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Cloning of a third nitrate reductase gene from the cyanobacterium *Anacystis nidulans* R2 using a shuttle cosmid library

(Photosynthesis; blue-green algae; mutants; complementation; recombinant DNA; phage λ vector; plasmid)

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SUMMARY

A strategy for gene cloning in the cyanobacterium *Anacystis nidulans* R2 was developed which made use of a gene library constructed in a shuttle cosmid vector. The method involved phenotypic complementation of mutants with pooled cosmid DNA. The development of the procedure and its application to the cloning of a third gene involved in nitrate reduction are described.

INTRODUCTION

Cyanobacteria form a group of prokaryotes characterized by the capacity to perform an oxygen-evolving, plant-like type of photosynthesis. Both cyanobacteria and plants can fulfil their nitrogen requirement by the assimilation of inorganic nitrogen, notably nitrate (Guerrero et al., 1981; Beevers and Hageman, 1983). The key enzyme in nitrate assimilation is nitrate reductase, a molybdo-protein, which catalyses the first and rate-limiting step in the pathway, the conversion of nitrate into nitrite. Nitrate reduction has been studied in some detail in the unicellular cyanobacterium *Anacystis nidulans* (Guerrero et al., 1981). The enzyme nitrate reductase consists of a single polypeptide with an M_r of 75 000 and contains a molybdenum cofactor. Reduction of nitrate can be performed in isolated thylakoids with H_2O as the electron donor and thus is truly photosynthetic in nature. Approx. 20% of the reducing power generated by photosynthesis is used for the reduction of nitrate, the rest is consumed during carbon dioxide fixation (Losada et al., 1981). Ammonium inhibits enzyme activity, not directly, but after its conversion to glutamine by the enzyme glutamine synthetase (Herrero et al., 1981). The activity is stimulated by carbon dioxide and a regulation mechanism monitoring the C/N balance in the cell has been proposed (Flores et al., 1983).

It is our aim to perform a genetic study of nitrate reduction in *A. nidulans* R2. Mutants affected in nitrate reduction are readily isolated from cyanobacteria, either after chemical mutagenesis or by

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Abbreviations: Ap, ampicillin; Cm, chloramphenicol; EtBr, ethidium bromide; kb, kilobase pairs; LB, Luria broth; ^{R} (superscript), resistance; SDS, sodium dodecyl sulfate; T/0.1E, see MATERIALS AND METHODS, section c; Tc, tetracycline; Tn, transposon; [ ], indicates plasmid-carrier state.

direct selection for chlorate resistance (Herdman, 1982; Bagchi and Singh, 1984). They exhibit a characteristic yellow colour due to breakdown of phycobilisomes, the so-called nitrogen chlorosis (Allen and Smith, 1969). We recently reported the isolation of nitrate reductase mutants and the cloning of two nitrate reductase genes from *A. nidulans* R2 (Kuhlemeier et al., 1984). One gene was cloned by complementation of the corresponding mutant, the other via selection for the transposon which caused the mutation. Transformation of a series of other mutants using the two cloned wild-type genes showed that all but one were transformed by the cloned genes. One mutant, Nar19, was not transformed by any of the cloned genes and thus represents a third locus (Kuhlemeier et al., 1984). Here we present the cloning of the third gene involved in nitrate reduction by an improved complementation procedure. The methods described previously have several disadvantages. Cloning a gene by first isolating the inactive gene is only possible for transposon mutants (Tandeau de Marsac et al., 1982; Kuhlemeier et al., 1984). Even with transposon-induced mutants complications can arise from DNA instability accompanying transposition. Complementation of mutants using pUC13 was complicated by the fact that this vector is unable to replicate in *E. coli* (Kuhlemeier et al., 1984). Our new procedure relies on complementation of mutants and involves the use of a shuttle cosmid library. This has several advantages compared to the previous procedures. The method is not restricted to transposon-induced mutants and the use of cosmids ensures that each vector molecule contains a large piece of chromosomal DNA. Furthermore, once the desired recombinant cosmid has been selected in the mutant, it can easily be transferred to *E. coli* because of the shuttle character of the cosmid vector.

MATERIALS AND METHODS

(a) Strains and plasmids

Strains used in this study were *A. nidulans* R2 (PCC7942) and its derivative R2-SPc, which is cured of the small resident plasmid pUH24 (Kuhlemeier et al., 1983). Culture conditions were as described (Van den Hondel et al., 1979). Nitrate reductase mutants (Kuhlemeier et al., 1984) were grown in BG-11 medium supplemented with 7 mM sodium nitrite. All mutants exhibited slow growth and yellow colour on BG-11 medium, complete reversion of phenotype upon the addition of nitrite and no detectable nitrate reductase enzyme activity in cell-free extracts. The three distinct loci, inferred from transformation experiments, have been named *narA*, *narB* and *narC*. A list of strains and plasmids is given in Table I. The nomenclature is such that the mutant and the corresponding plasmids have the same numbers (e.g., Nar19, pNRT192, pNR193). pNR denotes plasmids with wild-type genes, pNRT plasmids with mutant genes.

(b) Transformation of *A. nidulans* R2

Transformation conditions were essentially as described (Van den Hondel et al., 1980; Kuhlemeier et al., 1981). Cells were grown to 5×10^7/ml in BG-11 medium, washed and concentrated. The medium was supplemented with 7 mM sodium nitrite for growth of Nar mutants. In 0.3 ml, 10^9 cells were incubated with 200 ng of DNA, unless stated otherwise. Nar^+ transformants were scored as green colonies on a faint-yellow lawn. For Cm^R selection the plates were incubated overnight under growth conditions before the addition of the antibiotic. Cm (0.5 ml with a concentration of 0.75 mg/ml) was underlayered to allow slow adaptation of the transformants to increasing concentrations of the drug. For the construction of Nar mutants in R2-SPc (see RESULTS, section **b**) varying concentrations of Ap were used (0.5 ml with a concentration ranging from 20 to 70 μg/ml was underlayered) as this antibiotic is not an easy selective agent (Kuhlemeier et al., 1981). In this way, the lowest concentration not giving colonies on control plates was established. The plates always contained at least 55 ml of agar to prevent desiccation. Nar^+ colonies became visible after 3-5 days, Cm^R Nar^+ colonies one day later. Transformation frequencies were expressed as the ratio of transformants to total colony forming units.

(c) Isolation of *A. nidulans* DNA

For chromosomal DNA isolation the cells were grown to 2×10^8 cells/ml in batches of 50 to

TABLE I

Strains and plasmids

Strain or plasmid	Relevant characteristics	Reference
***A. nidulans* strains**		
R2	Wild-type strain, contains two cryptic plasmids, pUH24 (8.0 kb) and pUH25 (50 kb)	Van den Hondel et al. (1980)
R2-SPc	R2 cured of pUH24	Kuhlemeier et al. (1983)
R2[pCH1]	R2 in which pUH24 is replaced by pCH1	Van den Hondel et al. (1980)
Nar1	nitrate reductase mutant, R2-SPc *narA*1, selected by NTG-mutagenesis of R2-SPc	Kuhlemeier et al. (1984)
Nar6	nitrate reductase mutant, R2[pCH1]*narB*6, selected by Tn*901* mutagenesis	Kuhlemeier et al. (1984)
Nar6/4	derivative of Nar6, constructed in R2-SPc	Kuhlemeier et al. (1984)
Nar19	nitrate reductase mutant, R2[pCH1]*narC*19, selected by Tn*901* mutagenesis	Kuhlemeier et al. (1984)
Nar19/2	derivative of Nar19, constructed in R2-SPc	This study
Nar19/6	as Nar19/2	This study
Plasmids		
pCH1	pUH24::Tn*901*	Van den Hondel et al. (1980)
pACYC184	4.0-kb, Cm^R Tc^R, *E. coli* vector	Chang and Cohen (1978)
pPUC29	14.0-kb, Cm^R Ap^R, shuttle cosmid vector	Tandeau de Marsac et al. (1982)
pNR12	48-kb, Cm^R Ap^R, cosmid containing *narA*$^+$ gene	Kuhlemeier et al. (1984)
pNR63	50-kb, Cm^R Ap^R, cosmid containing *narB*$^+$ gene	Kuhlemeier et al. (1984)
pNRT631	Cm^R Ap^R, pACYC184 with 9.2-kb *Sal*I fragment containing *nar*B6::Tn*901*	Kuhlemeier et al. (1984)
pNRT13-16	cosmids containing *narA*$^+$ gene	This study
pNRT191	Ap^R Tc^R, pACYC184 with 2.8-kb and 0.75-kb *Eco*RI fragments from Nar19 total DNA	This study
pNRT192	as pNRT191 but with 2.8-kb insert only	This study
pNRT194	as pNRT191 but with 2.8-kb and 1.5-kb inserts	This study
pNR193	49-kb, Cm^R Ap^R, cosmid containing *narC*$^+$ gene	This study
pNR1934	Cm^R, pACYC184 with 6.1-kb *Sal*I fragment containing *narC*$^+$ gene	This study

4000 ml. Cultures were spun down, washed with 0.9% NaCl/50 mM EDTA and incubated for 1 h at 37°C with lysozyme (5 mg/ml in 25 mM Tris · HCl, pH 8.0, 10 mM EDTA, 50 mM glucose). Subsequently, SDS was added to a final concentration of 3% and after 1 h at 37°C, sodium perchlorate was added to give a concentration of 0.5 M. The lysed cells were extracted three times with chloroform/isoamyl alcohol (24:1 v/v) and the remaining aqueous phase was precipitated with ethanol. The pellet was dissolved in T/0.1 E (10 mM Tris pH 8.0, 0.1 mM EDTA) and banded in a CsCl + EtBr gradient. The upper band was collected, diluted threefold with T/0.1 E and ethanol-precipitated. The yield was approx. 1 mg of DNA per liter of culture.

Isolation of plasmid DNA from transformants followed the small-scale method by Kuhlemeier et al. (1981). For pUH24-derivatives, usually about 0.5 μg of plasmid was derived from 5×10^9 cells.

(d) Recombinant DNA procedures

Manipulations with recombinant DNA followed standard procedures. Restriction fragments were recovered from agarose gels by electro-elution. Nick-translation of probes with [α-^{32}P]dCTP was to a specific activity of at least 10^7 dpm/μg DNA. The preparation of a cosmid DNA library of partially cleaved *Sal*I fragments in the shuttle vector pPUC29 has been described (Kuhlemeier et al., 1984). Plasmid DNA was isolated from a mixture of 4000 colonies, which represents 40–50 genome equivalents. Transduction of cosmids was as follows. DNA was packaged into λ particles and diluted to 0.5 ml. A volume of 0.1 ml was added to 0.2 ml of *E. coli* HB101 cells grown to late log phase and 0.5 ml of LB medium was added. After 30 min at 37°C for phenotypic expression 0.1 ml was plated on LB plates with 100 μg Ap/ml. For transformation of *E. coli* strain

K-12-803 was used (Tandeau de Marsac et al., 1982).

(e) Nitrate reductase assay

Nitrate reductase was assayed essentially as described (Herrero et al., 1981). Mutants were grown for three days in BG-11 medium supplemented with 7 mM sodium nitrite. Cells were then washed with BG-11 medium and resuspended in an equal volume of BG-11. 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. Enzyme activities were expressed as nmol nitrite formed/10^{10} cells/min. Blank values were obtained by incubation of the reaction mixture under identical conditions except that the electron donor, methyl viologen, was oxidized before the addition of the fraction to be tested.

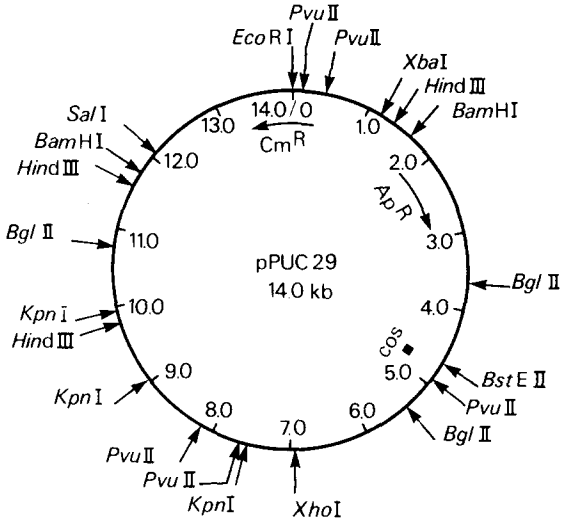

Fig. 1. Restriction map of the shuttle cosmid vector pPUC29. The vector is composed of pUC104 (Kuhlemeier et al., 1981) and a 1.5-kb *Bgl*II fragment from pHC79 (Hohn and Collins, 1980) containing the λ cos site. pPUC29 has five unique restriction sites, for *Eco*RI, *Xba*I, *Bst*EII, *Xho*I and *Sal*I. The construction has been described in Tandeau de Marsac et al. (1982).

RESULTS

(a) Development of the procedure

To ensure that a shuttle cosmid library could indeed be employed for the cloning of genes, the whole complementation procedure was first tested on the *narA* gene, which had been cloned previously (Kuhlemeier et al., 1984). Subsequently, the method was applied to the *narC* gene which had been resistant to cloning so far. The procedure was as follows. (1) Preparation of a cosmid gene library in the shuttle vector pPUC29 (Fig. 1), i.e., a plasmid capable of replication in *A. nidulans* and in *E. coli* and containing the λ cos site. (2) Transformation of the mutant of interest using plasmid DNA from a mixture of cosmids, selection being made for both vector and restoration of wild-type phenotype. (3) Isolation of plasmid DNA from the transformants and transfer of the DNA to *E. coli* via in vitro packaging into λ particles. (4) Isolation of plasmid DNA from the transductants and reintroduction into the mutant to confirm the presence of the wild-type gene.

For a successful operation of this cloning procedure several criteria had to be met a priori. In the first place no small plasmid should be present in the mutant strain. As all our vectors have homology with pUH24, recombination would probably take place and the physical linkage between vector and wild-type marker would be lost (Kuhlemeier et al., 1981). This requires the use of plasmid-cured strains. In the second place, simultaneous selection for vector and wild-type must be possible to minimize recombination of the insert into the chromosome (Kuhlemeier et al., 1984). As most of our mutants were generated by transposon Tn*901* (Ap^R) mutagenesis, Ap could not be used for vector selection. Therefore, a cosmid gene bank was used which has R2-chromosomal fragments inserted into the *Sal*I site of the vector. This leaves the Cm^R gene intact for vector selection.

To test the system, mutant Nar1 was transformed with the mixture of cosmid DNAs. This mutant is devoid of a small resident plasmid as it was isolated after chemical mutagenesis of R2-SPc. Cm^R Nar^+ transformants were obtained with Nar1 as well as with Nar6/4, a cured derivative of Nar6 (Table II). Five of the Nar1 transformants were further analysed and compared with the previously isolated cosmid pNR12. Plasmid DNAs were prepared from 50-ml cultures and run on an agarose gel. A putative cosmid would probably comigrate with the

TABLE II

Transformation of Nar mutant strains with cosmid DNA isolated from an *A. nidulans* R2 gene library

Transformation was carried out as described under MATERIALS AND METHODS, section **b**. Approx. 5 μg of DNA isolated from a pooled *A. nidulans* R2 cosmid library was used per transformation. Values in parentheses indicate the actual number of transformants per plate.

Recipient strain[a]	Transformation frequency ($\times 10^{-6}$)	
	Nar$^+$ selection	Nar$^+$ Cm^R selection
Nar1	>100	0.1 (9)
Nar6/4	16	0.03 (7)
Nar19/2	9.1	2.5 (160)
Nar19/6	21	2.3 (280)

[a] See Table I.

indigenous plasmid pUH25 (50 kb) and indeed no additional bands were seen. Transformation of Nar1 by the DNA preparation yielded green transformants in all cases (Table III). Subsequently, the DNA was packaged into λ particles and used to transduce *E. coli* HB101. Four out of five preparations gave transductants at a variable frequency (Table III).

TABLE III

Transformation and transduction with DNA isolated from Nar$^+$ Cm^R transformants

Transformation and transduction were carried out as described under MATERIALS AND METHODS, section **b,c**. Plasmid DNA was isolated from *A. nidulans* Nar$^+$ Cm^R transformants (Nar1-1 through Nar1-5) and used to transform Nar1. DNA from these preparations was also packaged into λ particles and used to transduce *E. coli* HB101. As the concentration of recombinant cosmids in the DNA preparation was unknown, transduction frequencies were expressed as the number of transductants obtained with the DNA isolated from approx. 4×10^9 cells. The value for pNR12 was obtained by transducing 100 ng of the plasmid under identical conditions.

DNA donor	Transformation into Nar1 strain [frequency ($\times 10^{-6}$)]	Transduction into HB101 strain (frequency of Ap^R)
Nar1-1	55	12
Nar1-2	110	1.8×10^3
Nar1-3	55	<8
Nar1-4	110	1.2×10^4
Nar1-5	55	16
pNR12	>300	1.6×10^4

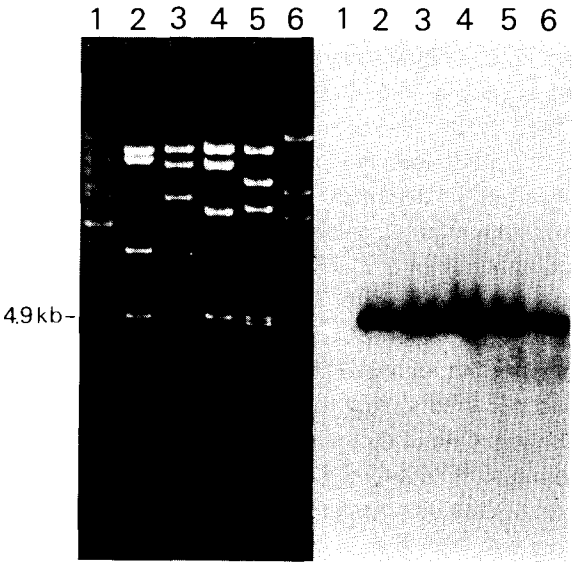

Fig. 2. Southern blot analysis of pNR12-like cosmids. DNAs were digested with *Xho*I and run on a 0.8% agarose gel (left panel) transferred to nitrocellulose paper and hybridized to the 4.9-kb insert fragment of pNR1211 (right panel). M_r markers were a λ *Hin*dIII digest combined with a *Hin*dIII + *Eco*RI double digest. Lane 1: R2 chromosomal DNA; lanes 2–5: pNR13–16; lane 6: pNR12. The 4.9-kb band in lane 1 of the right panel became more clearly visible after overexposure of the autoradiogram.

From each of the four positive transductions, plasmid DNA was analysed. All plasmids were 45–50 kb and contained a 4.9-kb *Xho*I fragment which hybridized with the corresponding band from pNR12 (Fig. 2). Each plasmid transformed Nar1 with a frequency comparable to pNR12 (not shown).

These experiments proved that the cloning procedure could work properly. The cosmids transformed the mutant at a frequency high enough to enable the selection of a particular cosmid from the total library and after passage through the cyanobacterium and transduction of *E. coli*, the intact fragment could be recovered.

(b) Cloning of the *narC* gene

To apply the same method to Nar19, one additional problem had to be settled beforehand. Mutant Nar19 was generated by transposon mutagenesis of *A. nidulans* R2 and contains plasmid pCH1. Therefore, the first step was to transfer the *narC* mutation to a small-plasmid-less strain. Total DNA was isolated from Nar19, digested with *Eco*RI, inserted into

pACYC184 and transformed to *E. coli* K-12-803. Ap^R Tc^R colonies were found containing a 2.8-kb insert and in some cases a second small fragment (Table I). Because the transposon is 4.2 kb in length and *Eco*RI does not cut it, a deletion must have occurred. To determine whether this 2.8-kb fragment was present in the same form in the Nar19 genome, a Southern blot of *Eco*RI-digested Nar19 DNA was performed. A pBR322 fragment containing the Ap^R gene hybridized with an over 20-kb fragment of Nar19 (and with pCH1), indicating that a deletion had occurred during transformation of *E. coli* (not shown). One of the plasmids, pNRT194, was used to transform R2-SPc. Despite careful Ap^R selection (see MATERIALS AND METHODS, sections **b** and **d**), only two Ap^R Nar^- transformants were obtained, whereas in the control experiment with pNRT631 hundreds of SPc-Nar6 transformants were found. The two mutants, designated Nar19/2 and Nar19/6 were not transformed by pNR12 or pNR63, suggesting that the mutation was not in *narA* or *narB*. Nar19/2, Nar19/6 and Nar19 had a nitrate-reductase enzyme activity of less than 1% of the wild-type.

The same procedure as for Nar1 was then followed for Nar19/2, and three cosmids, pNR192-194, were isolated. Cosmids pNR192 and 193 were identical, while pNR194 differed in the orientation of the vector. They transformed Nar19 as well as Nar19/2, but not Nar1 or Nar6. To locate the *narC* gene on the cosmid, pNR193 was digested with *Sal*I, the fragments were purified and separately used to transform Nar19/2. The transforming activity was found to reside on a 6.1-kb fragment, which was subcloned into pACYC184 to give pNR1934. A restriction map of this fragment is given in Fig. 3. Southern blot analysis was performed with the insert of pNR1934 as a probe (Fig. 4). The probe hybridized with a fragment of the same size in the chromosomal R2 DNA digested with *Sal*I, illustrating that the intact fragment was isolated. This fragment must be derived from the chromosome as none of the indigenous plasmids contains a band of this size (Laudenbach et al., 1983). With Nar19 DNA, the probe hybridized with a 22-kb *Sal*I fragment, indicating that the mutation did not arise from a simple insertion of the transposon (4.2 kb) into the wild-type fragment (6.1 kb). In *Eco*RI digests of Nar19 as well as Nar19/2 hybridization was at a position of approx. 22 kb. No hybridization was found with pNRT192 and with pNR12 or pNR63.

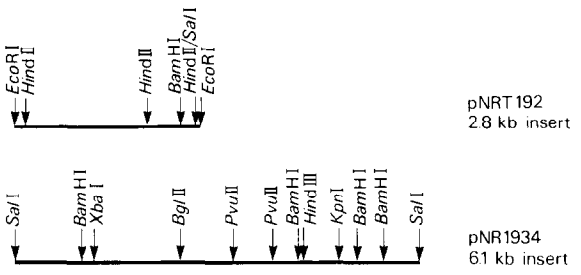

Fig. 3. Restriction maps of pNRT192 and pNR1934 insert fragments.

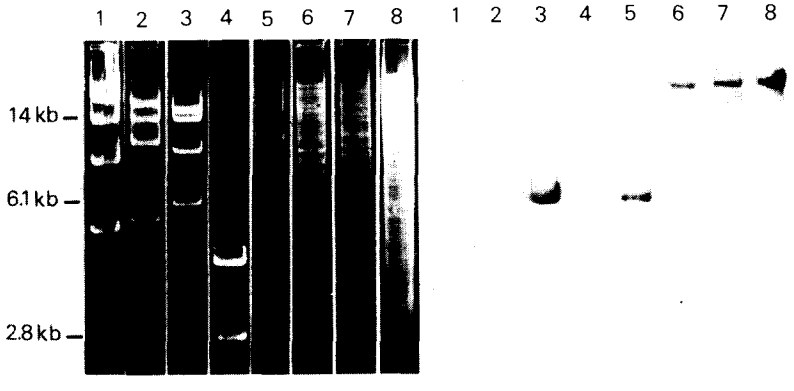

Fig. 4. Southern blot analysis of the *narC* gene. Restriction digests were run on 0.8% agarose gels (left panel), transferred to nitrocellulose paper and hybridized to the 6.1-kb insert of pNR1934 (right panel). Markers were the same as in Fig. 2. Lanes 1–3: pNR63, pNR12 and pNR193, respectively, digested with *Sal*I; lane 4: pNRT192 with *Eco*RI; lanes 5 and 6: R2 and Nar19 chromosomal DNAs with *Sal*I; lanes 7 and 8: Nar19 and Nar19/2 chromosomal DNAs with *Eco*RI. R2 DNA digested with *Eco*RI gave a signal at a position corresponding to a fragment of approx. 30 kb (not shown).

TABLE IV

Transformation of *nar* plasmids into Nar mutant strains

Transformation was carried out as described under MATERIALS AND METHODS, section **b**. Selection was for Nar^+ transformants. A frequency of 0 means an absolute value of $< 0.02 \times 10^{-6}$.

Recipient strains	Transformation frequency ($\times 10^{-6}$) with plasmids:			
	pNR12	pNR63	pNR193	pNR1934
Nar1	180	0	0	0
Nar6	0	370	0	0
Nar19	0	0	24	41
Nar19/2	0	0	630	320

Transformation experiments were carried out to confirm that pNR193 represented a third locus. Mutant Nar19 was transformed to wild-type by pNR193 and pNR1934, but not by pNR12 or pNR63. On the other hand, pNR193 and pNR1934 did not transform mutants Nar 1 and Nar 6 (Table IV). No in vitro complementation was achieved by mixing of mutant extracts (Kuhlemeier et al., 1984).

DISCUSSION

Gene cloning by complementation of mutants is in principle a relatively simple and straightforward procedure. Wild-type DNA, randomly inserted in a vector, is transferred to the particular mutant and the recombinant plasmid carrying the wild-type gene is selected. Recombination may, however, present a problem, as *A. nidulans* R2 has been shown to have a very active system for homologous recombination (Williams and Szalay, 1983). A first requirement is the availability of vectors without homology with host sequences. Cloning vectors have so far been constructed by modification of indigenous cryptic plasmids and recombination with the resident plasmid will interfere with their stability (Kuhlemeier et al., 1981). This problem was solved by curing the host of the indigenous plasmid (Kuhlemeier et al., 1983) and transferring the mutation to this cured strain. Homology between a gene to be cloned and the mutant gene present on the chromosome remains, however. Our data confirm that if selection is simultaneously carried out for both the vector part and the gene to be cloned, an intact recombinant containing the vector and the wild-type chromosomal fragment, can be recovered. The cloning strategy outlined at the beginning of RESULTS was shown to operate as expected in the case of the Nar1 mutant, which was chosen as a model system. Subsequent application of the method to the Nar19 mutant gave rise to several complications. No relationship could be demonstrated between the cloned 2.8-kb *Eco*RI fragment derived from Nar19 and the 6.1-kb *Sal*I fragment from R2 (Figs. 3 and 4). An assumption in line with the available data would be that the transposon initially inserted inside or near the 6.1-kb *Sal*I fragment and that afterwards a deletion occurred removing part of the 6.1-kb *Sal*I fragment. During transfer to *E. coli* a further deletion took place by a specific mechanism, since several independent isolates contained the same 2.8-kb *Eco*RI fragment. The lack of homology between the Ap^R fragment from the mutant and the wild-type *narC* fragment excluded cloning of the gene by colony hybridization, but the complicated nature of the mutation did not interfere with cloning via complementation. If it would have been impossible to obtain a cured Nar19 strain using pNRT194, an alternative would be to transform R2-SPc directly with Nar19 chromosomal DNA. However, in general this method is not advisable because the frequency of Ap^RNar^- transformants is expected to be very low.

Plasmids pNR193 and pNR1934 transformed Nar19 and its derivatives, but not Nar1 and Nar6, to wild-type. On the other hand, cosmids pNR12 and pNR63, which are representative for the *narA* and *narB* loci, did not transform Nar19 (Table IV). Together with the blotting data, these results confirm that at least three distinct single-copy chromosomal genes are involved in nitrate reduction in *A. nidulans* R2. About the function of each of the genes no conclusions can be drawn, except that they are not involved in nitrate uptake. Studies with the respiratory nitrate reductase system of *E. coli* (Stewart and MacGregor, 1982) and with the assimilatory nitrate reductases from fungi (Scazzocchio, 1980) have shown that besides the structural and regulatory genes several additional genes are involved in biosynthesis, processing and insertion of the molybdenum cofactor. Also in *Nicotiana*, genetic and

physiological experiments revealed the presence of several genetic loci (Negrutiu et al., 1983). Future research will be directed towards the characterization of the cloned genes by in vitro complementation and immunological methods.

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