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# Insertional Inactivation of Genes To Isolate Mutants of *Synechococcus* sp. Strain PCC 7942: Isolation of Filamentous Strains†

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**We have developed a simple procedure for generating mutants of the cyanobacterium *Synechococcus* sp. strain PCC 7942 in which the site of the lesion can be readily identified. This procedure involves transforming *Synechococcus* sp. strain PCC 7942 with a library of its own DNA that was fully digested with *Sau*3A and ligated into the plasmid vector pUC8. The homologous integration of the recombinant plasmid into the genome will often result in the disruption of a gene and the loss of gene function. We have used this method to generate many mutants of *Synechococcus* sp. strain PCC 7942 which grow as multicellular filaments rather than as unicells. Since the gene harboring the lesion was tagged with pUC8, it was easily isolated. In this paper, we discuss the usefulness of this procedure for the generation of mutants, and we characterize one mutant in which the lesion may be in an operon involved in the assembly of lipopolysaccharides.**

Cyanobacteria are photosynthetic prokaryotes that grow in a wide range of environments. These organisms have been used for the study of nitrogen fixation (8), photosynthesis (40), and other physiological processes (37). A number of different cyanobacteria have been used for the generation of mutant strains (5, 10, 13, 19, 38, 39, 41). Most frequently, such mutants have been generated by UV illumination or the administration of chemical mutagens. In a few studies, transposon mutagenesis has been employed (38). Characterization of these mutants has been important for elucidating photosynthesis and light harvesting processes (6, 14, 18, 21, 28, 30, 36), nitrogen fixation (7, 8, 15, 23), and the acclimation of cyanobacteria to different nutrient conditions (22, 24, 25, 29). In some instances, the mutant phenotypes have been complemented and the complementing DNA has been characterized (7, 11, 23).

Targeted mutations have also been generated in various cyanobacteria and have been used to elucidate the function of specific gene products. In this procedure, specific cyanobacterial genes present on a plasmid are interrupted with genes encoding drug resistance markers. The interrupted gene is then introduced into the cyanobacterium by transformation or conjugation, and there it is exchanged, via a double homologous recombination event, for the normal gene on the cyanobacterial genome. This strategy has helped establish the functions of specific polypeptides involved in photosynthetic electron transport (26) and the acquisition and assimilation of $CO_2$ (24), sulfate (25), phosphate (32), and nitrate (29). DNA introduced into cyanobacteria can also integrate into the genome by a single homologous recombination event. In some instances, when only part of a gene is present on a recombinant plasmid that is introduced into the cyanobacterium, a single recombination event will result in the generation of two genomic copies of the specific gene, both of which are truncated.

In this paper, we describe a general strategy in which a library of small fragments of *Synechococcus* sp. strain PCC 7942 genomic DNA, present in the plasmid vector pUC8, is transformed into the cyanobacterium for the purpose of generating mutants. The consequence of this procedure is the insertion of plasmid DNA into genomic sequences. Since the plasmid contains a gene encoding the ampicillin resistance function, it is relatively easy to rescue the integrated plasmid, along with some flanking genomic DNA, by digesting the genomic DNA with a restriction endonuclease that does not digest vector sequences (or only cuts once in the polylinker), circularizing the fragments that are generated and transforming *Escherichia coli* to ampicillin resistance with the population of circularized fragments.

To determine the feasibility of using the small fragment plasmid library for generating mutants and quickly isolating aberrant genes, we employed this procedure for creating filamentous mutants. Numerous filamentous strains were isolated and partially characterized. It was demonstrated that integration of the plasmid into the cyanobacterial genome was the primary cause of the mutant phenotype and that this system is indeed of general utility.

## MATERIALS AND METHODS

**Generation of recombinant library.** *Synechococcus* sp. strain PCC 7942 DNA was isolated according to the method of Laudenbach and Grossman (25) and digested to completion with *Sau*3A. Most of the fragments generated were in the size range of 0.2 to 1.0 kbp. These fragments were ligated into the plasmid vector pUC8, which had been digested with *Bam*HI and treated with calf intestine alkaline phosphatase (33). Aliquots of the ligated DNA were used to transform *E. coli* DH5α, and the cells were grown overnight in Luria broth supplemented with 100 μg of ampicillin per ml prior to the isolation of plasmid DNA (33). The number of clones containing unique inserts was estimated to be 50,000 to 60,000.

**Transformation of *Synechococcus* sp. strain PCC 7942.** *Synechococcus* sp. strain PCC 7942 was grown exponentially in BG-11 medium (1). The cells were harvested at 5,000 × *g* for 5 min at room temperature and resuspended in a 1/10 volume of fresh BG-11 medium. Plasmid DNA containing the library of *Sau*3A genomic fragments from *Synechococcus* sp. strain

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PCC 7942 was placed into the cyanobacterial suspension at a concentration of 5 μg/ml. Approximately 5 ml of the cell suspension was placed into 50-ml Falcon tubes, gently shaken in the dark for between 4 and 6 h, and then placed in the light for 12 h. Following this incubation, the cells were spread onto solid BG-11 medium (0.5 ml of cells per petri dish [100 by 15 mm]) containing 2 μg of ampicillin per ml.

**Mutant selection and microscopy.** Transformed strains resistant to ampicillin were visually screened for filamentous growth on solid medium. The mutants exhibiting the filamentous phenotype were photographed at a ×20 magnification under a Bausch and Lomb Stereozoom 7 microscope. Mutant strains grown in liquid medium were placed onto glass microscope slides and photographed at a magnification of ×250 with an Axioplan (Zeiss) fluorescence microscope and Kodak color film, ASA 400.

**Rescuing the plasmid from the genome and isolation of the corresponding sequences from a genomic library.** Genomic DNA from the mutant strains was isolated, digested with various restriction enzymes, and hybridized to pUC8 to demonstrate the presence of plasmid DNA in the cyanobacterial genome. Once plasmid integration was established, the genomic DNA of the mutants was digested with *Pst*I and/or *Bam*HI, and the resulting fragments were diluted to a concentration of approximately 0.01 μg/μl, prior to ligation with T4 DNA ligase. The ligated material was transformed into *E. coli* DH5α, and transformants were selected on 100 μg of ampicillin per ml. DNA was isolated from the transformants with the

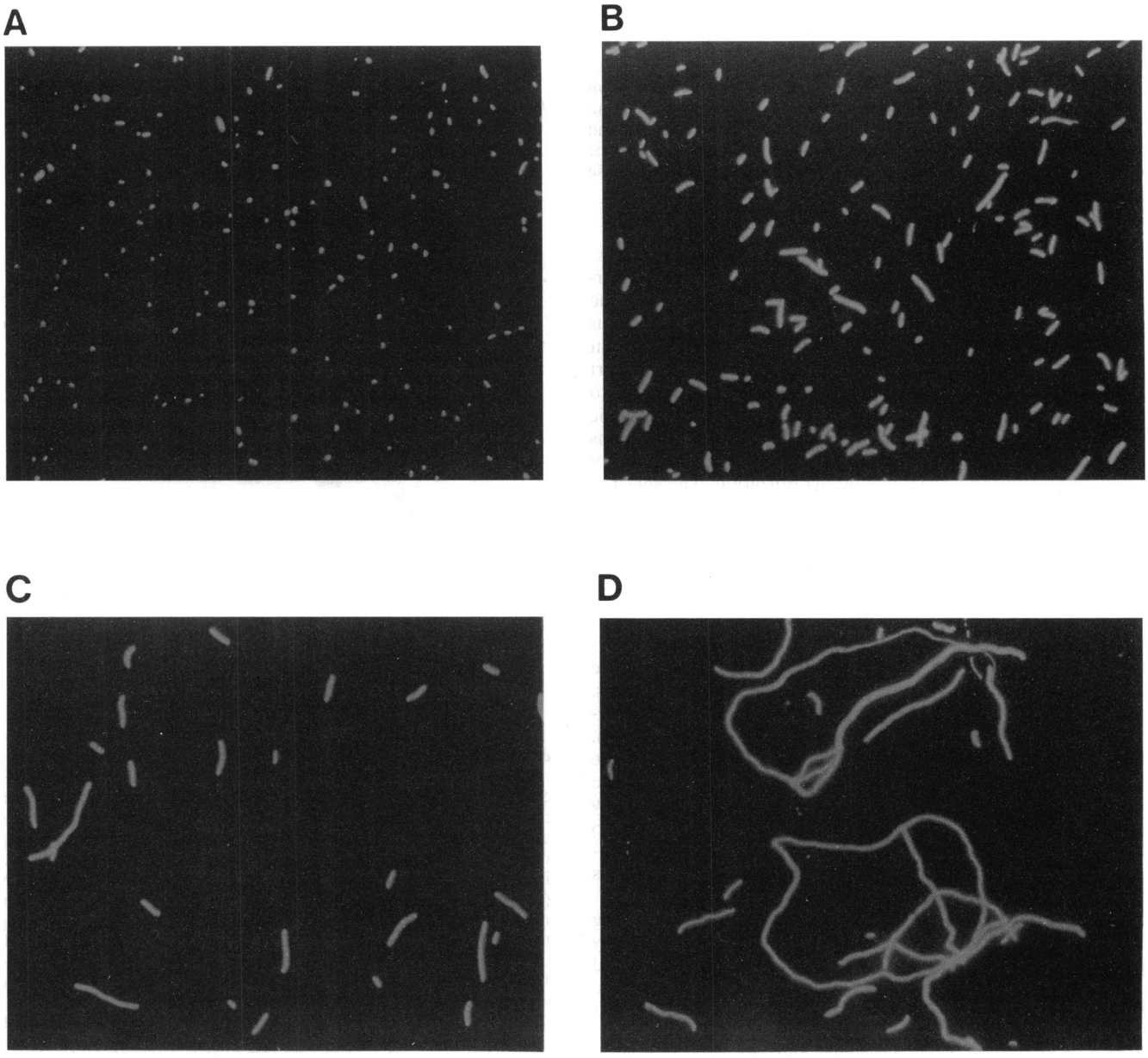



FIG. 1. Fluorescence micrographs of *Synechococcus* sp. strain PCC 7942 filamentous mutants. Cells were grown to mid-logarithmic phase at 30°C in BG-11 medium containing 1 μg of ampicillin per ml. The cells were washed in fresh BG-11 medium prior to examination under the Axioplan (Zeiss) fluorescence microscope. (A) Wild-type cells; (B) *flm3* strain; (C) *flm4* strain; (D) *flm11* strain. Magnification, ×250.

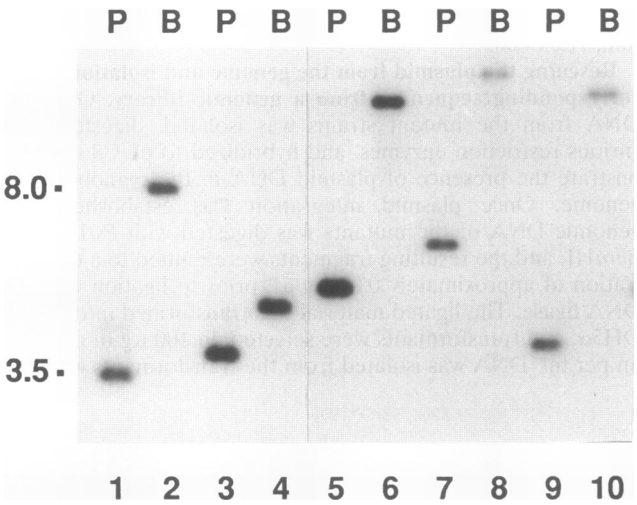



FIG. 2. Hybridization of pUC8 plasmid DNA to restriction digests of *Synechococcus* sp. strain PCC 7942 genomic DNA from the *flm* mutants. Genomic DNA was isolated from strains *flm14* (lanes 1 and 2), *flm11* (lanes 3 and 4), *flm9* (lanes 5 and 6), *flm4* (lanes 7 and 8), and *flm3* (lanes 9 and 10). The DNA was digested with either *Pst*I (P) or *Bam*HI (B). The positions of marker DNA fragments, in kilobase pairs, are given to the left of the autoradiogram.

Qiagen Plasmid Purification Kit according to the manufacturer's directions (Qiagen Inc., Chatsworth, Calif.). Once the plasmid was isolated, the insert from the plasmid DNA was mapped for restriction endonuclease sites. For the mutant *flm3*, a 1.2-kbp *Pst*I-*Eco*RI fragment was present in the insert DNA from the rescued plasmid. This fragment was used to isolate corresponding sequences from a λEMBL3 genomic library of *Synechococcus* sp. strain PCC 7942 (25) that contains genomic fragments of an average size of 15 kbp. Screening of the genomic library was by plaque hybridization. The selected λ clones were mapped relative to the position at which the pUC8 integrated into the genomic DNA in the original filamentous strain.

**DNA sequence determination and analysis.** DNA sequencing was by the dideoxy chain termination method (34) with single-stranded templates of Bluescript M13 phage vector. Both strands of the DNA were sequenced with subclones and oligonucleotide primers. Reactions were primed with phage M13 universal or reverse primers or synthetic oligonucleotides, and chain extension was initiated with Sequenase II polymerase (U.S. Biochemical Corporation, Cleveland, Ohio). Primer extension analyses were performed according to the method of Sambrook et al. (33). Analyses of the DNA and the deduced amino acid sequences of the polypeptides encoded by the DNA were performed with the Genetics Computer Group Incorporated software package (Madison, Wis.). The GenBank and EMBO data bases were searched for homologous DNA and protein sequences.

**Insertional inactivation.** The λEMBL3 clone containing *Synechococcus* sp. strain PCC 7942 DNA that was isolated by its homology to the 1.2-kbp *Pst*I-*Eco*RI fragment that flanked the site of insertion of the pUC8 DNA in the genome of the *flm3* mutant was used as a source of DNA for all subsequent constructions.

The plasmid pHP45Ω (31) was digested with *Sma*I to release the spectinomycin resistance gene (*aadA*). The DNA fragment containing the gene was resolved on a 1% agarose gel and recovered from the gel with the Qiaex gel extraction kit (Qiagen Inc.). The *aadA* gene was inserted directly either into a restriction site or into a site generated by deletion of a segment of the genomic DNA. Insertions were in the following locations (see the restriction map of Fig. 3): (i) into the *Sac*II site of the 1.2-kbp *Pst*I-*Xho*I fragment that had been cloned into pUC9 (the new plasmid was designated p49Ω), (ii) into the *Xho*I site of a 1.3-kbp *Kpn*I-*Sac*II fragment cloned into pUC19 (p47Ω), (iii) into the *Xho*I site of a 0.7-kbp *Kpn*I-*Hin*dIII fragment cloned into pUC19 (p42Ω), and (iv) as replacement of a 1-kbp region between two *Xho*I sites in the 1.9-kbp *Sac*II-*Hin*dIII fragment cloned into pUC19 (p17Ω). The *Xho*I site was lost in any construct that used an *Xho*I site for the insertion of the *aadA* gene. The ligation mixtures were transformed into DH5α and selected for growth on spectinomycin and ampicillin. The plasmids harbored by the transformed *E. coli* cells were isolated with the Qiagen plasmid purification kit and used to transform *Synechococcus* sp. strain PCC 7942 to spectinomycin resistance. Cyanobacterial trans-

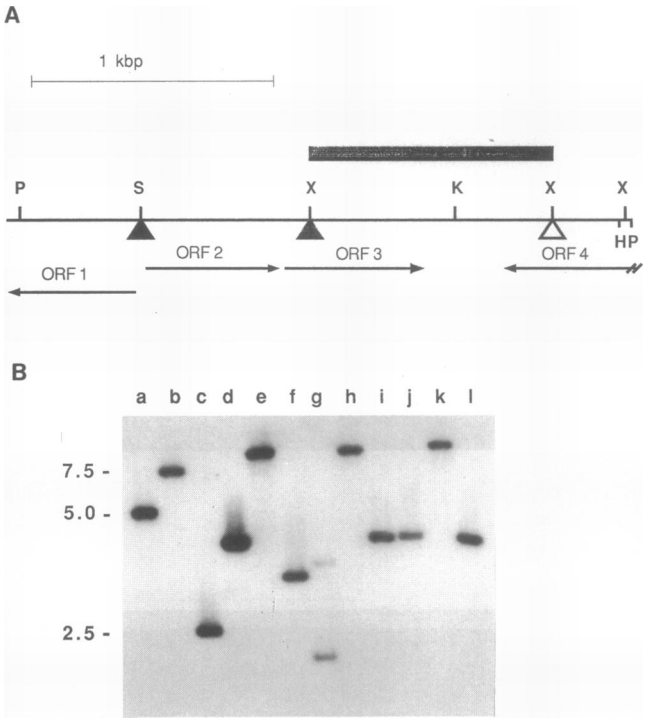



FIG. 3. (A) Restriction endonuclease map of the region of *Synechococcus* sp. strain PCC 7942 genomic DNA altered in *flm3*. The restriction sites are marked as follows: H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sac*II; X, *Xho*I. The positions of the ORFs and the direction in which they are transcribed are indicated, with arrows, below the restriction endonuclease map. The triangles positioned along the restriction map mark the sites into which the spectinomycin resistance gene was introduced: ▲-S (strain flm3p49Ω), ▲-X (strain flm3p47Ω), and △-X (strain flm3p42Ω). The large darkened rectangle above the restriction map delimits the region of a piece of DNA that was removed during the construction of the deletion strain flm3p17Ω. (B) Southern hybridizations of the 1.2-kbp *Xho*I-*Pst*I fragment (see panel A) to genomic DNA of flm3p49Ω, flm3p47Ω, and flm3p17Ω. Genomic DNA from the wild type (lanes a, b, and c), flm3p17Ω (lanes d, e, and f), flm3p49Ω (lanes g, h, and i), and flm3p47Ω (lanes j, k, and l) was digested with *Hin*dIII (lanes a, d, g, and j), *Xho*I (lanes b, e, h, and k), or *Pst*I (lanes c, f, i, and l). Sizes of the wild-type fragments in kilobase pairs are given to the left of the autoradiogram. The △-X strain flm3p42Ω was also shown to have the appropriate insertion, but the data are not presented in this figure.

formants were selected for growth on 25 μg of spectinomycin per ml and screened for sensitivity to 2 μg of ampicillin per ml. The presence and the position of the *aadA* gene were confirmed by Southern hybridizations.

**Primer extension assay.** Total RNA was isolated (20) and used for primer extensions as described by Sambrook et al. (33). The oligonucleotide 5′-GGCCAAAGGT GATTGGA TCG AAACTGCC AGGATAGATG GCG-3′, which annealed at nucleotide positions 9 to 49 of ORF2, was used as a primer.

**Nucleotide sequence accession number.** The GenBank accession number for the sequence described here is L19521.

## RESULTS

We have employed a method to mutagenize cells that generates mutant strains via insertional inactivation of genes. This method is based on the generation of a recombinant library of *Sau*3A fragments of *Synechococcus* sp. strain PCC 7942 genomic DNA in a plasmid vector and integration of the recombinant vectors into the cyanobacterial genome by homologous recombination. The integrated plasmid, plus flanking DNA, is easily isolated from the genome of the mutant strain, making this a powerful method for correlating phenotype with the loss of function of a specific gene. In our initial studies to demonstrate that this was a viable method for the rapid generation of mutants and the isolation of the mutated sequences, we mutagenized by transformation and screened for transformants that were altered either in morphology or in pigmentation. While both phenotypes were obtained, we pursued studies on those strains that exhibited multicellular, filamentous growth.

Wild-type *Synechococcus* sp. strain PCC 7942 is a unicellular cyanobacterium, as shown in fluorescence micrographs in Fig. 1A. Upon mutagenesis of the cyanobacterium by transformation, several strains were identified that formed short chains of cells, both on plates and in liquid; the septation between the cells of the filaments can be visualized by light microscopy (data not shown). Representative mutants grown in liquid medium are shown in Fig. 1B to D. The strain that exhibited the most pronounced filamentation was *flm11* (D). Other strains such as *flm3* (B) and *flm4* (C) exhibited intermediate levels of filamentation. A number of the mutants that grew as intermediate or short filaments in liquid medium appeared as long filaments when grown on solid medium (compare *flm3* in Fig. 1 and Fig. 4).

To demonstrate that the pUC8 vector had integrated into the genome of the mutated strains, genomic DNA from each of the strains was isolated, digested with *Pst*I and *Bam*HI, and probed with radiolabelled pUC8. As shown in the Southern analyses of Fig. 2, pUC8 was integrated into the genome of representative filamentous mutants. In the genomic DNA of the mutant strains that are presented in Fig. 2, the fragments that hybridized to the vector were all unique. However, Southern hybridizations of genomic DNA isolated from several independently isolated mutants often revealed hybridizing bands of identical size (for both the *Pst*I and the *Bam*HI digestions). For example, a number of filamentous strains exhibited hybridizing *Pst*I and *Bam*HI fragments that were identical in size to those of *flm3*. We chose to characterize the lesion in *flm3* in more detail.

To analyze the region of the genome altered in *flm3*, we rescued DNA from the mutant strain by cutting the genomic DNA with *Bam*HI and *Pst*I, diluting the solution of DNA fragments, circularizing the fragments by ligation, and transforming the ligated DNA population into *E. coli* DH5α. The

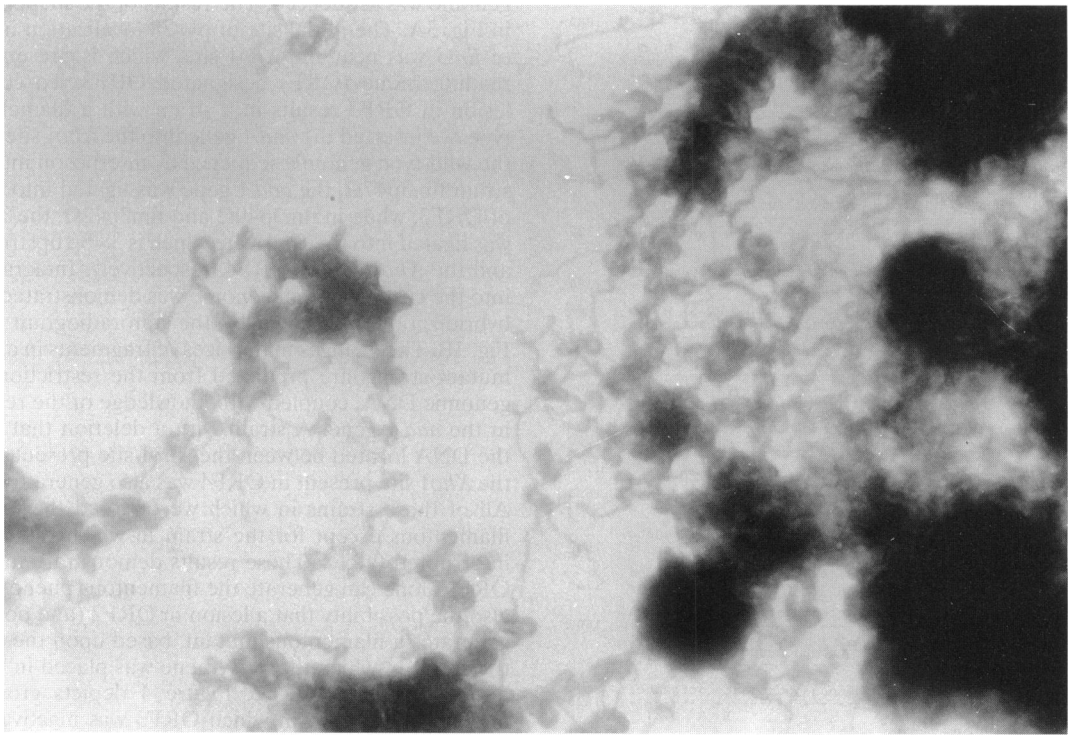
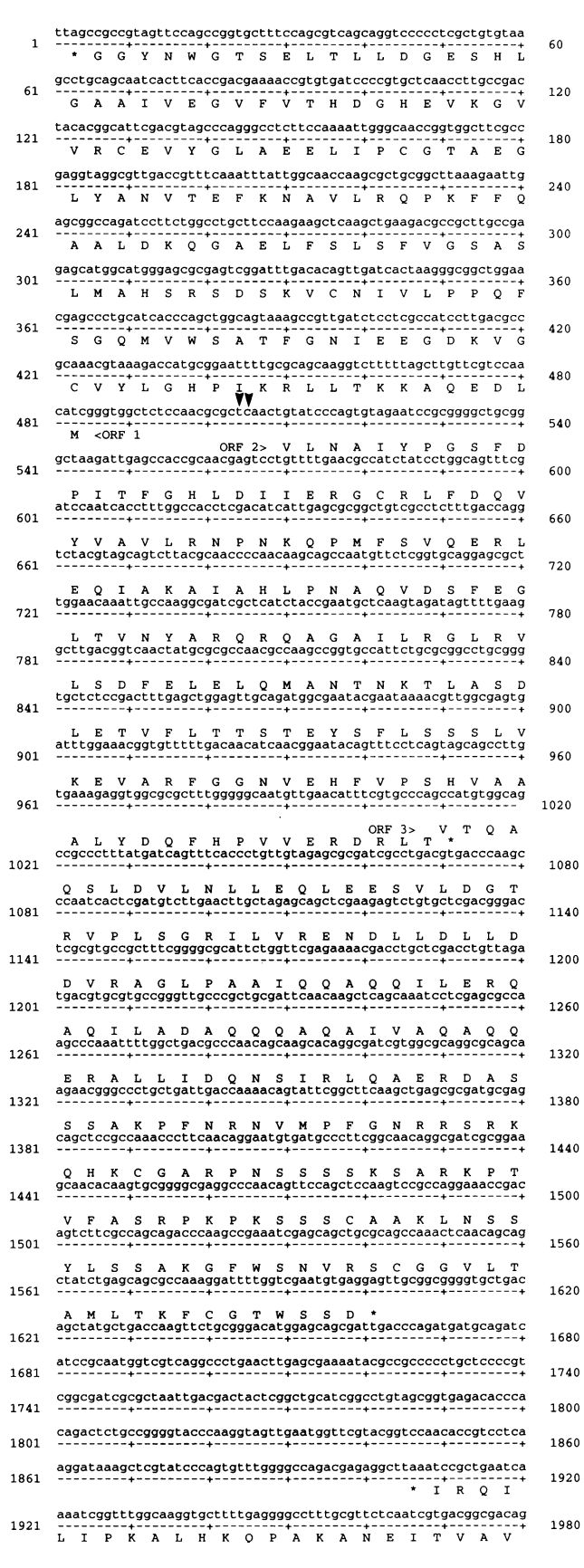


FIG. 4. Micrograph showing the filamentous growth of flm3p17Ω on solid medium. The 1-kbp *Xho*I fragment (Fig. 3) was replaced by the *aadA* cassette in this mutant strain. The transformants were grown on BG-11 solid medium containing 25 μg of spectinomycin per ml and examined under a Bausch and Lomb binocular microscope (Stereozoom 7) at a magnification of ×20.



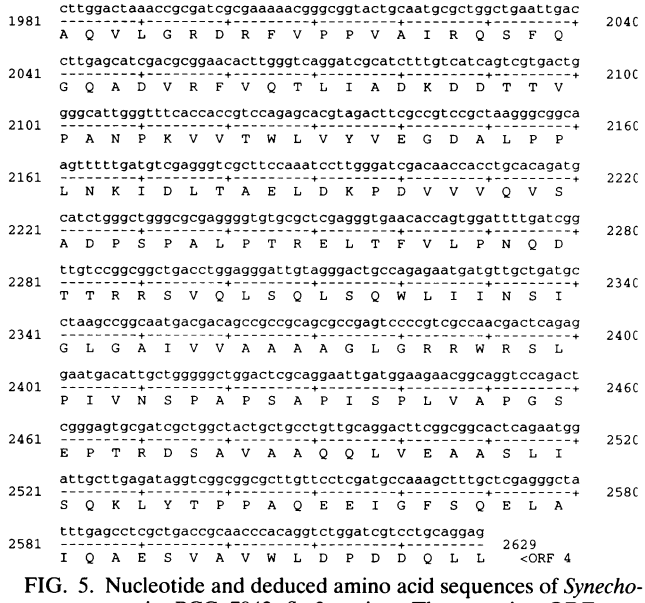


FIG. 5. Nucleotide and deduced amino acid sequences of *Synechococcus* sp. strain PCC 7942 *flm3* region. The putative ORFs are marked. The vertical arrowheads indicate the potential transcription start sites that precede ORF2.

plasmid isolated from DH5α had the appropriately sized insert DNA and was used to isolate a large genomic fragment containing the same sequences (but unaltered by the insertion of the plasmid) from a *Synechococcus* sp. strain PCC 7942 genomic library in λEMBL3. The DNA and the position of the pUC8 within that DNA in the mutant genome were mapped by restriction endonuclease digestion, and this region of the genome was sequenced. The results of the mapping are shown in Fig. 3A. The initial site of pUC8 localization in the genome of *flm3* was near the *Xho*I site, which is present in an open reading frame (ORF) designated ORF3. To confirm that a lesion in ORF3 results in a strain with a filamentous phenotype, we inserted the *aadA* gene into the *Xho*I site and mutated the wild-type genomic sequence by interposon mutagenesis. In strain flm3p47Ω, the *aadA* gene was ligated into the *Xho*I site of ORF3, while in flm3p49Ω and flm3p42Ω, the *aadA* cassette was ligated into the *Sac*II site which is 38 bp upstream of ORF2 and the *Xho*I site of ORF4, respectively. Integration of *aadA* into the cyanobacterial genome was demonstrated by Southern hybridizations as shown by the autoradiogram presented in Fig. 3B. The changes in the sizes of fragments in all three of the mutant strains are predicted from the restriction map of the genomic DNA, coupled with knowledge of the restriction sites in the *aadA* gene. A strain with a deletion that encompasses the DNA located between the *Xho*I site present in ORF3 and the *Xho*I site present in ORF4 was also generated (flm3p17Ω). All of these strains in which we targeted the mutation were filamentous except for the strain in which the *aadA* cassette interrupted ORF4. These results demonstrate that a lesion in ORF3 alone can generate the filamentous phenotype. There is also the possibility that a lesion in ORF2 (and possibly ORF1) generates a filamentous mutant, based upon the phenotype of the strain in which the *aadA* gene was placed in the *Sac*II site just upstream of ORF2. Figure 4 depicts growth on solid medium of a strain in which ORF3 was inactivated by interruption with the *aadA* gene. This mutant, when grown either in liquid medium or on solid medium, looks similar to the original *flm3* strain.

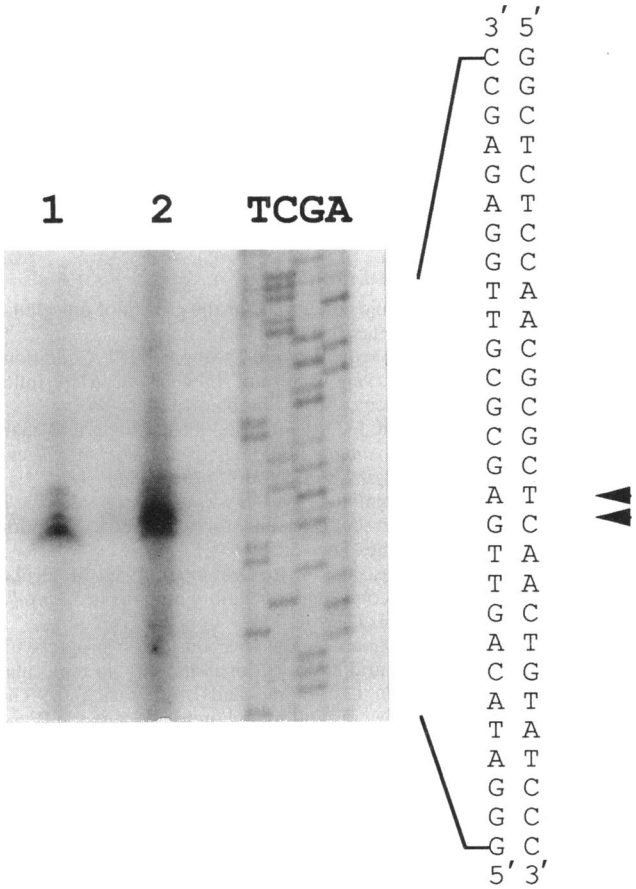



FIG. 6. Primer extension analysis of RNA from the *flm3* region. Total RNA was annealed to a 41-base oligonucleotide (complementary to bases 578 to 618 in Fig. 5). The primer, between 1 and 4 pmol, was extended with 35 U of avian myeloblastosis virus reverse transcriptase (lanes 1 and 2). Extension products were separated on a 6% polyacrylamide gel and sized by comparison with a sequencing ladder (TCGA) generated with the same primer extended on the 1.2-kbp *Xho*I-*Pst*I template (Fig. 3A). Lanes 1 and 2 were loaded with extension products from reaction mixtures containing 10 and 50 μg, respectively, of total RNA. The transcription start sites are indicated by arrowheads to the right of the nucleotide sequence.

The nucleotide sequence of the DNA fragment in the region altered in *flm3* and the amino acid sequences of the ORFs present on this fragment are given in Fig. 5. With various primers, no transcription start site was mapped immediately preceding ORF3. Furthermore, ORF2 may end just as ORF3 begins (position 1070) or slightly downstream (position 1127). This suggests that ORFs 2 and 3 are transcribed as a polycistronic mRNA, although no clear transcript from this region of the genome was detected by Northern (RNA) analyses (data not shown). The potential transcription start site preceding ORF2 was mapped by primer extension with a 41-bp oligonucleotide (see Materials and Methods) annealed to total RNA isolated from wild-type *Synechococcus* sp. strain PCC 7942. Three or four extension products, differing in one nucleotide each, were generated, as shown in Fig. 6. This places the 5′ end of the transcript derived from the ORF2-ORF3 region at a position 65 to 68 bp upstream of the putative translation start site (positions −65 and −66 are marked with arrowheads in Fig. 5).

The protein encoded by ORF2 has strong homology to a gene encoding an 18-kDa polypeptide (Fig. 7A) that may be involved in some aspects of lipopolysaccharide assembly in *E. coli* (12). ORF3 has no convincing similarity to sequences in the GenBank data base. The ORF1 amino acid sequence shows strong homology to a gene present in an operon characterized in *Streptomyces coelicolor* that is required for the synthesis of the antibiotic actinorhodin (16) (Fig. 7B).

## DISCUSSION

Many methods have been used to generate random mutations in cyanobacteria. These methods have employed the chemical mutagens *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine and diethyl sulfate for generating mutants of *Anabaena* strain ATCC 29413 (10) as well as UV mutagenesis of *Synechocystis* strain 6714 (*Aphanocapsa* strain 6714) and *Synechocystis* strain PCC 6803 (2, 17, 41). To isolate the aberrant genes in mutant strains generated by these methods requires complementation of the mutant phenotypes with wild-type DNA. Transposon (38) and ectopic (9) mutageneses have also been employed. For example, methionine auxotrophs of *Synechococcus* sp. strain PCC 7942 were generated by transposition of Tn*901* (38). However, the frequencies of transposition in *Synechococcus* sp. strain PCC 7942 are not very high, and in some cases, the insert DNA was unstable and lost upon replication.

In this paper, we describe a method to generate mutants by randomly inactivating genes via insertion of a plasmid. This is a relatively easy procedure that allows for the rapid isolation of the gene that has been inactivated. Using a standard transformation procedure, we were able to generate 300 to 800 transformants per $10^9$ cells, although the number could be quite variable. A better frequency of gene transfer can be achieved by conjugation of cyanobacteria with an *E. coli* strain

**A**

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flm3.orf2   1   VLNAIYPGSFDPITFGHLDIIERGCRLFDQVYVAVLRNPNKQPMFSVQE  49
                 ||||| ||||| ||:||: |:  :||:| :|:   | | ||| ::|
eco 18kD    1   MQKRAIYPGTFDPITNGHIDIVTRATQMFDHVILAIAASPSKKPMFTLEE  50

flm3.orf2  50   RLEQIAKAIAHLPNAQVDSFEGLTVNYARQRQAGAILRGLRVLSDFELEL  99
                |:     | ||| | :| :| :|  |:||  :|  ::|||| : ||| |:
eco 18kD    51   RVALAQQATAHLGNVEVVGFSDLMANFARNQHATVLIRGLRAVADFEYEM  100

flm3.orf2 100   QMANTNKTLASDLETVFLTTSTEYSFLSSSLVKEVARFGGNVEHFVPSHV  149
                |:|: |: |  :|| ||| | |:||:|||||||||  |:| ||:| :|
eco 18kD  101   QLAHMNRHLMPELESVFLMPSKEWSFISSSLVKEVARHQGDVTHFLPENV  150

flm3.orf2 150   AAALYDQFHPVVERDRLT 167
                  ||  :
eco 18kD  151   HQALMAKLA 159
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**B**

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flm3.orf1   1   MLDEQAKKTLLRKIPHGLYVCGVKDGEEI.NGFTASWVMQGSFQPPLVI  49
                 |:   :  : ::| |: :   | ::: :|||||  :  |::||| :
sco.orf6    1   MAADQGMLRDAMARVPAGVALVTAHDRGGVPHGFTASSFVSVSMEPPLAL  50

flm3.orf1  50   NCVKSDSRSHAMLSASGVFSLSFLEAGQKDLAAQFFKPQRLVANKFETVN  99
                 |:    | :::  :| | :| |  :: ||| |   |  |:||
sco.orf6   51   VCLARTANSFPVFDSCGEFAVSVLREDHTDLAMRF---ARKSADKFAG-G  100

flm3.orf1  100  AYLGEATGCPILEEALGYVECRVVGKVEHGDHTVFVGEVIAAGLHSEGDL  149
                 ::  | |  :|:::|:: ||| | ::   ||| :::||  ::| |:
sco.orf6   101  EFVRTARGATVLDGAVAVVECTVHERYPAGDHIILLGEV--QSVHVEEKG  150

flm3.orf1  150  LTLESTGWNYGG 160
                :  :: :::
sco.orf6   151  VPAVYVDRRFAALCSAAGACPSATGRGVPAHAG 177
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FIG. 7. (A) Amino acid sequence similarity between ORF2 and the 18-kDa polypeptide encoded by a gene of the 3-deoxy-D-manno-octulosonic-acid transferase operon of *E. coli* (12). (B) Amino acid sequence similarity between ORF1 and *S. coelicolor* ORF6 polypeptide (16), which is involved in the synthesis of actinorhodin. The alignments were performed with the TFASTA and Best-Fit programs of the Genetics Computer Group software package. Identical amino acids are indicated by vertical bars, and conservative amino acid differences are indicated by dots. Dashes denote gaps introduced into the sequence to maximize the alignments.

harboring the cyanobacterial library. Mutagenesis by insertion of endogenous DNA into the cyanobacterial genome also has advantages over a number of other procedures that have been used since it employs a plasmid library that can be propagated in *E. coli*, and in the process of generating the mutants, the gene with a lesion is tagged and can be readily isolated. This procedure depends upon the transformability of the cyanobacterium and the efficiency at which the introduced DNA is integrated into the genome by homologous recombination. There are several naturally transformable cyanobacteria with which this procedure can be used. They include *Synechocystis* sp. strains PCC 6803 and PCC 6714, *Synechococcus* sp. strain PCC 7942, and the marine cyanobacterium *Synechococcus* sp. strain PCC 7002. In cyanobacteria that are not naturally transformable, the same results may be achieved if a library is introduced into the cyanobacterium by conjugation. The strategy that we have used for random gene disruption does have some disadvantages. First, most of the mutants generated will be null for the gene that is disrupted. Hence, many of the interruptions will result in cell death. Second, if the *Sau*3A fragments from some regions of the genome are not small enough, a single recombination event will not generate a mutation. Therefore, a specific subset of mutants will never be generated with this library, and it may be necessary to make a new library in which the insert DNA is prepared with a variety of restriction enzymes that recognize 4-bp target sites or by randomly shearing the cyanobacterial genomic DNA.

In this study, we generated many mutants that showed altered cell filamentation both on plates and in liquid. The number of transformants that exhibited a filamentation phenotype was quite high and in the range of two to five per 1,000 transformed cells. The fact that filamentous mutants can be found at high frequencies suggests that inhibition of a number of different processes in the cell can result in that phenotype (35). Filamentation has been shown to be caused by lesions that affect DNA replication or protein secretion in *E. coli* (27). Mutations in genes important for the regulation of the cell cycle in *E. coli* and *Bacillus subtilis* (3, 4) may also yield filamentous strains, although inactivation of most of the cell cycle genes would be lethal, and isolation of such mutants would require the generation of conditional lesions (27).

One of the filamentation strains that we have isolated, *flm3*, was characterized in detail. From *flm3*, we isolated the DNA that flanked the site of pUC insertion, secured the uninterrupted DNA from this region from a wild-type recombinant library in λEMBL3, and established that a filamentous phenotype was observed if we targeted an interruption of the gene originally inactivated by pUC8. One gene that was interrupted and yielded a filamentous phenotype encoded a protein that was not homologous to any other sequence in the data base (ORF3 in the text). However, this gene is immediately downstream of, and probably cotranscribed with, a second gene containing an ORF (ORF2) that exhibits over 50% homology with an ORF from the operon encoding 3-deoxy-D-manno-octulosonic-acid transferase in *E. coli* (12). An insertion 38 bp upstream of this ORF, and probably located in the leader sequence of the mRNA that covers both ORF2 and ORF3, gives the same filamentous phenotype. The 3-deoxy-D-manno-octulosonic-acid transferase operon is involved in the synthesis of the cellular capsule. The 18-kDa polypeptide may be important for the assembly of the lipopolysaccharide components of the capsule, and a lesion in the gene may alter the cell wall, which in turn could retard the separation of cells following septation. ORF3 might have a related function. Further analysis of this and other filamentous mutants may lead us to genes that are important for diverse processes, and in some instances, to genes directly involved in cell division in cyanobacteria.

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