Gene, 20 (1982) 111–119
Elsevier Biomedical Press

A new approach for molecular cloning in Cyanobacteria: cloning of an *Anacystis nidulans met* gene using a Tn*901*-induced mutant

(Recombinant DNA; gene library; shuttle cosmid vector; transposition mutants; methionine gene)

N. Tandeau de Marsac, W.E. Borrias *, C.J. Kuhlemeier *, A.M. Castets, G.A. van Arkel * and C.A.M.J.J. van den Hondel *,[a]

*Unité de Physiologie Microbienne, C.N.R.S. E.R.A. 398, Département de Biochimie et Génétique Moléculaire, Institut Pasteur, 28, rue du Docteur Roux, 75724 Paris Cedex 15 (France), and * Department of Molecular Cell Biology, State University of Utrecht, Padualaan 8, P.O. Box 80.056, 3508 TB Utrecht (The Netherlands)*

(Received July 8th, 1982)
(Accepted August 11th, 1982)

SUMMARY

A new strategy for molecular cloning in the cyanobacterium *Anacystis nidulans* R-2 is described. This strategy involved the use of a transposon and was developed for the cloning of a gene encoding methionine biosynthesis. A *met*::Tn*901* mutant was isolated. Chromosomal DNA fragments were cloned in the *Escherichia coli* plasmid vector pACYC184. A recombinant plasmid carrying the inactivated *met*::Tn*901* gene was selected after transformation to *E. coli*. The cloned *met*::Tn*901* DNA fragment was used as a probe to select the corresponding *A. nidulans* R-2 wild-type *met* gene from a gene library prepared in *E. coli*, using the newly constructed shuttle cosmid vector pPUC29. When transformed into *A. nidulans* Met$^-$ mutants, this cloned gene allowed the mutants to grow prototrophically.

INTRODUCTION

Cyanobacteria, also called blue-green algae, are the only prokaryotes that perform an oxygenic, "higher-plant" type, photosynthesis. In addition, some species are able to fix molecular nitrogen even under aerobic conditions (Stanier and Cohen-Bazire, 1977). It is thus of particular interest to develop molecular genetic studies within this group of microorganisms. Until now, genetic analysis has been extremely limited, since transformation, the only currently available tool, is restricted to six cyanobacterial strains: *A. nidulans* 602 PCC7943 (Shestakov and Khyen, 1970), *A. nidulans* PCC6301 (Herdman, 1973), *Aphanocapsa* PCC6714 (Astier and Espardellier, 1976), *Gloeocapsa alpicola* PCC6308 (Devilly and Houghton, 1977), *Agmenellum quadruplicatum* PCC7002

[a] Present address: Medical Biological Laboratory, T.N.O., P.O. Box 45, 2288 GJ Rijswijk (The Netherlands).

Abbreviations: Ap, ampicillin; CCC, covalently closed circular; Cm, chloramphenicol; kb, kilobase pairs; L, linear; OC, open circular; SDS, sodium dodecyl sulfate; Tc, tetracycline; U, units; ::, indicates novel joint; [ ], indicates plasmid-carrier state.

0378-1119/82/0000–0000/$02.75

(Stevens and Porter, 1980) and *Synechocystis* PCC6803 (Grigorieva and Shestakov, 1982). Mutants from *A. nidulans* PCC6301 have been isolated and biochemically characterized, but only very restricted genetic linkage maps have been established (Delaney et al., 1976; Ladha and Kumar, 1978; Doolittle, 1979). As an alternative to classical genetic analysis, we have begun to develop a molecular cloning system in *A. nidulans* R-2 (Van den Hondel et al., 1980).

The cyanobacterium *A. nidulans* R-2 harbours two plasmids, pUH24 (7.9 kb) and pUH25 (50.8 kb) which have, thus far, remained cryptic. The small plasmid pUH24 has been genetically marked by in vivo insertion of the TEM β-lactamase transposon Tn*901* (Van den Hondel et al., 1980). This recombinant plasmid pCH1 (pUH24::Tn*901*) efficiently transforms *A. nidulans* R-2, is stably maintained, and confers on the cells resistance to 1–2 μg/ml of ampicillin. In Ap^R transformants, pCH1 replaces the small plasmid pUH24, while the large plasmid pUH25 remains (Van den Hondel et al., 1980). Recently, two hybrid shuttle cloning vectors, pUC104 and pUC105, capable of transforming *E. coli* and *A. nidulans* R-2, have been constructed in vitro (Kuhlemeier et al., 1981). These vectors consist of the *E. coli* plasmid pACYC184 and the cyanobacterial plasmid pUC1 derived from pCH1 (part of pCH1 had been deleted in vitro so that pUC1 still encoded ampicillin resistance and that Tn*901* could not transpose any more). Plasmid pUC104 was used further to construct a cosmid vector (pPUC29, 14.2 kb), by in vitro insertion of the cohesive end DNA sequence of bacteriophage λ (to be published elsewhere). After insertion of foreign DNA, cosmid pPUC29 can be used as a plasmid to transform both *E. coli* and *A. nidulans* R-2 or can be packaged in vitro into λ particles, and introduced into *E. coli* cells by infection.

At present two cyanobacterial genes, *nifH* and *glnA* from *Anabaena cylindrica* PCC7120, sharing sufficient homology with the corresponding enterobacterial genes, have been isolated by means of heterologous DNA hybridization (Mazur et al., 1980; Fisher et al., 1981). In addition, ribosomal RNA genes have been cloned from *A. nidulans* PCC6301. The cloned rDNAs have been characterized by DNA-RNA hybridization and the R-loop technique (Tomioka et al., 1981). In this report, we describe an alternative cloning strategy which involves the use of a transposon as a physical marker. This method has been successfully applied to the cloning of a gene involved in methionine biosynthesis in *A. nidulans* R-2, and theoretically can be extended to the cloning of any cyanobacterial gene into which a transposon is inserted.

MATERIALS AND METHODS

(a) Strains and plasmids (or cosmids)

The plasmids, bacterial and cyanobacterial strains used in these experiments are listed in Table I.

E. coli strains were grown in LB medium, at 37°C with the exception of the thermosensitive strains BHB2688 and BHB2690 which were grown at 30°C. Solid media contained 15 g agar per liter. Ampicillin (50 μg/ml), tetracycline (10 μg/ml) or chloramphenicol (25 μg/ml) were added when growing bacteria that contained plasmids.

A. nidulans strains were grown in BG11 minimal medium (Rippka et al., 1979) supplemented with methionine 30 μg/ml or 200 μg/ml for MET1 and 602 Met^-, respectively. *A. nidulans* R-2[pCH1] and MET1 were grown with ampicillin (1 μg/ml). Solid media contained 10 g agar per liter. Cells were incubated at 30°C, under a light intensity of 3000 lux (white fluorescent light).

(b) Isolation of Met^- mutants induced by Tn*901*

A. nidulans R-2 Met^- mutants induced by transposition of Tn*901* were isolated as follows: *A. nidulans* R-2[pCH1] cells were grown to a density of 8×10^7 cells/ml, in minimal medium supplemented with methionine. Cells were collected by centrifugation and, after three washings, were resuspended to a concentration of 2×10^6 cells/ml, and incubated for 18 h in minimal medium, to deplete the endogenous methionine pool. As Tn*901* conferred on *A. nidulans* R-2 cells resistance to only 1–2 μg/ml of ampicillin, the cell population was enriched for Met^- mutants by a further in-

TABLE I

Strains and plasmids used in this study

Strains/plasmids (or cosmids)	Relevant characteristics	Reference/source
Escherichia coli		
803	*recA hsdS supE supF*	P. Kourilsky (Institut Pasteur)
BHB2688	N205 (λ*E*am4 *b2 red3 imm*434 *c*Its *S*am7)/λ	Hohn (1979)
BHB2690	N205 (λ*D*am15 *b2 red3 imm*434 *c*Its *S*am7)/λ	Hohn (1979)
Plasmids and cosmids		
pACYC184	Cm^R Tc^R, with a unique *Eco*RI restriction site in the *cam* gene	Chang et al. (1978)
pBHT187	Recombinant plasmid pACYC184::(R-2 DNA::Tn*901*), Cm^S Tc^R Ap^R (Fig. 1a)	This work
pPUC29	Hybrid cosmid vector *E. coli*-*A. nidulans* R-2, Cm^R Ap^R (Fig. 1b)	Our laboratories (to be published)
pTH225	Recombinant cosmid pPUC29::R-2 DNA, Cm^S Ap^R (Fig. 1c)	This work
Anacystis nidulans		
R-2	Wild-type strain harbouring plasmids pUH24 (7.9 kb) and pUH25 (50.8 kb)	Our laboratories PCC7942
R-2 [pCH1]	R-2 strain harbouring pCH1 (= pUH24::Tn*901*) instead of pUH24, Ap^R	Van den Hondel et al., 1980
met-1	R-2 *met*::Tn*901* [pCH1], Ap^R Met^-	This work
602 Met^-	MET1 mutant of *A. nidulans* 602 harbouring two plasmids (7.9 kb and 50.8 kb)	S. Shestakov (Moscow University)

cubation for 16 h in minimal medium containing 150 μg/ml of ampicillin. After washing, surviving cells (approx. 1%) were plated on minimal medium supplemented with methionine. Met^- mutants were screened, by replica plating, on minimal medium with and without methionine. Two Met^- mutants resistant to ampicillin were obtained. The mutant MET1 was used further in this work.

(c) DNA preparation

Plasmids (or cosmids) from *E. coli* and from *A. nidulans* R-2 strains were purified by CsCl-ethidium bromide density gradients according to Davis et al. (1980) and Van den Hondel et al. (1979), respectively. For rapid screening of *E. coli* transformants, the technique devised by Birnboim and Doly (1979) was followed.

Chromosomal DNA from *A. nidulans* R-2 strains was extracted by Marmur's technique (Marmur, 1961) with slight modifications. 2–3 g of wet packed cells were washed once with 120 mM NaCl, 50 mM EDTA, pH 8.0 and resuspended in 40 ml of the same saline–EDTA solution. Lysis was performed by incubations with 3 mg/ml lysozyme (1 h, 37°C), then with 1% w/v SDS (1 h, 37°C). The lysed suspension was made 0.5 M with $NaClO_4$ and extracted three times with 1 vol. of chloroform–isoamyl alcohol (24 : 1). DNA was precipitated with ethanol, spooled and resuspended in 10 mM Tris · HCl pH 7.5, 0.1 mM EDTA (T/0.1E). The DNA solution was treated with 50 μg/ml ribonuclease B (1 h, 37°C). After phenol and chloroform–isoamyl alcohol extractions, DNA was again precipitated with ethanol, spooled and resuspended in T/0.1E buffer.

(d) Restriction analysis and DNA ligation

Restriction endonucleases *Eco*RI (Boehringer) and *Bam*HI (BRL) were used according to the manufacturer's instructions.

T4 DNA ligase was purified by R. Teertstra (Utrecht) using the strain KA936 from K. Murray (obtained from A. de Waard, Leiden) as described by Murray et al. (1979). The dilution buffer was 66 mM Tris · HCl, pH 7.6, 6.6 mM $MgCl_2$, 10 mM DTT and 0.4 mM ATP. Molar ratios of vector to DNA fragment cloned were 1 : 20 and 1 : 1 for cloning into pACYC184 and pPUC29, respectively. The reaction mixture, containing 1 unit of T4 DNA ligase/ml, was incubated at 16°C for 24 h.

(e) Southern transfers and DNA hybridizations

Restriction DNA fragments and undigested plasmid DNA were electrophoresed on a 0.7% agarose gel (16 h, 3 V/cm) in 89 mM Tris-borate buffer pH 8.3, containing 3 mM EDTA. DNAs were then transferred to nitrocellulose filters as described by Southern (1975).

The 11.1-kb *met*::Tn*901* DNA fragment used as a probe was purified from the *Eco*RI digested plasmid pBHT187 according to Tanaka and Weisblum (1975) and ^{32}P-labelled by nick translation (Rigby et al., 1977). The [α-^{32}P]dATP (410 Ci/mmol) was purchased from Amersham. Hybridizations were performed as described by Hanahan and Meselson (1980).

(f) *A. nidulans* R-2 gene library: construction and screening

After partial digestion with *Eco*RI, chromosomal DNA samples were subjected to velocity sedimentation on a sucrose gradient (Van den Hondel et al., 1979). The 30–40-kb fragments were collected and ligated to the *Eco*RI-digested pPUC29 cosmid DNA. Ligated DNA was packaged in vitro into the heads of bacteriophage λ (Collins, 1979) using the strains BHB2688 and BHB2690 described by Hohn (1979). *E. coli* strain 803 was then infected with this packaged DNA, Ap^R colonies were selected and tested by replica plating for sensitivity to chloramphenicol. Ap^R Cm^S colonies were screened by colony hybridization (Hanahan and Meselson, 1980) using the *met*::Tn*901* [^{32}P]DNA probe purified and labelled as described in section (e). In this experiment, cold pPUC29 DNA (10 μg per filter) was added during the prehybridization incubation, to decrease the background which might have resulted from the presence of a Tn*901* fragment in the cosmid vector (see Fig. 1b).

(g) Transformations

Transformation of *E. coli* was performed according to the calcium shock method (Cohen et al., 1972). Transformation of *A. nidulans* strains was performed as described by Van den Hondel et al. (1980).

RESULTS AND DISCUSSION

In the present work, we used *A. nidulans* R-2[pCH1] cells to isolate mutants induced by transposition of Tn*901*. Met^- mutants were recovered at a frequency of 10^{-4}. The reversion frequency for the Met^+ phenotype was lower than 10^{-9}, suggesting that a *met* gene had been inactivated by insertion of Tn*901*. An analysis of the plasmid content of two Met^- Ap^R mutants revealed the presence of plasmids pCH1 and pUH25. One of these mutants, MET1, was studied further.

The cloning of the methionine gene from *A. nidulans* R-2 proceeded as follows.

Step I. Total DNA of the mutant MET1 was partially digested with *Eco*RI and cloned into the *E. coli* plasmid vector pACYC184 linearized by the same restriction enzyme. The vector encoded chloramphenicol and tetracycline resistances, and cloning into its unique *Eco*RI site inactivated the *cam* gene. *E. coli* strain 803 was transformed with this cloned DNA, and selection for ampicillin-resistant colonies yielded recombinant plasmids harbouring DNA fragments which contained a Tn*901* insertion. pCH1 (pUH24::Tn*901*) has no *Eco*RI site and is not replicated in *E. coli* (Van den Hondel et al., 1980). Thus, the cloned DNA fragments could only have originated from pUH25 or chromosomal DNA. Three Ap^R Tc^R Cm^S colonies were obtained. Plasmid DNA from these transfor-

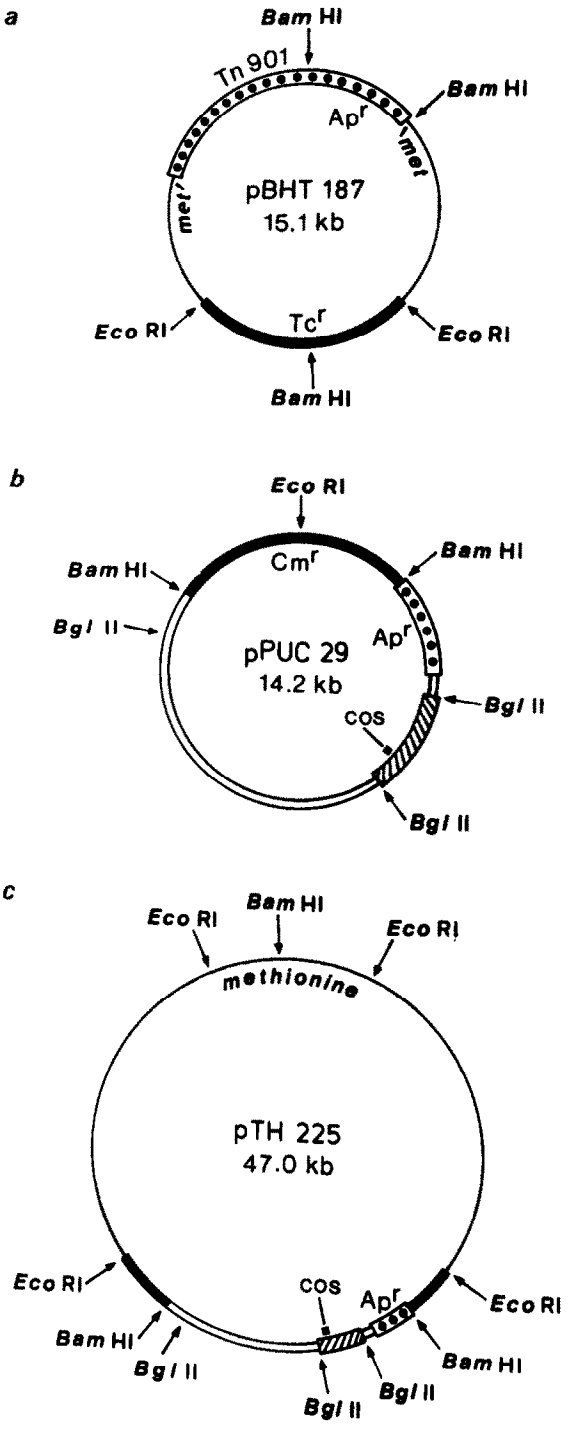

Fig. 1. Schematic representation of the plasmids pBHT187, pPUC29 and pTH225. (a) Recombinant plasmid pBHT187, obtained by cloning total DNA from the MET1 mutant of *A. nidulans* R-2 (thin line and dotted box), into the *Eco*RI site of *E. coli* plasmid vector pACYC184 (black box). (b) Cosmid pPUC29; hybrid *E. coli-A. nidulans* R-2 vector pUC104 (black box, pACYC184 part, open and dotted boxes, pUC1 part into which we have inserted, in vitro, the 1.7-kb *Bgl*II DNA fragment from pHC79 (hatched box; Hohn and Collins, 1980) housing the *cos* (black square) sequence of bacteriophage λ. Cosmid pPUC29 possesses a unique *Eco*RI site in the *cam* gene; Ap^R gene is carried on a 1.7-kb DNA fragment of Tn*901* which has lost the ability to transpose. (c) Recombinant cosmid pTH225 obtained by cloning a 32.8-kb chromosomal DNA fragment from wild-type *A. nidulans* R-2 partially digested with *Eco*RI (thin line) into the *Eco*RI site of the cosmid vector pPUC29. Within each plasmid or cosmids, the length of the restriction fragments were drawn according to their respective sizes. Within the DNA fragment cloned in pTH225, only two *Eco*RI and one *Bam*HI sites have been mentioned; the sizes of these restriction fragments and their respective locations have been determined from the sizes of the hybridizing bands (see Fig. 2, lanes 2 or 3, and Fig. 3a, lane 4).

mants was purified, digested with *Eco*RI and analyzed by agarose gel electrophoresis. All three contained a similar 11.1-kb *Eco*RI DNA fragment, termed "*met*::Tn*901*", inserted into the pACYC184 vector. One of these recombinant plasmids, pBHT187 (15.1 kb), was analyzed further. Restriction analysis of the purified *Eco*RI *met*::Tn*901* DNA fragment revealed the presence of two *Bam*HI sites, one located in the transposon Tn*901* (Van den Hondel et al., 1980), the second in the cloned *A. nidulans* R-2 DNA fragment. Fig. 1a shows a schematic representation of pBHT187.

To demonstrate that the cloned *met*::Tn*901* fragment originated from the chromosome of the mutant MET1, a Southern hybridization experiment was performed. Plasmid DNA from pUH24, pUH25, pCH1 and chromosomal DNA from both mutant MET1 and wild-type strain were tested using the ^{32}P-labelled *met*::Tn*901* *Eco*RI DNA fragment as a probe. As shown in Fig. 2, the ^{32}P-labelled probe hybridized with a 11.1-kb *Eco*RI fragment from the MET1 chromosomal DNA (lanes 5 and 6), as well as with a 6.2-kb *Eco*RI fragment from the wild-type chromosomal DNA (lanes 2 and 3). The difference in size between these two bands corresponded to the M_r-value of transposon Tn*901*. No hybridization was observed with undigested pUH24 (Fig. 2, lane 7) nor with pUH25 *Eco*RI fragments (Fig. 2, lane 9). These results show that the *met*::Tn*901* DNA fragment originated from the chromosomal DNA of the mutant MET1. The additional hybridization bands observed in Fig. 2 (lanes 5 and 6) resulted from the presence, in the chromosomal DNA prepara-

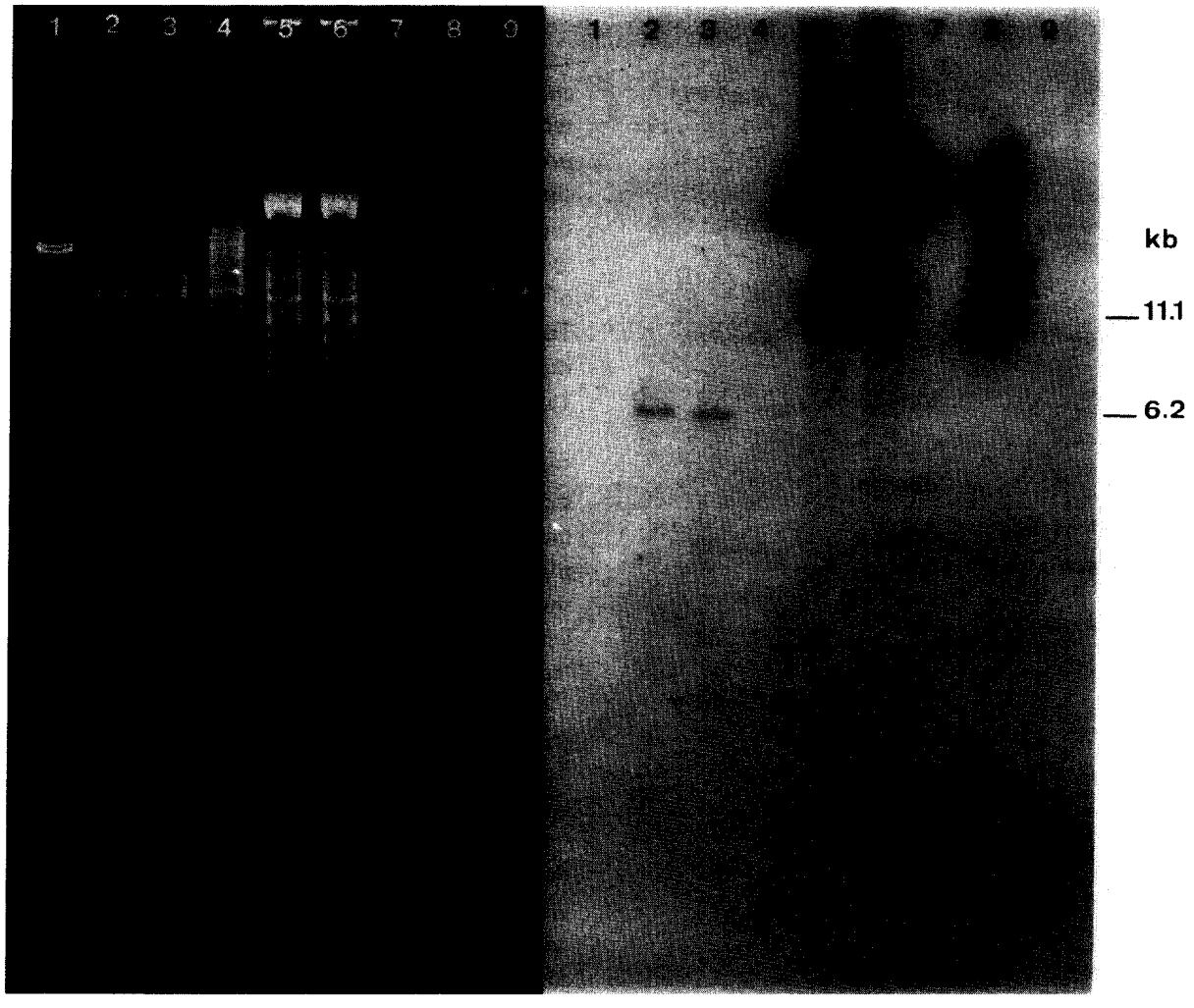

Fig. 2. Identification of the *Eco*RI DNA fragment from *A. nidulans* R-2 chromosomal DNA which contained a gene involved in methionine biosynthesis. Ethidium bromide-stained gel (left) and autoradiogram of the corresponding Southern blot after hybridization with the *met*::Tn*901* [^{32}P]DNA probe (right). Lane 1, M_r standards (λ DNA digested with *Hin*dIII + λ DNA doubly digested with *Hin*dIII and *Eco*RI). Lanes 2–4, chromosomal DNA of wild-type *A. nidulans* R-2 after digestion with *Eco*RI (2.0, 1.0 and 0.4 U per μg of DNA in lanes 2, 3 and 4, respectively). Lanes 5 and 6, chromosomal DNA of the *A. nidulans* R-2 mutant MET1 totally digested with *Eco*RI (2 U per μg of DNA); this DNA preparation contained L and OC forms of plasmid pCH1 (black arrows). Lane 7, undigested plasmid pUH24 (7.9 kb) from wild-type *A. nidulans* R-2 with CCC, L and OC plasmid DNA forms (from bottom to top). Lane 8, undigested plasmid pCH1 from the mutant MET1 with CCC, L and OC plasmid DNA forms (from bottom to top; black arrows). Lane 9, *Eco*RI-digested (partial digestion) plasmid pUH25 (50.8 kb) from wild-type *A. nidulans* R-2.

tion, of linear and open-circular forms of plasmid pCH1 which carries Tn*901* (cf. lane 8 showing CCC, L and OC forms of pCH1).

Step II. To select a DNA fragment that contains the corresponding wild-type *met* gene, we first prepared, in an *E. coli* strain, a gene library from wild-type *A. nidulans* R-2 chromosomal DNA (for construction and screening of the bank see MATERIALS AND METHODS). Our two main requirements were: first, to clone large DNA fragments, in order to preserve the authentic gene order, and to cover the entire genome, statistically, in a limited number of *E. coli* colonies; second, to clone DNA fragments into a vector that can be transferred back into the original organism. To meet these criteria, we prepared the gene library in our newly constructed cosmid vector pPUC29 (Fig. 1b) which is able to replicate either in *E. coli* or in *A. nidulans*

R-2 cells. Because of the length dependence of the DNA for in vitro packaging, a strong selection for hybrid DNA of 38–52 kb occurred (Hohn, 1979). By analogy with the taxonomically closely related *A. nidulans* PCC6301 (Rippka et al., 1979), the genome size of *A. nidulans* R-2 can be estimated to approx. M_r 2×10^9 (approx. 3×10^3 kb). As pPUC29 is 14.2 kb long, DNA fragments of approx. 24–38 kb can be inserted into this cosmid. Thus, the entire genome can be expected to be present in a few hundred colonies.

This *A. nidulans* R-2 gene library was screened by in situ colony hybridization using the *met*::Tn*901* *Eco*RI [^{32}P]DNA fragment as a probe. Of 300 Ap^R colonies analyzed, one hybridized with the probe and contained a 47-kb recombinant cosmid, which we called pTH225 (Fig. 1c). To confirm that this recombinant cosmid contains the wild-type *met* gene, DNA was analyzed by agarose gel electrophoresis after double digestion with *Bam*HI and *Eco*RI, and then tested with the *met*::Tn*901* [^{32}P]DNA probe. As shown in Fig. 3a, three of the *Bam*HI-*Eco*RI DNA fragments from pTH225 (10.2, 3.7 and 2.5 kb; lane 4) hybridized with the probe as well as three fragments (6.0, 3.7 and 1.4 kb; lane 3) from the *Bam*HI digest of the *Eco*RI *met*::Tn*901* fragment. The 10.2-kb hybridization band from pTH225 (Fig. 3a, lane 4) corresponded to the larger *Bam*HI-*Bam*HI restriction fragment of the cosmid vector pPUC29 (Fig. 3a, lane 2 and Fig. 1b and c) and the 3.7- and 2.5-kb bands to DNA fragments from the wild-type chromosomal DNA of *A. nidulans* R-2. Fig. 3b shows a schematic drawing of the *Eco*RI *met*::Tn*901* DNA fragment, and of the corresponding *Eco*RI wild-type "*met* DNA" fragment cloned into the recombinant cosmid pTH225. On the basis of the M_r-values of these fragments, we propose a structure in which Tn*901* has been inserted very close to the *Bam*HI site of the wild-type chromosomal DNA fragment. The 3.7-kb *Bam*HI-*Eco*RI restriction fragment was common to both the *met*::Tn*901* and the wild-type "*met* DNA" fragment, and thus corresponded to chromosomal DNA. The 6.0- and 1.4-kb pieces of the *met*::Tn*901* DNA represented wild-type "*met* DNA" (2.5 kb) into which Tn*901* (4.9 kb) had inserted.

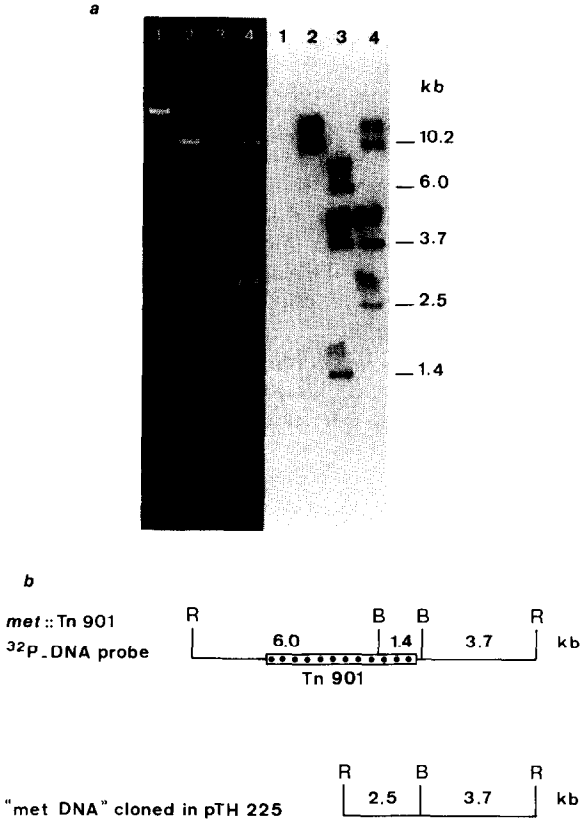

Fig. 3. Analysis of DNA from the recombinant cosmid pTH225. (a) Ethidium bromide-stained gel (left) and autoradiogram of the corresponding Southern blot after hybridization with the *met*::Tn*901* *Eco*RI [^{32}P]DNA fragment as a probe (right). Lane 1, M_r standards as in Fig. 2. Lane 2, DNA of cosmid vector pPUC29 digested with *Bam*HI and *Eco*RI. Lane 3, *Eco*RI *met*::Tn*901* DNA fragment digested with *Bam*HI. Lane 4, recombinant cosmid pTH225 DNA digested with *Bam*HI and *Eco*RI. (b) Schematic representation depicting the *Eco*RI (R) and *Bam*HI (B) sites in the 11.1-kb *met*::Tn*901* DNA fragment used as a probe and in the corresponding wild-type chromosomal "*met* DNA" fragment cloned in pTH225.

With the recombinant cosmid pTH225, we then transformed two Met$^-$ mutants, the original *A. nidulans* R-2 MET1 and a Met$^-$ mutant of a taxonomically closely related cyanobacterium *A. nidulans* 602 (obtained after *N*-methyl-*N*-nitroso-*N'*-nitroguanidine mutagenesis). Both yielded Met$^+$ colonies after transformation with pTH225 DNA and selection on minimal medium. The frequency of transformation, under conditions which gave no spontaneous Met$^+$ revertants, was approx. 5×10^{-5} and approx. 5×10^{-7} for the MET1 and the *A. nidulans* 602Met$^-$ mutants, respectively. These results demonstrated that we had

effectively cloned a gene involved in methionine biosynthesis. As schematically represented in Fig. 3b, the insertion of Tn*901*, which inactivated the *met* gene in MET1, occurred very close to the *Bam*HI site leading, after digestion, to two *Bam*HI-*Eco*RI pieces of 2.5 and 3.7 kb. Thus, if the coding capacity of the inactivated *met* gene is not greater than 2.5 kb, the corresponding wild-type gene must be present in its entirety in pTH225. In addition, as the two Met$^-$ mutants recovered prototrophic growth, these results suggested that the mutations had occurred either in the same gene or in closely linked genes.

An analysis of three Met$^+$ transformants of *A. nidulans* MET1 showed that the plasmids pCH1 (12.4 kb) and pUH25 (50.8 kb) were still present, whereas pTH225 was not found. Since the cosmid vector pPUC29 is incompatible with pCH1, this suggests that homologous recombination had occurred between the "*met* DNA" from pTH225 and the inactivated *met* gene on the chromosome of *A. nidulans* MET1. Similar results were found after analysis of three Met$^+$ transformants of *A. nidulans* 602 Met$^-$. The endogenous plasmids in this mutant (7.9 kb and 50.8 kb) were still present in the Met$^+$ transformants, and pTH225 was not found. In addition, the transformants were not ampicillin-resistant. These results suggested that recombination had occurred leading to the loss of the ampicillin-resistance gene carried by the cosmid cloning vector pPUC29.

In this work, we have shown that transposition-induced mutants can be obtained in the cyanobacterium *A. nidulans* R-2. The use of a shuttle cosmid vector allowed not only the construction of an *A. nidulans* R-2 gene library in *E. coli* but also the study of the cloned gene in the organism from which it originated. This is the first example of molecular cloning of a cyanobacterial gene which permits the restoration of a wild-type phenotype. These molecular genetic tools should provide the means to analyze the functional organization of the cyanobacterial genes, in cases where no direct selection nor heterologous DNA probes are available.

ACKNOWLEDGEMENTS

We thank Dr. G. Cohen-Bazire for advice and encouragement, Dr. J. Houmard for helpful discussions and critical comments during the preparation of the manuscript, Dr. E. Johnson and Dr. T. Kallas for careful reading of the manuscript, and Gérard Guglielmi for helping in the preparation of the photographs. This research was supported by C.N.R.S. grants ERA No. 398 and ATP No. A1-5053.

REFERENCES

Astier, C. and Espardellier, F.: Mise en évidence d'un système de transfert génétique chez une cyanophycée du genre *Aphanocapsa*. C.R. Acad. Sci. Paris 282 (1976) 795–797.

Birnboim, H.C. and Doly, J.: A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acids Res. 7 (1979) 1513–1523.

Chang, A.C.Y. and Cohen, S.N.: Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. 134 (1978) 1141–1156.

Cohen, S.N., Chang, A.C.Y. and Hsu, L.: Non-chromosomal antibiotic resistance in bacteria: genetic transformation in *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA 69 (1972) 2110–2114.

Collins, J.: *Escherichia coli* plasmids packageable in vitro in λ bacteriophage particles, in Colowick, S.P. and Kaplan, N.O. (Eds.), Methods in Enzymology, Vol. 68. Academic Press, New York, 1979, pp. 309–326.

Davis, R.W., Botstein, D. and Roth, J.R.: Isolation of plasmid and bacterial DNA, I. Large-scale isolation of *E. coli* plasmid DNA, in Advanced Bacterial Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1980, pp. 116–117.

Delaney, S.F., Herdman, M. and Carr, N.G.: Genetics of blue-green algae, in Lewin, R.A. (Ed.), The Genetics of Algae. Blackwell, Oxford, 1976, pp. 7–28.

Devilly, C.I. and Houghton, J.A.: A study of genetic transformation in *Gloeocapsa alpicola*. J. Gen. Microbiol. 98 (1977) 277–280.

Doolittle, W.F.: The cyanobacterial genome, its expression, and the control of that expression. Adv. Microb. Physiol. 20 (1979) 1–102.

Fisher, R., Tuli, R. and Haselkorn, R.: A cloned cyanobacterial gene for glutamine synthetase functions in *Escherichia coli*, but the enzyme is not adenylylated. Proc. Natl. Acad. Sci. USA 78 (1981) 3393–3397.

Grigorieva, G. and Shestakov, S.: Transformation in the cyanobacterium *Synechocystis* sp. 6803. FEMS Microbiol. Lett. 13 (1982) 367–370.

Hanahan, D. and Meselson, M.: Plasmid screening at high colony density. Gene 10 (1980) 63–67.

Herdman, M.: Mutations arising during transformation in the blue-green alga *Anacystis nidulans*. Mol. Gen. Genet. 120 (1973) 369–378.

Hohn, B.: In vitro packaging of λ and cosmid DNA, in Colowick, S.P. and Kaplan, N.O. (Eds.), Methods in Enzymology, Vol. 68. Academic Press, New York, 1979, pp. 299–309.

Hohn, B. and Collins, J.: A small cosmid for efficient cloning of large DNA fragments. Gene 11 (1980) 291–298.

Kuhlemeier, C.J., Borrias, W.E., Van den Hondel, C.A.M.J.J. and Van Arkel, G.A.: Vectors for cloning in cyanobacteria: construction and characterization of two recombinant plasmids capable of transformation to *Escherichia coli* K-12 and *Anacystis nidulans* R-2. Mol. Gen. Genet. 184 (1981) 249–254.

Ladha, J.K. and Kumar, H.D.: Genetics of blue-green algae. Biol. Rev. 53 (1978) 355–386.

Marmur, J.: A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. 3 (1961) 208–218.

Mazur, B.J., Rice, D. and Haselkorn, R.: Identification of blue-green algal nitrogen fixation genes by using heterologous DNA hybridization probes. Proc. Natl. Acad. Sci. USA 77 (1980) 186–190.

Murray, N.E., Bruce, S.A. and Murray, K.: Molecular cloning of the DNA ligase gene from bacteriophage T4, II. Amplification and preparation of the gene product. J. Mol. Biol. 132 (1979) 493–505.

Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg, P.: Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. 113 (1977) 237–251.

Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M. and Stanier, R.Y.: Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. 111 (1979) 1–61.

Shestakov, S.V. and Khyen, N.T.: Evidence for genetic transformation in blue-green alga *Anacystis nidulans*. Mol. Gen. Genet. 107 (1970) 372–375.

Southern, E.M.: Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98 (1975) 503–517.

Stanier, R.Y. and Cohen-Bazire, G.: Phototrophic prokaryotes: the cyanobacteria. Annu. Rev. Microbiol. 31 (1977) 225–274.

Stevens Jr., S.E. and Porter, R.D.: Transformation in *Agmenellum quadruplicatum*. Proc. Natl. Acad. Sci. USA 77 (1980) 6052–6056.

Tanaka, T. and Weisblum, B.: Construction of a colicin E_1-R factor composite plasmid in vitro: means for amplification of desoxyribonucleic acid. J. Bacteriol. 121 (1975) 354–362.

Tomioka, N., Shinozaki, K. and Sugiura, M.: Molecular cloning and characterization of ribosomal RNA genes from a blue-green alga, *Anacystis nidulans*. Mol. Gen. Genet. 184 (1981) 359–363.

Van den Hondel, C.A.M.J.J., Keegstra, W., Borrias, W.E. and Van Arkel, G.A.: Homology of plasmids in strains of unicellular cyanobacteria. Plasmid 2 (1979) 323–333.

Van den Hondel, C.A.M.J.J., Verbeek, S., Van den Ende, A., Weisbeek, P.J., Borrias, W.E. and Van Arkel, G.A.: Introduction of transposon Tn*901* into a plasmid of *Anacystis nidulans*: preparation for cloning in cyanobacteria. Proc. Natl. Acad. Sci. USA 77 (1980) 1570–1574.

Communicated by S. Brenner.