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Gene Map for the *Cyanophora paradoxa* Cyanelle Genome

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The genes for the following proteins were localized by hybridization analysis on the cyanelle genome of *Cyanophora paradoxa*: the α and β subunits of phycocyanin (*cpcA* and *cpcB*); the α and β subunits of allophycocyanin (*apcA* and *apcB*); the large and small subunits of ribulose-1,5-bisphosphate carboxylase (*rbcL* and *rbcS*); the two putative chlorophyll *a*-binding apoproteins of the photosystem I-P700 complex (*psaA* and *psaB*); four apoproteins believed to be components of the photosystem II core complex (*psbA*, *psbB*, *psbC*, and *psbD*); the two apoprotein subunits of cytochrome *b*-559 which is also found in the core complex of photosystem II (*psbE* and *psbF*); three subunits of the ATP synthase complex (*atpA* and *atpBE*); and the cytochrome *f* apoprotein (*petA*). Eighty-five percent of the genome was cloned as *Bam*HI, *Bgl*II, or *Pst*I fragments. These cloned fragments were used to construct a physical map of the cyanelle genome and to localize more precisely some of the genes listed above. The genes for phycocyanin and allophycocyanin were not clustered and were separated by about 25 kilobases. Although the *rbcL* gene was adjacent to the *atpBE* genes and the *psbC* and *psbD* genes were adjacent, the arrangement of other genes encoding various polypeptide subunits of protein complexes involved in photosynthetic functions was dissimilar to that observed for known chloroplast genomes. These results are consistent with the independent development of this cyanelle from a cyanobacterial endosymbiont.

Cyanophora paradoxa is a flagellated protozoan belonging to a taxonomically diverse group of cyanelle-containing organisms (42). Cyanelles, to the extent that they have been characterized, are the metabolic (16, 17, 27, 42) and genetic (25, 28) equivalents of chloroplasts. However, their thylakoid organization, outer wall composition, and photosynthetic pigments are similar to those of cyanobacteria rather than those of typical chloroplasts (1, 18, 42). This is consistent with the belief that cyanelles and red algal chloroplasts (rhodoplasts) developed by the degeneration of endosymbiotic cyanobacteria independently of the development of green plant chloroplasts from a *Prochloron*-like intermediate (20).

Because the cyanelle of *C. paradoxa* has a thick peptidoglycan wall, pure and intact cyanelle DNA is easily obtained. Consequently, *C. paradoxa* has been studied as a representative of those organisms with phycobiliprotein-containing plastids. Cyanelle DNA is similar to chloroplast DNA in size (25, 28), in its G+C content of 36% (25), and in the presence of rRNA genes enclosing isoleucine and alanine tRNA genes on inverted repeats separated by a small spacer region (30). In contrast to green plants, the order of the rRNA genes of *C. paradoxa* is reversed relative to that of the small unique-sequence spacer (28). Furthermore, the distribution of tRNA genes on the cyanelle genome does not resemble that observed for chloroplasts (28, 35, 44). Unlike chloroplast genomes, the gene encoding the small subunit of *C. paradoxa* ribulose-1,5-bisphosphate carboxylase is immediately adjacent to the gene encoding the large subunit of this enzyme on the cyanellar genome rather than in the nuclear genome (24, 40a). This is similar to the situation observed in cyanobacteria (36, 38) and red algae, in which both subunits appear to be encoded together on the plastid genome (41). The *C. paradoxa* small subunit is more similar in amino acid sequence (40a) and secondary structure (D. H. Lambert and S. E. Stevens, unpublished data) to the cyanobacterial proteins than to those of green algae and higher plants (40a).

Results of a number of studies suggest that the genes for phycobiliproteins in red algae and cyanelles are encoded by the plastid genome (6, 14, 21, 41). Correspondingly, genes for the β subunit of phycocyanin (29) and the α and β subunits of allophycocyanin (10) have been located on the cyanelle genome. The gene for another phycobilisome component, the high-molecular-mass linker phycobiliprotein, which anchors the phycobilisome to the thylakoid membrane, is believed to be encoded on the cyanellar genome (21).

Establishment of polyphyletic origins for plastids is considered a major proof for the endosymbiont hypothesis, i.e., that plastid genomes are derived from endosymbionts rather than from the partition of a single preexisting genome (20). The foregoing evidence suggests that cyanelles and chloroplasts have developed convergently from substantially different endosymbionts into functionally similar plastids with a superficially similar genome architecture. Further investigation of the cyanelle genome is not only of evolutionary interest, but may indicate common or dissimilar mechanisms by which plastid genomes and their photosynthesis genes are organized, stabilized, and expressed. With this in mind, we substantially cloned the cyanelle genome of *C. paradoxa* and mapped the genes that encode a wide variety of proteins involved in photosynthetic functions.

MATERIALS AND METHODS

Cyanelle DNA preparation. The *C. paradoxa* strain used in this study was originally obtained from the Pasteur Culture Collection, Institut Pasteur, Paris, France, by Jessup M. Shively, and is apparently identical to the standard LB555 University of Texas Culture Collection strain (31). Cells were grown on Allen medium (2) modified by the addition of 20 mg

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TABLE 1. Probe homologies with cyanelle DNA restriction fragments

Probe[a]	Restriction fragment size (kb)[b]
cpcA	B, 41.9; G, 12.4; P, 1.2
cpcB	B, 41.9; G, 12.4; P, 2.4
apcA	B, 41.9; G, 2.0; P, 7.8
apcB	B, 41.9; G, 2.0; P, 7.8
rbcL	B, 15.8; B-S, 9.5; C, 0.6, 2.4; E, 0.3, 0.6, 2.4; G, 3.6, 8.3; H, 2.2; P, 13.5; S, 20.3; X, 27.4
rbcS	B, 15.8; B-S, 9.5; C, 2.4; E, 0.3, 8.2; G, 3.6; H, 2.2, 1.5; P, 13.5; S, 20.3; X, 27.4
psaA psaB	B, 41.9; B-S, 40.1; G, 4.0, 7.0; P, 4.5, 8.8; S, 35.2; X, 4.8
psbA	B, 15.8; B-S, 9.5; C, 0.8; G, 3.6; P, 13.5; S, 20.3; X, 27.4
psbB	B, 39.4; B-S, 26.9; G, 16.4; P, 1.3, 2.1; S, 20.3; X, 27.4
psbC	B, 10.4; B-S, 10.4; G, 8.3; P, 6.8; S, 20.3; X, 27.4
psbD	B, 10.4; B-S, 10.4; G, 8.3; P, 6.8; S, 20.3; X, 27.4
psbE	B, 39.4; G, 16.4; P, 18.4; H, 8.5; E, 0.75
psbF	B, 39.4; G, 16.4; P, 18.4; H, 8.5; E, 0.75
atpA	B, 11.2; B-S, 6.3; G, 7.7; H, 0.6, 4.0; P, 0.5, 1.4, 2.5; S, 18.8; X, 22.4; E, 5.6
atpBE	B, 10.4, 15.8; B-S, 9.5, 10.4; E, 1.2, 2.4; G, 8.3; H, 1.8; P, 1.9, 13.5; S, 20.3; X, 27.4
petA	B, 10.4; B-S, 10.4; G, 8.3; H, 4.3; P, 1.2, 1.9; S, 20.3; X, 27.4
petB	B, B-S, G, P, S, and X, negative
petC	B, B-S, G, P, S, and X, negative
petD	B, B-S, G, P, S, and X, negative
petF	B, B-S, G, P, S, and X, negative
*Pst*I 1.9	B, 10.4, 15.8; B-S, 9.5, 10.4; G, 8.3; P, 1.9; S, 20.3; X, 27.4
*Pst*I 7.8	B, 39.4, 41.9; B-S, 1.8, 27.9, 40.1; G, 2.0, 7.3, 16.4; P, 7.8; S, 28.7, 35.2; X, 9.0, 21.7
*Pst*I 10.0	B, 11.2, 41.9; B-S, 6.3, 12.5; G, 5.0, 6.3, 7.7; P, 10.0; S, 18.8; X, 22.4
*Pst*I18.5	B, 39.4; G, 10.8, 16.3, P, 18.5
*Bam*HI 14.6	B, 14.6; P, 0.6, 2.5, 4.5, 12.0, 14.4

[a] Descriptions of probes are provided in the text.
[b] Abbreviations: B, *Bam*HI; C, *Hin*cII; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; X, *Xho*I.

of ferric ammonium citrate per ml and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (pH 7.2) in constant light at ca. 28°C with continuous bubbling of 1% CO_2 in air. Cyanelles were obtained by freezing cell pellets overnight followed by numerous wash and minimum speed centrifugation cycles with 0.1% sodium dodecyl sulfate, 50 mM Tris hydrochloride, 5 mM EDTA (pH 8.0). DNA was extracted by the lysozyme-sodium dodecyl sulfate method of Godson and Vapnek (19) as described in Maniatis et al. (32). The lysed cyanelle pellet was extracted an additional one or two times with 1% sodium dodecyl sulfate in buffer if partial chlorophyll removal indicated incomplete DNA extraction. These extractions were followed by phenol extraction, cesium chloride-ethidium bromide density gradient centrifugation, dialysis, and ethanol precipitation.

DNA isolation. The isolated cyanelle DNA was digested with the restriction enzymes *Bam*HI and *Pst*I for cloning into the vector pBR322 (9) with *Escherichia coli* RDP145 (11). In addition, several smaller *Pst*I fragments were cloned into pUC vectors (43) with *E. coli* JM103 (33); and single *Bgl*II, *Bgl*II-*Bam*HI, and *Hin*dIII fragments encoding ribulose-1,5-bisphosphate carboxylase genes were cloned into pBR322 or pUC vectors. In most cases, these digests were first electrophoresed into low-melting-point agarose to obtain specific bands or groups of bands. After isolation, phenol extraction, ethanol precipitation, and cloning, small libraries of these fragments were either characterized by size and restriction enzyme digestion patterns or were screened with radiolabeled probe DNAs. For use as hybridization probes, oligonucleotides were labeled at their 5' ends with [γ-^{32}P]ATP with T4 polynucleotide kinase; plasmid DNAs and purified restriction fragments were labeled by nick translation with DNA polymerase I and [α-^{32}P]ATP. Hybridizations were performed as described previously (23).

Gene identification. *C. paradoxa* cyanelle DNA was digested with the restriction enzymes *Bam*HI, *Bgl*II, *Pst*I, *Sal*I, *Xho*I, and *Bam*HI-*Sal*I and in some cases with *Hin*dIII and *Eco*RI; the digested DNAs were separated by agarose gel electrophoresis, and the restriction fragments were transferred to nitrocellulose filters by the method of Southern (40). Southern blots of subdigests of the larger *Pst*I and smaller *Bam*HI fragments were also prepared. Sequences homologous to the genes listed in Table 1 were localized on these blots, using radiolabeled plasmid DNA, restriction digest fragments, or synthetic oligonucleotides as probes. Cyanelle DNA restriction fragments exhibiting hybridization to these probes are also listed in Table 1. The identification of the genes and the sources of the probes employed are as follows. *cpcA* and *cpcB* are genes encoding the α and β subunits of phycocyanin (probes were subclones derived from the genes cloned from *Agmenellum quadruplicatum* PR-6 [12]). *apcA* and *apcB* are genes encoding the α and β subunits of allophycocyanin (the probe was a oligonucleotide mixture matching a highly conserved region of the β subunit of allophycocyanin and subclones generated during sequence analysis of the allophycocyanin coding region [10]). *rbcL* was the gene encoding the large subunit of ribulose-1,5-bisphosphate carboxylase (the probe was the subclone carrying a portion of the coding sequence for the *Chlamydomonas reinhardtii* large subunit [13]). *rbcS* was the gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase (the probe was the subclone carrying a portion of the coding sequence for the small subunit from pea [5]). *psaA* and *psaB* were the genes encoding the P700 chlorophyll *a* proteins of photosystem I (probes were subclones carrying the coding sequences for the *Zea mays* chloroplast genes [15]). *psbA* was the gene encoding the 32-kilodalton (kDa) herbicide-binding protein of photosystem II (the probe was a subcloned fragment carrying the coding sequence for the spinach *psbA* gene obtained from J. Williams). *psbB* was a gene encoding the 51-kDa chlorophyll *a*-binding protein of photosystem II (P680; the probe was gene-internal restriction fragment from the spinach chloroplast gene obtained from R. Herrmann [34]). *psbC* was the gene encoding the 44-kDa chlorophyll *a*-binding protein of photosystem II (the probe was the gene-internal restriction fragment of the spinach chloroplast gene obtained from R. Herrmann [3]). *psbD* was the gene encoding the D2 protein of photosystem II (the probe was the gene-internal restriction fragment from a subclone of the chloroplast-encoded *Chlamydomonas reinhardtii* gene described by Rochaix et al. [37]). *psbE* and *psbF* were genes for the apoprotein subunits of cytochrome *b*-559 which is a part of the photosystem II core complex (26, 46) (probes were synthetic 81-mer oligonucleotides synthesized from the sequences of the spinach genes

as determined by Herrmann et al. [26] and were kindly supplied by Himadri Pakrasi, DuPont Experimental Station, Wilmington, Del.). *atpA* was a gene encoding the α subunit of coupling factor (the probe was the gene-internal restriction fragments from subclone carrying the spinach chloroplast gene described by Westhoff et al. [47]). *atpBE* were genes for the β and ε subunits of coupling factor (the probe was restriction fragments derived from the subclone carrying the coding sequences for the β and ε subunits of *Anabaena* sp. [S. Curtis, unpublished results]); *petA* was the gene encoding cytochrome *f* apoprotein (the probes were restriction fragments derived from a subclone carrying the pea chloroplast cytochrome *f* gene described by Willey et al. [48]). *petB* and *petD* were genes encoding the apoprotein of cytochrome b_6 and polypeptide subunit IV of the cytochrome b_6-*f* complex, respectively (the probe was the gene-internal fragment of the gene encoding the mitochondrial cytochrome *b* of *Kluyveromyces lactis* [L. A. Grivell, University of Amsterdam, unpublished data]). *petC* was the gene encoding the apoprotein of the Rieske Fe-S protein, an integral component of the cytochrome b_6-*f* complex (the probe was the gene-internal fragment of the *Rhodopseudomonas capsulata* gene [Fevzi Daldal, Cold Spring Harbor Laboratory, unpublished data]). *petF* was the gene encoding soluble ferredoxin (the probe was the synthetic oligonucleotide 29-mer [D. A. Bryant, unpublished data]). 20 kDa was the putative gene for a 20-kDa membrane-associated polypeptide in pea chloroplasts as described by Willey et al. (48) (the probe was a restriction fragment derived from a subclone of pea chloroplast DNA which also carries the *petA* gene [48]).

Genome mapping. To demonstrate restriction fragment overlaps, genomic digests were also probed as described above with the following restriction fragments (in kilobases [kb]): *Pst*I, 1.9; *Pst*I, 7.8; *Pst*I, 10; *Pst*I, 19; *Bam*HI, 15; *Hin*dIII, 2.2, which was a fragment encoding the *rbcL* gene and a portion of the *rbcS* gene (Table 1). By using the information obtained and the previously published data of Bohnert et al. (8) and Kuntz et al. (28), a complete restriction map of the *C. paradoxa* cyanelle genome was assembled.

RESULTS

Genome map. Including the inverted repeat region twice, 85% of the cyanelle genome was cloned. The data shown in Table 1 were compiled to produce the linear map of the cyanelle genome in Fig. 1, which proceeds clockwise from the leftmost 16S rRNA gene on the circular map in Fig. 2. The map agrees with the data of Kuntz et al. (28) and Bohnert et al. (8), except for the placement of *Bgl*II sites in the small spacer region (cf. Lemaux and Grossman [29]) and the presence of additional restriction sites. The total length of the map, derived by the addition of fragment lengths, is 133.4 kb, which is somewhat larger than the 126.5 kb previously reported (8, 28). This difference is one that is due to proportion rather than to the existence of additional sequences, because our estimates of various restriction fragment lengths are uniformly higher than those previously reported. Restriction sites or fragments included in our map which are not fixed include those in the noncloned *Pst*I 5.2-kb fragment (map positions 50.3 to 55.5), in noncloned portions of the *Bam*HI 15.8-kb fragment (74.2 to 84.1), and in the small spacer region (125.6 to 132.1). Sites in these regions were assigned by the extrapolation of adjacent fragments of known length, and these sites were confirmed by the mapping of other sites (8, 28, 29). The relative positions of the *Pst*I 1.3- and 2.1-kb fragments which encode the *psbB* gene (28.4 to 31.8) were not determined, so their common *Pst*I site (30.1) is actually 0.4 kb to the left or right of the position at which it was centered. The relative positions of the *Pst*I 1.2- and 2.4-kb fragments encoding the phycocyanin genes (122.0 to 125.6) were not determined, and we relied on the data of Lemaux and Grossman (29) who have mapped partial *Sau*3A clones of this region.

Small spacer inversion. Genomic digests of several DNA preparations made over the period of a year did not show the reversed orientations of the small spacer region reported by Bohnert et al. (7). Substoichiometric restriction digest fragments were not observed; for example, our *Bam*HI digest produced six large fragments rather than the eight that resulted from two small spacer orientations (8). Hence, our culture appears to contain a single cyanelle DNA species, suggesting that inversion of the small spacer may not occur in all *C. paradoxa* strains.

Gene locations. Comparative circular maps for the cyanelle and spinach chloroplast genomes are shown in Fig. 2. These show the opposite orientations of the rRNA genes relative to the small spacer region and the relative positions of various protein-encoding genes. The genes for phycocyanin and allophycocyanin are widely separated (approximately 25 kb separate the two gene pairs). The genes for the ribulose-1,5-biphosphate carboxylase large subunit and ATPase (coupling factor) β and ε subunits are located together as in chloroplasts (47). The gene for the small subunit of ribulose-1,5-bisphosphate carboxylase, which is cotranscribed with the large subunit gene (40), is located beyond the 3′ end of the large subunit gene, as in cyanobacteria (36, 38). Although the *psbC* and *psbD* genes are immediately adjacent, as reported previously for spinach, the relative locations of other genes show little relation to those of spinach (45) or *Euglena gracilis* (22) chloroplast-encoded genes. Of the genes mapped onto the spinach chloroplast genome (Fig. 2), only those for *psaAB* have tRNA genes (for leucine and serine) which might correspond to those around the same cyanelle gene (28, 45), and it has not been established that these tRNA genes are actually in equivalent positions relative to the *psaAB* genes. An open reading frame adjacent to the cytochrome *f* gene in pea chloroplasts, which could code for a 20-kDa protein of unknown function, was not found on the cyanelle genome. Hybridizations performed with an oligonucleotide probe for ferredoxin (*petF*), which hybridized to specific restriction fragments of a wide variety of cyanobacterial DNAs (D. A. Bryant, unpublished data), suggest that this gene is not encoded by the cyanelle genome as previously reported for higher plants (39). Hybridizations performed with probes for the *petB*, *petC*, and *petD* genes were also negative (Table 1).

DISCUSSION

The results of the studies reported here indicate that the *C. paradoxa* genome encodes, in general, the same set of genes found in chloroplasts. In addition, it encodes some, but not all (14) of the phycobilisome structural genes. Control of phycobilisome assembly, like that of other multiprotein complexes (4, 44–46), appears to involve the coordination of gene expression from both nuclear and cyanelle genomes. In contrast, both subunits of ribulose-1,5-bisphosphate carboxylase are encoded in the cyanelle. This is the only indication at present that the cyanelle might be more independent (42) than the chloroplast of its eucaryotic host. The transfer of the small subunit gene to the nucleus apparently was an event which occurred in the development of green plant chloroplasts but not in the development of equivalent

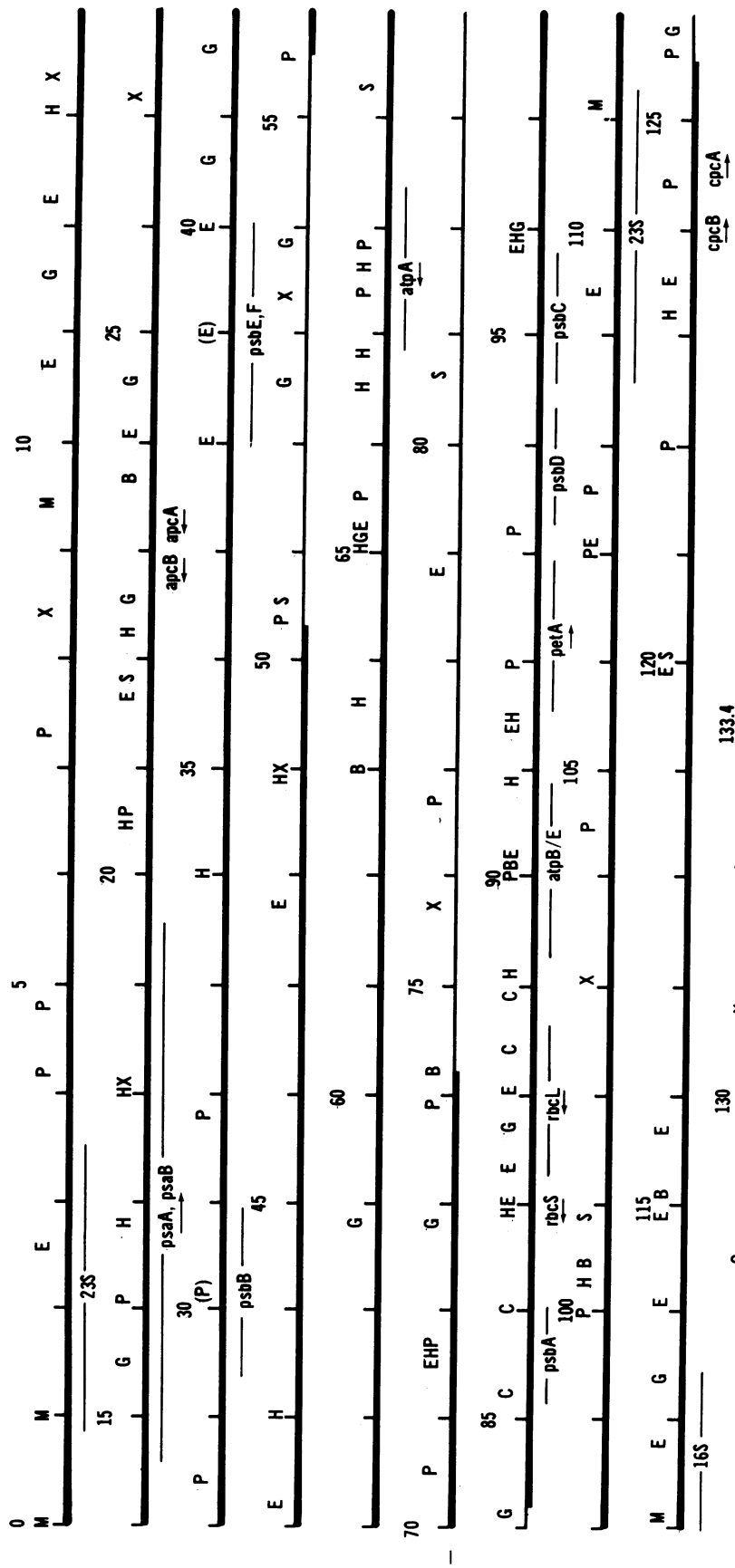

FIG. 1. *C. paradoxa* cyanelle genome map. Restriction site codes are as follows: B, *Bam*HI; C, *Hin*cII; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; M, *Sma*I; P, *Pst*I; S, *Sal*I; X, *Xho*I. Distances are given in kilobases, cloned regions are indicated by thick horizontal lines, and arrows denote the expected direction of transcription. The positions of the 16S and 23S rRNA genes are taken from Kuntz et al. (28). Other gene loci are as in Table 1; most loci are approximate since localization is by hybridization analysis and not by sequence analysis.

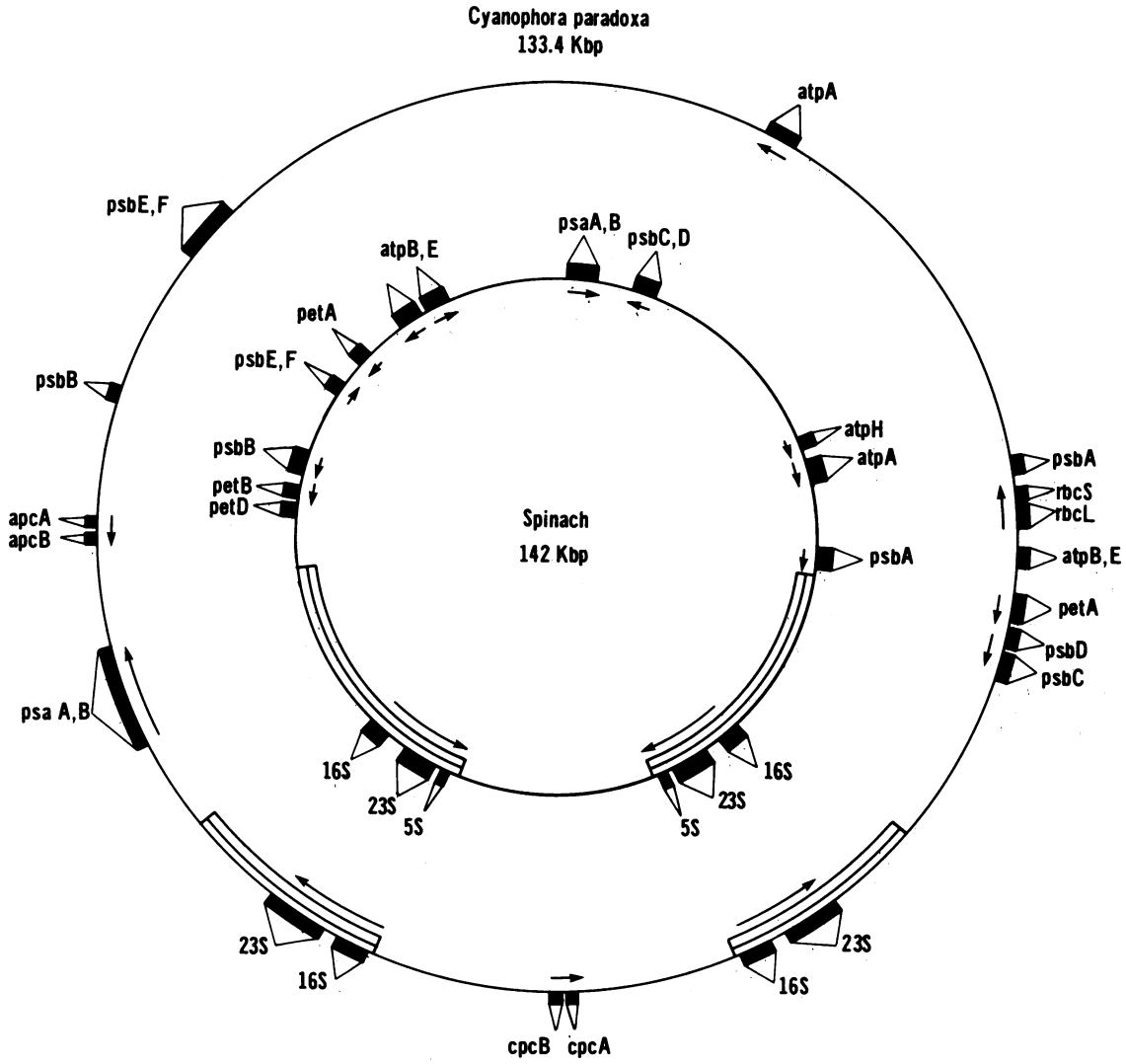

FIG. 2. Comparative circular maps for the plastid genomes of *C. paradoxa* and spinch (44). Cyanelle gene loci are as in Table 1. Bars indicating gene loci and sizes are approximate.

plastids in biliprotein-containing eucaryotes. This difference in small subunit location, the major differences in gene arrangements, the presence of a peptidoglycan layer surrounding the cyanelle, and the analogous but not homologous organization of the rRNA-containing inverted repeats are all consistent with an independent but convergent development of photosynthetic plastids in green and phycobiliprotein-containing eucaryotes.

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