Phycologia (2001) Volume 40 (2), 147-152 Published 10 July 2001

Plastid genome size and heterogeneous base composition of nuclear DNA from *Ochrosphaera neapolitana* (Prymnesiophyta)

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A.G. Sáez, H. Engel, L.K. Medlin & V.A.R. Huss. 2001. Plastid genome size and heterogeneous base composition of nuclear DNA from *Ochrosphaera neapolitana* (Prymnesiophyta). *Phycologia* 40: 147-152.

We have studied the plastid genome of *Ochrosphaera neapolitana* (Hymenomonadaceae, Prymnesiophyta), a member of the chlorophyll a + c-containing algae. Total DNA from *O. neapolitana* was fractionated in a CsCl density gradient and the 'lighter' upper band (which is typically of plastid origin) was unusually abundant relative to the 'heavier' nuclear one. Denaturation and renaturation experiments done with DNA isolated from the upper band showed that it had a base composition of 43.6 mol% G + C (molar fraction of guanosine plus cytosine), compared to 60.5 mol% G + C in the lower band, and that it lacked a substantial fraction of repetitive sequences; this DNA had a size of *c*. 10 Mb, which is more than an order of magnitude larger than has been reported for any plastid genome. Using Pulse Field Gel Electrophoresis (PFGE) of total DNA, we resolved a plastid genome size of approximately 165 kb for *O. neapolitana*. Although this is within the range reported for other plastid genomes, there is an increase of about 40 kb compared to related algae. Using the upper band from the CsCl gradient as a probe for the PFGE blots, we showed that this upper band is apparently a mixture of nuclear DNA with a small fraction of plastid DNA. The nuclear genome of *Ochrosphaera neapolitana* thus appears to have a distinctive heterogeneity in G + C content, with two DNA types differing by *c*. 17 mol%.

INTRODUCTION

The endosymbiotic hypothesis of plastid evolution maintains that chloroplasts were originally acquired by eukaryotic heterotrophs through the endocytotic capture of photosynthetic prokaryotes and their subsequent conversion into organelles (Schimper 1883; Mereschkowsky 1905; Raven 1970; Margulis 1981). The plastids of rhodophyte, chlorophyte and glaucocystophyte algae and higher plants have plastids that are surrounded by only two membranes and are therefore assumed to have resulted from a primary endosymbiotic event, in which a eukaryotic host engulfed a prokaryotic cell. The host organisms associated with the primary endosymbioses have been proposed to have arisen as independent plastid-bearing lineages within the crown group radiation of the eukaryotes (Bhattacharya & Medlin 1995). However, there is increasing evidence for a monophyletic origin of the rhodophyte and chlorophyte lineages, and possibly also the glaucocystophyte lineage (Burger *et al*. 1999; Moreira *et al*. 2000). The genomes of these plastids are typically 89–292 kb in size and the smaller ones among them usually lack an inverted repeat unit of the ribosomal rRNA genes (Sugiura *et al*. 1998).

Other algae have plastids surrounded by three or four membranes and these are hypothesized to have arisen through secondary endosymbiotic events, in which a heterotrophic eukaryote host engulfed and reduced a photosynthetic eukaryote cell to a plastid. The additional outer membranes surrounding the plastid reflect its secondary origin, representing the host vacuole membrane and the plasmalemma of the eukaryotic endosymbiont (Gibbs 1993). Algae resulting from secondary endosymbioses include the euglenophytes and chlorarachniophytes, which contain chlorophylls *a* and *b*, as well as the heterokont chromophytes, haptophytes, dinoflagellates and cryptophytes, most of which contain chlorophylls *a* and *c* (Gibbs 1978, 1981; Cavalier-Smith 1989; Jeffrey 1989; Rowan 1989; Kowallik 1992; Valentin *et al*. 1992). These plastids are 118–154 kb in size and have an inverted repeat of the ribosomal RNA genes. Other differences between primary and secondary endosymbiotic lines also include the types and numbers of genes transferred to the nucleus (Martin *et al*. 1998).

There is continued interest in the evolution of plastids, with recent studies documenting the evolution of the highly reduced plastids of parasitic Apicomplexa, such as *Plasmodium* (McFadden & Waller 1997), and the remarkable diversity of plastids in the dinoflagellates, including some that represent tertiary endosymbioses (Tengs *et al*. 2000) and others that posses individual ring structures (replicons) for each gene (Zhang *et al*. 1999). In addition, the timing of endosymbioses events (Medlin *et al*. 1997) and the numbers and types of plastid genes transferred to the nucleus (Martin *et al*. 1998) have been studied. Here we study the base composition and size of the plastid genome of the chlorophyll a + c-containing alga *Ochrosphaera neapolitana* Schussnig. Although the group of algae to which *Ochrosphaera* belongs – the coccolithophorids – is a very large and ecologically important one, its plastid genomes have been poorly investigated.

MATERIAL AND METHODS

Cultures

Ochrosphaera neapolitana HAP13 and *Pavlova lutheri* (Droop) J.C. Green (Pavlovales, Prymnesiophyta) HAP44 (=PLY75) were obtained from the Algobank collection of the University of Caen, France (http://www.unicaen.fr/unicaen/ufr/ibba/lbbm/algobank/). Both species were grown in approximately 500 ml (for PFGE) or *c*. 10 litres (for CsCl gradients) of ES-Tris II medium (Cosson 1987), aerated by bubbling (approximately one bubble per second; 0.2% CO_2 in air bubbled through media). Cells were harvested during the exponential growth phase and centrifuged at 2500 × g for 5 min at 4°C. At this speed we noticed that most of the algae sedimented to the bottom of the tubes, whereas most of the bacteria remained in the supernatant. We washed the cells in 100 mM Tris-HCl pH 7.5, 100 mM NaCl and 50 mM EDTA (at 4°C) by resuspending the cells in this buffer and centrifuging under the same conditions as above. We performed three washes, after which we observed almost no bacteria, either in the supernatant or among the collected algae. Cultures of *Chlorella sorokiniana* Shih. & Krauss (Trebouxiophyceae, Chlorophyta) and *Escherichia coli* were grown as described in Huss *et al*. (1986).

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DNA extraction and CsCl gradients

DNA was extracted and purified according to Huss *et al*. (1986). Reference algae and bacteria were homogenized with glass beads and the cell extracts separated from the beads by washing with 10 mM Tris-HCl, 1 mM EDTA pH 8.0 and suction through a coarse sintered filter. Lysis of *Ochrosphaera* cells could be achieved by adding distilled water, leaving most of the remaining contaminating bacteria unaffected. DNA was purified by a modification of Marmur's (1961) method, using CTAB, RNAse A (Serva), and proteinase K (Merck) for the removal of polysaccharides (Darby *et al*. 1970), RNA, and proteins, respectively. CsCl density gradient centrifugation was performed for 40 h at 42,000 rpm and 20°C in a VTi 50 rotor of a Beckman L8-60M ultracentrifuge, as described by Huss *et al*. (1988). Fifty microlitres of bisbenzimide (Hoechst dye 33258) per mg DNA were added for better separation of AT- and GC-rich DNA bands (Müller & Gautier 1975) and visualization in UV-light at 312 nm. Dye removal was effected by several extractions of the recovered DNA with CsCl-saturated isopropanol. To remove CsCl, the DNA solutions were dialysed twice against large volumes of standard saline citrate (SSC: 0.15 M NaCl, 0.015 M trisodium citrate pH 7.0). If necessary, DNA was concentrated in dialysis tubes over dry saccharose with subsequent dialysis.

DNA reassociation kinetics and c_0t analyses

DNA reassociation was followed optically in a Gilford Response ™ spectrophotometer as described by Britten *et al*. (1974). The conditions and parameters used for the reassociation kinetics and c_0t analyses, as well as for second order rate plots (Wetmur & Davidson 1968), were as described by Dörr & Huss (1990).

Pulse Field Gel Electrophoresis (PFGE)

We followed mainly the method of Cole & Williams (1988) for the preparation of the PFGE plugs (cells embedded in agarose). One volume (0.5 ml) of 2% low melting point agarose (FMC) in 90 mM Tris-borate, 2 mM EDTA pH 8.0 (TBE) at 40°C was mixed with the same volume of harvested algae using a 1 ml syringe. The mixture was cooled on ice for 5 min within the syringe. The agarose plug was then removed from the syringe into 30 ml of 500 mM EDTA, 10 mM Tris-HCl pH 7.5, 1% Sarcosyl and 1 mg ml^{-1} proteinase K (lysis buffer) at 50 °C in a horizontally rotating tube and incubated overnight. Then the buffer was discarded and a second wash performed under the same conditions. The agarose plugs were stored at 4°C in fresh lysis buffer lacking proteinase K. Approximately 2 mm of a cross-section of these plugs were inserted into the wells of a 1% agarose gel used for PFGE. Electrophoresis conditions in 0.5 × TBE buffer were 2 h at 140 volts with a constant field, followed by 36 h at the same voltage but with periodic rotations of 120° and increasing electrical pulse times from 60 to 120 s between rotations (initial and final pulses respectively). After 26 h, the run was interrupted to obtain a better resolution of the plastid genomes (in previous runs we observed that shorter runs improved the resolution).

Southern blots

A standard protocol from Ausubel *et al*. (1992: alkaline method) was followed for obtaining the Southern blots. Positively-charged nylon membranes were used (Boehringer, Mannheim). The prehybridization lasted for 2 h in 7% SDS, 500 mM sodium phosphate, 1 mM EDTA, pH 7.2, at 65°C. Hybridization to the labelled probes was done under the same conditions for approximately 15 h. Blots were washed three times in twofold concentrated SSC, 0.1% SDS, at 65°C for 20 min.

RESULTS

We used CsCl gradient centrifugation to study the plastid and nuclear genomes of *O. neapolitana*. Total DNA extracted from the algae was centrifuged in a CsCl solution at high speed. Because AT pairs are less dense than GC pairs in double-stranded DNA, and because plastid genomes are usually less GC-rich than nuclear genomes, CsCl centrifugation separates a light plastid fraction from a heavier nuclear fraction (Ausubel *et al*. 1992). As expected, CsCl centrifugation produced two distinct bands of DNA in *O. neapolitana*. Typically, plastid DNA accounts for about 10% of the total DNA (Bastia *et al*. 1971; Coleman & Goff 1991) but, surprisingly, in *O. neapolitana* the lighter upper band was almost as prominent as the heavier one. Thermal denaturation of these two DNA species (De Ley 1970) showed a base composition of 43.6 mol% G + C for the lighter band (hereafter designated L-DNA) and 60.5 mol% G + C for the heavier band. The purified L-DNA was then used in DNA reassociation analyses, in which the kinetics of DNA renaturation can be studied (Britten *et al*. 1974). These analyses offer an overall assessment of the proportion of single vs repetitive copy sequences, and allow a rough estimate of DNA length. Using *Chlorella sorokiniana* and *Escherichia coli* as controls, we found that isolated L-DNA from *O. neapolitana* had a size greater than 10 Mb (Fig. 1A). This would be at least an order of magnitude larger than has been described for any plastid genome (which range from 89 to 292 kb: Sugiura *et al*. 1998). Moreover, a second-order rate plot of the reassociation data showed that the L-DNA of *O. neapolitana* was almost completely composed of single copy genes (Fig. 1B). These results suggest either that *O. neapolitana* possesses an exceptionally large plastid genome, or that the L-DNA is a mixture of plastid and mainly nuclear DNA, in which case the plastid genome of *O. neapolitana* might be of normal size. These two hypotheses were tested.

The most straightforward technique to resolve the size of any chromosome is PFGE. This is an agarose-gel electrophoresis with periodical changes in the direction, strength, and pulse time of its electrical field, allowing separation of DNA fragments larger than 50 kb (Cantor *et al*. 1988). Total DNA from *O. neapolitana* and *Pavlova lutheri* was separated by PFGE, transferred to a nylon membrane, and probed with ^{32}P-labelled DNA fragments of three typical plastid genes isolated, either from *O. neapolitana* alone (*ATPase*B and 23S rDNA), or from both *O. neapolitana* and *P. lutheri* (*rbc*L). *Pavlova lutheri* was included in our experiments because it represents a closely related evolutionary lineage within the Prymnesiophyta (Edvardsen *et al*. 2000) and because its plastid genome size (115 kb, i.e. well within the normal range) has been previously reported using PFGE as well (Scaramuzzi *et al*. 1992). The three probes revealed DNA bands of approximately 165 kb for *O. neapolitana* (Fig. 2) and of 120 kb for *P. lutheri*. The *ATPase*B probe from *O. neapolitana* did not produce a signal with *P. lutheri* DNA under the conditions specified in the Material and Methods (Fig. 2), although a 120 kb band was visible after a very prolonged exposure (not shown). No larger chromosomes hybridized with the 23S rDNA and *ATPase*B probes (not even with a tenfold greater exposure time). However, the *rbc*L probe reacted with two large chromosomes (> 1120 kb) from *O. neapolitana*, in what might be a nonspecific hybridization. A nuclear 18S rDNA probe (isolated via PCR from both species) hybridized to several chromosomes larger than 1 Mb in the two algae (Fig. 2). In *P. lutheri*, a fragment of approximately 70 kb appeared, which was most likely a degradation product. An additional PFGE blot was incubated with total L-DNA from *O. neapolitana* as a probe. Because of the many DNA fragments contained in this probe, a very intense signal was observed in the autoradiographs. In X-ray films exposed for a short time it was possible to see that most of the signal originates from chromosomes larger than 1.12 Mb (Fig. 2).

DISCUSSION

The PFGE results indicate that the AT-rich L-DNA from *O. neapolitana* is composed largely of a nuclear DNA, but also a small amount of plastid DNA. We know that L-DNA contains plastid sequences because specific genes from the plastid genome, such as the rRNA- and the *atp* gene clusters, were isolated and characterized from *O. neapolitana* L-DNA (Giovannoni *et al.* 1992; Tietze 1996). The nuclear genome is heterogeneous with respect to its base composition in many taxa, e.g. insects (Carulli *et al.* 1993), plants (Barakat *et al*. 1998) and yeast

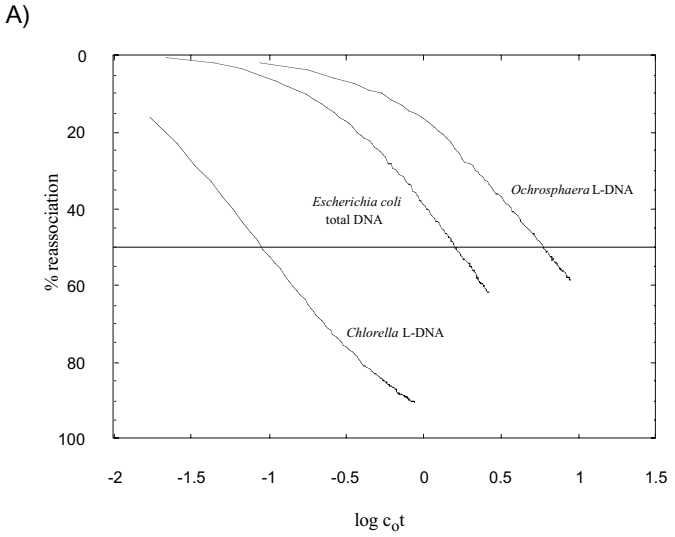

Fig. 1. DNA renaturation kinetics of L-DNA from *Ochrosphaera neapolitana* and *Chlorella sorokiniana*, and of total DNA from *Escherichia coli*.
(A) Log c_ot diagram (Britten *et al.* 1974): the fraction of reassociated single stranded DNA is plotted against the logarithm of the product of DNA concentration and time. The log c_ot values at 50% reassociation (straight horizontal line) were taken to calculate the kinetic complexities as 0.25 Mb (*Chlorella*) and 15.9 Mb (*O. neapolitana*) with *E. coli* as a reference (4.2 Mb; Cairns 1963).
(B) Second-order rate plot (Wetmur & Davidson 1968; data are taken from Fig. 1A): straight lines indicate the absence of significant amounts of repetitive sequences. For details, see Dörr & Huss (1990).

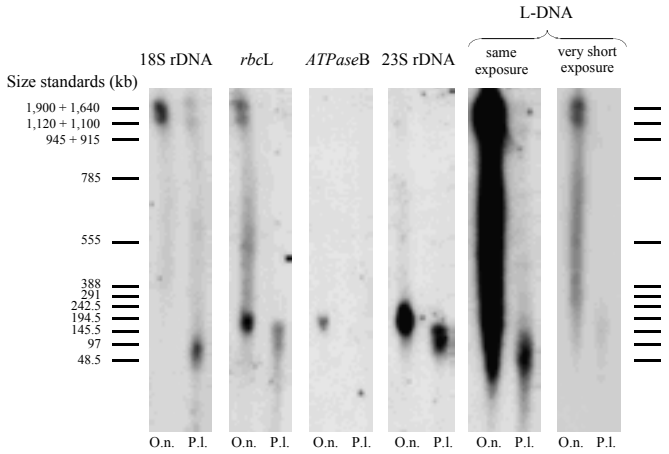

Fig. 2. Southern blot analysis of total DNA from *Ochrosphaera neapolitana* (O.n.) and *Pavlova lutheri* (P.l.) to identify the size of their plastid genomes. Total DNA from both species was separated by PFGE. The different probes used are indicated on the top (see text). The blot in the far right lane was exposed at room temperature (instead of –70°C) and for only one fifth of the time compared to the others.

(Bradnam *et al*. 1999), and the range of GC content is similarly wide as in *O. neapolitana* (17%). One possible explanation is that pervasive selective constraints are acting upon coding regions by forcing them to maintain a distinct base composition relative to other, more freely evolving regions, whose GC ratio is determined mainly by mutational bias (Li 1997). A second possibility, not mutually exclusive with respect to the previous, is that mutational properties vary among chromosomes or chromosome segments; such a phenomenon has recently been confirmed for mammals (Matassi *et al*. 1999). What appears to be exceptional about the nuclear genome of *O. neapolitana* is the markedly 'bimodal' heterogeneity of its DNA base composition, with two clearly distinct types of DNA sequences. Where CsCl gradients have been obtained from other species, a single band of nuclear DNA has been observed, even though the overall GC range may be similar to that found in *O. neapolitana*. For example, it has been suggested that most genes cluster within a small fraction of the GC range in maize (1–2%: Carels *et al*. 1995), barley and rice (0.8 and 1.6%, respectively: Barakat *et al*. 1997), and *Arabidopsis thaliana* (8%: Barakat *et al*. 1998); however, CsCl profiles in these species are continuous and exhibit only one predominant peak. The fact that we obtain, instead, two clearly distinct bands suggests that there is a strong compartmentalization of the GC ratio among chromosomes. This, in turn, favours the idea expressed above that chromosome-specific mutational bias is responsible for the GC heterogeneity.

There is a third, highly speculative possibility, which could explain both the heterogeneity in GC composition and its compartmentalization: a massive, recent lateral gene transfer from another organism. Sequencing genome projects on several microbes are revealing that horizontal or lateral gene transfer is much more frequent than had previously been thought (Fraser *et al*. 2000). It has been suggested, for example, that approximately 25% of the genes of two bacteria have been transferred from archaebacteria, based on their higher similarity to archaeal rather than to bacterial genes (Aravind *et al*. 1998).

We discard alternative explanations based on two sources of potential artifacts: DNA methylation and significative amounts of bacterial DNA in our preparations. Methylation has been shown to have no significant effect on the melting point of DNA (Huss & Jahnke 1994), which was the parameter used in calculating the base composition of the upper and the lower DNA bands in the CsCl gradients. Although our cultures were not axenic, the algae taken for DNA extraction did not contain substantial amounts of bacteria (see Methods). We carried out two experiments to verify this. First, we grew the bacteria isolated from the supernatant of the *O. neapolitana* culture. Their genome size was 2.55×10^9 Da, as compared to 6.92×10^9 Da of *O. neapolitana* L-DNA, both determined via reassociation kinetics (data not shown). And second, we could amplify the plastid 16S rRNA gene from *O. neapolitana* L-DNA by PCR, whereas no bacterial 16S rRNA genes were detected in this way. We thus conclude that bacterial DNA, if present at all in *O. neapolitana* L-DNA, could not significantly influence our results.

The distinctly heterogeneous GC composition may be a feature shared by other prymnesiophytes, perhaps excluding members of the Pavlovophyceae (Cavalier-Smith) J.C. Green & Medlin, since the sole representative of this class that we studied – *Pavlova lutheri* – showed the standard eukaryote pattern (data not shown). We have examined another coccolithophorid of the same family as *O. neapolitana*, *Hymenomonas globosa* (Magne) Gayral & Fresnel (Hymenomonadaceae, Prymnesiophyta), and this showed a pattern very similar to *O. neapolitana*: DNAs isolated from CsCl gradients revealed 36 and 58 mol% GC for the light and heavy DNA bands, respectively, with sizes of approximately 17 Mb for L-DNA as indicated by reassociation experiments (data not shown). It would be worthwhile to extend these studies to other coccolithophorids and find out if the same

'bimodal' heterogeneity of GC content is present throughout.

Our PFGE results reveal a plastid genome size in *O. neapolitana* of approximately 165 kb and confirm the size reported for *P. lutheri* (*c*. 120 kb vs 115 kb determined by Scaramuzzi *et al*. 1992). Thus, *O. neapolitana* has a significantly larger plastid genome than other chlorophyll a + c-containing algae. It is approximately 45–50 kb larger than the plastid genomes of *P. lutheri* or the diatom *Odontella sinensis* (119,704 bp); *O. sinensis* is the most closely related alga from which the whole plastid genome has been completely sequenced (Kowallik *et al*. 1995). The plastid genome of the chlorophyll a + c containing cryptophyte *Guillardia theta* Hill & Wetherbee (Cryptomonadaceae, Cryptophyta) also falls in the same range (121,524 bp: Douglas & Penny 1999). *Ochrosphaera neapolitana* is in fact more similar in this respect to red algae, the proposed ancestors of chlorophyll a + c-containing plastids (Valentin & Zetsche 1990): *Cyanidium caldarium* Geitler (Bangiophyceae, Rhodophyta) and *Porphyra purpurea* (Roth) C. Agardh (Bangiophyceae, Rhodophyta) have plastid genomes of 164,921 bp and 191,028 bp, respectively (GenBank accessions AF022186 and U38804). However, given the smaller plastid genome sizes of *Pavlova lutheri*, *Odontella sinensis* and *G. theta*, the three closest relatives of *Ochrosphaera neapolitana* whose plastid genomes have been characterized for size so far, we would predict that the larger plastid genome in *O. neapolitana* is a derived character, and not an ancestral one shared with red algae. It would be interesting to know if the plastid genome from *O. neapolitana* contains additional genes not present in other chlorophyll a + c-containing algae. However, if the larger plastid genome is a derived characteristic, it is more likely that the extra DNA in *O. neapolitana* is 'selfish' or 'junk' DNA (e.g. transposons).

ACKNOWLEDGEMENTS

We thank Chantal Billard for sending the strains studied in this report and for her advice in growing them. We also thank Klaus Valentin, David G. Mann and two anonymous reviewers for many useful comments or corrections to the manuscript. And finally to Friedel Hinz for helping us to take the picture in Fig. 2. This work was funded by the EU CODENET project, ERBFMXCT-970113.

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Accepted 10 February 2001

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