PARTIAL CHARACTERIZATION OF THE GENOME OF THE 'ENDOSYMBIOTIC' CYANELLES FROM *CYANOPHORA PARADOXA*

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1. Introduction

Cyanophora paradoxa, a flagellate of uncertain taxonomic position, is capable of growing photoautotrophically and its cyanelles are generally accepted to be endosymbiotic blue-green algae [1]. In electron micrographs these 'endosymbionts' are characterized by a rudimentary cell wall which differentiates them clearly from any known type of chloroplast [2]. Furthermore the cyanelles have been shown to be sensitive to lysozyme [3] and to contain *N*-acetyl muramic acid and 2,6-diaminopimelic acid in their envelopes [4].

Unlike those from other host organisms the cyanelles from *Cyanophora paradoxa* to date cannot be cultivated in vitro. It thus appears that these cyanelles represent semiautonomous organelles which could be envisaged as being intermediates between free living blue-green algae and chloroplasts [5]. The use of reassociation kinetics [6] to determine a genome size of 117 megadaltons for the DNA of these cyanelles seems to underline the above. This value is comparable to genome sizes of ~90–130 megadaltons observed for ctDNAs of green algae and higher plants [7]. However the kinetic complexities from free-living Cyanophyceae are in the range of $1.6–3 \times 10^9$ daltons [8] and hence 1 order of magnitude larger.

Here the isolation and purification of the cyanelle DNA and the results of its cleavage by restriction endonucleases are reported. We find a size 115 ± 5 megadaltons and the results show in addition that the basic principles of genome organization of this endosymbiont appear to be similar to that of known ctDNAs.

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2. Materials and methods

2.1. *Growth of* Cyanophora paradoxa

Cells were grown at 24°C under continuous light (1100 Lux) in a mineral medium with nitrate as the sole source of nitrogen [9]. Cultures were harvested in the late logarithmic phase.

2.2. *Isolation of cyanelle DNA*

Cells were lysed osmotically by transfer from 0.5 M sucrose to 0.2 M sucrose [6]. The cyanelle pellet obtained by centrifuging at 2000 × *g*, 10 min was used for the isolation of DNA by the method developed for DNA from spinach chloroplasts [10]. In some cases the isolated cyanelles were treated with DNAse before the isolation procedure.

The DNA thus obtained was then centrifuged in CsCl or CsCl–ethidium bromide density gradients by which procedures the host DNA could be removed due to the significant density differences [6]. The gradients were fractionated using an ISCO gradient fractionator equipped with an ultraviolet monitor. Analytical ultracentrifugation of purified cyanelle and host nuclear DNAs was performed in a Beckman model E ultracentrifuge at 44 000 rev./min and 20°C. Total *Cyanophora* DNA was prepared according to [12]. DNA was determined fluorimetrically using 3,5-diaminobenzoic acid [11].

2.3. *Restriction endonuclease cleavage of cyanelle DNA*

The endonucleases *Sal* I, *Bam* HI, *Sma* I, *Hpa* I, *Hpa* II, *Eco* RI, *Hin*dIII, *Hin*dII, *Pst* I and *Bgl* II have been used. They were purchased from Boehringer, Mannheim. Digestions by the restriction endonucleases were done according to manufacturer's instruction. The total volume was 60–80 μl (single and double digestions, respectively). The DNA fragments obtained by these digestions were separated by electrophoresis on 0.5–1.6% agarose slab gels (MCI, Rockville) in 40 mM Tris–HCl buffer (pH 7.8), 20 mM sodium acetate, 1 mM EDTA at 50 mA and 10°C for 16 h. After staining of the DNA with ethidium bromide fragment bands could be seen under ultraviolet-light and the gels were photographed on Polaroid positive/negative film. The following marker DNAs were included in agarose gels routinely: undigested DNA of phage λ, DNA fragments of λ DNA digested with *Hin*dIII (Boehringer), φX 174 DNA digested with *Hae* III (Bio Labs, Beverly, MA) and fragments of the DNA from plasmid pBR 322 digested with *Hpa* II [13].

DNA fragments after separation in 0.6–1.6% agarose gels were transferred to nitrocellulose filters according to [14]. To these the radioiodinated [15] rRNAs isolated from spinach chloroplast ribosomal subunits (16 S, 23 S and 5 S rRNA, respectively) have been hybridized [16]. Hybrid bands were visualized by autoradiography.

3. Results

Analytical ultracentrifugation (fig.1) shows the distribution of DNA components in whole-cell DNA and the separation of the cyanelle DNA from the other DNA components. DNA peaks can be seen at positions in the gradients corresponding to 1.691 g/cm^3 (32% G+C), 1.704 g/cm^3 (45% G+C) and 1.725 g/cm^3 (66% G+C). These fractions comprised 5%, 2% and <90% of total DNA, respectively. The % of the DNA species with 1.691 g/cm^3 was increased in preparations derived from isolated cyanelles. 30% without DNAase treatment and ⩽90% after treatment with increasing concentrations of DNAase (⩽200 μg/ml). The origin of the 1.704 g/cm^3 species is uncertain. Routinely the cyanelle DNA, with or without DNAase treatment of the cyanelles, was purified by two cycles of preparative CsCl density gradient centrifugation and fig.1c illustrates the first step.

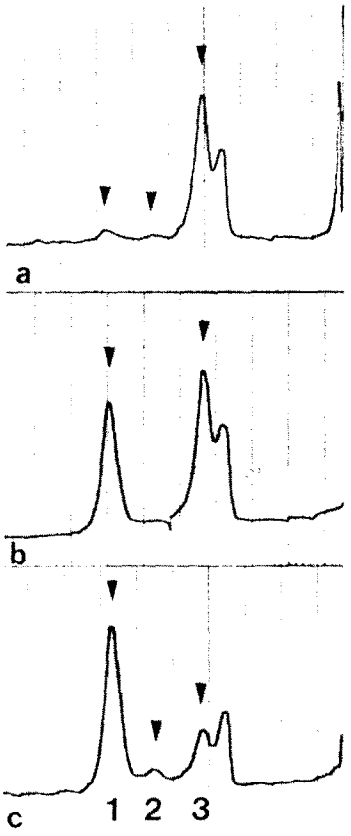

Fig.1. Photoelectric scans from analytical ultracentrifugations of *Cyanophora* DNAs. (a) Whole-cell DNA; (b) DNA from isolated cyanelles treated with DNase (50 μg/ml); (c) DNA from isolated cyanelles not treated with DNase after the first preparative CsCl density gradient centrifugation. The lighter of the two DNA components thus obtained was taken for analytical ultracentrifugation. The DNA species have been identified as: (1) cyanelle DNA, (2) unknown or possibly mitochondrial; (3) host nuclear DNA. The DNA component at the right is marker DNA from *Micrococcus lysodeicticus* (1.731 g/cm^3).

Fluorometric quantitative measurements [11] resulted in a DNA amount of 8.2×10^{-15} g/cyanelle. From the genome size [6] of 117 megadaltons one may calculate that the 1.691 g/cm^3 cyanelle DNA should be present in ~40–60 copies/organelle. For the free living blue-green algae *Anacystis nidulans* [17] and *Agmenellum quadruplicatum* [18] $6–22 \times 10^{-15}$ g DNA/cell has been reported.

Of the two DNA components separated by preparative CsCl density gradient centrifugation only the species ascribed to the cyanelles could be digested

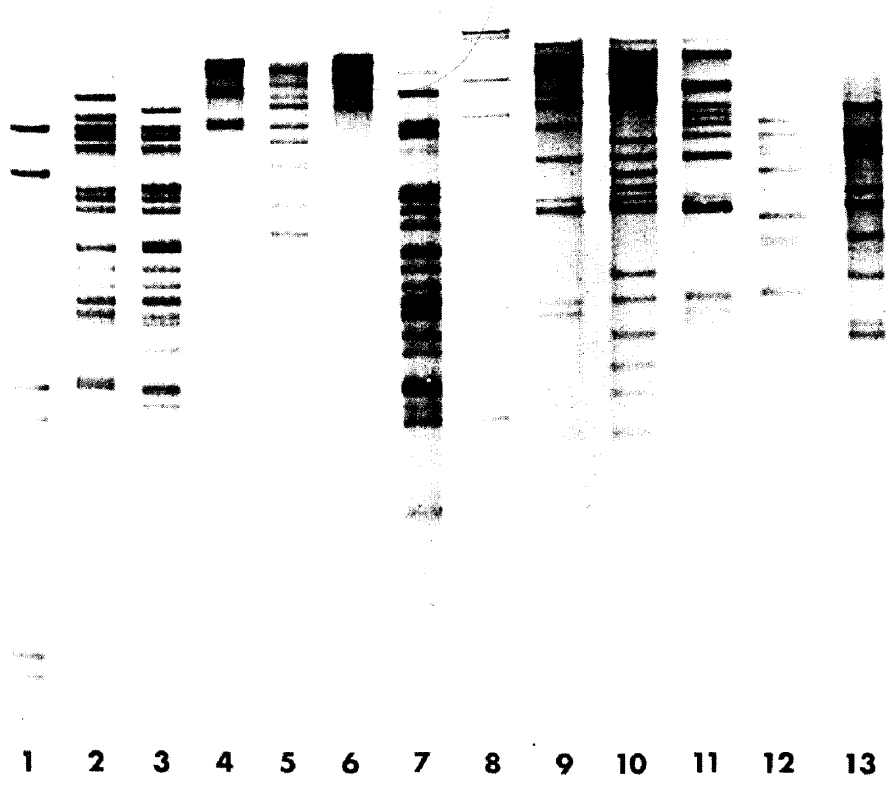

Fig.2. Agarose gel electrophoresis of cyanelle DNA after cleavage with various restriction endonucleases: (1) marker (λ DNA digested with *Hin*dIII + plasmid pBR 322 digested with *Hpa* II); (2) *Eco* RI, (3) *Bam* HI + *Eco* RI; (4) *Bam* HI; (5) *Bam* HI + *Sal* I, (6) *Sal* I; (7) *Eco* RI + *Sal* I (all on the same 0.8% agarose gel); (8) marker (λDNA digested with *Hin*dIII); (9) *Bgl* II; (10) *Bgl* II + *Bam* HI; (11) *Bgl* II + *Sal* I; (12) *Bgl* II + *Eco* RI (all on the same 0.9% agarose gel); (13) *Bgl* II cleavage pattern of cyanelle DNA isolated without DNase treatment showing the 25 megadalton fragment (0.6% agarose gel).

with restriction endonucleases resulting in a pattern of DNA fragments specific for each enzyme (fig.2; table 1). The major DNA component, *Cyanophora paradoxa* nuclear DNA, yielded a smear throughout the gel.

Addition of the DNA fragment molecular weights showed a considerable variation of the total molecular weight depending on the restriction endonuclease used. The molecular weight was especially low when the enzymes *Sal* I and *Bam* HI with only few recognition sites were used. This is due to the fact that the cyanelle DNA isolated is not of genome size but only ~1/3–1/2 leading to a reduced amount or even to the absence of DNA fragments close to or above 30 megadaltons. Without taking into account those fragments we found 106 megadaltons as the highest molecular weight for cyanelle DNA when the enzyme *Bgl* II was used. With other enzymes (e.g., *Eco* RI, *Pst* I, *Hin*dIII and *Hpa* I) the genome size was ~90 megadaltons but there was always an indication for the existence of a faintly visible additional DNA fragment of ~30 megadaltons.

The 106 megadaltons to which the DNA fragments add up following a *Bgl* II digestion could be corroborated by double digestions using the enzyme combinations *Bgl* II/*Bam* HI, *Bgl* II/*Sal* I and *Bgl* II/*Eco* RI

Table 1

Molecular weights of DNA fragments resulting from single and double digestions of the cyanelle DNA from *Cyanophora paradoxa* with various restriction endonucleases

Restriction endonuclease		*Sal* I	*Bam* HI	*Eco* RI	*Bgl* II	*Bam* HI/*Sal* I	*Bgl* II/*Sal* I	*Bgl* II/*Bam* HI	*Bgl* II/*Eco* RI	*Eco* RI/*Sal* I	*Bam* HI/*Eco* RI
Predicted[a] no. fragments		14	14	62	62						
Actual no. fragments		6–7	7–8	39–40	19	13	23	26	42	38	35
Fragment no.	A	>30[b]	>30[b]	20[b]	25	18	25	13	12.5	12.5	9.7
	B	28	25	12.5	10.7(2)	13	10.7	10.7	6.8	8.6	7.1
	C	16	15.5	8.6	8.3	12	8.3	8.3	5.6(2)	6.2	6.2
	D	14	14.0	6.8	7.3	9	7.4	7.9	4.5	5.6	5.6
	E	11.2	8.4	6.2	6.8(2)	7.9	5.2(2)	6.8(2)	4.0(2)	3.8	5.0
	F	11.0	8.0	5.6	5.0	7.6	5.0	6.5	3.7	3.65	3.8(2)
	G	7.5	6.4	5.1	4.4(2)	6.2	4.05	6.0	3.5(2)	3.6	3.6
	H		6.2	3.8	3.65	5.6	3.85	4.4(2)	3.4	3.4	3.4
	I			3.6	3.0	4.1	3.65	3.3	3.2	3.05	3.0
	J			3.4	2.47	3.1	3.35	3.12	3.0	2.75(2)	2.85(2)
	K			2.9(2)	2.3(2)	2.85(2)	3.0(2)	3.0	2.9(2)	2.6	2.55
	L			2.6	1.47	1.1	2.47	2.78	2.6	2.4	2.35
	M			2.4	1.37		2.3(3)	2.56	2.55	2.25(2)	2.20(2)
	N			2.25(2)	0.75		1.45(2)	2.4	2.4	2.1(2)	2.12
	O			2.1(2)	0.61		1.37	2.3(2)	2.25(2)	1.8	1.95
	P			2.05			1.2	1.62	2.05	1.68	1.7
	Q			1.56			0.75	1.47	2.0(2)	1.52(2)	1.48(2)
	R			1.52(2)			0.61	1.16	1.52(2)	1.50	1.35
	S			1.50				1.0	1.49	1.40	1.25
	T			1.40				0.9	1.45	1.33	1.15
	U			1.33				0.75	1.28	0.98	0.95
	V			0.98				0.61	1.12(2)	0.90(2)	0.88(2)
	W			0.9(2)				0.46	1.08	0.88	0.86
	X			0.88					1.03	0.82	0.78
	Y			0.82					0.9	0.63	0.66
	Z			0.63					0.88	0.55(2)	0.62
	A I			0.55(2)					0.75	0.53(2)	0.57(2)
	B I			0.53(2)					0.61	0.48	0.50(2)
	C I			0.48					0.55(2)	0.46	
	D I			0.46					0.53(2)	0.28	
	E I			0.28					0.48	0.19	
	F I			0.19					0.40		
Total mol. wt		88–118	85–115	95–115	106	93	105	104	105	86	86

[a] Calculated on the basis of a genome size of 180 000 basepairs, a 32% (G+C) content and a random base distribution

[b] These bands were not obtained in all experiments. They often occur with a stoichiometry <1 which is probably due to the reduced size of the cyanelle DNA preparations (~50 megadaltons). The exact assessment of sizes >30 megadaltons was not possible with the agarose gels used

The sizes are given in megadaltons; no. in parentheses indicate the stoichiometric amount of the particular fragment if it is >1. In double digestions original DNA fragments from either enzyme are underlined. DNA fragments ≲400 basepairs are not considered in this study

(fig.2, table 1). Very small fragments from the *Bgl* II/*Eco* RI digestion, which were resolved on 4% polyacrylamide gels (data not included) amount of ~2 megadaltons. Cyanelle DNA can thus be estimated as 107 megadaltons total as the lowest value. When the weak bands of high molecular weight obtained with *Sal* I, *Bam* HI and *Eco* RI are also included in the addition a maximum genome size of 115–118 megadaltons may be calculated (table 1), which is in good agreement with values obtained from reassociation kinetics [6].

DNA bands with a stoichiometry >1 were observed with a variety of enzymes (fig.2, table 1). Some of these bands are due to the closeness of otherwise unrelated DNA fragments. This may be seen in cases when only one of these double bands was cleaved by a second enzyme (e.g., *Bgl* II-B in *Bgl* II/*Bam* HI double digestions). Alternately both components were split yielding an entirely different subfragment pattern of bands with single stoichiometry (e.g., *Bgl* II-G in *Bgl* II/*Sal* I double digestions). There were however bands, which were cleaved by the second enzyme into subfragments once more showing a stoichiometry of 2 (e.g., *Bgl* II-K in *Bgl* II/*Eco* RI double digestions). This is an indication for repetitive parts in the cyanelle DNA known also for most chloroplast DNAs [7]. Further evidence for the presence of a repeat unit came from hybridization of radioiodinated ribosomal RNAs from spinach chloroplast [10] to fragments present in stoichiometries of 2. The 16 S rRNA hybridized to the DNA fragments *Eco* RI-N and W, whereas the 23 S rRNA hybridized to the DNA fragments N and R (fig.3). The observed hybridization of both rRNAs to the same DNA fragment points to a clustering of rRNA genes on repetitive sequences of cyanelle DNA. This too is an analogy with ctDNAs [7].

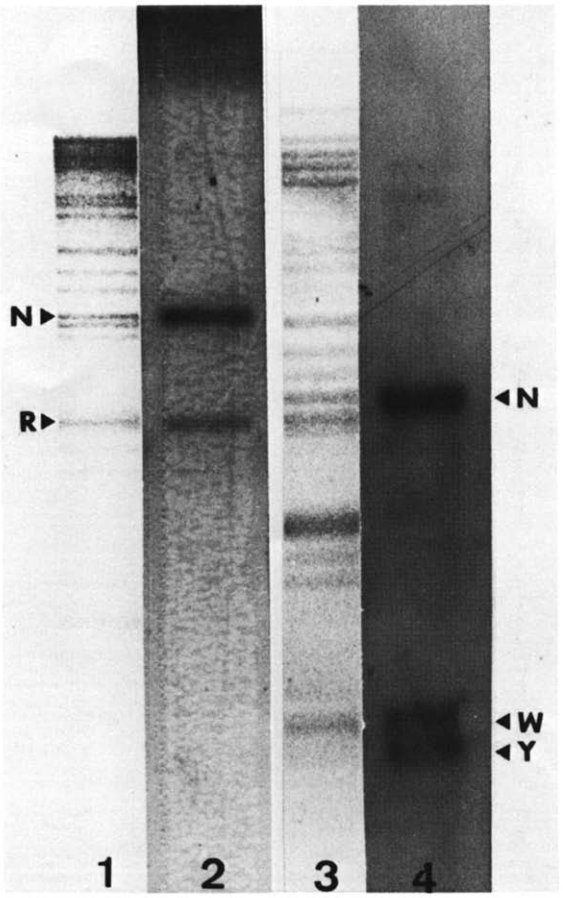

Fig.3. Hybridization of radioiodinated rRNAs from spinach chloroplasts to *Eco* RI fragments of cyanelle DNA: *Eco* RI fragments from a 1.5% agarose gel (1) have been hybridized with 23 S rRNA (2); *Eco* RI fragments from a 0.8% agarose gel (3) have been hybridized with 16 S rRNA (4).

4. Discussion

When isolated cyanelle DNA was treated with a variety of endonucleases, DNA fragments could be resolved on agarose gels which added up to ~115 ± 5 megadaltons. The number of cleavage sites for an individual enzyme were in most cases in the range expected for a DNA of this size and (G+C) content [19].

The size of the cyanelle genome leads to a comparison with chloroplast DNAs rather than with those from blue-green algae [7]. Also the cyanelle DNA shares another feature with most ctDNAs since it contains a repeated segment of ~10 megadaltons. In the chloroplast genomes of spinach, maize and *Chlamydomonas* the genes for the chloroplast rRNAs are located on an inverted repeat whereas in *Euglena gracilis* 3 tandem repeats contain the genes of the rRNAs. In our hybridization experiments we used rRNAs from spinach chloroplasts. This was justified since the structure of rRNAs is phylogenetically highly conservative as shown in [20]. Thus successful DNA:RNA hybridizations are possible for a wide range of organisms [16]. It is not yet known if the repeat in the cyanelle DNA is inverted. The 16 S and 23 S rRNAs (from spinach chloroplasts) hybridized both partially to one *Eco* RI DNA fragment with a

stoichiometry of 2. The size of this DNA fragment (2.25 megadaltons) and the fact that each of the rRNAs hybridizes to another DNA fragment in addition make a location of the rRNA genes likely which is comparable to that in spinach ctDNA [10].

On the other hand the cyanelles from *Cyanophora* resemble free living blue-green algae in morphological [2] and metabolic [21] respects. A strong indication for a Cyanophycean origin of these cyanelles is the rudimentary cell wall containing peptidoglycan. Thus one may ascribe to the cyanelles from *Cyanophora* a position between free-living Cyanophyceae and chloroplasts in support of the endosymbiont hypothesis [22].

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