Isolation and Complementation of Nitrogen Fixation Mutants of the Cyanobacterium *Anabaena* sp. Strain PCC 7120

WILLIAM J. BUICKEMA* and ROBERT HASELKORN

Department of Molecular Genetics and Cell Biology, University of Chicago, 920 East 58th Street, Chicago, Illinois 60637

Received 7 November 1990/Accepted 4 January 1991

Approximately 140 mutants of *Anabaena* sp. strain PCC 7120 unable to grow aerobically on media lacking fixed nitrogen (Fix−) were isolated after mutagenesis with diethyl sulfate and penicillin enrichment. A large cosmid library of wild-type *Anabaena* sp. strain PCC 7120 DNA was constructed in a mini-RK-2 shuttle vector, and seven mutants representing several morphologically abnormal heterocyst phenotypes were complemented. One of these mutants, 216, failed to differentiate heterocysts. All of these mutants except 216 reduced acetylene under anaerobic conditions, indicating that they are not defective in nitrogen fixation per se. Several cosmids were isolated from each complemented mutant and in most cases showed similar restriction patterns. Comparisons of the complementing cosmids from mutant 216 and two other phenotypically distinct mutants by restriction enzyme analysis identified a common region. This region, when present in either a cosmid or a 9.5-kb *NheI* subclone, is capable of efficiently complementing all three mutants. A 2.4-kb subclone of this region complements mutant 216 only.

*Anabaena* sp. strain PCC 7120 is one of many species of cyanobacteria that carry out both oxygenic photosynthesis and oxygen-sensitive nitrogen fixation simultaneously. In heterocystous cyanobacteria such as *Anabaena* spp., this feat is accomplished by cellular differentiation. Under nitrogen-replete conditions, all of the 100 or more cells in an *Anabaena* filament have the same morphology. These vegetative cells fix CO2 by using ribulose bis-phosphate carboxylase, contain thylakoid membranes with both of the plant-type photochemical reaction center complexes photosystem I (PSI) and photosystem II (PSII), and contain phycobiliproteins that function as light-harvesting antennae. When combined nitrogen (usually nitrate or ammonia) is absent from the growth medium, specialized cells called heterocysts differentiate from vegetative cells at regular intervals along the filament. Heterocysts lack phycobiliproteins, PSI, and ribulose bisphosphate carboxylase activity. They have a unique external envelope with an outer polysaccharide layer and an inner glycolipid layer that serves to attenuate O2 penetration. Within the heterocyst the nitrogenase enzyme complex is synthesized; this synthesis is accompanied by further changes in the photosynthetic apparatus and the enzymes of carbon metabolism to provide ATP and a low-potential reductant for nitrogen fixation (2, 8, 16, 19).

Intercellular communication is also modified as a consequence of heterocyst differentiation. In differentiated filaments, carbon compounds from photosynthesizing vegetative cells are transported into heterocysts (18). Glutamine, bearing in its amide group the ammonia created by nitrogen fixation, is transported from heterocysts to vegetative cells (12). In *Anabaena* sp. strain PCC 7120, heterocysts are spaced approximately 10 to 20 cells apart, depending on growth conditions. Heterocysts, possibly as a consequence of glutamine export, inhibit the differentiation of adjacent vegetative cells. In fact, the vegetative cell selected for differentiation is usually located midway between two heterocysts (17). Thus, the heterocyst spacing pattern is maintained during growth on N2 as the nitrogen source.

The development of a system for DNA transfer to *Anabaena* spp. by conjugation from *Escherichia coli* (21) has provided a powerful instrument for the study of heterocyst differentiation. By using complementation, it has become possible to isolate the wild-type gene(s) corresponding to any mutation, with the few exceptions being dominant mutations or deletions larger than the amount of DNA clonable in a cosmid vector. Wolk et al. have in fact isolated a gene involved in the synthesis of the polysaccharide layer of the heterocyst by this procedure (20). Here we describe the isolation and complementation of *Anabaena* mutants defective in heterocyst differentiation and nitrogen fixation and the identification of several closely linked mutations.

### MATERIALS AND METHODS

**Strains and culture conditions.** *Anabaena* sp. strain PCC 7120 has been previously described (13). Mutant strains 216, 415, 416, 541, 129, 53, and 522 are described in Results.

For liquid culture, *Anabaena* sp. strain PCC 7120 (wild type) and its mutants were grown in a modified Kratz and Myers medium C (K&M medium) or BG-11 medium (9, 13). The modified K&M medium contains 1.125 mM Na2HPO4 and 1.125 mM K2HPO4 (instead of only Na2HPO4) and 3 to 5 mM MOPS (morpholinepropanesulfonic acid) buffer, pH 7.5. For growth in the presence of nitrogen (N2 growth), K&M medium was supplemented with 2.5 mM (NH4)2SO4 and BG-11 was supplemented with 17.6 mM NaNO3. For growth on plates, 1.3% agar (BBL* purifed*) was added prior to autoclaving. Cultures were typically grown under 30 to 40 microEinsteins of cool white fluorescent lighting per m2 per s at 25 to 30°C in the presence of 2% CO2 (large-scale liquid cultures were bubbled with a 2% CO2-air mixture). For selective growth of exconjugants, media were supplemented with 25 to 30 μg of neomycin per ml. Mid-log-phase cells refer to cultures containing 2 to 6 μg of chlorophyll per ml, corresponding to 0.7 × 107 to 2.0 × 107 cells per ml.

*E. coli* strains were typically grown in Luria broth (LB)
(11) for liquid culture and LB solidified with 1.3% agar for plate culture. For selective growth, the media were supplemented with antibiotics at the following concentrations: 100 μg of ampicillin or carbenicillin per ml, 50 μg of kanamycin per ml, and 10 μg of chloramphenicol per ml. For plasmid isolation, *E. coli* strains were grown in LB supplemented with M9 salts (11), 2 g of glucose per liter, 1 g of sodium succinate per liter, and the appropriate antibiotic.

**Construction of pDUCAT.** The 0.4-kb *psl* fragment from *pSUP205* was cut with *SalIII* and transferred to *E. coli* HB101(AD) containing *pUCcosl9*. The neomycin phosphotransferase gene (*neo*) from *Tn5* was isolated from pBR322 carrying a Tn5 insertion by using *AvaI* and *HindIII*, and the fragment was end filled with the Klenow fragment and purified from an acrylamide gel. This fragment was inserted into the *Smal* site of *pUC cosl9* to form pUCK19. The polynuker of pUCK19 containing the *cos* site and *neo* gene was isolated by digestion with *KpnI* and *HindIII* and was cloned into the *HindIII* and *KpnI* sites of the mini RK-2 replication vector pOCA6 (obtained from N. Olszewski) to form pDUC6. The Nostoc replicon (pDU1) from pRL25C (20) was isolated with *NheI* and *EcoRI*, end filled with the Klenow fragment, and cloned into the end filled *Stul* and *HindIII* sites of pDUC6 to form pDUC6.5. Finally, the 122-bp *Tn* terminator fragment of bacteriophage T7 was isolated as a BamHI-BgII fragment from pAR2529 (obtained from F. W. Studier) and cloned into the BamHI site of pDUC6.5 to form pDUC7. These elements are shown in the map of pDUC7 included in Fig. 3.

**Construction of the cosmid shuttle bank.** The cosmid shuttle bank was constructed as follows. Total genomic DNA from *Anabaena* sp. strain PCC 7120 was partially digested with *Sau3AI*, and a 50- to 50-kb fragment was isolated from a preparative agarose gel and ligated into the dephosphorylated BamHI site of pDUC7. The ligated DNA was packaged in vitro into bacteriophage lambda (11), which was then used to infect *E. coli* HB101 containing the *Aval* and Eco47III methylase helper plasmid pRL528 (5). Infected cells were plated onto LB plates containing 50 μg of kanamycin per ml, and approximately 2 × 10⁹ colonies were combined and frozen in aliquots at −70°C.

**Mutagenesis and isolation of mutants.** *Anabaena* sp. strain PCC 7120 was mutagenized essentially as described elsewhere (3). Mid-log-phase cultures were treated with 5 μl of diethyl sulfate per ml for 5 to 20 min in K&M *N*⁺ medium. The cells were washed several times to remove the diethyl sulfate and then transferred to fresh *N*⁺ medium. These cultures typically bleached in a day and required 5 to 7 days to recover to mid-log-phase growth. Cultures were then transferred to fresh K&M nitrogen-free (*N*⁻) medium and allowed to grow for 2 to 3 days. Nongrowing cells were selected by adding 200 μg of ampicillin per ml of culture and incubating the culture for 3 days. The surviving cells were transferred to fresh *N*⁺ medium and allowed to recover for several days. This enrichment was repeated twice more, and the survivors were transferred to solid K&M *N*⁺ medium. Small green colonies were visible after 1 week, and after 3 weeks some colonies bleached yellow as the residual nitrogen in the plates was exhausted. These colonies were picked onto *N*⁺ plates, allowed to recover, and then restested for a Fix⁺ phenotype on *N*⁻ plates. Colonies that displayed a high reversion frequency, indicated by the subsequent development of green sectors, were discarded.

**Complementation of Fix⁺ mutants.** Complementation of the *Anabaena* sp. strain PCC 7120 Fix⁺ mutants with the wild-type cosmid bank was performed as follows. *Anabaena* cells were grown to mid-log phase in K&M *N*⁺ medium, washed in K&M *N*⁺ medium with 5% LB added (K&M LB), and resuspended in the same medium at approximately 2 × 10⁹ cells per ml. A portion of the frozen cosmid bank was grown in LB containing 50 μg of kanamycin per ml for 3 to 4 h, washed in K&M LB, and resuspended in the same medium at approximately 5 × 10⁸ cells per ml. A mid-log-phase culture of *E. coli* HB101(RP-1) grown in LB containing 50 μg of kanamycin per ml was similarly washed and resuspended. These three suspensions were then mixed in equal proportions, and approximately 0.5 ml was placed onto nitrocellulose filters (HATF 085 50; Millipore Corp.) on K&M LB agar plates. After 2 days of incubation under standard *Anabaena* growth conditions, the filters were transferred to K&M *N*⁻ plates containing 30 μg of neomycin per ml and incubated until small green colonies appeared, typically within 3 to 4 weeks. Four single colonies from each complementation were picked and streaked onto similar selective plates and allowed to grow for 2 weeks. Cells were scraped from the plates and washed several times in K&M *N*⁺ medium by low-speed centrifugation to remove the remaining *E. coli* cells (which remained in the supernatant), and plasmid DNA was prepared as described above. This plasmid DNA was used to transform *E. coli* HB101.

Subsequent confirmatory complementations were done as follows. A liquid mid-log-phase culture of the *Anabaena* mutant strain was centrifuged and resuspended in K&M LB at approximately 5 × 10⁹ cells per ml. Samples (50 to 100 μl) of cells were placed as long streaks onto filters on K&M LB plates and allowed to dry. Cultures of the two donor *E. coli* strains, one carrying RP-1 and the other carrying the complementing plasmid together with pRL528, were prepared as described above for the cosmid bank conjugations. The *E. coli* strains were mixed, and approximately 50 μl of the mixture was dropped at several locations on the streak of *Anabaena* cells. The filters were grown as described above and scored for growth after 2 to 3 weeks. Successful complementation was evident as circular areas containing a green lawn or with heavier green rings of colonies where the *E. coli* donor cells had been plated.

**Molecular biological techniques.** The general molecular biological techniques used were as described previously (11). Plasmid isolation from *E. coli* and *Anabaena* sp. used the alkaline lysis method. Before lysis of *Anabaena* sp., the cells were washed in 500 mM NaCl in BG-11 medium and then treated with 1 mg of lysozyme per ml in 50 mM glucose-10 mM EDTA-25 mM Tris (pH 8.0) for 30 min at 37°C.

**Microscopy.** Photomicroscopy of *Anabaena* sp. strain PCC 7120 wild-type and mutant cells was performed with a Zeiss Axiopt microscope equipped with phase contrast and Nomarski (differential interference contrast) optics. Photomicrographs were taken with Kodak Technical Pan film at 50 ASA.

**RESULTS**

In order to identify potentially interesting developmental mutants of *Anabaena* sp. strain PCC 7120, we sought to generate a large number of aerobic Fix⁺ mutants by using the mutagen diethyl sulfate (3). Mutants were amplified with three sequential rounds of penicillin enrichment, which selected for cells unable to grow in *N*⁻ medium. Survivors were spread on agar plates containing a minimal amount of fixed nitrogen. During the first 2 weeks of growth, all colonies that appeared were uniformly green. After 3 weeks
of incubation, approximately 50% of the colonies began to yellow as the fixed nitrogen was exhausted, an expected phenotype for Fix" mutants. Several hundred of these colonies were rescued by transfer to N" plates. Approximately 140 of these mutants were characterized as having a stable phenotype with a low reversion frequency and were screened for obvious developmental phenotypes by light microscopy. Most of these mutants displayed no obvious morphological defects and are not considered further here. However, seven mutants representing four morphologically defective classes were identified and chosen for further study.

A comparison of the selected mutants with wild-type *Anabaena* sp. strain PCC 7120 by using Nomarski optics is shown in Fig. 1. Each panel in Fig. 1 shows representative cells 72 h after transfer from N" to N" medium. Mutant 216 fails to initiate any visible early stages of heterocyst differentiation (Fig. 1B). These filaments do not show the usual clumping because of the formation of the outer polysaccharide layer of the heterocyst envelope, one of the earliest visible events of heterocyst development. Randomly ordered nongranular cells are visible and may represent cells that have degraded their internal stores of nitrogen more fully than other cells. Mutants 415, 816, and 522 initiate differentiation, but the differentiating cells detach from the vegetative cells, resulting in very short fragments that clump together (Fig. 1C through E). Mutant 53 displays normally spaced heterocysts, but they show early and extensive formation of apparent vacuolated regions that increase in size with the age of the heterocyst (Fig. 1F). Mutant 541 differentiates the normal number of heterocysts with the normal spacing pattern, but the heterocysts display a thickened envelope that increases with age (Fig. 1G). This thickening may be caused by a modified glycolipid layer in the heterocysts. The vegetative filaments are more rigid and the cells are more rectangular than in the wild type. Finally, mutant 129 exhibits vegetative filaments that are weak and easily broken into small (one- to four-cell) pieces. Upon infection in N" medium, heterocysts may form from the longer filaments, but they are easily dislodged, and most of the filaments fragment into single cells (Fig. 1H).

Many strains of cyanobacteria can be induced to fix N₂ anaerobically without the need to differentiate heterocysts. Anaerobic inductions are typically performed by placing a culture in N-free medium, adding the herbicide dichlorophenyl-dimethylurea to inactivate O₂ evolution from PSII, and sparging the medium with an O₂-free gas mixture (14). All of the mutant strains described here, with the exception of 216, reduce acetylene when grown anaerobically (data not shown), suggesting that their Fix" phenotypes are probably caused by defects in heterocyst development or maintenance rather than in the nif genes themselves. The failure of 216 to fix N₂ when grown anaerobically may be a symptom of a mutation in a general regulatory pathway leading to heterocyst formation and nitrogen fixation.

For complementation of these mutants, we constructed a cosmid shuttle bank that could be transferred from *E. coli* to *Anabaena* sp. strain PCC 7120 and replicate there. The original conjugation system described by Wolk et al. consisted of a shuttle vector, a plasmid to provide trans-acting mobilizing functions for the shuttle vector, and a broad-host-range plasmid to provide the transfer functions and apparatus (21). The shuttle vector contained a small *Anabaena* plasmid for replication in *Anabaena* sp., a portion of pBR322 for replication in *E. coli*, and antibiotic resistance genes for selection in both hosts. *Anabaena* sp. is the source of a number of restriction endonucleases such as *AvaI* and *AvaII*, and it was originally noted that removal of the corresponding cleavage sites from the shuttle vector improved the efficiency of conjugation. Later, Elhai and Wolk introduced the step of modifying the DNA to be transferred with the *AvaI* and *Eco47II* methylases (5). This modification increases the efficiency of conjugal transfer considerably.

The feasibility of isolating cloned genes by complementation rests on the ability to construct a fully representative library and to transfer it en masse by conjugation. Our early experience with libraries of *Anabaena* DNA fragments cloned in high-copy-number plasmid or cosmids vectors indicated that some fragments were strongly countersel ected in *E. coli*. Subsequent cloning of individual genes revealed that some *Anabaena* proteins, such as those that make up the photosynthetic apparatus, are toxic to *E. coli* (4, 10). The cosmid vector pDUCA7 was constructed with these principles in mind. The cloning sites are protected on one side by a transcriptional terminator that prevents readthrough into the cloned fragment from the vector neo promoter. The replication of pDUCA7 in *E. coli* is dependent on the ori region derived from RK-2, which ensures that the copy number will be low in *E. coli*. In addition, the RK-2 element in the vector includes an origin-of-transfer site that provides for conjugal mobilization by RP-1. A pDUI replicon permits replication in *Anabaena* sp. (21). The basic features of pDUC7 are included in Fig. 3. Our cosmid shuttle bank was prepared by ligating a sized partial *Sau3AI* digest of wild-type *Anabaena* sp. strain PCC 7120 DNA into the *BamHI* site of pDUCA7. A bank of approximately 20,000 members was introduced into HB101(pRL528), pooled, and frozen in aliquots for later use.

Complementation of the selected mutants was carried out by transfer of the cosmid bank to each mutant via conjugation. Each of the seven mutants already described was separately added to a mixture of the cosmid bank in HB101(pRL528) and HB101 containing the conjugative plasmid RP-1. Following 3 to 4 weeks of growth on selective plates containing no fixed nitrogen and 30 μg of neomycin per ml, presumptive complemented cells appeared as green colonies on a brown background of dying mutant cells. For each mutant, these colonies varied in number, size, time of appearance, and growth rate.

Four of these green colonies from each of the seven mutants were picked and spread onto selective plates. After 2 weeks, the cells were subjected to a modified alkaline lysis procedure to isolate the complementing cosmids. This DNA was used to transform *E. coli*, from which plasmid DNA was isolated for restriction analysis.

For six of the seven mutants, each of the cosmids isolated from the four complemented colonies contained overlapping or identical DNA fragments, as determined by restriction enzyme analysis using *HindIII* (Fig. 2). This result provided evidence that the colonies from which the cosmids were isolated were actually being complemented and did not arise by reversion of the original mutations. For mutant 816, however, none of the four isolated cosmids contained overlapping DNA, suggesting that the colonies arose by reversion of the mutation in some of the recipient cells that had received a cosmid via conjugation. This would both allow the cells to grow under selective pressure (neomycin resistance) and result in the isolation of different cosmids from each colony. Indeed, 816 had a significantly higher reversion frequency than the other selected mutants (data not shown). However, we found later that one of the four cosmids
actually was capable of complementing mutant 816 (Fig. 2, lane 16), as described below.

The eight cosmids isolated after complementation of mutants 216 and 415 and one cosmid isolated from a Fix− derivative of mutant 816 contained overlapping DNA fragments, indicating that the three mutations are linked (compare lanes 9 to 12 with lane 16 and lanes 17 to 20 in Fig. 2). Strains 816 and 415 have similar phenotypes, suggesting that their lesions may in fact lie within the same gene. To determine whether these similar cosmids could complement the three mutants, we transferred them via conjugation into strains 216, 415, and 816 and determined their phenotypes under N− conditions. In most cases, each of the nine overlapping cosmids was able to complement all three mutant strains. However, one cosmid isolated from complemented mutant 216 failed to complement the same mutant when reintroduced, even though it had the restriction pattern expected of a 216-complementing plasmid and could complement the linked mutations in mutants 816 and 415. This difficulty was not observed with the cosmids that complemented other mutations. Since wild-type Anabaena sp. strain PCC 7120 is recombination proficient, it is likely that genetic exchange between the wild-type cosmids and the mutant chromosome had taken place in this instance. This result is discussed below. As expected, the three dissimilar cosmids isolated from Fix+ derivatives of mutant 816 did not complement any of the mutants.

The cosmid families that complement mutants 522, 53, 541, and 129 do not overlap each other or the cosmids that complement mutants 216, 415, and 816. In addition, none of these cosmids contain restriction fragments from the known nif gene regions of Anabaena sp. strain PCC 7120. Thus, the mutants described here comprise five unlinked loci required for heterocyst differentiation.

One cosmid that could complement mutant 216, pWB216C5, was chosen for further study. Its restriction map is shown in Fig. 3. In an effort to localize the region of DNA required for complementation of mutant strains 216, 415, and 816, we subcloned the three Nhel fragments of pWB216C5 into the Nhel site of pDUCA7 and transferred the resulting plasmids into these strains. The rightmost 9.5-kb Nhel fragment (subcloned in pWB216N9.5 [Fig. 3]) was able to complement all three mutants, while no complementation was seen with plasmids containing the other two fragments (data not shown).

In order to delineate further the genetic regions essential for the complementation of these three mutants, the 9.5-kb Nhel fragment in pWB216N9.5 was partially digested with Sau3AI, and fractions of 1 to 2, 2 to 4, and 4 to 8 kb were ligated into the BamHI site of pDUCA7. Pools of several hundred plasmids containing DNA fractions of these size categories were constructed in E. coli and subsequently transferred into strains 216, 415, and 816, selecting for growth on N− plates. Plasmid DNA was prepared as before from eight complemented colonies of each strain. On the basis of restriction digestion patterns, several related plasmids were found to complement each mutant, with the same plasmids appearing in 415 and 816 but different ones appearing in 216. This result indicates that mutants 415 and 816 probably contain mutations in the same gene or two closely linked genes, while 216 contains a lesion in a nearby gene. The smallest plasmid that complements 216 (pWB216S2.4) contains a 2.4-kb partial Sau3AI insert shown in Fig. 3. The plasmids that complement both 415 and 816 contain most of the region to the left of the 2.4-kb Sau3AI fragment.

DISCUSSION

Using chemical mutagenesis, we produced a large number of aerobic Fix− mutants of Anabaena sp. strain PCC 7120. Most mutants displayed no obvious morphological defects under light microscopy. Several mutants displayed such defects, including strain 216, which forms no heterocysts under any conditions. We selected representatives of several morphologically defective classes for complementation and

FIG. 1. Light micrographs of wild-type Anabaena sp. strain PCC 7120 and mutants defective in aerobic nitrogen fixation. Strains were incubated for 3 days in N-free medium and photographed by using Nomarski (DIC) optics. (A) Wild type; (B) strain 216, defective in heterocyst development; (C) strain 415, in which filaments break apart under N− conditions and form clumps; (D) strain 816, which has a phenotype similar to that of 415; (E) strain 522, which has a phenotype similar to those of 415 and 816; (F) strain 53, in which heterocysts show vacuolated regions; (G) strain 541, in which heterocysts show thickened walls and vegetative cells are rectangular; (H) strain 129, in which filaments display smaller cells and break easily under N+ or N− conditions. Bar, 10 μm.

FIG. 2. Restriction digests of complementing cosmids isolated from Fix− mutants of Anabaena sp. strain PCC 7120. Cosmids isolated from complemented mutant colonies and used to transform E. coli were prepared as described in Materials and Methods. Each DNA was cut with HindIII. Three-digit numbers over the lane numbers refer to mutant designations. For each mutant, digests of four independent complementing cosmids are shown. The size markers in lanes marked λ are a BstEII digest of λ DNA. Four bands of 430, 500, 810, and 1,100 bp present in all the lanes are vector fragments (v). The four cosmids isolated from each mutant except 816 clearly overlap each other. Additionally, the cosmids isolated from mutants 216 and 415 overlap with each other and with a single cosmid isolated from 816 (lane 16). Four overlapping cosmids were also isolated from mutant 53 (data not shown).
in most cases found distinctive DNA fragments in the complementing cosmids. It is noteworthy that in most instances cosmids could be efficiently transferred between E. coli and Anabaena sp. strain PCC 7120 without any structural or functional changes. However, in one instance, an apparently complementing cosmid that could no longer complement mutant 216 upon reintroduction was isolated from 216. It is possible that this cosmid had undergone genetic recombination with the mutant chromosome, resulting in the conversion of its wild-type DNA sequence to that of mutant 216.

The three mutants 216, 415, and 816, representing two distinctly different phenotypes, are complemented by a single 9.5-kb DNA fragment. Mutant 216 is blocked at a very early stage of differentiation, while the other mutants appear to progress further. This result suggests that some genes required for heterocyst function are tightly linked. However, most of the mutants described here appear to have lesions in unlinked loci, suggesting that genes required for heterocyst differentiation are present in several locations around the chromosome. This result agrees with physical mapping results that have shown that two genes involved in heterocyst envelope formation are unlinked from each other as well as from the nif/HDK gene cluster (1).

All of the mutants, with the exception of 216, fix nitrogen anaerobically. This means that whichever lesions cause the failure of aerobic fixation do not significantly affect the function of the nif gene apparatus. In this regard, these mutants must be able to express those genes involved in nitrogen fixation, including those involved in the two nif gene rearrangements that occur in Anabaena sp. strain PCC 7120 (6, 7), separately from those required for functional heterocyst formation. Also, some of these mutants may have lesions affecting filament or vegetative cell integrity, which only indirectly affects heterocyst function. In the case of mutant 216, however, the gene affected may be required at such an early stage in differentiation as to affect nif gene expression as well as heterocyst formation. Most of the mutants that were originally isolated display no morphologically abnormal phenotypes. We expect that many of these mutations will be found within genes more directly involved in nitrogen fixation and can be identified by their inability to fix nitrogen anaerobically.

The method we have described for isolating heterocyst-specific genes takes advantage of a large, stable cosmid bank. There are several advantages to using a large cosmid bank for complementation. An obvious one is that a gene of interest is more likely to be represented in a large bank, especially if it is present in a region of DNA that would normally be underrepresented because of restriction or adverse gene expression. Another advantage is that the region of the genome represented by a group of cosmids is likely to be larger and more easily mapped when a larger number of unique cosmids can be isolated. This mapping facilitates detection of linked regions and will be useful when a more detailed physical map of the Anabaena sp. strain PCC 7120 genome is constructed.

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FIG. 3. Restriction map of cosmid pWB216C5 and subclones. The main features of pDUCA7 are shown on the top line. The origins of the subclones of pWB216C5 and pWB216N9.5 are indicated by the diagonal dotted lines. Only selected restriction sites are indicated. Restriction sites that have been inactivated are in parentheses.