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Cloning of Nitrate Reductase Genes from the Cyanobacterium *Anacystis nidulans*

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***Anacystis nidulans*, a non-nitrogen-fixing cyanobacterium, can fulfill its nitrogen requirement by the assimilation of nitrate. The first step in the pathway, the reduction of nitrate to nitrite, is catalyzed by the molybdo-protein nitrate reductase. In this study, newly developed techniques for gene cloning in *A. nidulans* R2 were used for the isolation of two genes involved in nitrate reduction. One gene was cloned by complementation of the corresponding mutant; the other gene was picked up from a cosmid gene library by using a restriction fragment containing the transposon-inactivated gene as a probe. Both genes were unlinked single-copy chromosomal genes. Transformation studies provided evidence for the existence of a third locus involved in nitrate reduction.**

Organisms that are capable of an oxygen-evolving type of photosynthesis, such as cyanobacteria, eucaryotic algae, and higher plants, have only a limited number of growth requirements. Besides light and carbon dioxide, only a nitrogen source is needed in appreciable quantities. In the case of the non-nitrogen-fixing cyanobacterium *Anacystis nidulans*, nitrogen is normally supplied in the form of nitrate (32). The conversion of nitrate into organic carbon compounds proceeds via nitrite and ammonium (20); the latter compound is incorporated into glutamate to form glutamine. Reducing equivalents are donated by ferredoxin, and thus nitrate reduction is intimately linked to photosynthesis (4, 5, 28).

Nitrate reduction in cyanobacteria has been the subject of a number of biochemical and physiological studies. The enzyme nitrate reductase is a molybdo-protein consisting of a single polypeptide with a molecular weight of 75,000 (19). Ammonium serves as a repressor, not directly but via the action of the enzyme glutamine synthetase. Antibiotic inhibition studies suggest that this repression takes place at the level of transcription (23, 38). However, similar studies with the green alga *Chlorella* demonstrate that a more complex regulation cannot be excluded (9, 10).

Mutants deficient in nitrate assimilation have been generated by using conventional techniques of mutagenesis and enrichment (22, 36, 37). They grow slowly on nitrate medium and display a characteristic yellow color (21). This yellow color has been observed previously under nitrogen starvation and is caused by a shifted chlorophyll-to-phycocyanin ratio (1). It has been well documented that under conditions of nitrogen starvation, a protease is induced that specifically breaks down the phycobiliproteins (7, 43, 44). In this way, protein synthesis can be sustained at the expense of phycobiliproteins (27).

The genetic analysis of nitrate assimilation in cyanobacteria has been greatly hampered by the lack of a suitable gene transfer system. No data are available on the number of genes involved or their organization and regulation. The recent development of a gene cloning system for the unicellular cyanobacterium *A. nidulans* R2 (25, 26, 41) allows a genetic study of nitrate reduction. Here we report on the isolation and characterization of a series of mutants with no detectable nitrate reductase activity. For two mutants, the corresponding wild-type genes were cloned on cosmid vectors. They represent single-copy, unlinked chromosomal genes. Transformation data indicated that the mutants described here constitute at least three distinct loci.

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MATERIALS AND METHODS

Strains and plasmids. Cyanobacterial strains used in this study were *A. nidulans* R2 (PCC7942) and R2-SPc; the latter strain is R2 cured of the small resident plasmid pUH24 (26). For transposon mutagenesis, R2 strain A6 was used in which pUH24 had been replaced by pCH1 (41). *Escherichia coli* K-12 strain 803 (*recA hsdS lac gal met supE supF*) was used for transformation, and HB101 (3) was used for transduction of cosmids. A list of plasmids is given in Table 1.

Mutagenesis. Mutants were generated either by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or by transposon Tn*901* mutagenesis (40). In both cases, ampicillin-enriched cultures were plated for single colonies on BG-11 medium, which contained sodium nitrate as the nitrogen source (32). Yellow colonies were picked and streaked on various supplemented media. Colonies that turned green on 7 mM sodium nitrite were purified and analyzed further. In liquid BG-11 medium, they grew very slowly but were indistinguishable from the wild type if 7 mM $NaNO_2$ was added. On solid BG-11 medium, green colonies were formed which turned yellow after prolonged incubation. Plating of cells at high density resulted in a very slow-growing yellow lawn of cells. Surprisingly, addition of $NaNO_2$ to plates impaired the colony formation from single cells. All mutants had an in vitro nitrate reductase activity of <3% compared with the wild type, which was not significantly above the background. A list of mutants with relevant characteristics is given in Table 2.

Transformation of Nar mutants. Transformation of wild-type strains R2 and R2-SPc was according to published procedures (25, 26). When antibiotics were used for selection, the number of cells that could be plated was limited to 5×10^7 to 2×10^8, depending on the antibiotic. The use of higher cell concentrations resulted in incomplete killing of untransformed cells. With auxotrophic mutants, the number of cells that could be plated was limited only by the reversion frequency; therefore, 5×10^8 cells could be applied per plate

TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristics	Reference or source
pUH24	8.0 kb; indigenous plasmid of *A. nidulans* R2	41
pUH25	50 kb; indigenous plasmid of *A. nidulans* R2	41
pCH1	12.4 kb; Ap^r; pUH24::Tn*901*	41
pPUC29	14.2 kb; Ap^r Cm^r; shuttle cosmid vector	40
pUC13	6.9 kb; Sm^r; cyanobacterial vector	26
pUC303	10.9 kb; Sm^r Cm^r; shuttle vector	26
pACYC177	3.6 kb; Ap^r Km^r	6
pACYC184	4.0 kb; Tc^r Cm^r	6
pDD47	pBR322 with 4.8-kb insert with *E. coli chlC* locus	2
pDD48	pBR322 with 11.6-kb insert with *E. coli chlC* locus	2
pDD100	pBR322 with 2.9-kb insert with *E. coli chlA* locus	13
pNR1	Sm^r; pUC13 with a *Sal*I fragment from R2 cloned into the *Xho*I site; transforms Nar1 to wild type	This study
pNR12	48 kb; Ap^r Cm^r; cosmid containing a 19-kb *Sal*I fragment which transforms Nar1 to wild type	This study
pNR1211	Ap^r; pACYC177 with a 4.9-kb *Xho*I fragment from pNR12, which transforms Nar1 to wild type	This study
pNRT63	24 kb; Tc^r Ap^r; pACYC184 with a 20-kb *Eco*RI fragment from Nar6 containing Tn*901*	This study
pNRT631	Ap^r Cm^r; pACYC184 with 9.2-kb *Sal*I fragment from pNRT63 which contains Tn*901*	This study
pNR63	50 kb; Ap^r Cm^r; cosmid containing a 5.1-kb *Sal*I fragment of R2 which transforms Nar6 to wild type	This study
pNR631	Cm^r; pACYC184 with the 5.1-kb *Sal*I fragment from pNR63, which transforms Nar6 to wild type	This study

without background problems. Mutants were grown in supplemented medium to a concentration of 5×10^7 cells per ml, washed in BG-11 medium, and concentrated. Usually, 10^9 cells were incubated with 200 ng of DNA in a total volume of 0.3 ml. A volume of 0.1 ml was then plated. For transformations with chromosomal DNA, R2 DNA was digested with *Bam*HI, *Bgl*II, *Sal*I, or *Xho*I, either completely or partially. The digests were size fractionated on sucrose gradients, and each fraction was transformed separately. Transformants were scored as fast-growing green colonies in a lawn of untransformed cells. Transformation frequencies were expressed as the ratio of transformants to total CFU.

Enzyme assays. Nitrate reductase activity was assayed either in whole cells or in sonicated extracts essentially as described previously (23). Mutants were grown for 3 days in BG-11 medium with 7 mM sodium nitrite. Cells were washed and resuspended in BG-11 medium. After overnight incubation under growth conditions, the cultures turned yellow, indicating that the nitrite reserves were exhausted. The cell concentration was measured in a cell counter. Sonicated extracts and subcellular fractions were prepared according to Manzano et al. (28). Enzyme activities were expressed as nanomoles of nitrite formed per 10^{10} cells per minute. Blank values were obtained by incubation of the reaction mixtures under identical conditions, except that the electron donor, methyl viologen, was oxidized before the addition of the fraction to be tested.

Recombinant DNA procedures. Gel electrophoresis, blotting, and cloning of fragments were performed according to standard procedures. DNA fragments to be used as probes were separated from the vector on 0.8% agarose gels and recovered by electroelution. Nick-translation of probes was with [α-^{32}P]dCTP to a specific activity of at least 10^7 dpm/μg of DNA.

A cosmid gene bank was constructed by the vector arms method of Ish-Horowicz and Burke (24). The shuttle cosmid pPUC29 (40) was digested with *Eco*RI or *Kpn*I and dephosphorylated with calf intestine alkaline phosphatase. The two digests were combined and, after digestion with *Sal*I, were ligated with *Sal*I-digested, size-fractionated R2 chromosomal DNA. The ligation mixture was packaged into λ particles and transduced into HB101. Transductants were obtained with an efficiency of 40,000 per μg of insert DNA. Colony hybridizations were done according to Grosveld et al. (18), except that in the prehybridization buffer, denatured pPUC29 was present to minimize hybridization between probe and cosmid vector.

RESULTS

Transformation of Nar1 and Nar6 with chromosomal DNA; shotgun cloning of the *nar-1* wild-type gene. *A. nidulans* R2 is easily transformed by chromosomal DNA (35). This means that DNA can be taken up as linear molecules and integrated in the chromosome via homologous recombination. Alternatively, a gene can be introduced into the mutant cell on an autonomous replicon, and the wild-type phenotype can be restored by complementation. In our strategy, mutants were first transformed with various fractions of wild-type chromosomal DNA, selection being made for the wild-type phenotype. Subsequently, a fraction with high transformation efficiency was ligated into a cyanobacterial vector, transformed into the mutant with selection for both the antibiotic resistance of the vector and the wild-type phenotype, thus selecting for complementation of the mutation by the corresponding wild-type gene present on the vector.

Chromosomal DNA from R2, digested completely or partially with four enzymes and fractionated as described above, was transformed into strains Nar1 and Nar6. The only selection applied was for Nar$^+$. Fractions giving the highest transformation frequencies were ligated into the cyanobacterial vector pUC13 and again transformed into the two mutants. In this case, there was an additional selection for streptomycin resistance (Sm^r), the plasmid marker. With Nar1, transformants were obtained at a frequency of 10^{-8}. With Nar6, no such Sm^r Nar$^+$ transformants were found, despite considerable effort. From six Nar1 transformants, plasmid DNA was isolated and retransformed into Nar1. The appearance of streptomycin-resistant green colonies at high frequency indicated that the *nar-1* wild-type gene was on the plasmid. The next step concerned the physical characterization of these plasmids. However, they were isolated in such low quantity that a direct analysis proved impossible. Direct transfer of the recombinant plasmids to *E. coli* would be an easy way to overcome this difficulty, as plasmid DNA can readily be isolated from this organism. Unfortunately, unlike the recently constructed shuttle vector pUC303, pUC13 is unable to replicate in *E. coli*; therefore, transfer of the recombinant plasmids to *E. coli* was impossible (26). Instead of repeating the experiment with pUC303, we chose an alternative approach for obtaining large quantities of the fragment with the *nar-1* gene. One of the Sm^r *nar-1*$^+$

plasmids, pNR1, was nick translated and used as a probe to search among approximately 1,000 colonies from a cosmid bank of R2 chromosomal DNA. Three colonies were found that contained DNA capable of transforming Nar1. One of these cosmids, pNR12, was studied further. *Sal*I fragments were isolated, and the transforming activity was located on the 19-kilobase (kb) A fragment. Further analysis of this fragment showed that the transforming activity resided in a 4.9-kb internal *Xho*I fragment, which was subsequently subcloned into pACYC177 to give pNR1211 (Fig. 1). This 4.9-kb fragment hybridized to genomic blots of R2 DNA with bands of the expected size (Fig. 2).

Cloning of the *nar-6* wild-type gene. As shotgun cloning failed to give positive clones in the case of the Nar6 mutant, we chose an alternative method, which can be used for transposon-mediated mutations (40). The strategy is as follows: (i) digestion of the chromosomal DNA of the mutant with an enzyme that does not cut in Tn*901*; (ii) cloning of the fragments in an *E. coli* vector and, after transformation, selection for the antibiotic resistance of the transposon in *E. coli*; and (iii) isolation of the wild-type gene from a cosmid gene bank of *Anacystis* DNA, using the fragment containing the transposon-inactivated gene as a probe. Total DNA from mutant Nar6 was digested with *Eco*RI, which does not cleave in the transposon. The fragments were cloned into pACYC184, selection being made for ampicillin resistance (Ap^r), the transposon marker. In this way, pNRT63 was obtained which contained a 20-kb *Eco*RI insert. The transposon was located in a 9.2-kb *Sal*I fragment which was subcloned into pACYC184 to give pNRT631 (Fig. 1). Evidence that these plasmids carried the *nar-6* mutation and did not originate from a secondary transposition event came from the following experiment. pNRT63 was transformed into *A. nidulans* R2-SPC. After ampicillin selection, approximately 75% of the colonies had the same phenotype as Nar6. One such colony was kept so that we would have a Nar6 strain without resident small plasmid. The strain was designated R2-SPC Nar6.

With pNRT63 as a probe, several cosmids were found that were able to transform Nar6 to the wild-type phenotype. One of them, pNR63, was analyzed further. It contained a *Sal*I fragment with the expected size of 5.1 kb, i.e., the size of the pNRT631 insert minus the size of the transposon. The 5.1-kb fragment was cloned into pACYC184 to give pNR631

TABLE 2. Relevant characteristics of *A. nidulans* R2 nitrate reductase mutants

Strain	Mutagen	Small plasmid content	Nitrate reductase activity[b]	Proposed genetic designation
Wild type (R2)		pUH24	191	
Nar1	NTG[a]	None	1.2	*narA1*
Nar4	NTG	None	0.0	*narA4*
Nar6	Tn*901*	pCH1	0.1	*narB6*
Nar7	Tn*901*	pCH1	1.2	*narB7*
Nar8	Tn*901*	pCH1	4.2	*narA8*
Nar10	Tn*901*	pCH1	0.0	*narA10*
Nar13	Tn*901*	pCH1	0.5	*narA13*
Nar15	Tn*901*	pCH1	0.4	*narB15*
Nar18	Tn*901*	pCH1	0.0	*narB18*
Nar19	Tn*901*	pCH1	4.0	*narC19*

[a] NTG, *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine.

[b] Nitrate reductase activity was measured in sonicated extracts as described in the text; activity is expressed as nanomoles of nitrite formed per 10^{10} cells per minute.

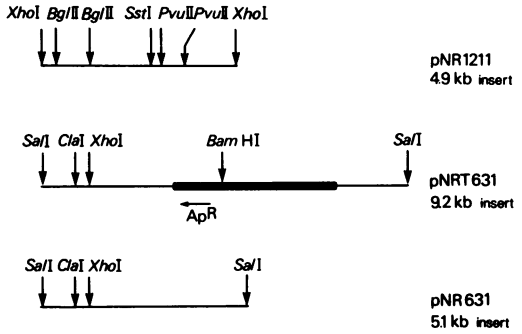

FIG. 1. Restriction map of cloned nitrate reductase genes.

(Fig. 1). This plasmid also transformed Nar6 to wild-type phenotype.

Southern analysis showed that the insert of pNR631 hybridized to a unique R2 chromosomal fragment of the expected size (Fig. 3).

Characterization of the mutants. The other Nar mutants listed in Table 2 were transformed with pNR12 and pNR63 to yield information about the relationship between the mutations (Table 3). Mutants Nar1, 4, 8, 10, and 13 were transformed to wild type by pNR12 and its subclone, and mutants Nar6, 7, 15, and 18 were transformed to wild type by pNR63 and its subclone. Mutant Nar19 was transformed by neither plasmid, but was transformed by wild-type chromosomal DNA and therefore is not a transformation-negative mutant. Thus, the mutants can be grouped into three classes representing distinct loci. For two loci, the corresponding wild-type genes are present on pNR12 and pNR63, respectively. Southern analysis showed that both genes were located on unique chromosomal fragments with no homology to each other (Fig. 2 and 3).

Experiments were carried out to assign a specific function to each of the genes. At first, plasmids containing the *E. coli* genes *chlA* and *chlC* were transformed to Nar1, Nar6, and Nar19; no transformants were obtained. Mutations could affect either the structural gene for the apo-enzyme or the biosynthesis, processing, or insertion of the molybdenum cofactor. In other organisms, it has been shown that cofactor mutants exhibit pleiotropic effects by the fact that other molybdo-enzymes are also affected (29, 33, 39). Our mutants grew readily in the presence of nitrite, which could argue against cofactor mutations. However, no data are available on the presence of other molybdenum-containing enzymes in cyanobacteria. In tobacco, *Aspergillus*, and *E. coli*, extracts of different mutants are capable of in vitro complementation (11, 14–16, 30). Furthermore, some cofactor mutants can be supplemented by increasing the molybdenum concentration in the medium (16, 31). Cell-free extracts of mutants Nar1, Nar6, and Nar19 were not capable of in vitro complementation. Also, no stimulation of enzyme activity was seen when the mutants were grown in 1 mM Na_2MoO_4 (data not shown).

DISCUSSION

Nitrate reductase mutants were isolated after chemical or transposon Tn*901* mutagenesis. For two mutants, the corresponding wild-type genes were cloned by using different procedures. In the case of Nar1, wild-type DNA inserted in a cyanobacterial vector was transformed into the mutant, and after double selection for both vector marker and Nar^+ phenotype, plasmids were obtained carrying the wild-type

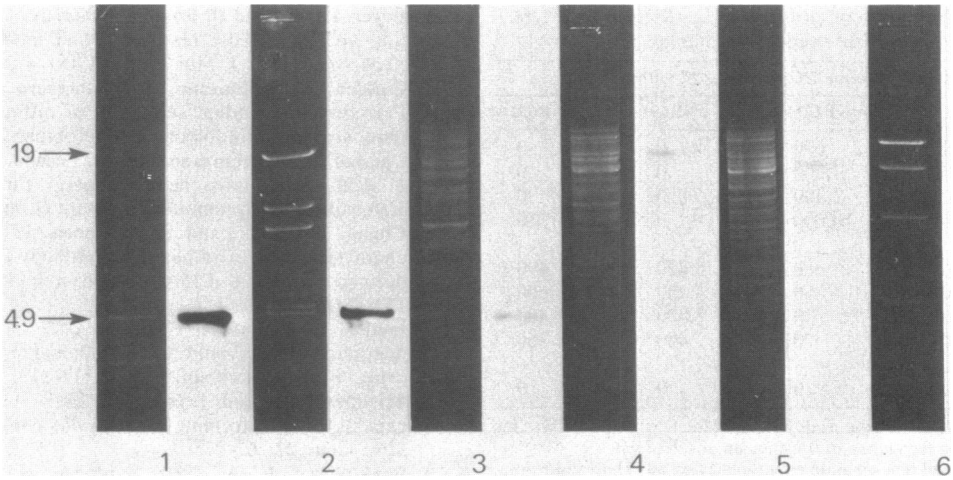

FIG. 2. Southern blot analysis of the *nar-1* gene. The left part of each panel represents a 0.8% agarose gel; the right part represents an autoradiogram of the Southern imprint, probed with the insert fragment of pNR1211. Lanes: 1, pNR1211, *Xho*I; 2, pNR12, *Xho*I; 3 to 5, R2 chromosomal DNA digested with *Xho*I, *Sal*I, and *Eco*RI, respectively; 6, pNR63, *Sal*I. No hybridization was observed with pUH24 and pUH25 (data not shown).

nar-1 gene. One such recombinant plasmid was used to identify cosmid pNR12. This procedure was unsuccessful in the case of Nar6, most likely because the presence of plasmid pCH1 interferes with an incoming recombinant plasmid (25, 26). For the Tn*901*-induced mutant Nar6, the transposon-containing fragment was cloned into pACYC184 in *E. coli*. This fragment was then used to search for the wild-type gene in a cosmid gene library, yielding cosmid pNR63. Transformation of the two cloned genes into a set of Nar mutants showed that all but one were transformed either by pNR12 or by pNR63. Only mutant Nar19 was transformed by neither plasmid and thus represents a third locus.

Some physiological aspects of the nitrate reductase mutants were analyzed. They all showed the following characteristics: (i) slow growth and yellow color on nitrate medium, (ii) complete restoration of growth and color after addition of nitrite to the medium, (iii) no detectable activity of the enzyme nitrate reductase, (iv) no stimulation of enzyme activity by 1 mM Na_2MoO_4 in the medium, and (v) no in vitro complementation of enzyme activity after mixing of cell-free extracts. In *E. coli* and *Aspergillus*, the two organisms in which the genetics of nitrate reduction has been studied in great detail, it has been established that besides structural genes for the apo-enzyme and regulatory genes, a number of genes are involved in the biosynthesis and insertion of the molybdenum-containing cofactor. As our preliminary physiological experiments with the *Anacystis* mutants have not yet provided evidence for the nature of the affected genes, further analysis is needed. Especially, the probing of mutants and cloned genes with specific antibodies seems feasible.

Cloning of a gene by selection for phenotypic complementation of a mutant is in principle a very simple and straightforward approach. It requires the stable maintenance of the cloned wild-type gene on the plasmid vector, the mutant gene being present on the chromosome. In a recombination-proficient host such as *Anacystis*, all kinds of interaction between the plasmid and the chromosome are conceivable. Our studies show that the *nar* genes transform the mutants with equal efficiency, irrespective of whether the genes are present on an *E. coli* vector or on a hybrid vector with the capacity of autonomous replication in *A. nidulans* (Table 3). Because such an additional capacity of autonomous replication does not cause an increase in transformation frequency,

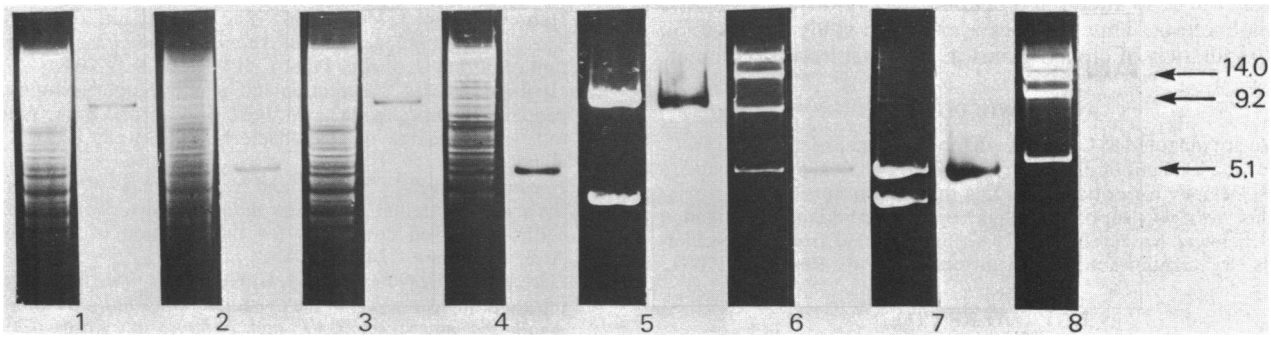

FIG. 3. Southern blot analysis of the *nar-6* gene. The left part of each panel represents a 0.8% agarose gel; the right part represents an autoradiogram of the Southern imprint, probed with the insert fragment of pNR631. Lanes: 1 to 4, chromosomal DNAs of Nar6, R2, SPc-Nar6, and SPc; 5, pNRT631; 6, pNR63; 7, pNR631; 8, pNR12. All DNAs were digested with *Sal*I. The hybridization observed in lane 8 is at the position of pPUC29 and is due to contamination of the probe with vector DNA. No hybridization was observed with pUH24 and pUH25 (data not shown).

TABLE 3. Transformation of cloned *nar-1* and *nar-6* wild-type genes into nitrate reductase mutants

Mutant	Transformation frequency (10^6)[a] with plasmid:			
	pNR12	pNR1211	pNR63	pNR631
Nar1	1,300	690	0	0
Nar4	4,200	4,200	0	0
Nar8	4,200	2,300	0	0
Nar10	8,300	10,000	0	0
Nar6	0	0	420	490
Nar7	0	0	3,200	5,600
Nar15	0	0	7,800	4,400
Nar18	0	0	480	360
Nar19	0	0	0	0

[a] Transformation was carried out as described in the text; selection was for Nar^+. A transformation frequency of 0 means an absolute value of $<0.05 \times 10^{-6}$. Nar13 was screened in a separate experiment and was shown to belong to the Nar1 group.

it seems likely that integration into the chromosome is the preferred mechanism by which transformation to wild type takes place. This is confirmed by a previous study with a methionine gene cloned on a shuttle vector (40). It was shown that if selection was carried out only for the wild-type marker, the vector could not be recovered and rapid integration of the wild-type gene into the chromosome had to be assumed. Williams and Szalay (42) have performed a detailed study on the integration of foreign DNA interrupting *Anacystis* chromosomal sequences; in a great majority of the cases, the DNA was integrated via an allele replacement mechanism. These observations imply that for stable maintenance of a cloned gene on a plasmid vector, double selection for both the vector and the gene to be cloned is needed. Because in shotgun cloning only a very small portion of the transformants that take up the vector will contain the desired chromosomal fragment cloned into that vector, shuttle vectors with a high transformation capacity are obligatory. Therefore, the usefulness of the recently constructed cloning vectors depends directly on their transformation efficiency (8, 12, 17, 25, 26, 34).

This study shows the successful application of molecular cloning techniques to the analysis of the nitrate reductase system of *A. nidulans* R2. In principle, any gene for which a mutant is available can be cloned in this way. The techniques described here allow not only the cloning of cyanobacterial genes, but also their modification and reintroduction into cyanobacteria. This provides a means to study the function and regulation of cloned genes in their natural background.

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