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Identification of the *chlB* Gene and the Gene Product Essential for the Light-Independent Chlorophyll Biosynthesis in the Cyanobacterium *Plectonema boryanum*

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We cloned a 6.0-kb *Hin*dIII fragment from the cyanobacterium *Plectonema boryanum* using the chloroplast *chlB* (ORF513) gene of the liverwort (*Marchantia polymorpha*) as a probe. An open reading frame (ORF508) encoding a polypeptide of 508 amino acid residues was found within the nucleotide sequence of the 4,437-bp *Hin*dIII-*Eco*RV subfragment. The deduced amino acid sequence of ORF508 shows very high similarity to that encoded by the liverwort *chlB* gene (72.7%). A mutant, YFB14, in which ORF508 was inactivated by the insertion of a kanamycin-resistance cartridge, was unable to synthesize chlorophyll, accumulating protochlorophyllide in darkness while synthesizing chlorophyll normally in the light. Thus, the *chlB* gene is the third gene that is essential for the light-independent reduction of protochlorophyllide. The other two genes are *chlL* and *chlN*, and the results suggest that the light-independent protochlorophyllide reductase consists of at least three subunits, which are encoded by *chlL*, *chlN* and *chlB*. Using an antiserum prepared against a ChlB-6xHis fusion protein expressed in *Escherichia coli*, we detected a protein with an apparent molecular weight of 58,000 in the membrane fraction of the cyanobacterium. These results indicate that either the cytoplasmic or thylakoid membranes could be the site of the light-independent reduction of protochlorophyllide.

Key words: *chlB* — Chlorophyll biosynthesis — Cyanobacterium — *Plectonema boryanum* — Protochlorophyllide reductase.

The reduction of Pchlide is a key step in the biosynthesis of Chl during the greening of phototrophic organisms. When seedlings of angiosperms grow in darkness, the synthesis of Chl is arrested prior to the reduction of Pchlide, because, in angiosperms, Pchlide is reduced exclusively by a light-dependent enzyme. NADPH:Pchlide oxidoreductase (POR; EC 1.3.1.33), which catalyzes this reduction, has an absolute requirement of light for the catalytic reaction (for review, see Griffiths 1991). Once etiolated seedlings are illuminated, the reduction of the accumulated Pchlide to chlorophyllide occurs as the first step in the greening process, and Chl is formed by esterification of the propionate moiety of chlorophyllide group with phytol. POR, the key enzyme in light-dependent greening, consists of a single polypeptide, and cDNAs encoding POR have recently been cloned from several plant species, namely, barley (Schulz et al. 1989), oat (Darrah et al. 1990), *Arabidopsis* (Benli et al. 1991), pea (Spano et al. 1992a), pine (Spano et al. 1992b), wheat (Teakle and Griffiths 1993), and cucumber (Kuroda et al. 1995).

By contrast to the angiosperms, some land plants (e.g., pines, ferns and liverwort), green algae (e.g., *Chlamydomonas* and *Chlorella*) and cyanobacteria (e.g., *Plectonema, Anabaena*) have the capability of greening even in darkness. This observation suggested that they have an alternative system for reduction of Pchlide that operates in a light-independent manner. However, very little information was available about such light-independent systems because suitable organisms for investigations by molecular genetic techniques have not been available.

Cyanobacteria are prokaryotes with a photosynthetic apparatus similar to that of higher plants. Some cyanobacterial species can grow heterotrophically utilizing sugar and they synthesize Chl even in darkness (Fujita et al. 1992, Mannan and Pakrasi 1993). These characteristics suggest that these microorganisms might provide a suitable system for investigations not only of the organization of the oxygenic photosynthetic apparatus (Berry et al. 1994, Golbeck 1994) but also of the biosynthesis of Chl including the light-independent reduction of Pchlide. Thus, in a series of studies of the cyanobacterium *Plectonema boryanum* (Fujita et al. 1992, 1993), two novel genes, *chlL* and *chlN* (formerly *frxC* and ORF467, respectively), were shown to be involved in the light-independent reduction of Pchlide. The amino acid sequences encoded by *chlL* and *chlN* exhibit significant similarities to those of subunits of nitrogenase and not to that of POR, suggesting that the light-independent Pchlide reductase might have a molecular structure similar to that of nitrogenase (Fujita et al. 1992, 1993, Burke et al. 1993c).

The *chlL* and *chlN* genes have been found in the chloroplast DNAs from a variety of plants (Lidholm and Gustafsson 1991, Ohyama et al. 1988, Reith and

Abbreviations: 6xHis, six consecutive histidine residues; IPTG, isopropyl-1-thio-β-D-galactoside; kb, kilobase; ORF, open reading frame; Pchlide, protochlorophyllide; POR, NADPH: protochlorophyllide oxidoreductase.

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Munholland 1993, Richard et al. 1994, Tsudzuki et al. 1992, Yamada et al. 1992a, b) and algae (Choquet et al. 1992, Li et al. 1993, Liu et al. 1993, Richard et al. 1994, Suzuki and Bauer 1992) but not in angiosperms (Hiratsuka et al. 1989, Shinozaki et al. 1986, Wolfe et al. 1992). It has been established that the presence of these genes is a prerequisite for greening in darkness. Molecular genetic studies using the green alga *Chlamydomonas reinhardtii* have confirmed that the two genes, *chlL* (Suzuki and Bauer 1992) and *chlN* (Choquet et al. 1992), are involved in the light-independent reduction of Pchlide in chloroplasts. In addition, it has been shown that the *chlB* gene in chloroplast DNA is also involved in this reduction (Li et al. 1993, Liu et al. 1993).

To our knowledge no information has been reported about the cyanobacterial homologue of *chlB*. In the present study, we cloned the *chlB* gene from the cyanobacterium *P. boryanum* and identified the gene product for the first time in the membrane fraction of the cyanobacterial cells as part of our efforts to characterize the light-independent Pchlide reductase.

Materials and Methods

Cultivation of the cyanobacterium—The cyanobacterium *Plectonema boryanum* IAM-M101 was cultivated as described previously (Fujita et al. 1991). In this study, a dark-adapted strain "*dg5*" was used. The strain *dg5* was isolated by the long-term incubation (40 d) of the original parent strain IAM-M101 in darkness in the presence of 30 mM glucose. In darkness, the doubling time of the *dg5* strain was about 66% of that of the original strain.

Southern blot analysis—The genomic DNA of *P. boryanum* was probed with the *chlB* (ORF513) gene of liverwort. Plasmid pMP795 (Ohyama et al. 1988), carrying the chloroplast *chlB* gene of liverwort was kindly provided by Dr. Kanji Ohyama (Kyoto University), and the 918-bp *Hin*dIII fragment containing the *chlB* gene was labeled with digoxigenin-dUTP (DNA Labeling and Detection Kit Non-radioactive; Boehringer Mannheim Yamanouchi, Tokyo, Japan). Genomic DNA (2 μg) from *P. boryanum dg5*, prepared as described in Fujita et al. (1992), was digested with *Hin*dIII or *Ssp*I. The DNA fragments were fractionated by electrophoresis in an agarose gel (0.8%) and transferred to Hybond-N membrane (Amersham, Arlington Heights, IL, U.S.A.) by the standard capillary method (Sambrook et al. 1989). Hybridization was carried out overnight at 50°C according to the instructions supplied with the kit. The filter was washed twice in 2 × SSC and 0.1% SDS at room temperature and twice at 50°C, and hybridization signals were visualized with alkaline phosphatase-conjugated antibodies against digoxigenin and appropriate reagents.

Cloning of the cyanobacterial chlB gene and DNA sequencing—A partial genomic library was constructed to clone the 6.0-kb *Hin*dIII fragment that included the *chlB* gene. About 100 μg of genomic DNA from the *dg5* strain were digested with *Hin*dIII and fractionated by agarose gel (0.8%) electrophoresis. DNA fragments of 4 to 7 kb were excised and ligated into the dephosphorylated *Hin*dIII site of pBluescript II SK+ (Stratagene, La Jolla, CA, U.S.A.). The ligation mixture was used to transform *E. coli* DH5α. Plasmid pYFA45, carrying the 6.0-kb *Hin*dIII fragment, was isolated by screening 320 recombinant clones by colony hybridization with the 918-bp fragment of the *chlB* gene from liverwort as the probe.

The sequences of both strands of the 4.4-kb *Hin*dIII-*Eco*RV fragment (Fig. 1A) were determined with a series of nested deletion clones that were constructed by the standard method using exonuclease III (Sambrook et al. 1989). The template DNAs were isolated by "Easypreps" (Berghammer and Auer 1993), and sequenced with DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA, U.S.A.). Specific oligonucleotide primers were used for sequencing of some regions in cases where appropriate deletion clones were not obtained.

The deduced amino acid sequence encoded by the *chlB* gene was aligned with those encoded by the other *chlB, bchB, bchZ, chlN* and *nifK* genes according to the results of a homology search with the FASTA program (Pearson and Lipman 1988).

Targeted mutagenesis of P. boryanum—The kanamycin-resistance (*neo*) cartridge was excised from pMC19 (Fujita et al. 1992) by digestion with *Bam*HI, and it was subcloned into the *Bam*HI site of pUC12 in the same direction as that of the transcription of *lacZ'* to generate pUCK122. The *neo* gene fragment excised from pUCK122 by digestion with *Eco*RI was ligated with pYFA45 that had been partially digested with *Eco*RI. Three plasmids, pYFB11, pYFB14 and pYFB16, were obtained. They carried the *neo* gene at one of the five *Eco*RI sites in the 6.0-kb *Hin*dIII fragment, oriented in the same direction as that of the transcription of the *chlB* gene (Fig. 1A).

The plasmids were linearized by digestion with *Kpn*I and introduced into cells of *P. boryanum dg5* by electroporation as described by Fujita et al. (1992, 1993). Transformants were selected on kanamycin-containing plates and were checked for sensitivity to ampicillin (0.1 μg ml^{-1}) to distinguish double recombinants from single recombinants which had the wild-type target gene in addition to the disrupted target gene. Genomic DNA was isolated from the various transformants obtained, and insertion of the *neo* gene at the expected site was confirmed by Southern blot analysis with digoxigenin-labeled pYFA45 as the probe.

Characterization of mutants and analysis of pigments—Growth of the mutants was evaluated by measurements of turbidity (OD_{730}) and Chl contents were determined as described previously (Fujita et al. 1992). Absorbance and fluorescence spectra of methanol extracts of the mutants and *dg5* were recorded with a spectrophotometer (UV-365; Shimadzu, Kyoto) and a spectrofluorometer (RF-1500; Shimadzu), respectively, at room temperature.

Overexpression of the chlB gene in E. coli and purification of the gene product—A gene for the ChlB-6xHis fusion protein was constructed by the insertion of a PCR-amplified fragment of the *chlB* gene into an *E. coli* expression vector pQE-70 (QIAGEN Inc., Chatsworth, CA, U.S.A.), which provides a 6xHis affinity tag at the carboxy-terminus of the protein encoded by the inserted fragment. A pair of oligonucleotides, designated QEB1 (forward primer; 5'-CGCTGCATGCTGAAATTAGCTTATTGGA-3') and QEB2 (reverse primer; 5'-TTAGGATCCAGCACCCACTGCT TCTTT-3'), was used to amplify a DNA fragment (1,538 bp) that included the entire coding region of *chlB* connected to *Sph*I and *Bam*HI sites (underlined). PCR was conducted under standard conditions using pYFA45 that had been linearized by digestion with *Bam*HI as the template. The amplified 1.5-kb DNA fragment was digested with *Sph*I and *Bam*HI, and ligated into the *Sph*I and *Bam*HI sites of pQE-70 to generate pQEB4. *E. coli* JM105 carrying pQEB4 was cultivated at 37°C for 2 h in LB medium that contained 50 μg ml^{-1} ampicillin after inoculation, at a dilution of 1 to 100, with an overnight culture. IPTG was added to a final concentration of 2 mM to induce the expression of the ChlB-6xHis fu-

sion protein, and cultivation was continued for a further 5 h. The *E. coli* cells were harvested by centrifugation and disrupted by sonication (Sonifier 350; Branson Sonic Power Co., Danbury, CT, U.S.A.). The lysate was centrifuged at 20,000 × *g* for 10 min. The ChlB-6xHis protein was extracted from the pellet in 50 mM sodium phosphate buffer (pH 6.8) that contained 8 M urea and 10 mM 2-mercaptoethanol. The solubilized proteins were loaded onto a HiTrap affinity column (HiTrap Chelating; Pharmacia LKB Biotechnology, Piscataway, NJ, U.S.A.) that formed part of an FPLC system (Pharmacia LKB Biotechnology). The protein that absorbed to the column was eluted with a linear gradient of imidazole from 0 to 0.25 M in the sodium phosphate buffer. The pooled fractions containing the ChlB-6xHis fusion protein were further fractionated by gel filtration on a HiLoad column (HiLoad 26/60 Superdex 200pg; Pharmacia LKB) that had been equilibrated with the sodium phosphate buffer.

Preparation of antibodies—Polyclonal antibodies against the ChlB-6xHis fusion protein were raised by immunization of a white rabbit. The purified ChlB-6xHis protein (1 mg) was emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, MI, U.S.A.) and injected subcutaniously into the rabbit. A second injection of the protein emulsified with Freund's incomplete adjuvant, as a booster, was given three weeks after the initial injection. For Western blot analysis, the ChlB-specific polyclonal antibodies were purified from the rabbit serum as described by Fujita et al. (1989). Antiserum against the 33-kDa protein of spinach was kindly provided by Dr. Takashi Ideguchi (Osaka University).

Preparation of a membrane fraction from P. boryanum cells and Western blot analysis—*P. boryanum* cells, grown in BG-11 medium supplemented with 20 mM HEPES-NaOH (pH 7.5) and 30 mM glucose in the light for 10 d at 30°C, were harvested by centrifugation (3,000 × *g*, 10 min) and stored at −80°C. The cells were disrupted by sonication (Sonifier 350) in 50 mM Tris-HCl (pH 8.0). The suspension was centrifuged at 3,000 × *g* for 10 min at 4°C to remove cell debris. Soluble and insoluble membrane fractions were prepared by ultracentrifugation at 180,000 × *g* for 1 h. Proteins separated by SDS-PAGE were electro-transferred from the gel to a PVDF membrane (Immobilon P; Millipore, Bedford, MA, U.S.A.) as described by Fujita et al. (1989). The PVDF membrane was incubated with affinity-purified antibodies and then with alkaline phosphatase-conjugated antibodies raised in goat against rabbit IgG (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The specific proteins were visualized by the generation of the colored reaction products the alkaline phosphatase conjugate in the standard manner.

Results

Cloning of the cyanobacterial chlB gene and its nucleotide sequence—Southern blot analysis of the genomic DNA from *P. boryanum* was performed with the fragment of the liverwort *chlB* (ORF513) gene as the probe. Clear positive signals were detected in several restriction digests and a DNA fragment of about 6 kb hybridized with the probe in the *Hin*dIII digest (data not shown). The 6.0-kb *Hin*dIII fragment was cloned from a partial library of *P. boryanum dg5* (Fig. 1A) to yield pYFA45. Southern blot analysis of several subfragments of the 6.0-kb *Hin*dIII insert indicated that the hybridizing region was localized in the "left" part of the *Hin*dIII fragment (data not shown). Therefore, the nucleotide sequence of 4,437 bp from the left *Hin*dIII site to the unique *Eco*RV site was determined (Figs. 1, 2). We found two ORFs, ORF508 and ORF306, and two partial ORFs, URF4 and URF5.

ORF508 encoded a polypeptide of 508 amino acid residues with a molecular weight of 56,819. The deduced amino acid sequence encoded by ORF508 was very similar (61–73%) to those encoded by the chloroplast *chlB* genes from liverwort, *Chlamydomonas*, pine and ginkgo (Fig. 3).

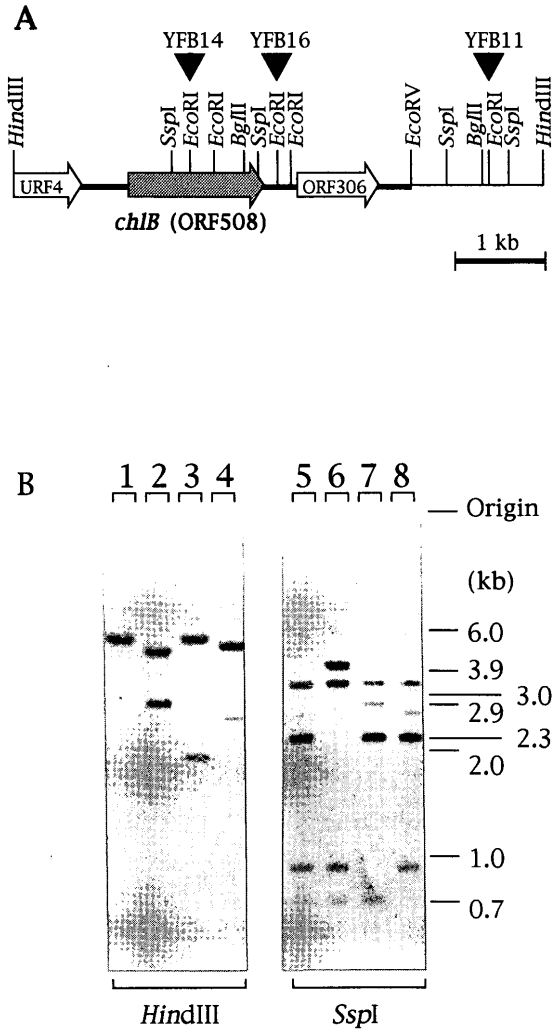

Fig. 1 (A) A physical map of the 6.0-kb *Hin*dIII insert of pYFA45 including the *chlB* (ORF508) gene. The thick line indicates the region whose nucleotide sequence was determined in this study. ORFs found in this region are shown by the thick arrows (ORF508 is shaded). Solid triangles indicate the sites at which the *neo* gene (1.9 kb) was introduced in each mutant. (B) Southern blot analysis of the genomic DNA of the wild type (*dg5*; lanes 1 and 5) and the mutants, namely, YFB11 (lanes 2 and 6), YFB14 (lanes 3 and 7) and YFB16 (lanes 4 and 8). Genomic DNA (0.4 μg each) from *dg5* and the mutants was digested with *Hin*dIII (lanes 1–4) and *Ssp*I (lanes 5–8) and fractionated on an agarose gel (0.8%). After transfer to a Hybond-N membrane, the fragments of DNA were probed with linearized pYFA45 that had been labeled with digoxigenin-dUTP.

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      AAGCTTGCAGAAAAGCGAGGATTTGGTCTTACTGATGGGTTCTGGAATCGCTTTGGTGTAGTTCTCATCGAAGCTCCAATTCACCTTTTTACTTCTACTC  100
URF4> K  L  A  E  K  R  G  F  G  L  T  D  G  F  W  N  R  F  G  V  V  L  I  E  A  P  I  H  L  F  T  S  T  L

      TTGCTAACTACTTTCGAGCAGAATATGATGCCGACATCTTTCCTGCATCATTTAATGAACAGGAGAAACATTTTCTTTGGCAATATGCTGGACATACCTG  200
        A  N  Y  F  R  A  E  Y  D  A  D  I  F  P  A  S  F  N  E  Q  E  K  H  F  L  W  Q  Y  A  G  H  T  W

      GACAGTTTGGTGGGCATTTTCGTCTGAAGAGGTAGCATTTGCACTTGCTCTTTTCCTAGAAACCAAGGCTATTGTCATTACTCATCAAGGAACTAGTGAA  300
       T  V  W  W  A  F  S  S  E  E  V  A  F  A  L  A  L  F  L  E  T  K  A  I  V  I  T  H  Q  G  T  S  E

      TGGAGTACTGTAAAAATCTTCTATCAGGACAGGTGGACTGAATACTATCACTTCGGATCTGATGACTATGATGACCCTAATGAGAAGATTGGCGATCGCG  400
      W  S  T  V  K  I  F  Y  Q  D  R  W  T  E  Y  Y  H  F  G  S  D  D  Y  D  D  P  N  E  K  I  G  D  R  G

      GTTGGGGTGCTGGCTACTGGGATCTTGAAATAACTTATGAATACTTCGTATCTCAACAGTCTACTTTCCCAATTCAGACGCGGCATTTGTTCAGCAGCGT  500
        W  G  A  G  Y  W  D  L  E  I  T  Y  E  Y  F  V  S  Q  Q  S  T  F  P  I  Q  T  R  H  L  F  S  S  V

      CATGCGAAAAGTTACTGAATCTGAAATGAAATCAGTGCTTAAATCTGGAACAGGCAAATTTGGTTTTCTTCATGCAACTCTTCAGCACTATGGAGCTTAT  600
       M  R  K  V  T  E  S  E  M  K  S  V  L  K  S  G  T  G  K  F  G  F  L  H  A  T  L  Q  H  Y  G  A  Y

      CTTCCCGATTGCAATGAAACGCCCCTTAACTACTCACGCTCCGATTTTGACTTTCATCCAGTTCGGTCAGACTTTGAGCGAGTTGATGCTTTAGTCTTAC  700
      L  P  D  C  N  E  T  P  L  N  Y  S  R  S  D  F  D  F  H  P  V  R  S  D  F  E  R  V  D  A  L  V  L  P

      CCAAAGAAACGTTTTATTGGCGAAATATGCTGCCTGTTCCAGAGCATGTAGCACCATAACAATCCCAATGCACACCGACCGCCGAGAAGTTATCGGTTAG  800
        K  E  T  F  Y  W  R  N  M  L  P  V  P  E  H  V  A  P  *

      AGGTCGAGCTTATCTGCGGCGGGTGATTGGGAACGTTAGGCTGAGTCATCGTTTAGTATATAAGTACGAATAACTTGCACGAAGTATTTTCTGATGGCAA  900

      TGCAAAAGGTGAGCTTAACTATTATTTGTGCAATTCTGAGCAAGAGATATTCACGCTCGTGTGCTTGTGAAACTGCGATCGACAGAACTAGTTCACCACG 1000

      ATAAATCTGATAGGAAGATAGGGATTACTCTAATTCTAGGTTCTCCCCGCTCCAAATTCATTCATAGAGTTTGTTGTAGTTTAGCAACGAACTATTAAGA 1100

      ATCACTGGCTGTGAAATCACGCTCTAGCCGCTCCTGAGAACGGTTTGCAGCTTTTTTAATGGCAGTGCGATCGCGTAACACATCACAAAGCAAGCGCAAA 1200

      CGCGGTCAGTAACTTTGAGAGAATGCAAGAGCAATCTCCACATCATCATTTCACGCTTCATGAAATTAGCTTATTGGATGTACGCAGGTCCCGCGCATAT 1300
                                                    chlB(ORF508)>M  K  L  A  Y  W  M  Y  A  G  P  A  H  I

      TGGCACTCTCCGCATTGCCAGCTCTTTTAAAAATGTTCATGCCATCATGCACGCGCCGCTCGGCGATGACTATTTCAACGTCATGCGATCGATGTTGGAA 1400
       G  T  L  R  I  A  S  S  F  K  N  V  H  A  I  M  H  A  P  L  G  D  D  Y  F  N  V  M  R  S  M  L  E

      CGAGAGCGTGATTATACTCCAGTCACAACCAGTGTCGTCGATCGACATGTGCTGGCGCGGGGTTCTCAAGAGAAAGTCGTTGATAACATCACCCGGAAAG 1500
      R  E  R  D  Y  T  P  V  T  T  S  V  V  D  R  H  V  L  A  R  G  S  Q  E  K  V  V  D  N  I  T  R  K  D

      ATGCTGAAGAACATCCTGACTTAATTGTGCTGACTCCGACTTGTACCTCTAGTATTTTGCAGGAAGATTTGCAGAATTTCGTCGAGCGAGCACAACTCGA 1600
        A  E  E  H  P  D  L  I  V  L  T  P  T  C  T  S  S  I  L  Q  E  D  L  Q  N  F  V  E  R  A  Q  L  E

      AGCCAAAGGCGATGTGATGTTAGCGGATGTGAATCACTACCGCGTCAATGAGCTACAAGCCGCCGATCGCACGTTACAGCAAATCGTTCAGTTCTATATC 1700
       A  K  G  D  V  M  L  A  D  V  N  H  Y  R  V  N  E  L  Q  A  A  D  R  T  L  Q  Q  I  V  Q  F  Y  I

      GCCAAAGCTCGCAAGCAAGGCAATCTCGTCACTGAGAAAACCGAAAAACCTTCCGTCAATATTTTCGGAATGACGACGCTCGGATTCCATAACAATCACG 1800
      A  K  A  R  K  Q  G  N  L  V  T  E  K  T  E  K  P  S  V  N  I  F  G  M  T  T  L  G  F  H  N  N  H  D

      ATGCGACTGAACTGAAAAAGTTAATGTCGGATTTGGGCATTGAAGTCAATGCGATTGTGCCTGCAGGCGCAAGTGTTCATGAACTAAAATCCCTTCCTCG 1900
        A  T  E  L  K  K  L  M  S  D  L  G  I  E  V  N  A  I  V  P  A  G  A  S  V  H  E  L  K  S  L  P  R

      GGCTTGGTTTAACCTGGTGCCTTATCGCGAAACAGGGTTACTAGCAGCCGAATTTTTACAGCAAGAATTCAACATGCCTTATGTTGACATTACGCCGATC 2000
       A  W  F  N  L  V  P  Y  R  E  T  G  L  L  A  A  E  F  L  Q  Q  E  F  N  M  P  Y  V  D  I  T  P  I

      GGCATTGTGGAAACGGCACGCTGTATTCGCAAAATTCAGCAAGTGATCAATGCTCAAGGTGCAAATGTTGACTATGAACCCTTCATTGATCAGCAAACGC 2100
      G  I  V  E  T  A  R  C  I  R  K  I  Q  Q  V  I  N  A  Q  G  A  N  V  D  Y  E  P  F  I  D  Q  Q  T  R

      GATTTGTGTCTCAAGCGGCTTGGTTTTCTCGATCGATTGACTGCCAAAATCTCACAGGCAAAAAAGCAGTCGTTTTCGGAGATAACACTCATGCAGCAGC 2200
        F  V  S  Q  A  A  W  F  S  R  S  I  D  C  Q  N  L  T  G  K  K  A  V  V  F  G  D  N  T  H  A  A  A

      CTTGACCAAGATTCTGGCACGCGAGATGGGAATTCACGTATTGCTGGCAGGAACTTATTGCAAATACGATGAAGCATGGTTTAGAGAACAAGTCAGCGAA 2300
       L  T  K  I  L  A  R  E  M  G  I  H  V  L  L  A  G  T  Y  C  K  Y  D  E  A  W  F  R  E  Q  V  S  E

      TACTGTGATGATGTCTTAGTCAGCGATGACAATGGACAAATTGCAGATGCGATCGCGCGGCTTGAACCTGCTGCAATCTTCGGAACTCAGATGGAACGCC 2400
      Y  C  D  D  V  L  V  S  D  D  N  G  Q  I  A  D  A  I  A  R  L  E  P  A  A  I  F  G  T  Q  M  E  R  H

      ACGTTGGAAAACGGCTCGATATTCCGTGTGGTGTGATTGCGGCTCCCATTCACATTCAAAACTTCCCAGTGGGCTACAAGCCATTTGTTGGCTACGAAGG 2500
        V  G  K  R  L  D  I  P  C  G  V  I  A  A  P  I  H  I  Q  N  F  P  V  G  Y  K  P  F  V  G  Y  E  G

      AAGCAATCAGATTGTGGATTTGATCTACAACTCGTTTACTTTGGGCATGGAAGATCATCTGCTGGAGATCTTTGGCGGTCACGATACAAAAGAAGTGATT 2600
       S  N  Q  I  V  D  L  I  Y  N  S  F  T  L  G  M  E  D  H  L  L  E  I  F  G  G  H  D  T  K  E  V  I

      ACCAAATCTGTTTCAGCCGACTCTGATTTGAACTGGAGTAAAGACGGACTGGCAGAACTGAACCGTATTCCGGGCTTTGTGCGCGGTAAGGTGAAGCGGA 2700
      T  K  S  V  S  A  D  S  D  L  N  W  S  K  D  G  L  A  E  L  N  R  I  P  G  F  V  R  G  K  V  K  R  N

      ACACTGAGAAATTTGCCCGCGATCGCAATATTACTCAGATCACTGCCGAAGTTCTTTACGCTGCGAAAGAAGCAGTGGGTGCTTAGTTGATCAATCAGTC 2800
        T  E  K  F  A  R  D  R  N  I  T  Q  I  T  A  E  V  L  Y  A  A  K  E  A  V  G  A  *

      GCCGCTAGTATTCGATTTTGCGGCGACTGATGGAACTATGCGATCGTGTTTTAGATGATGTTTGAACAGTACCGTTCATTGCAGTTCACCCGCCCCAGAA 2900

      AAGTAATCGTTCATCGCATTCACCCGCCCCGGAATGGAATTCGGGGCTAGTTGAACGAAGTCCACTCAAGGGACTAACAGCCAATTAGAATCAAGTCTTC 3000

      AGTCCTTTTAAGGACTTCGCACCGTTAGCCCTGGAATTCCATTCCGGGGCGAGATAGAACGGAGCAAGAAAACTAGCACTCTCTCAAGCGAATCTGCGCA 3100
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Fig. 2A

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ATTCTCTACTCGCTTCGATTACACTGGATCTCTGTCTTTCACAAGCGCTGTCCATGTCTGTTTTCGCTGAGTTAATCGGGCAATCTCAAGCTGTCGAATT 3200
                                          ORF306>M  S  V  F  A  E  L  I  G  Q  S  Q  A  V  E  L

GCTAGAAAGTGCGATCGCGCAAGATCGTCTCGCTCCGGCTTATCTATTTGTGGGTTCTCCGGGCATTGGGAAAAGTCTAGCTGCTGAATGTTTTCTCGAA 3300
 L  E  S  A  I  A  Q  D  R  L  A  P  A  Y  L  F  V  G  S  P  G  I  G  K  S  L  A  A  E  C  F  L  E

ACCTTACTCGCATCGTCTCGATCGCGGATTCTCAACCGTAATCATCCTGATTGGTTTGGGGTTGCACCGACTTATCTCCATCAAGGTAAAAGACTAAGTG 3400
T  L  L  A  S  S  R  S  R  I  L  N  R  N  H  P  D  W  F  G  V  A  P  T  Y  L  H  Q  G  K  R  L  S  A

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  A  E  A  E  A  A  G  V  K  R  K  T  P  P  M  I  R  L  E  Q  I  R  E  V  S  Q  F  L  S  R  P  P  L

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 E  A  V  R  S  T  V  V  I  E  Q  A  E  T  M  P  E  A  A  A  N  A  L  L  K  T  L  E  E  P  G  R  A

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T  I  I  L  L  A  P  S  L  E  S  V  L  P  T  I  V  S  R  C  Q  R  I  P  F  S  R  L  D  A  T  S  M  A

CAGAAGTCTTGAGTAAAACTGGACATGAAGCGATTCTGGCACAGCCGCAGTTACTAGCACTAGCACAGGGCAGTCCTGGAGCCGCGATCGCACATCAGCA 3800
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T  D  S  E  T  Q  L  W  L  I  D  Y  L  Q  Q  G  Y  W  A  Q  M  Q  H  Q  E  R  P  L  Q  L  L  E  Q  A

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  K  T  M  L  L  A  Y  V  Q  P  Q  L  V  W  E  V  T  W  M  Q  L  I  N  *

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                                                  URF5>M  T  E  Q  V  Y  D  V  V  V  V  G  G  G  V  A

CAGGAGCCGCTTTACTTTATTCCCTGGCAACGTTCACGGATTTGAAACGTGTGGCACTGATTGAAAAATACTCACAGGTTGCGACGGTCAATTCCAAATC 4400
  G  A  A  L  L  Y  S  L  A  T  F  T  D  L  K  R  V  A  L  I  E  K  Y  S  Q  V  A  T  V  N  S  K  S

AACGAACAATAGTCAAACCATTCATTGCGGCGATATC 4437
 T  N  N  S  Q  T  I  H  C  G  D  I
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Fig. 2B

Fig. 2 Nucleotide sequence of the 4,437-bp *Hin*dIII-*Eco*RV fragment and the predicted amino acid sequences of URF4, *chlB* (ORF508), ORF306 and URF5. The nucleotide sequence is shown in the direction of transcription. Candidates for ribosome-binding sites are underlined. The sequence data will appear in the DDBJ/EMBL/GenBank Nucleotide Sequence Databases under the accession number D78208.

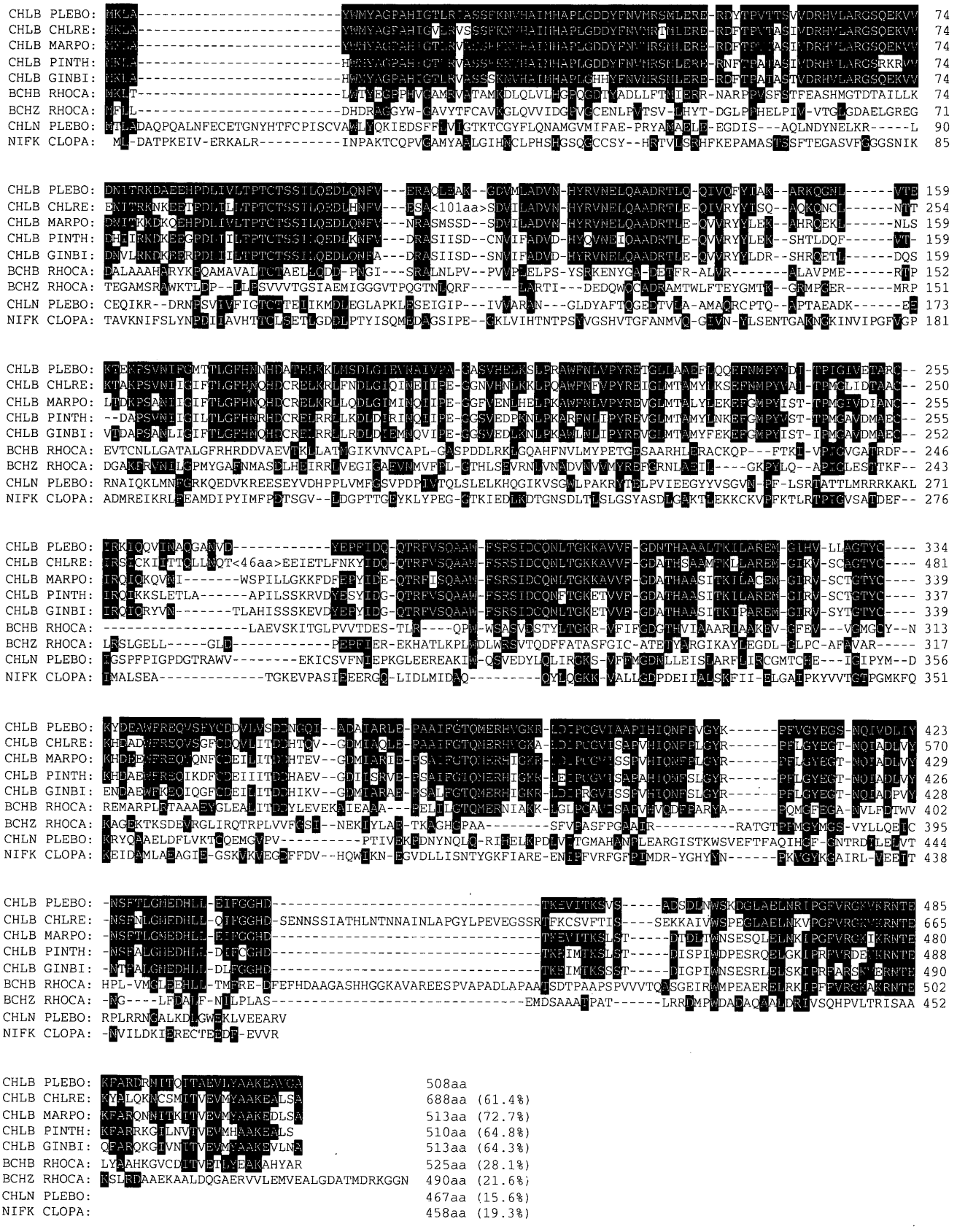
Thus, it appeared that ORF508 was a homologue of *chlB*. In a comparison with the *chlB* homologue of a photosynthetic bacterium, *bchB* from *Rhodobacter capsulatus*, the extent of similarity was found to be only 28.1%.

ORF306, located 367 bp downstream of ORF508, encoded a polypeptide of 306 amino acid residues with a molecular weight of 33,745. The deduced amino acid sequence of ORF306 showed similarities to those of some subunits of DNA polymerase III from *E. coli* (Flower and McHenry 1986, Yin et al. 1986, Carter et al. 1993, Dong et al. 1993) and *Bacillus subtilis* (Alonso et al. 1990). The extent of sequence similarity was 24.9% to the amino-terminal region (residues 1-217) of the γ and τ subunits of the enzyme from *E. coli*, and 29.0% to the central region (residues 94-270) of the δ' subunit. A hypothetical 37.6-kDa protein found in the *xpaC-abrB* intergenic region of *B. subtilis* (Ogasawara et al. 1994) was found to show 21.1% similarity to ORF306 over the entire respective stretch of polypeptide. The protein encoded by ORF306 had an ATP-binding motif (GxxxxGKS) in the amino-terminal region, as is also found in the γ and τ subunits of DNA polymerase III and the hypothetical 37.6-kDa protein.

Targeted mutagenesis of ORF508—To identify the function of the protein encoded by ORF508, we performed targeted mutagenesis with a kanamycin-resistance cassette. Plasmid pYFB14 was constructed by the insertion of the *neo* gene cartridge into the *Eco*RI site at the 237th codon of ORF508 in the same direction as that of the transcription of ORF508. In addition, two other plasmids were also constructed. Plasmids pYFB11 and pYFB16 carried a *neo* gene cartridge at different *Eco*RI sites outside the coding region of ORF508 (Fig. 1A).

The three plasmids pYFB11, pYFB14 and pYFB16 were linearized and electroporated into cells of the parent strain *dg5*. After screening on kanamycin-containing agar plates, three mutants, namely, YFB11, YFB14 and YFB16, were isolated. Insertion of the *neo* gene cartridge at the expected sites in the genomic DNA of the mutants was confirmed by Southern blot analysis (Fig. 1B).

To examine whether these mutants could synthesize Chl in darkness, the mutant cells were cultivated in BG-11 medium supplemented with glucose in darkness. The wild-type (*dg5*) strain and the *dg5*-derived mutant YFC2 that carried the *neo* gene cartridge in the *chlL* gene, which was identical to YFC1004 (Fujita et al. 1992), were also examined as controls. As shown in Figure 4A, all of the mutants



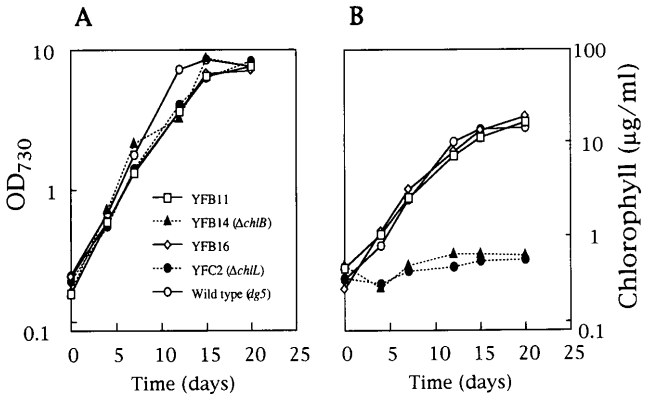


Fig. 4 The time course of chemoheterotrophic growth of *dg5* cells and the mutants cells (YFB11, YFB14, YFB16 and YFC2) in darkness. (A) Growth was monitored by measuring the optical density at 730 nm (OD_{730}). (B) Growth was monitored in terms of the levels of Chl in the cultures.

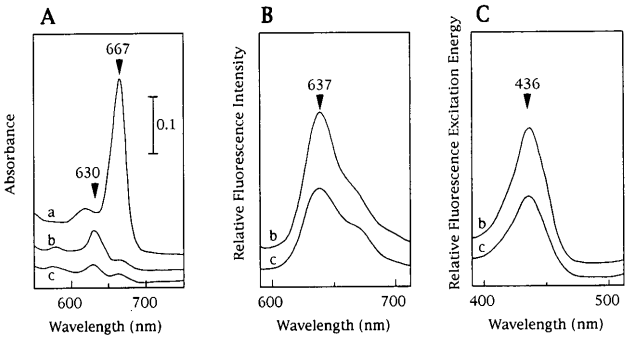


Fig. 5 Absorption (A), fluorescence emission (B) and excitation (C) spectra of the methanol extracts of dark-grown *dg5* (a), YFB14 (b) and YFC2 (c) cells. The emission spectra were elicited by excitation at 436 nm (B). The excitation spectra were recorded at 637 nm (C).

grew as well as the *dg5* strain in terms of increase in turbidity (OD_{730}). However, the levels of Chl in YFB14 and YFC2 did not increase, while those in YFB11, YFB16 and *dg5* increased with increases in OD_{730} (Fig. 4B). The culture of the dark-grown YFB14 cells was an anomalous bluish color, as was that of YFC2, contrast to the normal blue-green color of the cultures of the other strains (data not shown).

Methanol extracts of the dark-grown YFB14, YFC2 and *dg5* cells were analyzed spectroscopically to compare their pigments. Figure 5 shows the absorption spectra obtained from YFB14, YFC2 and *dg5* (panel A) and the fluorescence emission and excitation spectra obtained from YFB14 and YFC2 (panels B and C). The methanol extract of dark-grown YFB14 cells had the same spectroscopic properties (peaks of absorbance, emission and excitation at 630, 637 and 436 nm, respectively) as those of YFC2, which accumulates Pchlide instead of Chl as the major pigment (Fujita et al. 1992). This result indicated that the dark-grown YFB14 cells accumulated Pchlide. When YFB14 was cultivated in the light, no such accumulation of Pchlide occurred irrespective of the presence or absence of glucose (data not shown). From the phenotype of YFB14, which is indistinguishable from that of the *chlL*-disrupted mutant YFC2, we concluded that ORF508 corresponds to the third gene involved in the light-independent reduction of Pchlide, together with two other genes, *chlL* and *chlN* (Fujita et al. 1992, 1993).

Immunological analysis of the ChlB protein—To raise antibodies against the ChlB protein, we expressed the *chlB* gene in *E. coli* as a fusion protein with a 6xHis tag. A DNA fragment corresponding to the entire coding region of the *chlB* gene was amplified by PCR and introduced between the *Sph*I and *Bam*HI sites of an *E. coli* expression vector, pQE-70. The constructed plasmid pQEB4 encoded a fusion protein (ChlB-6xHis) with the 6xHis tag fused to the carboxyl-terminus of the ChlB protein. *E. coli* cells harboring pQEB4 overexpressed a protein with a molecular weight of about 59,000 only after induction by IPTG (data not shown). This molecular weight is in good agreement with the theoretical value for the ChlB-6xHis fusion protein (58,141). This protein was recovered in the insoluble fraction of a lysate of bacterial cells after sonication, and it was solubilized in 8 M urea. The solubilized fraction was loaded onto a chelating affinity column. The ChlB-6xHis protein was eluted from the column by a gradient of imidazole (0 to 0.25 M), and it was further purified by gel filtration to give a preparation that yielded a single band in SDS-PAGE. An antiserum against the ChlB-6xHis protein was prepared by immunization of a rabbit with the purified ChlB-6xHis protein.

Fig. 3 Comparison of the amino acid sequences deduced from various homologues of the *chlB* gene, the *bchZ* gene from *Rhodobacter capsulatus*, the *chlN* gene from *P. boryanum* and the *nifK* gene from *Clostridium pasteurianum*. CHLB PLEBO, *chlB* of *P. boryanum*; CHLB CHLRE, *chlB* of *C. reinhardtii* (Li et al. 1993, P36437); CHLB MARPO, *chlB* (ORF513) of *Marchantia polymorpha* (Umesono et al. 1988, P26238); CHLB PINTH, *Pinus thunbergii* (Tsudzuki et al. 1992, Q00846); CHLB GINBI, *chlB* of *Ginkgo biloba* (Richard et al. 1994, P36208); BCHB RHOCA, *bchB* of *R. capsulatus* (Burke et al. 1993a, P26163); BCHZ RHOCA, *bchZ* of *R. capsulatus* (Burke et al. 1993b, P26179); CHLN PLEBO, *chlN* of *P. boryanum* (Fujita et al. 1993, D12973) and NIFK CLOPA, *nifK* of *C. pasteurianum* (Wang et al. 1988, P11347). Accession numbers are given after names of authors. These sequences were aligned according to the results of a homology search with the FASTA program (Pearson and Lipman 1988). Amino acid residues identical to those in ChlB from *P. boryanum* are indicated by white letters on a black background. Percent similarities between the cyanobacterial ChlB and the respective protein are given in parentheses. The ChlB protein from *C. reinhardtii* has two regions for which homologous regions are not found in the other ChlB proteins. These regions are indicated only by the number of amino acid residues and are omitted from this alignment.

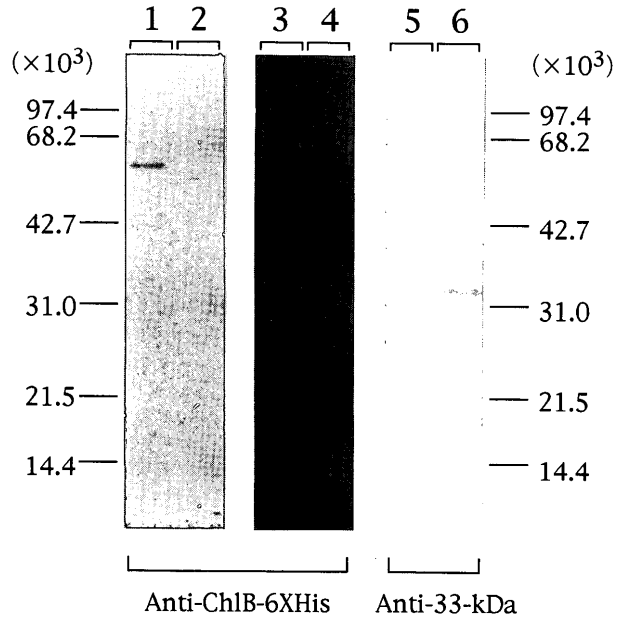


Fig. 6 Western blot analysis of the ChlB protein of *P. boryanum*. Total extracts from *dg5* (lane 1) and YFB14 (lane 2) cells, and the soluble (lanes 3 and 5) and membrane (lanes 4 and 6) fractions of *dg5* cells were subjected to SDS-PAGE. Proteins were transferred to a PVDF membrane, which was incubated with antiserum against ChlB-6xHis (lanes 1–4) or the 33-kDa protein of spinach (lanes 5 and 6). For other details, see Materials and Methods.

Cells of *P. boryanum* were fractionated into soluble and membrane fractions, and Western blot analysis was performed using the antiserum raised against ChlB-6xHis (Fig. 6). As a control for assessment of cell fractionation, an antiserum against the 33-kDa protein of spinach was also used. The 33-kDa protein is a thylakoid protein found on the lumen side of the membrane (Berry et al. 1994). The anti-ChlB-6xHis antiserum reacted with a single major protein with a molecular weight of about 58,000 in the total extract (Fig. 6, lane 1) and also in the insoluble membrane fraction (lane 4), in which the 33-kDa protein was also detected (lane 6). The apparent molecular weight is in good agreement with that of the ChlB protein (56,819) deduced from the nucleotide sequence of the corresponding gene. Furthermore, this signal was not detected in a total extract of YFB14, with an inactivated *chlB* (Fig. 6, lane 2). This immunochemical analysis indicated that the ChlB protein is accumulated in the membranes of the cyanobacterial cells.

Discussion

The cyanobacterial chlB gene and the light-independent reduction of Pchlide—In this study, we cloned a 6.0-kb *Hin*dIII fragment from the cyanobacterium *P. boryanum* using the chloroplast *chlB* gene from liverwort as the probe. An open reading frame contained in the fragment, ORF508, encoded a protein that was homologous to those encoded by the chloroplast *chlB* genes from a variety of plants and algae. The cyanobacterial mutant YFB14, in which ORF508 had been specifically inactivated, lost the ability to synthesize Chl in darkness. Its phenotype was essentially the same as that of a *chlB*-disrupted mutant of *Chlamydomonas* (Li et al. 1993, Liu et al. 1993). Thus, ORF508 was concluded to represent the cyanobacterial *chlB* gene.

Liu et al. (1993) noted that the ChlB proteins from two green algae (*C. reinhardtii* and *C. moewusii*) have four "highly variable sequence domains" designated V1, V2, V3 and V4, that are absent from plant forms of ChlB. In the case of ChlB from *C. reinhardtii* shown in Figure 3, the two domains designated V1 and V3 are indicated only the number of the amino acid residues, "<101aa>" and "<46aa>", respectively, and V4 corresponds to the 32 amino acid residues in carboxyl-terminal region (SENN—GSSR). V2 is too short to be identified in the alignment shown in Figure 3. The molecular weight of ChlB from *C. reinhardtii* is 76,822, namely, about 20,000 greater than those of plant and cyanobacterial forms of ChlB, as a result of the presence of these domains. Since it seems likely that such long domains would interfere with the structure of ChlB, the transcripts for these domains might be removed by splicing. If, however, they are indeed included in the ChlB protein, they might be located between two functional domains on the surface of the protein (Liu et al. 1993), and they might help us to understand the structure-function relationships of ChlB. With respect to the evolution of chloroplast, Richard et al. (1994) assumed that these domains might have formed part of the primitive chloroplast gene and have been deleted during the evolution of land plants. No such the domains were found in the cyanobacterial ChlB, suggesting that these domains might have been added during the evolution of green algae after branching from the land-plant lineage.

The two genes, *chlL* and *chlN* (formerly *frxC* and ORF467, respectively), form a single operon and are involved in the light-independent reduction of Pchlide in the cyanobacterium *P. boryanum* (Fujita et al. 1992, 1993). Therefore, it is concluded that the cyanobacterium harbors a light-independent system for the reduction of Pchlide that consists of the products of at least the three genes. These three genes, *chlB, chlL* and *chlN*, have been found in a wide variety of photosynthetic organisms, such as green algae (*C. reinhardtii*, and *C. moewusii*; Liu et al. 1993, Li et al. 1993), red alga (*Porphyra purpurae*, Reith and Munholland 1993), liverwort (*Marchantia polymorpha*, Ohyama et al. 1988), ferns (*Adiantum capillus-veneris*, Yamada et al. 1992a; *Pteridium aquilinum*, Yamada et al. 1992b), pines (*Pinus thunbergii*, Tsudzuki et al. 1992, Wakasugi et al. 1994; *Pinus contorta*, Lidholm and Gustafsson 1991), and ginkgo (*Ginkgo biloba*, Richard et al.

1994). By contrast, there are no homologous genes in the chloroplast DNAs from angiosperms (tobacco, Shinozaki et al. 1986; rice, Hiratsuka et al. 1989, and beechdrop, Wolfe et al. 1992) and *Euglena gracilis* (Hallick et al. 1993), all of which become etiolated in darkness. In these organisms Pchlide is reduced by the light-dependent enzyme, POR, which seems to be distributed in essentially all organisms from cyanobacteria to higher plants. Thus, the distribution of the *chlB, chlL* and *chlN* genes in photosynthetic organisms seems to be very well correlated with that of greening ability in darkness. The coexistence of light-independent and light-dependent systems for reduction of Pchlide has been conserved from cyanobacteria to gymnosperms during the evolution of photosynthetic organisms, and the light-independent system might have been lost during the evolution from gymnosperms to angiosperms. Purple non-sulfur bacteria, such as *Rhodobacter capsulatus*, have the ability to synthesize bacteriochlorophyll in a light-independent manner and they have *bchB, bchL* and *bchN* genes as homologues of *chlB, chlL* and *chlN*, respectively (Burke et al. 1993a, Yang and Bauer 1990). However, the light-dependent system is not present in the synthesis of bacteriochlorophyll.

Similarity between light-independent Pchlide reductase and nitrogenase—Three proteins, ChlL, ChlN, and ChlB, exhibit significant similarity to the three subunits of nitrogenase; ChlL is homologous to NifH protein (Fe-protein; Fujita et al. 1989, Burke et al. 1993c), ChlN is homologous to the NifD and NifK proteins (α and β subunits of MoFe-protein, respectively; Fujita et al. 1993), and ChlB is homologous to NifK (Fig. 3). Limited similarity (15.6%) was found between ChlB and ChlN, as is the case when the two subunits of MoFe-protein are compared (Holland et al. 1987). These similarities between subunits of the Pchlide reductase and nitrogenase suggest that the light-independent Pchlide reductase has a molecular structure similar to that of nitrogenase (for review, see Burris 1991); ChlL might function as a specific reductase for the other component that consists of ChlN and ChlB, which might provide the catalytic site for the reduction of Pchlide.

The ChlB protein exhibited significant similarity to another protein (Fig. 3), namely, the protein encoded by the *bchZ* gene of *R. capsulatus* (Burke et al. 1993b) whose sequence exhibits 21.6% similarity to that of ChlB. This protein is involved in the reduction of the chlorin B-ring in the synthesis of bacteriochlorophyll, and this reduction requires two proteins encoded by *bchX* and *bchY*, which exhibit similarity to ChlL and ChlN, respectively. Thus, it appears that the chlorin reductase has a molecular structure similar to those of Pchlide reductase and nitrogenase (Burke et al. 1993b).

Localization of the light-independent Pchlide reductase—We detected the ChlB protein for the first time in cyanobacterial cells using polyclonal antibodies against the ChlB-6xHis fusion protein. Our immunochemical analysis indicated that the ChlB protein was located in the membrane fraction of the cyanobacterial cells, even though no typical transmembrane domains were found in a hydropathy profile of ChlB (data not shown). This localization of ChlB seems to be consistent with the observations of Peschek et al. (1989) who reported that the activity of the light-independent reduction of Pchlide was associated with the cytoplasmic membranes of the cyanobacterium *Anacystis nidulans*. Further studies are required to determine whether ChlB is distributed in the cytoplasmic or thylakoid membranes.

The ChlL (FrxC) protein can be recovered in the soluble fraction of chloroplasts from liverwort (Fujita et al. 1989). If the ChlL protein is also present in the soluble fraction of cyanobacterial cells, it seems likely that both soluble and membrane fractions are required for the light-independent reduction of Pchlide in cyanobacteria. For clarification of the molecular structure of the light-independent Pchlide reductase and its subcellular localization, *P. boryanum* appears to provide a promising system since a series of mutants that lack the *chlB, chlL* and *chlN* genes, respectively, has been isolated and a genetic complementation system using a shuttle vector has recently been established (Takagi et al. unpublished results).

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