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The isolation of high molecular mass DNA from the prochlorophyte *Prochloron didemni*

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Using a protocol known to inactivate endogenous nucleases in other organisms we have been able to isolate high molecular mass DNA from *Prochloron didemni*. This DNA is shown to be suitable for restriction endonuclease analysis and the generation of genomic libraries in the λ cloning vector EMBL3.

Prochlorophyte; Evolution; DNA isolation; Cloning; (*Prochloron didemni*)

1. INTRODUCTION

The Prochlorophyta are eubacteria which, like the chloroplasts of euglenoids, chlorophytic algae and plants, contain chlorophylls *a* and *b*, but lack phycobilins [1]. Cyanobacteria, by contrast, lack chlorophyll *b* and possess phycobilins. On the basis of this pigment composition, it has been suggested that the prochlorophytes are descendants from the evolutionary lineage that gave rise to the green chloroplasts [2]. An alternative hypothesis to explain prochlorophyte origins is that they are cyanobacteria which have, independently of chloroplasts, acquired the ability to produce chlorophyll *b* and lost their phycobilins.

The first prochlorophyte identified was *Prochloron didemni* [1], growing ectosymbiotically in the cloacal cavity of didemnid ascidians [3]. Based on 16 S rRNA oligonucleotide catalogue comparisons for *Prochloron* with cyanobacteria and chloroplasts [4], it was concluded that this prochlorophyte is more closely related to the cyanobacteria than to chloroplasts. This conclusion has however been disputed [5]. The compared catalogue binary association coefficients (S_{AB} values) obtained in this study were low and very similar for *Prochloron* with chloroplast and cyanobacterial species. In this situation S_{AB} does not give an accurate indication of actual sequence differences (and hence phylogenetic relatedness) between species [6,7]. Analysis of the 5 S RNA sequence determined for *Prochloron* [8] is likewise inconclusive. Phylogenetic estimation under the probabilistic model of Bishop and Friday [9] shows that neither tree topology is significantly favoured (Holmes, E.C., personal communication). Furthermore, recent analysis of 16 S rRNA [10] and *psb*A gene sequence data [11] from *Prochlorothrix hollandica* (one of two free-living prochlorophytes identified) has given conflicting phylogenies for this organism.

Further nucleic acid sequence and gene organisation data should allow clarification of the evolutionary relationship between *Prochloron* and *Prochlorothrix*, chloroplasts and cyanobacteria. However, previous attempts to obtain this information from *Prochloron* have failed due to an inability to establish laboratory cultures (despite one successful report [12] to the contrary) or to isolate high molecular mass DNA from naturally occurring material [13].

We compare here three methods routinely used

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for the isolation of high molecular mass DNA from other sources ([14,15] and Brisco, M., personal communication) and show that at least one of them consistently yields high molecular mass DNA from naturally occurring material, which is suitable for restriction enzyme analysis and cloning into λ EMBL3.

2. MATERIALS AND METHODS

2.1. *Source of DNA isolates*

Collection of the ascidian host *Lissoclinum patella* was made in shallow water surrounding Heron and One Tree Islands near Gladstone on the Great Barrier Reef, Australia. Due to its size and the large number of epizoic *Prochloron* cells it contains, we have found *Lissoclinum* to be a good source of *Prochloron*. Ascidians were removed from their coral substrate on the reef, kept in fresh seawater and *Prochloron* cells isolated within a few hours of harvesting the host.

In all isolations, *Prochloron* cells were expressed into buffered seawater (0.1 M Tricine, pH 8.3) and collected by centrifugation (3000 × *g*, 2 min) at room temperature. Nucleic acid extractions were then carried out immediately. Samples were checked microscopically for other species and found to show very low levels of contamination, as reported in [4,16].

2.2. *Extraction in proteinase K/SDS*

This protocol is a modification of the method of Gross-Bellard et al. [14]. Pelleted cells were gently resuspended in an equal volume of homogenisation buffer (100 mM Tris-borate, pH 8.0, 50 mM Na_2EDTA). SDS and proteinase K were added to final concentrations of 1% (w/v) and 200 μg/ml respectively, and the suspension incubated for 1 h at 37°C. To remove pigment/protein complexes from the DNA, the suspension was extracted three times with an equal volume of Tris-HCl-buffered phenol (pH 8.0) and this procedure repeated with an equal volume of buffered phenol/chloroform (1:1, v/v). The DNA was then precipitated by addition of 1/10 vol. of 3 M sodium acetate and 2 vols ethanol. Samples were transported on ice as precipitates under ethanol to the Department of Biological Sciences, University of Sydney, for further processing. There they were centrifuged (10000 × *g*, 2 min) to pellet nucleic acids and the ethanol decanted and drained onto absorbant paper. Nucleic acids were gently resuspended in TE (10 mM Tris-HCl, 1 mM Na_2EDTA, pH 8.0). Ethidium bromide solution (10 mg/ml) was added to a final concentration of 600 μg/ml and aliquots loaded onto a caesium chloride density gradient for ultracentrifugation (200000 × *g*, 16 h). After centrifugation a single fluorescing band was seen using long-wavelength (365 nm) ultraviolet light. The DNA in this band was removed with a pasteur pipette and the ethidium bromide extracted into water-saturated butan-1-ol. After 2-fold dilution in TE, the DNA was precipitated by addition of 1/10 vol. of 3 M sodium acetate and 2 vols ethanol. After centrifugation (10000 × *g*, 5 min) the pellet was drained and air-dried before being resuspended in TE to a final concentration of 0.5 μg/μl. Unlike DNA isolated using the protocols described in sections 2.3 and 2.4, it was not possible to spool the DNA precipitate which formed in the ethanol.

2.3. *Osmotic lysis in high concentrations of SDS*

Prochloron cells were resuspended in 3 vols homogenisation buffer, and SDS added to a final concentration of 7% (w/v). The suspension was then heated at 50°C for 10 min prior to dilution with an equal volume of deionised water. Extraction with phenol and subsequent treatment was carried out as described in section 2.2.

2.4. *Extraction in guanidinium isothiocyanate*

Cells were gently resuspended in 5 vols homogenisation solution (6 M guanidinium isothiocyanate, 2% (v/v) β-mercaptoethanol and 0.1 M Tris-HCl, pH 7.2) similar to that used for the isolation of nucleic acids from bull testes [15]. SDS was then added to a final concentration of 1% (w/v) and the suspension gently inverted to mix. At this stage the sample was transported to Sydney in a sealed container kept at room temperature. DNA was found to be stable in this solution for at least 8 weeks. Cell debris was removed by centrifugation (3000 × *g*, 2 min) at room temperature and the supernatant extracted with phenol/chloroform. Nucleic acids were precipitated by addition of 1 vol. ethanol and separated on a caesium chloride gradient as described in section 2.2.

2.5. *Restriction digests and gel electrophoresis of* Prochloron *DNA*

Restriction endonuclease digestion and gel electrophoresis were carried out as in [17]. DNA size fractions were collected from agarose gels onto strips of dialysis tubing or from ammonium acetate concentration gradients in an elution chamber. DNA obtained in this way was extracted once with phenol and phenol/chloroform, then precipitated with 1/10 vol. of 3 M sodium acetate and 2 vols ethanol prior to subsequent manipulations (e.g. ligation, endonuclease digestion).

2.6. *Repair of* Prochloron *DNA*

Nick repair reactions on DNA isolated using proteinase K were carried out with *E. coli* DNA ligase which should not (under the conditions used) ligate together duplexes with non-cohesive ends [18]. Samples were run on 0.8% agarose gels, and denaturing (8 M urea) and non-denaturing 5% acrylamide gels to assess the degree of nicking and the success of repair.

3. RESULTS AND DISCUSSION

The proteinase K protocol (section 2.2 and [14]), commonly used for DNA extraction from algae, did not consistently produce high molecular mass DNA from *Prochloron* (fig.1A). When size fractions of 5–10 kilobase (kb) of the partially degraded DNA isolated using this method (fig.2A) were run on denaturing acrylamide gels (fig.2B), their mobility was indicative of much smaller fragment sizes, suggesting extensive nicking of the DNA. Presumably this degradation results from an endogenous nuclease activity from within the *Pro-*

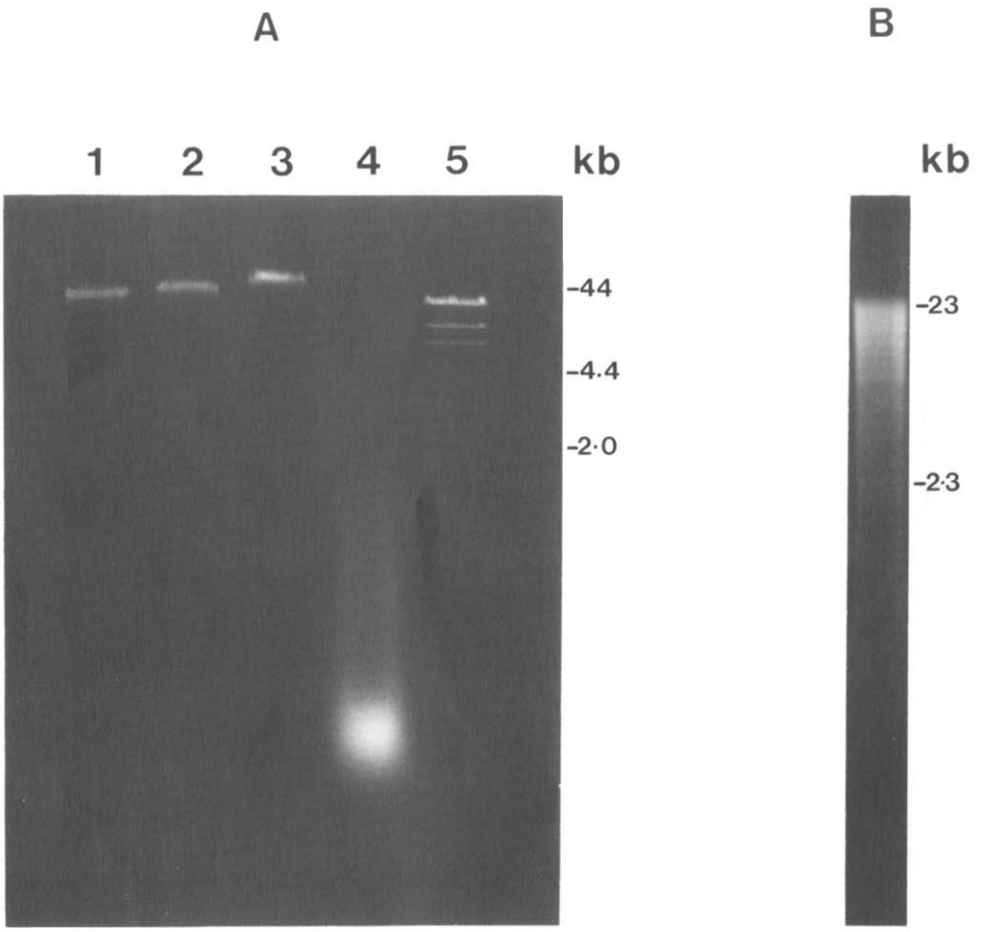

Fig.1. (A) *Prochloron* DNA isolates obtained using three methods of extraction. Lanes: 1, uncut λ EMBL3 DNA; 2, DNA obtained with the guanidinium isothiocyanate method; 3, DNA obtained using the hot SDS method; 4, DNA obtained using the proteinase K protocol; 5, λ DNA digested with *Hin*dIII. (B) *Prochloron* DNA from the guanidinium isothiocyanate method digested with *Hin*dIII.

chloron cells. DNA could be repaired with *E. coli* DNA ligase (fig.2A,B) so that it could be cloned with *Eco*RI linkers (albeit at a low efficiency) into pUC18 and λgt10. *Acc*I, *Pst*I and *Sau*3AI digests of both repaired and untreated DNA generated smears with no visible banding on agarose gel electrophoresis.

The hot SDS (section 2.3) and guanidinium isothiocyanate protocols (section 2.4) should rapidly denature DNases and consistent with this expectation, both methods were found to yield DNA molecules at least as large as uncut monomeric λ EMBL3 (44 kb, fig.1A). We have found that DNA from guanidinium isothiocyanate isolations could be cleaved to completion with a number of restriction enzymes including *Sau*3AI, *Pst*I, *Acc*I, *Eco*RI and *Hin*dIII. Characteristically for a prokaryote, endonuclease-digested DNA produced banding on agarose gels (fig.1B). DNA isolated using this method could also be cloned by partial digestion with *Sau*3AI and ligation of 15–20 kb size fractions into the λ replacement vector EMBL3.

Since the guanidinium isothiocyanate protocol provides a convenient means for maintaining nucleic acid integrity under conditions where there is limited research facility, we have adopted it as a preferred method for DNA isolations from *Prochloron*. Given a protocol for obtaining high molecular mass DNA for Southern blotting and

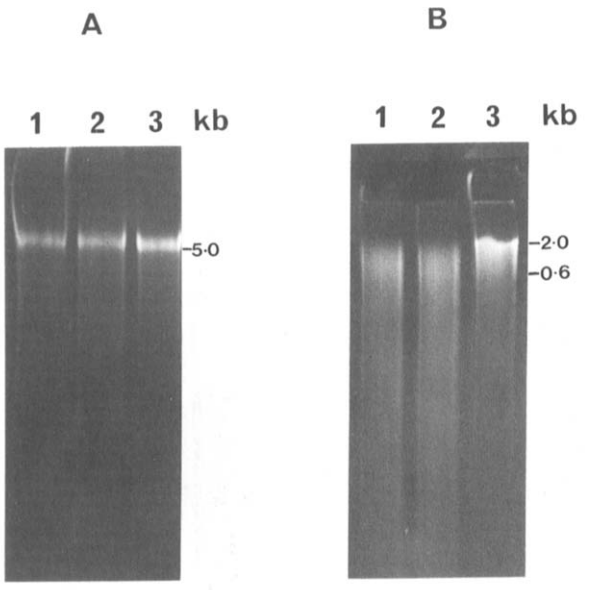

Fig.2. Size fractions (5–10 kb) of *Prochloron* DNA isolated using the proteinase K protocol and electrophoresed on 5% acrylamide non-denaturing (A) and 8 M urea denaturing (B) gels. Lanes: 1, untreated DNA; 2, DNA incubated for 12 h in ligation buffer; 3, DNA incubated in ligation buffer with *E. coli* DNA ligase for 12 h.

cloning, it should now be readily possible to obtain gene sequence and organisation data. We are currently cloning and identifying genes of phylogenetic interest, and hope this will offer insight into the significance of *Prochloron* in the evolution of green chloroplasts.

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REFERENCES

[1] Lewin, R.A. (1976) Nature 261, 697–698.
[2] Margulis, L. (1981) Symbiosis in Cell Evolution, Freeman, San Francisco.
[3] Cox, G.C. (1986) New Phytol. 104, 429–445.
[4] Seewaldt, E. and Stackebrandt, E. (1982) Nature 295, 618–620.
[5] Van Valen, L.M. (1982) Nature 298, 493–494.
[6] Ochmann, H. and Wilson, A.C. (1987) J. Mol. Evol. 26, 74–86.
[7] Woese, C.R. (1987) Microbiol. Rev. 51, 221–271.
[8] MacKay, R.M., Salgado, D., Bonen, L., Stackebrandt, E. and Doolittle, W.F. (1982) Nucleic Acids Res. 10, 2963–2970.
[9] Bishop, M.J. and Friday, A.E. (1985) Proc. Roy. Soc. Lond. B226, 271–302.
[10] Turner, S., Burger-Wiersma, T., Giovannoni, S.J., Mur, L.R. and Pace, N.R. (1988) Nature 337, 380–382.
[11] Morden, C.W. and Golden, S.S. (1988) Nature 337, 382–385.
[12] Patterson, G.M.L. and Withers, N.W. (1982) Science 217, 1034–1035.
[13] Stackebrandt, E., Seewaldt, E., Fowler, V.J. and Schleifer, K.-H. (1982) Arch. Microbiol. 132, 216–217.
[14] Gross-Bellard, M., Oudet, P. and Chambon, P. (1973) Eur. J. Biochem. 36, 32–38.
[15] Krawetz, S.A., States, C. and Dixon, G.H. (1986) J. Biochem. Biophys. Methods 12, 29–36.
[16] Herdmann, M. (1981) Arch. Microbiol. 129, 314–316.
[17] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[18] Modrich, P. and Lehman, I.R. (1970) J. Biol. Chem. 245, 3626–3631.