.

Thiel, 1991), is poorly understood and is repressed in normal growth medium like AA; 4) phenotypes of antibiotic resistance are discussed separately (see Appendix F).

Fix$^-$ Unable to fix dinitrogen under anaerobic conditions (Ar/CO_2).

Sample strains: *Anabaena* Y34, and *Anabaena* Y46 (A. Ernst, Y. Cai, and C. P. Wolk, unpublished results); *Anabaena* P9 (A. Ernst, J.-M. Panoff, and C. P. Wolk, unpublished results)

Fox$^-$ Unable to fix dinitrogen in the presence of oxygen.

Sample strains: those that are Fix$^-$, Hdi$^-$, Hem$^-$, Hen$^-$, Hep$^-$, Het$^-$, Hgl$^-$, or Nif$^-$ (see above and below)

Hdi$^-$ Unable to initiate, or carry heterocyst differentiation to a stage in which *nifHDK* genes are expressed under anaerobic conditions (Ar/CO_2). This definition is based on the conclusion that expression of the *nifHDK* operon is developmentally regulated (Elhai and Wolk, 1990) in filamentous cyanobacteria such as *Anabaena* spp.

Sample strains: *Anabaena* α41 (T. Black and C. P. Wolk, unpublished results); *Anabaena* WJB216 (Buikema and Haselkorn, 1991a; the mutant 216 described in the article is presented as WJB216 here)

Hem$^-$ Heterocyst maturation mutant; unable to form morphologically mature heterocysts which have envelope deposition.

Sample strain: (a typical mutant will be one that forms only proheterocysts)

Hen$^-$ Defective in the heterocyst envelope.

Sample strain: *Anabaena* M17 (A. L. Ernst and C. P. Wolk, unpublished results)

Hep$^-$ Defective in the synthesis of heterocyst envelope polysaccharides.

Sample strain: *Anabaena* EF116 (Wolk et al., 1988); *Anabaena* PS256-17

Het$^-$ Unable to form proheterocysts discernible by bright-field microscopy.

Sample strain: *Anabaena* sp. strain PCC 7118 (Elhai and Wolk, 1990)

Hgl$^-$ Defective in the synthesis of heterocyst glycolipids.

Sample strain: *Anabaena* EF114 (Wolk et al., 1988)

Nif$^-$ A phenotype resulting from a mutation in the *nif* genes (this definition makes this phenotype more of a genotype because specific genetic mutation is required).

Sample strain: *Anabaena* PS263-1

Pat$^-$ Altered in the spatial pattern of heterocysts along the filament.

Sample strain: *Anabaena* N10 (D. N. Tiwari and C. P. Wolk, unpublished results)

Suc^S Unable to grow on agar medium containing 5% sucrose because of the presence of a functional *sacB* gene.

Sample strain: *Anabaena* sp. strain PCC7120(pRL250)

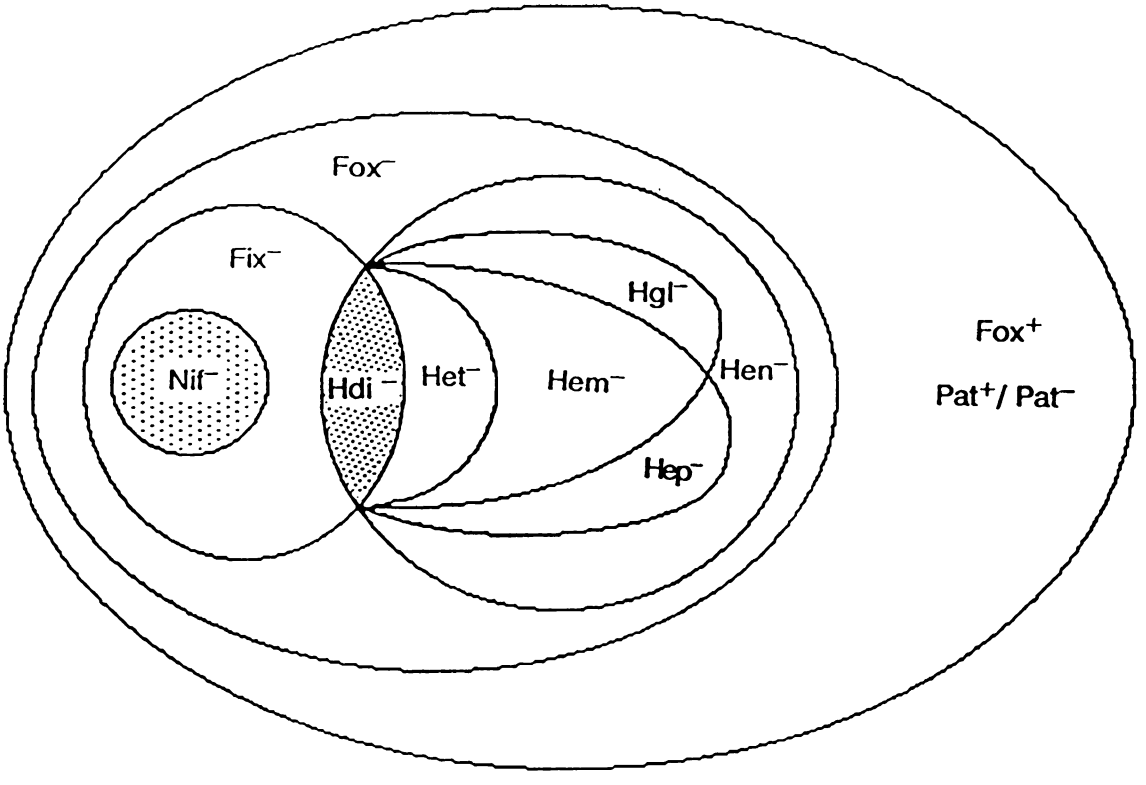

Figure A.1. Probable inclusion/exclusion relationships between mutants of different phenotypes.

APPENDIX E

LIST OF GENES DISCUSSED IN THIS STUDY

aadA This name appears to be used specifically for genes that encode an aminoglycoside 3"(9)-*O*-nucleotidyltransferase, abbreviated as **AAD(3")(9)**. Among the many characterized aminoglycoside nucleotidyltransferases (ANTs; often referred to as aminoglycoside adenylyltransferases because of their strong preference for ATP as substrate, and abbreviated as AADs), the AAD(3")(9) enzymes, which form a unique group, are able to inactivate streptomycin by adenylylation of the 3"-hydroxyl group on the amino-hexose III ring **and** to inactivate spectinomycin by adenylylation of the 9-hydroxyl group on the actinamine ring (Davies and Smith, 1978; Fling et al., 1985).

atp The 9 genes encoding the 9 polypeptides of the ATP synthase of *Anabaena* sp. strain PCC 7120 (Curtis, 1988)

bla Encodes ß-lactamase which detoxifies ß-lactam antibiotics (penicillins and ampicillin, etc.) by hydrolysis, and therefore opening, of the ß-lactam ring. The *bla* gene we used is originally from transposon Tn*3*

and encodes a 286-amino acid prepeptide. The first 23 amino acid residues form a hydrophobic leader peptide for secretion of the 263-amino acid mature, active ß-lactamase into the periplasmic space and sometimes out of the cells (Davies and Smith, 1978; Balbás et al., 1986)

ble Encodes a function conferring resistance to bleomycin. The resistance mechanism encoded by *ble* of Tn*5* is not known (Berg, 1989)

cat Encodes the chloramphenicol acetyltransferase (CAT) which inactivates chloramphenicol (Cm) by acetylation of its hydroxyl groups (Davies and Smith, 1978)

conA A gene 3' to *hetA* in the chromosome of *Anabaena* sp. strain PCC 7120, which could encode a protein of at least 280 amino acids. This gene is constitutively expressed during growth with NO_3^- and during heterocyst differentiation (partially characterized, and denoted ORF2 in Holland and Wolk, 1990; D. Holland, personal communication)

dam Encodes a methyltransferase that methylates at the N^6 position of the adenine in the sequence 5' GATC 3'. Such methylation, present both in *E. coli* and *Anabaena* spp., blocks digestion of DNA by many restriction endonucleases, e. g., *Xba* I, when their recognition sequences overlap the 5' GATC 3' sequence (Padhy et al., 1988; Nelson and McClelland, 1989)

endA1 Encodes endonuclease 1. A defect in this gene in *E. coli* strain DH5α is considered to be one of the reasons that this strain gives somewhat higher frequency of transformation and better quality of miniprep DNA

erm Genes conferring resistance to erythromycin (Em). From the literature it appears that this name is used specifically for genes that encode an rRNA N^6-amino adenine N-methyltransferase (NMT) that transfers one or two methyl group(s) to a particular adenine residue of the 23S rRNA, and thereby reduces its binding affinity for Em (Horinouchi and Weisblum, 1982). A different Em^r gene, *ereA*, encodes an erythromycin esterase that hydrolyzes the lactone ring of Em (Ounissi and Couvalin, 1985)

glnA Encodes glutamine synthetase (GS) which, with expenditure of ATP, combines ammonium and glutamate to form glutamine (Tumer et al., 1983)

gyrA Also referred to as *nalA*. This gene encodes one of the two subunits of the DNA gyrase, function of which is required for DNA synthesis to proceed. A particular mutation (*gyrA96*) in the *E. coli* strain DH5α renders the product of this gene resistant to the action of nalidixic acid (Lewin, 1985)

hetA A single-copy chromosomal gene in *Anabaena* sp. strain PCC 7120 that could code for a protein of 601 amimo acids. Mutation of this gene results in the formation of heterocysts with a defective polysaccharide layer (Wolk et al., 1988; Holland and Wolk, 1990; also see chapter 2)

hetR A single-copy gene in *Anabaena* sp. strain PCC 7120 that encodes a 299-amino acid protein. Mutaion of this gene gives a Hdi^- phenotype (Het^- Fix^-), and overexpression of this gene in the wild-type strain leads to formation of pairs and groups of heterocysts (Buikema and Haselkorn, 1991a and 1991b; T. Black and C. P. Wolk, unpublished results)

hsd Host-specificity determinant. Loci in *E. coli* that govern host-specified restriction function such as restriction by Type I or Type II endonucleases (Yuan, 1981)

lacZ Encodes the *E. coli* ß-galactosidase. The gene *lacZ* is widely used as a marker. Often a fragment of the *E. coli lac* regulatory region (*lacI*) is used along with an incomplete *lacZ* gene, *lacZ'*, which 1) encodes ca. 150 amino acids of the aminoterminal portion of the ß-galactosidase; 2) is able to complement (α complementation) a defective ß-galactosidase gene present usually in the chromosome to produce active ß-galactosidase; and 3) contains a region of multiple cloning sites (cloning of a fragment into this region destroys the α complementation). Derepression of the *lacI* region by the inducer isopropyl-thiogalactoside (IPTG) allows expression of *lacZ* (or *lacZ'*). Activity of functional ß-galactosidases turns the

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colorless chromogenic substrate 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (Xgal) blue (Maniatis et al., 1982)

luxAB Structural genes encoding for a bacterial luciferase (Meighen, 1991)

mcrA Encodes the type A modified cytosine restriction function (McrA), one of the three known methylation-dependent restriction systems (MDRS) of *E. coli*. McrA restricts DNA that contains the 5' $C^{m}CGG$ 3' methylated sequence (Raleigh and Wilson, 1986; Grant et al., 1990)

mcrBC Encode a different type of cytosine restriction function (McrBC), one of the MDRS of *E. coli*. McrBC restricts DNA that contains the 5' GmC 3' methylated sequence (Raleigh and Wilson, 1986)

mrr Encodes the function of methylated adenine recognition and restriction (Mrr), a third MDRS of *E. coli*. Mrr restricts DNA containing the 5' $G^{m}AC$ 3' and/or 5' $C^{m}AG$ 3' methylated sequences (Heitman and Model, 1987)

mys The name given to members of the IS*895* family before one of them was proven mobile (Alam and Curtis, 1985)

nif Nitrogen fixation genes. While many genes are involved in nitrogen fixation in one way or another, only genes that are homologs of defined *nif* genes of the extensively studied diazotroph, *Klebsiella pneumoniae*, are

denoted *nif* genes. To date, only six *nif* genes have been proposed in *Anabaena* sp. strain PCC 7120, and these are present in a *nif*-gene cluster. Unlike other diazotrophs, two insertions of unknown function are found in this *nif* gene cluster in vegetative cells: an 11-kb insertion (the *nifD* element) interrupting *nifD*, and a 55-kb insertion (the *nifS* element) 5' to *nifS*. In mature heterocysts both insertions are excised from the chromosome by site-specific recombinations to form a "cured", functional *nif*-gene cluster (Haselkorn, 1986; Haselkorn, 1989)

nifD Encodes the α subunit of dinitrogenase (MoFe protein) of the molybdenum-dependent (conventional) nitrogenase. In *Anabaena* sp. strain PCC 7120 this gene is interrupted by the 11-kb *nifD* element. After excision of the 11-kb element, the "cured" *nifD* gene codes for a protein of 497 amino acids (Lammers and Haselkorn, 1983; Golden et al., 1985)

nifH Encodes nitrogenase reductase (Fe protein). In *Anabaena* sp. strain PCC 7120 there is another *nifH* homolog (*nifH*$_2$ or *nifH**) of unknown function that is located distant from the *nif* gene cluster (see Fig. 4.5) (Mevarech et al., 1980; Rice et al., 1982)

nifK Encodes the ß subunit of dinitrogenase (MoFe protein) (Mazur and Chui, 1982)

npt Encodes a neomycin phosphotransferase which is an alias for some of the aminoglycoside phosphotransferases (APHs). Two *npt*

genes, *nptI* and *nptII*, were used in this study. The gene *nptI*, originally from transposon Tn*903* (also known as Tn*601*), encodes a type I aminoglycoside 3'-phosphotransferase, abriviated as APH(3'). The gene *nptII*, originally from transposon Tn*5*, encodes a type II APH(3'). Products (sharing significant homology at amino-acid level) of both genes (sharing moderate similarity at their 3' regions) inactivate deoxystreptamine aminoglycosides (e.g., neomycin and kanamycin) by phosphorylation of the 3'-hydroxyl group on the amino-hexose I ring (Davies and Smith, 1978; Beck et al., 1982)

oriV Origin of (vegetative) replication, sometimes called *oriR*. A site in a replicon at which DNA repliction is initiated and, often after completing the replication, terminated

oriT Origin of transfer (also called the *Bom* site for basis of mobilization). A site in a mobilizable replicon at which product(s) of *tra* gene(s) nick one strand of the DNA, and initiate conjugal transfer

prcA A single-copy gene in the chromosome of *Anabaena* sp. strain PCC 7120 that encodes a calcium-dependent protease (Maldener et al., 1991)

psbA Encodes the D1 protein, one of the five (or more) polypeptides comprising the core complex of the reaction center of photosystem II. More than one copy of the gene may be present in the genome

rbc The two genes encoding the large (*rbcL*) and small (*rbcS*) subunits of ribulose-1,5-bisphosphate carboxylase. In *Anabaena* sp. strain PCC 7120 and Anacystis nidulans, *rbcLS* are cotranscribed in a polycistronic transcript (Curtis and Haselkorn, 1983; Nierzwicki-Bauer et al., 1984; Shinozaki and Sugiura, 1985)

recA Encodes a function essential to DNA homologous recombination. The RecA protein is a dual-function protein that is 1) a specific DNA-dependent protease that cleaves certain repressors, and 2) a DNA-dependent ATPase that promotes homologous pairing of DNA molecules (Radding, 1985)

rpoT7 Gene ϕ1, encoding RNA polymerase, of coliphage T7

rpsL Encoding a ribosomal protein. In *E. coli* strain HB101, a particular mutation (*rpsL20*) of this gene greatly reduces the affinity of the product of the gene to binding by Sm, and thereby confers resistance to Sm

sacB Encodes the secretory levansucrase (sucrose:2,6-ß-D-fructan 6-ß-D-fructosyltransferase; E.C. 2.4.1.10) of the unicellular Gram-positive bacterium, *Bacillus subtilis*. When bearing a functional *sacB*, many Gram-negative bacteria, including *Anabaena* spp., are killed on solid media containing 5% sucrose which induces the expression of *sacB* (Gay et al., 1983 and 1985; chapter 2)

sigA Encodes the housekeeping σ factor (cofactor of an mRNA polymerase) (Brahamsha and Haselkorn, 1991)

sigB A *sig* homolog proposed on the basis of hybridization with *sigA*. Insertional mutation of this gene, which is transcribed only under nitrogen-fixing conditions, does not cause an observable change of phenotype (Brahamsha, 1991; B. Brahamsha, personal communication)

str Genes that confer resistance to streptomycin (Sm). Several such genes, however, are named *aad* because the function of their products are known to be aminoglycoside nucleotidyltransferases (see *aad*, above). The *str* gene in transposon Tn*5* encodes an aminoglycoside phosphotransferase (APH), possibly an aminoglycoside 6-*O*-phosphotransferase, abriviated as APH(6). APH(6) inactivates streptomycin by phosphorylation of the 6-hydroxyl group on the streptidine ring. In the mycelial Gram-positive bacterium, *Streptomyces griseus*, the gene encoding an APH(6) enzyme is named *aphD*, and the one encoding an aminoglycoside 3"-*O*-phosphotransferase (APH3") is named *aphE*. A mutated *str* gene of Tn*5*, *str**, from plasmid pPM111* , confers substantially greater resistance to Sm than the wild-type gene (Davies and Smith, 1978; Mazodier et al., 1985; Mazodier et al., 1986; Heinzel et al., 1988)

tet A Tc^r determinant. This gene encodes a protein that modifies the cell membrane and thereby blocks the transport of tetracycline into the cell (Maniatis et al., 1982)

tln2 A single-copy gene in the chromosome of *Anabaena* sp. strain PCC 7120, expression of which increases rapidly following removal of nitrate (as a source of fixed nitrogen) from the growth medium (see chapter 4)

tln6 A single-copy gene in the chromosome of *Anabaena* sp. strain PCC 7120, expression of which increases within 4 hour following removal of nitrate or ammonium (as a source of fixed nitrogen) from the growth medium (see chapter 4)

tra Genes that determine conjugation. These genes can be divided into two groups: those whose products are involved in the formation of mating pairs, and those involved in DNA transfer

xisA Present at the *nifK*-proximal end of the 11-kb *nifD* element, this gene encodes a protein of 354 amino acids that is required for excision of the *nifD* element from the *nifD* gene via site-specific recombination (Lammers et al., 1986; Golden and Wiest, 1988)

APPENDIX F

RESISTANCE-CONFERRING GENES AND DOSAGE OF CORRESPONDING ANTIBIOTICS

A number of genes that confer resistance to specific antibiotics were used in this study. The dosages used for selection of functions of those genes are listed in Table A.4.

Table A.4. Resistance-conferring genes and dosage of corresponding antibiotics.

Cassette [1]	Gene [2]	Antibotics used	Concentration used (μg/ml) [3] AA + NO_3^-	AA/8 + NO_3^-	LB [4]	Note
C.A1	*bla*	Ap	--	--	50	5
(Tn*5*-1063)	P_{psbA}-*ble*	Bm	1-4			6
C.CE1	*cat, erm*	Cm	--	--	25	7
		Em	10-40	5	(1,000)	8
C.CE2	*cat, erm*	Cm	15		25	9
		Em	10-40	5	(1,000)	8
C.CE3	*cat, erm*	Same as C.CE2				
C.K1	*nptII*	Km	--	--	50	10
		Nm	25	3		
C.K2	*nptI*	Same as C.K1				10
C.K3 and	P_{psbA}-*nptII*	Km	--	--	50	10
(Tn*5*-1063)		Nm	200-400	25-45		11
C.S3 (Ω)	*aadA*	Sm	2.5	1.5	25	12
		Sm/Sp	2.5/2.5	1.5/1.5 or 1.0/1.0	25	
		Sp	10	1.0	100	
C.S4	P_{rbc}-*aadA*	Same as C.S3				
(Tn*5*-1063)	P_{psbA}-*str**	Sm	5-10	2-5	(25)	13
C.T1	*tet*	Tc	--	--	10	14

Notes to Table A.4:

1. For nonmenclature and construction of most of the drug-resistance cassettes see Elhai and Wolk (1988a). Construction of cassettes C.CE2 and C.CE3 is described in Chapter 2 and 4, respectively. Cassette C.S4 was described by Bancroft and Wolk (1989).

2. For a more detailed description of these genes please see Appendix E. For a comprehensive review concerning antibiotics please see Davies and Smith (1978) and references therein.

3. All antibiotics used in this study were purchased from Sigma Chemical Co., St. Louis, MO. The concentration of an antibiotics presented here is specific to the chemical form of the antibiotics (listed below).
   - Am: ampicillin, sodium salt ($C_{16}H_{18}N_3O_4S{\cdot}Na$, FW 371.4)
   - Bm: bleomycin sulfate (mixture, chemical content not clear; Sigma catalog number B-5507)
   - Cm: chloramphenicol, crystalline ($C_{11}H_{12}Cl_2N_2O_5$, FW 323.1)
   - Em: erythromycin ($C_{37}H_{67}NO_{13}$, FW 733.9)
   - Km: kanamycin monosulfate (95% kanamycin A plus 5% kanamycin B; $C_{18}H_{36}N_4O_{11}{\cdot}H_2SO_4$, FW 582.6)
   - Nm: neomycin sulfate (90-95% neomycin B plus 10-5% neomycin C; $C_{23}H_{46}N_6O_{13}{\cdot}3H_2SO_4$, FW 908.9)
   - Sm: streptomycin, sulfate salt (FW 1457.4, which suggests a formula of $C_{21}H_{39}N_7O_{12}{\cdot}9H_2SO_4$ [Bérdy, 1980])
   - Sp: spectinomycin dihydrochloride ($C_{14}H_{24}N_2O_7{\cdot}2HCl$, FW 405.3)
   - Tc: tetracycline hydrochloride ($C_{22}H_{24}N_2O_8{\cdot}HCl$, FW 480.9)

4. AA-based media containing nitrate are most often used for culture of *Anabaena* spp. When media free of fixed nitrogen are used, or resistances to two or more antibiotics are simultaneously selected, the concentration of antibiotics used is usually reduced 50 to 80%, depending on the particular antibiotic(s) and the combination used. Concentrations of antibiotics used in both liquid and solidified LB media for culture of *Escherichia coli* are presented.

5. *Anabaena* sp. strain PCC 7120 produces ß-lactamase that degrades ampicillin as well as penicillin (Kushner and Breuil, 1977), but apparently not at a high level. However, secretion of ß-lactamase at higher pH envirnoment (Kushner and Breuil, 1977; also see *bla* in Appendix E) by a donor strain could protect a recipient strain. Ap therefore may not be suitable for use for selection in conjugation experiments with *Anabaena* sp. strain PCC 7120 (Wolk et al., 1984).

6. Bm was very effective for selection of *Anabaena* sp. Nonetheless, because it is very expensive, it was used only occasionally. For the same reason it was rarely used with *E. coli* (C. P. Wolk, personal communication).

7. The natural promoter of the cat gene in cassette C.CE1 was removed during construction of the cassette (Elhai and Wolk, 1988a). This cassette confers very weak resistance to Cm in *Anabaena* sp. strain PCC 7120, but

is useful in *Anabaena* sp. strain M-131 (C. P. Wolk, personal communication).

8. Em is used very infrequently with cells of *E. coli* because about 1 mg/ml is required for selection, and that is an inconveniently high level to use.

9. Cassettes C.CE2 and C.CE3, unlike C.CE1, retain the natural promoter of the *cat* gene, and confer greater resistance to Cm. Cells of *Anabaena* sp. strain PCC 7120 bearing either of these two cassettes can be selected with 15 μg/ml and 10 μg/ml of Cm on AA+NO_3^- and AA agar media, respectively.

10. The *nptI* and *nptII* genes confer resistance to both Km and Nm. Nm and Km were used in *Anabaena* sp. and *E. coli*, respectively, to select for resistance conferred by the *npt* genes (Wolk et al., 1984). Km is not used in cells of *Anabaena* sp. strain PCC 7120 because those cells show a high level of natural resistance to Km. Nm is not used in *E. coli* for a similar reason.

11. Cells of *Anabaena* sp. bearing the P_{psbA}-*nptII* construct show strong resistance to Nm and are usually selected with 200 to 400 μg/ml of Nm. However, such a high level of Nm is not suitable for use in experiments, e.g., the N-stepdown induction assay, in which a minimal content of fixed nitrogen is important, because the degradation products of neomycin appear to be used by cells of *Anabaena* sp. as fixed nitrogen. The neomycin sulfate used has a nitrogen (N) content of 9.24%, which translates into 2.64 mM and 0.132 mM N for 400 μg/ml and 20 μg/ml of neomycin sulfate, respectively. All of the nitrogen in neomycin is in the form of amino groups (Bérdy, 1980), which might be readily used by cells of *Anabaena* sp. Streptomycin is ideal for use in the N-stepdown induction assay because 1) the streptomycin sulfate salt that we use has a nitrogen content of 6.72%, which translates into 0.048 mM N for 10 μg/ml Sm, and 2) less than 43% of the nitrogen in streptomycin is in the form of amino groups (Bérdy, 1980).

12. The *aadA* gene in cassette C.S3, and P_{rbc}-*aadA* in C.S4, confer resistance to both Sm and Sp. Selection with both antibiotics (Golden and Wiest, 1988) gives lower background than with either alone. Cells selected on Sp alone tend to be healthier than those selected on Sm alone.

13. P_{psbA}-*str** (Mazodier et al., 1986) confers excellent resistance to Sm in *Anabaena* sp. but only very weak resistance to Sm in *E. coli.*

14. Tetracycline is light-sensitive and its action is antagonized by magnesium ions (Maniatis et al., 1982). It was therefore not used for selection of *Anabaena* sp. (Wolk et al., 1984) but was used for selection of *E. coli* in LB medium which contains virtually no magnesium. Preferably, tetracycline-containing media should be prepared fresh.

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