Cloning of the β-phycocyanin gene from *Anacystis nidulans*

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The β-phycocyanin gene, *pcyB*, of *Anacystis nidulans* was isolated from an *Escherichia coli* λ-phage bank by the use of synthetic oligonucleotides derived from the 170 amino acid sequence of the β-phycocyanin protein. Two positive, overlappling λ-clones were found. Partial DNA sequencing of one of the clones gave a deduced amino acid sequence which was in full agreement with a portion of the published sequence of *A. nidulans* β-phycocyanin. A comparison with the published DNA sequence for β-phycocyanin from *Agmenellum quadruplicatum* shows a DNA sequence homology of 70.4% over the sequenced region.

Cyanobacteria Anacystis nidulans *Phycobilisome* *β-Phycocyanin* *Photosynthesis gene* *DNA sequence* *Light-harvesting antenna*

1. INTRODUCTION

Cyanobacteria (blue-green algae) are prokaryotic organisms capable of photosynthetic [1,2]. The structure of their photosynthetic apparatus and their mode of photosynthesis resemble those of higher plants and eukaryotic algae [1,2]. Three major photosynthetic components can be defined in the cyanobacteria: two photosystems, PS I and PS II, and a light-harvesting complex, the phycobilisome [2–4], which is functionally analogous to the light harvesting chlorophyll-protein complex (LHCP) found in higher plants. The phycobilisome, which is built up of chromoproteins together with uncoloured linker polypeptides, is a water-soluble macromolecular complex, attached to the outer surface of the photosynthesic (thylakoid) membrane [4–6].

The phycobilisome of the unicellular cyanobacteria *Anacystis nidulans* 625 (*Synechococcus* 6301) can be divided into two major structures, the core and the rod [4,5]. The core consists of the chromophoric proteins allophycocyanin (α- and β-subunits), allophycocyanin B, and a high-M_r anchor protein which attaches the phycobilisome to the thylakoid membrane. The rod consists of the chromoprotein phycocyanin, composed of α- and β-subunits, and 3 uncoloured linker polypeptides [6]. *A. nidulans* as well as other cyanobacteria responds to changes in the light environment by modulating the length of the phycocyanin-containing phycobilisome rod.

Hybrid DNA technology has recently been used to isolate genes from *A. nidulans* and to study their gene organization. A cosmid library has been constructed in *E. coli* with *A. nidulans* R2 DNA, and a gene involved in methionine biosynthesis was isolated from the library by the marker rescue technique [7]. The genes coding for the large and small subunits of ribulose-1,5-bisphosphate carboxylase (Rubisco LS and SS) have also been isolated, utilizing the homology between these genes in higher plants and cyanobacteria [8,9]. The genes for Rubisco LS and SS were found to be closely linked on the cyanobacterial chromosome, thereby showing a marked difference in genome organization compared to higher plants [8,9]. It is possible to transform *A. nidulans* with foreign DNA and shuttle-vectors have been developed, indicating that complementation studies can be performed with cloned genes [10,11].

Here, we report on the successful isolation of the *A. nidulans* 625 β-phycocyanin gene, *pcyB*.

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2. MATERIALS AND METHODS

2.1. *Bacteria and phages*

A. nidulans 625 (*Synechococcus sp.* PCC 6301) [12] was obtained from the University of Texas strain collection. *E. coli* strain DP50 [13] and λ-phage Charon 28 (λ Ch28) [14] were used in the construction of the gene bank. *E. coli* strain JM103 and phage M13mp9 [15] were used for DNA sequencing.

2.2. *Media and growth conditions*

A. nidulans was grown under incandescent white light in Meyers medium [16], modified to contain 14.4 μM $FeCl_3 \cdot 6H_2O$ and 65 μM EDTA instead of Fe-*N*-hydroxyethyl-EDTA, utilizing equipment as described by Öquist [17]. Phage M13 was propagated on *E. coli* strain JM103 in YT medium [15]. Phage λ was propagated on *E. coli* strain DP50 as described [18].

2.3. *Enzymes and synthetic oligonucleotides*

Restriction endonucleases, T4 DNA ligase and DNA polymerase I (Klenow fragment) were obtained from New England Biolabs. Proteinase K was obtained from Merck, Darmstadt. RNase A and DNase I (DPFR) were supplied by Sigma and Worthington, respectively. All enzymes were used as described by the vendors. The synthetic oligonucleotides were supplied by Kabigen, Stockholm, and are described in fig.1.

2.4. *Preparation of chromosomal DNA*

A. nidulans cells were harvested by centrifugation, and treated with lysozyme (1 mg/ml) and EDTA (50 mM) for 90 min. SDS (0.5% w/v) and proteinase K (1 mg/ml) were added, and the sample was incubated for 30 min at 65°C, after which it was phenolextracted and ethanol precipitated. The precipitate was resuspended in TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and RNase A treated for 1 h at 37°C, and then for 30 min at 65°C. The sample was then phenol-chloroform extracted, ethanol precipitated, resuspended in 10-times diluted TE-buffer, and dialyzed against the same buffer. Visualization of DNA on agarose gels detected two plasmid bands in addition to the chromosome. The purified DNA from *A. nidulans* was kept frozen at −20°C.

2.5. *Construction of a λ Ch28 gene bank*

The procedure was essentially according to [18]. *A. nidulans* DNA (250 μg) was partially digested with 32 units of restriction endonuclease *Sau*3A. The digested and purified DNA was fractionated by velocity sedimentation through a 10–40% sucrose gradient and 6–20 kb DNA fragments were collected. *Bam*HI-digested λ arms (3 μg) were ligated to the *A. nidulans Sau*3A-digested DNA (1 μg), and packaged with an in vitro λ-packaging kit from Amersham. 0.8 μg ligated DNA was used per packaging reaction with the *E. coli* strain DP50 as indicator bacteria.

2.6. *Screening of the λ Ch28 gene bank*

The resulting plaques were restreaked, transferred to nitrocellulose filters (Schleicher and Schull BA85) and treated according to [18]. Colony hybridization was as described [19] with the modified hybridization temperature of 30°C and washing temperatures of 4°C followed by 15°C. ^{32}P-labelled synthetic oligonucleotides (fig.1) were used as probes.

2.7. *Isolation of λ DNA*

A plate lysate stock was made of the appropriate λ clones [18]. The bacteriophages were purified and their DNA extracted [18]. The isolated DNA was further purified by the use of Elutips (Schleicher and Schull) which were used as described by the supplier, with the substitution of 2 M NaCl instead of 1 M in the high-salt solution.

2.8. *DNA sequence determination*

*Sau*3A-digested λ 20:2 DNA was ligated to phage M13mp9, which was previously digested with *Bam*HI. The plaques obtained were screened using synthetic oligonucleotide P2 (fig.1) as probe. Single-stranded template DNA was isolated from positive plaques as described [15]. The partial DNA sequence was determined by the dideoxy chain termination method of Sanger et al. [20] using the synthetic oligonucleotide P2 as specific primer.

3. RESULTS

3.1. *Identification and characterization of λ-clones carrying the gene for β-phycocyanin* (pcyB)

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81                                        90
Cys+Leu-Arg-Asp-Met-Glu-Ile-Ile-Leu-Arg
            Probes P1 and P2

131                                       140
Arg-Lys-Met-Lys-Asp-Ala-Ala-Val-Ala-Ile
    Probe P3

Probes:      P1  AT AAT TTC CAT GTC
             P2  AT GAT TTC CAT GTC
             P3  AAA ATG AAA GAT GC
                   G       G
```

Fig.1. Portion of the amino acid sequence of *A. nidulans* phycobilisome protein β-phycocyanin [21]. The amino acid sequence is numbered from the carboxyl end. Cys^+ indicates one of the chromophore binding sites. The synthetic oligonucleotides P1–P3, used in the cloning of the β-phycocyanin gene are shown. Probes P1–P3 are complementary to the coding sequence. Probe P3 is a mixture of 4 oligonucleotides.

DNA was prepared from an axenic culture of *A. nidulans* 625 and was partially digested with restriction endonuclease *Sau*3A. The digested DNA was separated on a sucrose gradient and 6–20 kilobase pair (kb) DNA fragments were isolated and ligated to purified λ Ch28 arms. The ligated DNA was packaged in vitro and transfected to *E. coli* DP50 cells, giving rise to 1×10^5 plaque forming units per μg DNA. From the original plates, 2500 plaques, corresponding to more than a 95% probability of having a given DNA sequence represented in the library, were isolated and restreaked. The restreaked plaques were transferred to nitrocellulose filters and hybridized to a mixture of ^{32}P-labelled synthetic oligonucleotides P1–P3 (fig. 1). Two clones, λ 20:2 and λ 23:30, hybridized weakly to the probes (fig.2). The clones 20:2 and 23:30, together with a λ-clone containing the large subunit of ribulose-1,5-diphosphate carboxylate (Rubisco LS), were patched onto two plates and transferred to nitrocellulose filters.

Probes P1 and P2 were individually hybridized

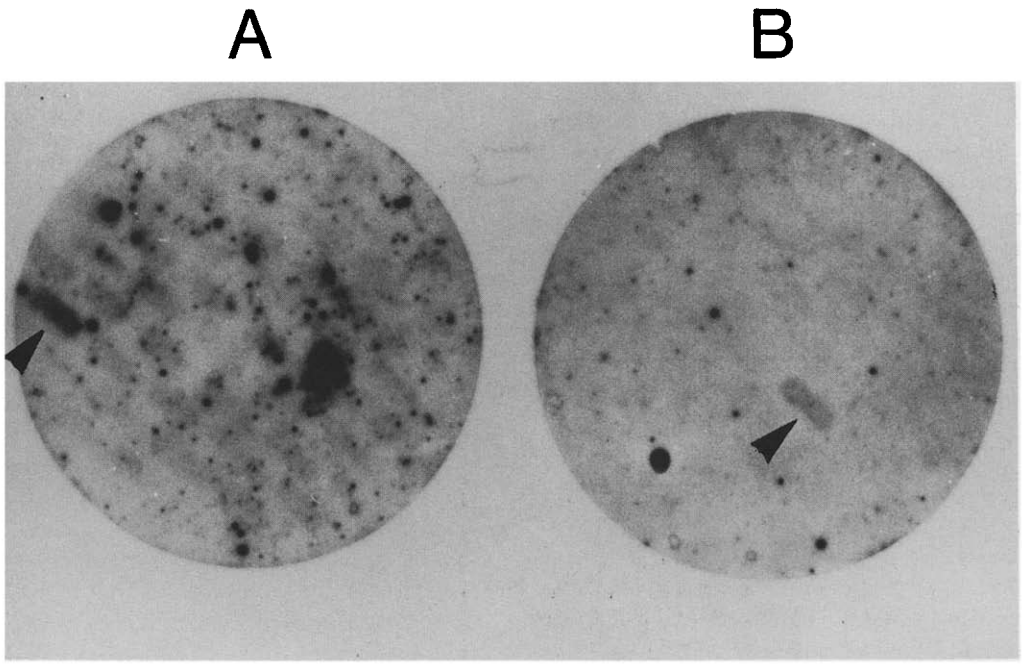

Fig.2. Hybridization of patched recombinant λ Ch28-*A. nidulans* plaques with a mixture of synthetic oligonucleotides P1–P3. Positive clones are indicated by arrows. Clones 20:2 and 23:30 are shown on plates A and B, respectively. Original recombinant plaques were restreaked, transferred to nitrocellulose filters and hybridized as described in section 2. The DNA-sequences of probes P1, P2 and P3 are shown in fig.1.

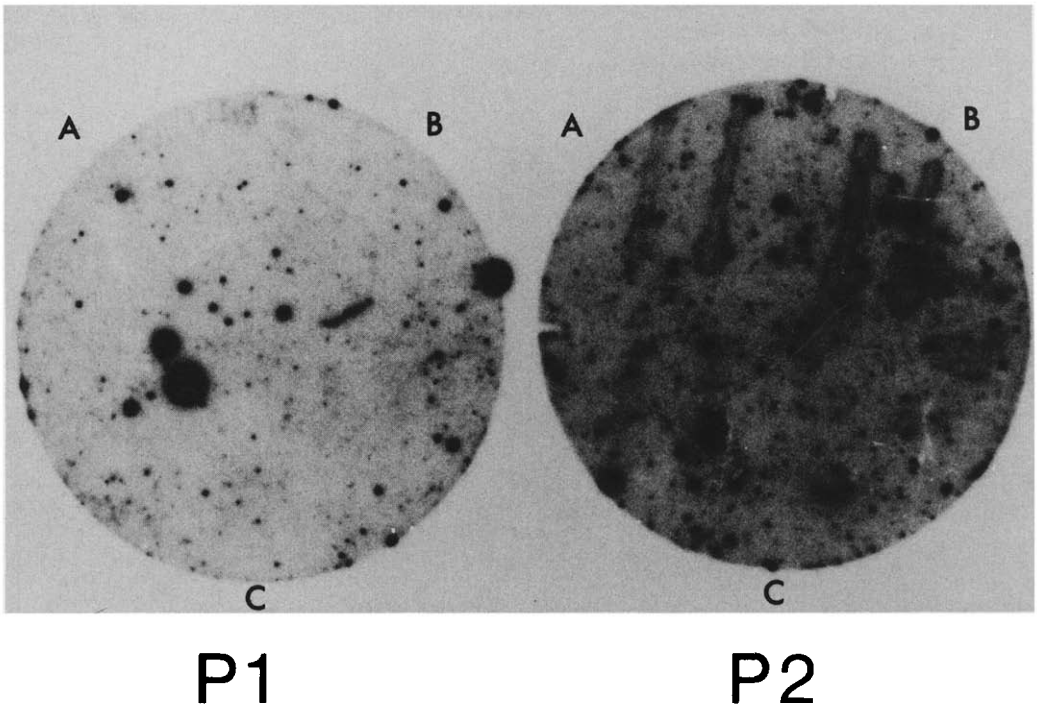

Fig.3. Hybridization of λ-clones 20:2 and 23:30 to probes P1 and P2. (A) λ Ch28 23:30, (B) λ Ch28 20:2, (C) λ Ch28 Rubisco LS. Probes P1 and P2 were used as indicated. Hybridization was performed as described in section 2.

to each of the two filters. From the result presented in fig.3, it can be seen that only probe P2 hybridized to λ clones 20:2 and 23:30. At a lower washing stringency, a weak hybridization was also detected between probe P1 and clones 20:2 and 23:30 (not shown). No hybridization was detected to the negative control represented by the Rubisco LS gene.

DNA from the λ clones 20:2 and 23:30 were prepared as described in section 2. The purified DNA was digested with different restriction endonucleases (*Eco*RI, *Hpa*I, *Hin*dIII, *Bam*HI, *Ava*I, *Sma*I and *Sac*II) and was analyzed on agarose gels. The fragment sizes obtained were compared to the restriction enzyme map of λ Ch28 [18], and from the data obtained, it was concluded that the *Bam*HI sites used to insert DNA in the clones λ 20:2 and λ 23:30 were not recreated. The lengths of the inserts in clones 20:2 and 23:30 were deduced to be 9 ± 1 and 13 ± 1 kb, respectively. The inserts in the two clones overlap by more than 8 kb. The β-phycocyanin gene was located on the inserted DNA using Southern hybridization technique with ^{32}P-labelled probe P2. The probe hybridized to an internal 7 kb *Ava*I fragment in both phages and also to an *Ava*I fragment of the same size in the complete *A. nidulans* genome (not shown), indicating that no rearrangements have occured during the construction of the λ-bank.

3.2. *Nucleotide sequencing of the β-phycocyanin gene*

The λ-clones 20:2 and 23:30 hybridized specifically to probe P2, indicating the potential existance of the β-phycocyanin gene. Isolated DNA from the λ clone 20:2 was digested with *Sau*3A and subcloned into the *Bam*HI site of phage M13mp9. Of the resulting M13 plaques, 650 plaques positive for insets, were restreaked and subsequently screened with probe P2. The hybridization yielded 4 positive clones, one of which (M13-747), contained a single *Sau*3A insert about 750 nucleotides long. A portion of the nucleotide sequence of this insert was determined by the dideoxy chain termination method, using probe P2 as primer. The resulting DNA sequence is 87 nucleotides in length, terminating at a *Sau*3A site at amino acid isoleucine at position 51 (fig.4).

```
 51                                          60
Ile Val Ala Asn Ala Ala Arg Ala Leu Phe
ATC GTC GCT AAC GCA GCG CGT GCA TTG TTT

                                            70
Ala Glu Gln Pro Ser Leu Ile Ala Pro Gly
GCA GAG CAA CCT TCT CTG ATT GCT CCT GGC

                                        77
Gly Asn Ala Tyr Thr Asn Arg
GGC AAC GCA TAC ACG AAC CGT
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Fig.4. The nucleotide sequence of part of the DNA insert of the M13 clone 747, hybridizing to probe P2. The numbering is in accordance with the amino acid sequence shown in fig.1. The DNA sequence shown is the reversed complement of the sequence read from the autoradiogram. The DNA sequence was determined with the dideoxy chain termination method using probe P2 as primer.

Comparison of the deduced amino acid sequence (fig.4) with the known sequence of β-phycocyanin [21] indicated that the two sequences are identical for 27 amino acids between positions 51 and 77 in the β-phycocyanin protein.

4. DISCUSSION

To reveal the control mechanisms optimizing the light-harvesting efficiency of photosynthetic organisms, it is of prime importance to isolate genes involved in the photosynthetic process. Here we describe the construction of a λ gene bank in *E. coli* which contains the genome of the cyanobacterium *A. nidulans* 625. We also describe the successful isolation of the gene encoding β-phycocyanin, which constitutes a major component of the light-harvesting complex, the phycobilisome.

To isolate the gene for β-phycocyanin, denoted *pcyB*, we constructed a λ-phage bank using the phage λ Charon 28, a nonlysogenic phage, whose use decreases the probability of having deletions or other rearrangements in the cloned *A. nidulans* DNA. It is interesting to note that the two λ-clones hybridizing to the β-phycocyanin probe have similar and overlapping restriction enzyme patterns, indicating that no DNA-reorganizations have taken place. In addition, the *Ava*I fragment hybridizing to the β-phycocyanin probe is of the same size in the two λ-clones as in the chromosome of *A. nidulans*.

The gene for β-phycocyanin was isolated using synthetic oligonucleotides corresponding to different regions of the amino acid sequence of the protein. Although different oligonucleotides were used, a strong hybridization signal was only obtained with the probe P2. Probe P1, covering the same region as probe P2, only gives a weakly detectable signal, despite the fact that it only differs from probe P2 by one base. We calculated the melting temperature for perfect matches of probes P1 and P2 under the hybridization conditions used, and found that they differ only by 2°C (36 and 38°C, respectively). With the 30°C hybridization temperature, provided perfect matches, both probes should have given positive signals, however, despite this the probe P1 did not hybridize. If probe P2 is presumed to match the DNA sequence for the β-phycocyanin gene, probe P1 would give an A–C mismatch. Such a mismatch decreases the hybridization temperature by at least 5°C [19], and this could explain the lack of hybridization with the P1 probe.

Phycocyanin has recently been cloned from the cyanobacteria *Agmenellum quadruplicatum* [22, 23]. From the DNA sequence and Northern blot experiments presented in those reports, it is clear that β- and α-phycocyanin are linked together on the chromosome and also co-transcribed. Preliminary data from our laboratory indicate that the same is true for *A. nidulans*.

The DNA sequence presented for β-phycocyanin in this report is located close to a cysteine residue involved in binding of one of the two phycobilin chromophores. This region has been thought to be conserved through evolution. A comparison between the *A. nidulans* and the *Ag. quadruplicatum* sequences also shows strong homology with 57 out of 81 compared bases being identical (70.4%). Preliminary data from the *A. nidulans* β-phycocyanin gene shows that the degree of homology towards *Ag. quadruplicatum* gene are lower in other regions of the DNA sequence.

Due to the lack of cloned genes, prior studies of the components of the phycobilisome have dealt with regulation of these components at the polypeptide level only. Future investigations in our laboratory will extend these studies to the interesting area of the gene regulation of these components. We intend to use the described region of the *A. nidulans* chromosome containing the β-phycocyanin gene, to study specific mRNA expression under different light conditions and during

shifts between different light environments. From these experiments, it should be possible to determine if the mRNA levels follow the fluctuations at the polypeptide level or whether other control systems are involved, thereby extending our knowledge of the regulation of phycobilisome biogenesis.

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REFERENCES

[1] Carr, N.G. and Whitton, B.A. (1982) The Biology of Cyanobacteria, Blackwell, London.
[2] Stanier, R.Y. and Cohen-Bazire, G. (1977) Annu. Rev. Microbiol. 31, 225–274.
[3] Glazer, A.N. (1982) Annu. Rev. Microbiol. 36, 173–198.
[4] Glazer, A.N. (1983) Annu. Rev. Biochem. 52, 125–157.
[5] Gantt, E. (1979) Int. Rev. Cytol. 66, 45–80.
[6] Yamanaka, G. and Glazer, A.N. (1981) Arch. Microbiol. 130, 23–30.
[7] Tandeau de Marsac, N., Borrias, W.E., Kuhlemeier, C.J., Castet, A.M., Van Arkel, G.A. and Van den Hondel, C.A.M.J.J. (1982) Gene 20, 111–119.
[8] Shinozaki, K., Chicko, Y., Takahata, N. and Sugiura, M. (1983) Proc. Natl. Acad. Sci. USA 80, 4050–4054.
[9] Shinozaki, K. and Sugiura, M. (1983) Nucleic Acids Res. 11, 6957–6964.
[10] Chauvat, F., Astier, C., Vodel, F. and Jiset-Espardellier, F. (1983) Molec. Gen. Genet. 191, 39–45.
[11] Kuhlemeier, C.J., Thomas, A.A., Van der Ende, A., Van Leen, R.W., Borias, W.E., Van den Hondel, C.A.M.J.J. and Van Arkel, G.A. (1983) Plasmid 10, 156–163.
[12] Rippka, R., Deruelles, J., Waterby, J.N., Herdman, M. and Stanier, R.Y. (1979) J. Gen. Microbiol. 111, 1–61.
[13] Leder, P., Tiemeier, D. and Engkvist, L. (1977) Science 196, 175–177.
[14] Rimm, D.L., Horness, D., Kucera, J. and Blattner, F.R. (1980) Gene 12, 301–309.
[15] Messing, J. and Vieira, J. (1982) Gene 19, 269–276.
[16] Siva, V., Rao, K., Brand, J.J. and Myers, J. (1977) Plant Physiol., 59, 965–969.
[17] Öquist, G. (1969) Physiol. Plant. 22, 516–528.
[18] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A laboratory manual, Cold Spring Harbor Laboratory, NY.
[19] Wallace, R.B., Johnson, M.J., Hirose, T., Miyake, T., Kawashima, E.H., and Itakura, K. (1981) Nucleic Acids Res. 9, 879–894.
[20] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[21] Freidenreich, P., Apell, G.S. and Glazer, A.N. (1978) J. Biol. Chem. 253, 212–219.
[22] Pilot, T.J. and Fox, L. (1984) Proc. Natl. Acad. Sci. USA 81, 6983–6987.
[23] De Lorimer, R., Bryant, D.A., Porter, R.D., Liu, W.-Y., Jay, E. and Stevens, S.F. jr (1984) Proc. Natl. Acad. Sci. USA 81, 7946–7950.