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A Hybrid Plasmid Is a Stable Cloning Vector for the Cyanobacterium *Anacystis nidulans* R2

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Anacystis nidulans R2 is a highly transformable strain which is suitable as a recipient for molecular cloning in cyanobacteria. In an effort to produce an appropriate cloning vector, we constructed a hybrid plasmid molecule, pSG111, which contained pBR328 from *Escherichia coli* and the native pUH24 plasmid of *A. nidulans*. pSG111 replicated in and conferred ampicillin and chloramphenicol resistance to both hosts. It contained unique sites for the restriction enzymes *Eco*RI, *Sal*I, *Sph*I, and *Xho*I, which could be used for the insertion of exogenous DNA. To demonstrate that a molecule like pSG111 could serve as a shuttle vector for the cloning of *A. nidulans* genes, we constructed a hybrid plasmid, pRNA404, containing an *A. nidulans* rRNA operon. This recombinant molecule was genetically and structurally stable during passage through *A. nidulans* and *E. coli*. The stability of the hybrid plasmid and the inserted rRNA operon demonstrates the feasibility of cloning in *A. nidulans* with hybrid vectors, with the subsequent retrieval of cloned sequences.

The cyanobacteria are procaryotic organisms that perform an oxygenic photosynthesis very similar to the process carried out by higher plants. The utility of the unicellular cyanobacterial strain *Anacystis nidulans* as a model for the study of photosynthesis has been enhanced by detailed analyses of the structure of the photosynthetic membrane (7, 8). In addition, procedures for the enrichment and isolation of photosynthesis-deficient mutants have been developed (6). However, the use of these organisms has been hampered by the lack of genetic transfer between individuals. The availability of a transformable cyanobacterial strain, *A. nidulans* R2 (15, 16, 22), provides a potential host for the use of molecular genetic techniques. Such an approach allows the selection in *A. nidulans* of cloned genes which complement mutations in photosynthetic function and the isolation of these genes for a study of their protein products. This can provide a direct correlation between their structural components and the function of the photosynthetic apparatus.

Workers at several laboratories have produced hybrid plasmid molecules which might serve as cyanobacterial cloning vectors. These hybrids contain all or part of the small endogenous *A. nidulans* plasmid (which confers maintenance in the cyanobacterium) and *Escherichia coli* sequences carrying antibiotic resistance genes (22). In some cases, the hybrid plasmids possess *E. coli* replicative ability (11, 15, 19). Until now, none of these plasmids has been shown to serve as a cloning vector for the stable passage of chromosomal genes in *A. nidulans*. Recently, a cosmid derivative of one such hybrid was used to carry a cloned *A. nidulans* methionine biosynthesis gene into Met$^-$ strains; however, the cloned gene could not be retrieved from transformed cells owing to a recombinational event in which the vector molecule was lost (19). We report here the construction of a hybrid plasmid, pSG111, which replicates in both *E. coli* and *A. nidulans*. To determine whether pSG111 can serve as a shuttle vector for the cloning of *A. nidulans* genes, we inserted an *A. nidulans* rRNA operon into the hybrid plasmid. This recombinant molecule was genetically and structurally stable during passage through *A. nidulans* and *E. coli*. This study showed that cloned chromosomal genes can be maintained extrachromosomally for retrieval and subsequent study, which is an important feature for the direct selection of *A. nidulans* genes in that organism, using hybrid shuttle vectors.

MATERIALS AND METHODS

Media and growth conditions. *A. nidulans* R2, obtained from G. van Arkel, University of Utrecht, Utrecht, The Netherlands, was grown axenically in BG-11 medium (1) as previously described (14). BG-11 medium containing 1.5% agar (Bacto-Agar; Difco Lab-

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oratories, Detroit, Mich.) was used for the plating of *A. nidulans* cells. At various times, 1 mM $Na_2S_2O_3$ was added to the solid medium to enhance growth when impurities were present in the water supply. *E. coli* HB101 was obtained as KA605 from P. van de Putte, University of Leiden, Leiden, The Netherlands. This strain was grown at 37°C in NZY medium containing, per liter, 5 g of NaCl, 2 g of $MgCl_2 \cdot 6H_2O$, 10 g of casein hydrolysate (Sigma Chemical Co., St. Louis, Mo.), and 5 g of yeast extract. The same medium plus 1.1% agar was used to obtain growth on plates.

A. nidulans plasmid pUH24 was isolated by the procedure described by van den Hondel et al. (21), except that isopropanol equilibrated with 20× SSC (3 M NaCl plus 0.3 M sodium citrate) was used to remove propidium iodide from plasmid DNA after buoyant density centrifugation. DNA was then dialyzed against TE buffer (10 mM Tris, 1 mM disodium-EDTA; pH 7.6) and ethanol precipitated. Sucrose gradient centrifugation (21) was sometimes used to separate *A. nidulans* plasmid pUH24 from pUH25.

Plasmid pBR328 (17) was a gift from F. Bolivar, and pAN4 (20) was generously provided by M. Sugiura. These plasmids and hybrid plasmid DNAs were isolated from *E. coli* as described previously (15), with the following exceptions: RNase treatment of cleared lysates was deleted, and ethanol-precipitated DNA preparations were banded in cesium chloride-ethidium bromide buoyant density gradients (1.57 g/ml in TE buffer) in a Beckman VTi65 ultracentrifuge rotor (Beckman Instruments, Inc., Fullerton, Calif.) for 24 h at 55,000 rpm. Isopropanol extraction and dialysis were as described for pUH24 isolation.

Restriction endonuclease cleavage and gel electrophoresis. Restriction endonucleases were purchased from New England BioLabs, Beverly, Mass., or Bethesda Research Laboratories, Rockville, Md. Restriction digests were carried out under conditions described by Davis et al. (4). A molecular weight standard mixture of λ *Hin*dIII and φX174 *Hin*cII fragments was purchased from P-L Biochemicals, Inc., Milwaukee, Wis. Agarose gel electrophoresis of DNA fragments was performed on 0.7 to 1.4% agarose (Bethesda Research Laboratories) in EB buffer (80 mM Tris, 20 mM sodium acetate, 2 mM disodium-EDTA; pH 8.0) on a horizontal, submerged gel electrophoresis apparatus (Bethesda Research Laboratories) at 3 V/cm for 3 to 5 h. Electrophoretic separation of DNA fragments of <1 kilobase (kb) was carried out on 5% polyacrylamide slab gels (3 mm by 15 cm) as described previously (2).

T4 DNA ligase was purchased from New England Biolabs and was used as suggested by the manufacturer for the ligation of DNA fragments. Vector molecules were treated with bovine intestinal alkaline phosphatase (type VII-S; Sigma Chemical Co.) as described previously (5). Target DNA fragments were sometimes purified by centrifugation through 10 to 40% exponential sucrose gradients (9). Sucrose stocks were prepared in E buffer (36 mM Tris, 30 mM NaH_2PO_4, 1 mM disodium-EDTA; pH 8.0), and gradients were subjected to centrifugation in a Beckman SW41 rotor at 25,000 rpm for 17 h at 20°C. Alternatively, fragments used for ligation to vector molecules were isolated from low-melting-temperature agarose gels (Seakem; Marine Colloids, Rockland, Maine) by the method of Maniatis et al. (12).

Southern analysis of electrophoretically separated DNA fragments was as described previously (18). The *Pst*I fragment of pAN4 comprising the rRNA operon was labeled with [^{32}P]dCTP by nick translation (13).

Transformation of *E. coli* and *A. nidulans*. *E. coli* was transformed with ligation products or hybrid plasmids by a standard Ca^{2+} treatment procedure similar to that described by Cohen et al. (3). Ampicillin and chloramphenicol (Sigma Chemical Co.) were added to NZY agar at 40 and 30 μg/ml, respectively, to select for growth of transformed cells. Transformants were rapidly checked for plasmid content by a single-colony screening procedure (10).

A. nidulans R2 cells were prepared for transformation by a wash with 10 mM NaCl and suspension at 5 × 10^8 cells per ml in BG-11 medium (1). Hybrid plasmid DNA (50 to 250 ng in TE buffer at less than a 20-μl volume) was added to 300 μl of cells and incubated in darkness at 30°C overnight with gentle shaking. Transformed cells were plated on 40-ml BG-11 agar plates in triplicate (100 μl per plate). After 6 h, the agar slab of each plate was lifted with a sterile spatula, and 400 μl of a 100× solution of a selective agent was dispensed underneath. Ampicillin (Polycillin-N; Bristol Laboratories, Syracuse, N.Y.) was used at 0.5 μg/ml, and chloramphenicol (Sigma Chemical Co.) was used at 5 μg/ml (final concentration). The plates were incubated at 30°C under the lighting conditions previously described (14). A detailed description of the transformation parameters for *A. nidulans* R2 will be published elsewhere (S. S. Golden and L. A. Sherman, manuscript in preparation).

RESULTS

In vitro construction of an *E. coli-A. nidulans* hybrid plasmid. *A. nidulans* R2 contains two endogenous plasmids, pUH24 (8 kb) and pUH25 (50 kb) (22), which carry no known genetic markers. To exploit the *A. nidulans* replicative ability of pUH24, we produced a hybrid plasmid containing pUH24 plus *E. coli* plasmid pBR328. The pBR328 portion of the molecule provided resistance to the antibiotics ampicillin and chloramphenicol and the ability to replicate in *E. coli*. This hybrid molecule, pSG111, was constructed by digestion of pUH24 and pBR328 with restriction enzyme *Bam*HI, which cleaves each molecule at a unique site. Linearized pBR328 (0.1 μg) was treated with bovine intestinal alkaline phosphatase and incubated with linearized pUH24 (1.0 μg) in the presence of T4 DNA ligase. The ligation mixture was used to transform *E. coli*, and transformants were selected for resistance to ampicillin and chloramphenicol. These transformants were then screened for sensitivity to tetracycline, since insertion of pUH24 into the *Bam*HI site of pBR328 inactivates the tetracycline resistance gene. Figure 1A shows restriction digests of plasmids (pSG111 and pSG210) from two of the transformants and the parent plasmid molecules. Lanes 2 through 4 are *Bam*HI digests of pBR328, pUH24 (with faint

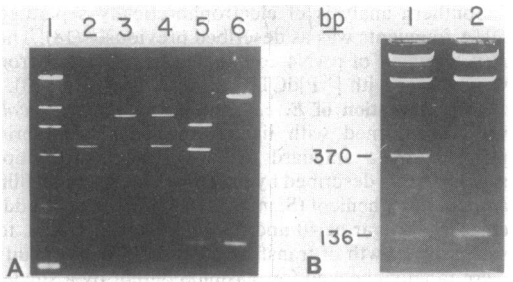

FIG. 1. (A) Gel electrophoresis on 0.7% agarose of pSG111, pSG210, and the parent plasmid molecules. Lane 1 is a λ *Hin*dIII-φX174 *Hin*cII molecular weight standard. *Bam*HI digests of pBR328 (lane 2), pUH24 with pUH25 bands also present (lane 3), and pSG111 (lane 4) and *Hin*dIII digests of pSG111 (lane 5) and pSG210 (lane 6) are shown. In all cases 200 ng of DNA was used, except in lane 2 in which 100 ng was used. (B) Electrophoresis on a 5% polyacrylamide gel of *Hin*dIII-*Bam*HI double digests of pSG111 (lane 1) and pSG210 (lane 2). A 2-μg sample of DNA was applied to each lane.

pUH25 bands also present), and pSG111, respectively. Lanes 5 and 6 are *Hin*dIII digests of pSG111 and pSG210, which indicated the orientation of pUH24 insertion into pBR328. Of 14 such transformants, 13 had pUH24 inserted in the orientation relative to pBR328 sequences shown in Fig. 2 (pSG111), and one harbored a hybrid plasmid (pSG210) of the opposite orientation.

A representative of each orientation (pSG111 and pSG210) was isolated and used as donor DNA for the transformation of *A. nidulans*. Chloramphenicol- and ampicillin-resistant transformants were obtained when either pSG111 or pSG210 was used as the donor DNA. Plasmid pSG111 was selected for further use in cloning experiments because subsequent restriction analysis of pSG210 showed that the *Hin*dIII site of the pBR328 portion of the molecule was missing. Figure 1B is a polyacrylamide gel of pSG111 and pSG210 DNAs digested with both *Hin*dIII and *Bam*HI, which demonstrated that the 370-base-pair *Hin*dIII-*Bam*HI fragment originating from pBR328 is missing in pSG210. Agarose gel electrophoresis of a pSG210 digest similar to that shown in Fig. 1B, lane 2 revealed that a fragment is produced which comigrates with *Bam*HI-linearized pBR328 (data not shown). No difference in size between pSG111 and pSG210 was detectable, indicating that loss of the *Hin*dIII site was not due to a gross deletion.

Hybrid plasmid pSG111 (12.9 kb) has unique restriction sites suitable for the insertion of DNA fragments (Fig. 2). *Eco*RI cleaves the molecule in the chloramphenicol resistance gene of the pBR328 segment. This site is suitable for screening clones by insertional inactivation. The enzymes *Sal*I and *Sph*I recognize unique sites in the vestigial tetracycline gene, whereas *Xho*I cleaves the plasmid in the pUH24 segment. The hybrid retains the replication origin for growth in *E. coli* and is capable of replication in *A. nidulans* because of the presence of pUH24. The genes coding for resistance to ampicillin and chloramphenicol were unaffected by the formation of the hybrid and functioned in both hosts. Plasmid pSG111 transformed *A. nidulans* cells to chloramphenicol resistance at an efficiency of 5×10^{-7} to 2×10^{-6} transformants per donor DNA molecule; however, the frequency of transformation with ampicillin selection was 50- to 100-fold lower. Plasmids pSG111 and pBR328 transformed *E. coli* cells at a comparable frequency with selection for either (or both) antibiotic(s). *E. coli* cells harboring the plasmid were resistant to at least 40 μg of ampicillin per ml and 30 μg of chloramphenicol per ml. *A. nidulans* transformants were resistant to 0.5 and 10 μg of these antibiotics per ml, respectively. Plasmid pUH24 is absent from *A. nidulans* cells which have been transformed by pSG111.

Restriction endonuclease digestion confirmed that no structural alteration of pSG111 occurred during passage through *A. nidulans*, regardless of the antibiotic (ampicillin or chloramphenicol) used for selection in transformation and propa-

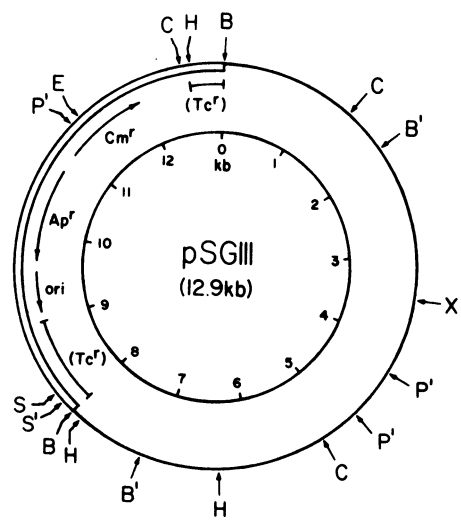

FIG. 2. Schematic representation of pSG111. The pBR328 sequence is shown as an open box and the solid line represents pUH24. Positions indicated are: Cm^r, pBR328 chloramphenicol resistance gene; Ap^r, ampicillin resistance gene; ori, *E. coli* replication origin; Tc^r, vestigial tetracycline resistance gene. Restriction sites are: *Bam*HI (B), *Hin*dIII (H), *Cla*I (C), *Eco*RI (E), *Pvu*II (P'), *Sal*I (S), *Sph*I (S'), *Bgl*II (B'), and *Xho*I (X).

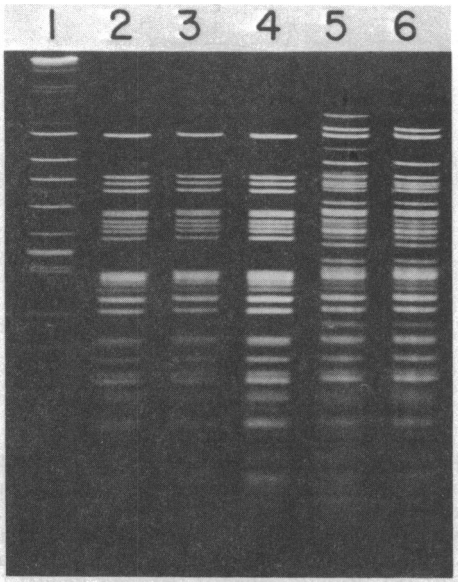

FIG. 3. Electrophoresis of pSG111 and pRNA404 on a 5% polyacrylamide gel. Lane 1 is the λ *Hin*dIII-φX174 *Hin*cII molecular weight standard. *Hae*III digests of: the original pSG111 isolate (lane 2); pSG111 after passage through *A. nidulans* with ampicillin selection (lane 3) or chloramphenicol selection (lane 4) followed by passage through *E. coli*. Lanes 5 and 6 show the *Hae*III digests of the original pRNA404 isolate (lane 5) and after passage through both hosts (lane 6).

gation. The tetranucleotide-recognizing enzyme *Hae*III was used to demonstrate at a fine level that pSG111 structural integrity is maintained during passage through the cyanobacterial host (Fig. 3). Figure 3, lane 2 shows an *Hae*III digest of pSG111 which was originally isolated from *E. coli*. When this DNA was passaged through *A. nidulans* in the presence of ampicillin (Fig. 3, lane 3) or chloramphenicol (lane 4) and reisolated from *E. coli*, identical *Hae*III patterns were obtained.

An rRNA operon of *A. nidulans* 6301 was cloned in pBR322 by Tomioka and colleagues (19) as a 6.5-kb *Pst*I fragment. Southern analysis of *Pst*I-digested chromosomal DNA from *A. nidulans* R2 probed with this clone revealed a high degree of homology to fragments of 6.5 and 8.0 kb (data not shown), the same sizes bearing the rRNA genes in *A. nidulans* 6301 (20). Because the restriction map of this fragment is known and the operon is homologous to one present in *A. nidulans* R2, this sequence was chosen as a target molecule for insertion into pSG111. The passage of pSG111 bearing a cyanobacterial chromosomal fragment through *A. nidulans* would demonstrate the feasibility of maintaining chromosomal genes on plasmid vectors in the cyanobacterium for subsequent retrieval. Extrachromosomal maintenance is central to the concept of the direct selection of particular genes in *A. nidulans*.

Plasmid pSG111 contains many sites for restriction enzyme *Pst*I. Therefore, the cloning strategy shown in Fig. 4 was used to insert the fragment bearing the rRNA operon into the hybrid plasmid. Plasmid pAN4 containing the rRNA operon (20) was digested with *Pst*I, and the 6.5-kb fragment was separated and purified from the pBR322 vector sequences by electrophoresis in low-melting-temperature agarose. This fragment (0.3 μg) was then ligated to pBR328 (1.0 μg) which had been cleaved by *Pst*I and treated with alkaline phosphatase. *E. coli* was transformed by the ligation mixture, and colonies resistant to chloramphenicol and tetracycline, but sensitive to ampicillin, were screened (10). The plasmid from one such colony, pRNA257, had the rRNA operon inserted into the pBR328 *Pst*I site. Regeneration of the

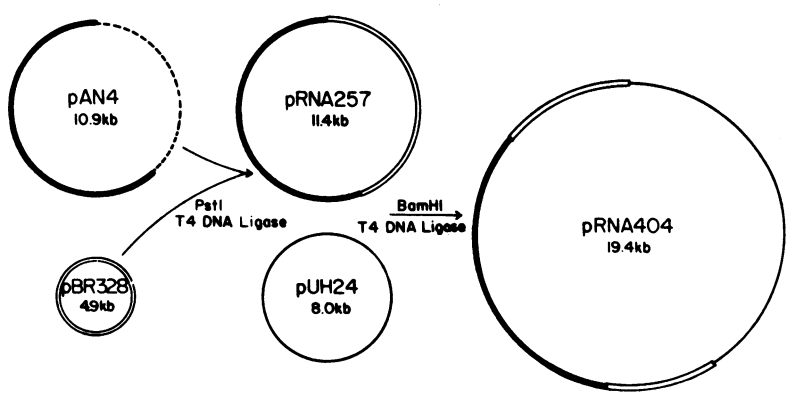

FIG. 4. Cloning strategy for the introduction of the *A. nidulans* rRNA operon into pSG111. Closed box, rRNA operon; dashed line, pBR322; open box, pBR328; single line, pUH24.

pSG111 vector around the insert was accomplished by digesting pRNA257 (0.6 μg) with *Bam*HI and ligating it to *Bam*HI-cleaved pUH24 (0.6 μg). The resulting plasmid, pRNA404, had the pUH24 molecule inserted into the tetracycline resistance gene of the pBR328 parent sequence but retained the gene coding for resistance to chloramphenicol. Lanes 7 and 8 of Fig. 5 show *Hin*dIII digests of pSG111 and pRNA404. The upper band marked by an arrow is a junction fragment of pUH24 and pBR328 sequences which identifies pUH24 insertion in pRNA404 as the same orientation as in the original pSG111 vector. The lower band indicated by an arrow is the internal *Hin*dIII fragment of the pUH24 molecule. Figure 6 represents the pRNA404 molecule, indicating the orientation of the rRNA genes and showing restriction sites used to construct and map the molecule.

Plasmid pRNA404 DNA transformed *A. nidulans* cells to chloramphenicol resistance at the same frequency as the parent molecule pSG111. Plasmid DNA was isolated from an *A. nidulans* culture which had been transformed to chloramphenicol resistance by pRNA404. This DNA was then passaged through *E. coli*, isolated, and examined by restriction mapping to determine the fate of the insert. The resulting plasmid

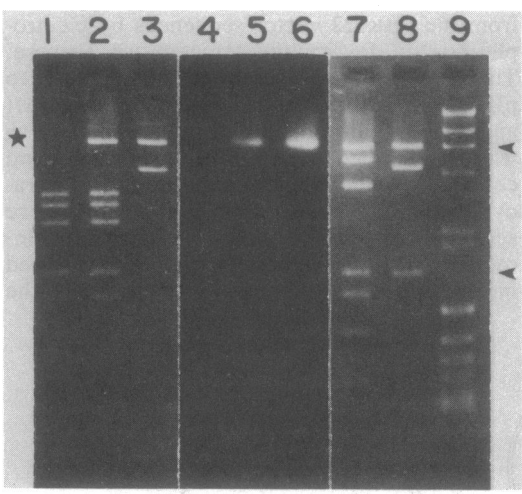

FIG. 5. Electrophoresis on a 1.4% agarose gel of restriction digests of pRNA404, pSG111, and pAN4. *Pst*I digests of pSG111 (lane 1), pRNA404 (lane 2), and pAN4 (lane 3) are shown. Southern analysis of lanes 1 through 3 is shown in lanes 4 through 6, with the large *Pst*I fragment of pAN4 used as the probe. *Hin*dIII digests of pRNA404 and pSG111 are lanes 7 and 8, respectively. Lane 9 is a λ *Hin*dIII-φX174 *Hin*cII molecular weight standard. The star marks the 6.5-kb *Pst*I fragment bearing the rRNA operon. The upper arrow marks a pUH24-pBR328 junction fragment and the lower arrow marks the internal pUH24 *Hin*dIII fragment.

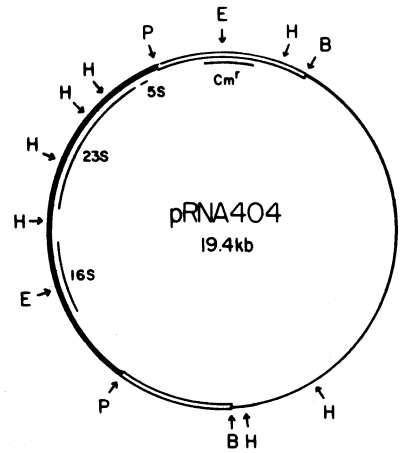

FIG. 6. Schematic representation of pRNA404. Closed box, rRNA operon; open box, pBR328; single line, pUH24. The locations of the rRNA genes (5S, 23S, and 16S) and the functional chloramphenicol resistance gene (Cm^r) are given. Restriction sites are: *Eco*RI (E), *Pst*I (P), *Hin*dIII (H), and *Bam*HI (B). Additional *Pst*I sites present in pUH24 are not shown.

DNA, after passage through both hosts, was identical to the original isolate of pRNA404. Figure 5, lane 2 shows a *Pst*I digest of the passaged DNA. All of the *Pst*I fragments arising from pSG111 (lane 1), as well as a 6.5-kb fragment (marked by a star) which comigrated with the rRNA operon fragment of pAN4 (lane 3), are present. Southern transfer of lanes 1 through 3, followed by hybridization to the larger pAN4 *Pst*I fragment which had been radioactively labeled, confirmed the identity of the 6.5-kb fragment as the intact rRNA operon. pSG111 sequences showed no hybridization to the probe (lane 4), whereas pRNA404 and pAN4 6.5-kb fragments (lanes 5 and 6, respectively) hybridized equally well.

*Hae*III digestion was used to compare pRNA404 structure before and after passage through both hosts. Lane 5 of Fig. 3 is the *Hae*III pattern of the original isolate of pRNA404. The pattern in lane 6 was obtained by using passaged pRNA404 DNA. The non-stoichiometric bands in lane 5, which are not present in lane 6, are due to the presence of a contaminating, covalently closed circular species which was present in the original DNA preparation and was not part of the pRNA404 molecule. No differences could be detected in pRNA404 *Hae*III fragments before or after serial passage through *A. nidulans* and *E. coli*.

DISCUSSION

The major contribution of this work to the field of cyanobacterial genetics is the demon-

stration that cloned genes which are homologous to the chromosome can be extrachromosomally maintained for selection and subsequent retrieval. Other researchers have reported the incorporation of cloned sequences into the cyanobacterial chromosome during passage through *A. nidulans*. Experiments in our laboratory have shown that vector stability is dependent upon the location at which deletions and insertions are made into the hybrid plasmid. Kuhlemeier and colleagues reported the instability in *A. nidulans* of a hybrid plasmid, pUC105 (11). This hybrid contains pUH24 that is interrupted at two positions by heterologous sequences. They suggested that the instability is due to recombination between the resident pUH24 molecule in *A. nidulans* and the interrupted sequence in the hybrid, resulting in loss of the heterologous inserts. This is consistent with our own results. When a 5.5-kb *Bgl*II fragment internal to the pUH24 portion of hybrid plasmid pSG111 is removed and the molecule is religated, passage through *A. nidulans* results in the regeneration of the parent pSG111 molecule (S. S. Golden, C. Vann, and L. A. Sherman, unpublished data). In this situation a resident copy of pUH24 seems to have "healed" its deleted analog in the hybrid. Chromosomal DNA from an *A. nidulans* mutant resistant to the herbicide 3,4-dichlorophenyldimethylurea transforms wild-type *A. nidulans* to 3,4-dichlorophenyldimethylurea resistance at a high frequency (Golden and Sherman, in preparation). This transformation apparently results from the efficient recombination of linear chromosomal DNA into the chromosome. With this phenomenon in mind, it is predictable that a chromosomal fragment displaced from a hybrid vector after interplasmid recombination would be incorporated into the chromosome. This is consistent with the report by Tandeau de Marsac and colleagues (19) that a methionine biosynthesis gene cloned in a hybrid cosmid vector recombines into the chromosome during *A. nidulans* passage. Their vector molecule contains multiply interrupted pUH24 sequences and would, therefore, be a substrate for the plasmid healing reaction described above. This would be followed by the incorporation of the displaced cloned DNA into the chromosome by homologous recombination.

The efficiency of *A. nidulans* transformation by the vector pSG111 is lower than that of some previously constructed hybrid plasmids (11, 15, 22), presumably because of the poor expression of the *E. coli* antibiotic resistance genes. *A. nidulans* RNA polymerase may weakly recognize the *E. coli* promoters or expression may be dependent upon readthrough from an *A. nidulans* promoter. The ligation of pUH24 sequences to the pBR328 molecule was made arbitrarily at the *Bam*HI site. If readthrough from *A. nidulans* genes is required to express the antibiotic resistance genes, pSG111 may have an alignment of these genes in a less favorable location than some of the other hybrid plasmids which have been constructed. pCH1 (22) was formed by the insertion of transposon Tn*901* (coding for ampicillin resistance) into the pUH24 molecule. Selection for ampicillin-resistant *A. nidulans* colonies would result in the isolation of a transposon insertion which efficiently expresses the antibiotic resistance gene. The use of transposons to form hybrid plasmids has an advantage over in vitro construction in that the location of selectable genes is not arbitrary. However, transposon-mediated cointegrate formation has the disadvantages of instability during formation (15). As discussed above, the rearrangement or interruption of pUH24 sequences may lead to a loss of cloned segments when the hybrid is used as a vector.

The relatively low expression of the antibiotic resistance genes of pSG111 should not pose a problem in the actual use of this molecule as a vector for the work we propose. Our overall goal is to use pSG111 as a vector for the selection of photosynthesis genes by complementation of conditional mutants defective in photosynthetic function. The primary selection of transformants will be for the complementing insert itself; transformants can then be screened for presence of the vector. This strategy is now being tested by "shotgun" cloning of an *A. nidulans* mutant gene coding for resistance to the herbicide 3,4-dichlorophenyldimethylurea. Cloning of the 3,4-dichlorophenyldimethylurea resistance gene should also provide a functional test for monitoring the reciprocal exchange of a cloned gene with its chromosomal allele.

Plasmid pUH24 preferentially inserted into the pBR328 molecule in the orientation shown for pSG111 (Fig. 2). A hybrid plasmid, pSG210, which has pUH24 inserted in a rare orientation, has a mutation which destroys the *Hin*dIII recognition site of the pBR328 molecule. When the pBR328 portion of pSG210 was released by *Bam*HI cleavage and religated, the resulting molecule transformed *E. coli* cells to ampicillin and chloramphenicol resistance. The transformants were, however, tetracycline sensitive, indicating that the mutation had destroyed the promoter activity of the tetracycline resistance gene. An identical treatment of the pBR328 molecule released from pSG111 resulted in transformants which were resistant to all three antibiotics. Perhaps the pSG210 orientation was obtained only when the tetracycline promoter of pBR328 was inactivated because a pUH24 gene product which would be inhibitory in *E. coli* was not produced from this molecule. The presence

or identity of genes on the pUH24 molecule has not yet been established.

The feasibility of using pSG111 as a vector in *A. nidulans* R2 has been demonstrated by stable maintenance of pRNA404, which contains all of the pSG111 sequence as well as an insert of *A. nidulans* chromosomal DNA. Unique restriction sites for the enzymes *Eco*RI, *Sal*I, and *Sph*I are present in the pBR328 portion of the hybrid, and these sites are suitable for the insertion of DNA without interruption of the pUH24 sequences. We are currently altering pSG111 to inactivate one of the *Bam*HI sites present at the junctions of *E. coli* and *A. nidulans* DNA. This will provide an additional unique restriction site for cloning in pSG111.

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