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On the Prokaryotic Nature of Red Algal Chloroplasts

(ribosomal RNA/oligonucleotide cataloging/evolution/endosymbiosis)

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ABSTRACT The sequences of oligonucleotides released by T1 ribonuclease digestion of ^{32}P-labeled 16S (chloroplast) and 18S (cytoplasmic) ribosomal RNAs from a marine species of *Porphyridium* (Rhodophyta) have been determined. The resultant catalogs have been compared to those obtained for three prokaryotes: *Escherichia coli*, *Bacillus subtilis*, and *Anacystis nidulans* (a blue-green alga). There is extensive sequence homology between the *Porphyridium* chloroplast 16S ribosomal RNA and each of the prokaryotic 16S ribosomal RNAs, but little homology between the *Porphyridium* cytoplasmic 18S ribosomal RNA and any of the 16S species. These data provide a measure of the evolutionary distance separating existing chloroplasts from contemporary bacteria and blue-green algae, and are discussed in terms of the hypothesis that these organelles evolved from endosymbiotic photosynthetic prokaryotes.

The notion that modern chloroplasts evolved from endosymbiotic prokaryotes (perhaps resembling blue-green algae) is an old one (1) which has gained considerable modern support (2–4), since the discovery of DNA in these semi-autonomous, self-replicating organelles (5). The argument rests on structural resemblances between chloroplasts and prokaryotic cells, strong similarities in structure and function between chloroplast and prokaryotic ribosomes (6), near identity of the pathways of photosynthetic electron flow and CO_2 fixation in chloroplasts and blue-greens, and the existence of modern true endosymbionts resembling the postulated ancestral endosymbiotic prokaryotes (2, 3). None of these types of evidence submits to *quantitative* analysis, and none can provide any measure of the evolutionary distance between contemporary chloroplasts and prokaryotes. It is thus still possible to maintain [as Klein (7) and Allsopp (8), for instance, recently have] that eukaryotes arose by "direct filiation" from prokaryotes, with subsequent or concomitant partitioning of the genome between nucleus and chloroplast.

In testing the endosymbiont hypothesis, it would be useful to show that the chloroplast DNA of a single eukaryotic cell carries genes that are more similar in nucleotide sequence to evolutionarily homologous prokaryotic genes than they are to homologous genes carried on nuclear DNA within the same cell. Few structural genes have been definitively localized within the chloroplast genome, but these fortunately include those for the major chloroplast ribosomal RNAs (16S and 23S rRNA), which have obvious evolutionary homologs in prokaryotic 16s and 23S and nuclear 18S and 28S rRNA genes (9, 10). Evidence for sequence relatedness of *Euglena* chloroplast rRNA genes to blue-green algal (but not bacterial) rRNAs has been obtained by Pigott and Carr (11) using molecular hybridization methods. These methods, however, provide considerably less quantitative information than does the technique of "oligonucleotide cataloguing," as it has been developed by Woese and his collaborators (12–14). This technique involves sequencing of all guanylate-terminated oligonucleotides released by T1 ribonuclease digestion of ^{32}P-labeled rRNAs and enumeration of those whose sequence is common to rRNAs of interest. We present below the results of such a sequence analysis of 16S (chloroplast) and 18S (cytoplasmic) rRNA from a species of *Porphyridium* (a unicellular marine red alga). A red alga was chosen since it is the chloroplasts of this eukaryotic group that most obviously resemble an existing line of prokaryotes, the blue-green algae (3, 4, 7, 8). Many supporters of the endosymbiont hypothesis indeed suggest that *only* red (and possibly cryptophytan) algal chloroplasts derive from blue-greens, while other chloroplasts arose separately from one or more now extinct lines of oxygenic photosynthetic prokaryotes (2–4). Elsewhere in this issue, Zablen *et al.* present the results of a similar analysis of chloroplast 16S rRNA from *Euglena gracilis* (15). Together these data show extensive homology between chloroplast 16S and prokaryotic 16S rRNAs, while failing to demonstrate homology between cytoplasmic 18S rRNA and any 16S species.

MATERIALS AND METHODS

An axenic culture of a unicellular species of *Porphyridium* (Bangiophycidae) was kindly provided by J. S. Craigie (National Research Council Atlantic Regional Laboratory, Halifax, N.S.). This strain was originally obtained from R. Lewin and is not distinguishable from known isolates of *P. cruentum*. It was grown in dim light at 22–25° in a medium containing 0.05 mmol of NaH_2PO_4, 1 mmol of $NaNO_3$, 2 μmol of Fe_2SO_4, 5 mmol of glycylglycine, 100 μg of thiamine·HCl, 0.5 μg of vitamin B12, and 0.5 μg of biotin per liter of Millipore-filtered sea water (adjusted to pH 7.5 before autoclaving). Culture purity was repeatedly confirmed by microscopic examination.

Cultures were labeled by incubation with 30–50 mCi of carrier-free [^{32}P]orthophosphate (New England Nuclear)/100 ml for 10–12 days. Cells were harvested by centrifugation, resuspended in 10 mM Tris·HCl (pH 7.3) containing 1 mM $MgCl_2$, and lysed by passage through an Aminco French pressure cell at 18,000 psi (124 kPa). Lysates were extracted three times with redistilled, water-saturated phenol (containing 0.1% 8-hydroxyquinoline), and RNA was precipitated from the final aqueous phase (made 0.2 M in sodium acetate) with ethanol. After resolution on 2.8% polyacrylamide gels, ^{32}P-labeled RNA was eluted from gel slices, extracted three times with phenol and twice with ether, and then passed through columns of Whatman CF-11 cellulose powder (16),

with final elution being performed in 15% ethanol. Eluted RNA was lyophilized and then digested with T1 ribonuclease (14). Guanylate-terminated oligonucleotides in digests were resolved by two-dimensional electrophoresis, as described by Sanger and coworkers (17), with modifications developed by Woese and his collaborators (12–14, Sogin and Woese, unpublished). Sequence determinations on individual spots cut from these "primary" electrophoretic fingerprints were performed by "secondary" and "tertiary" digestion using procedures of Uchida *et al.* (13) and the "T3 technique" of Bonen and Woese (in preparation). Molar yields of oligonucleotides were determined by measuring radioactivity in each spot prior to secondary digestion, with the assumption that 16S and 18S rRNA contain 1650 and 1925 nucleotide residues, respectively. Ribonucleases were purchased from Calbiochem (T1 and U_2) and Worthington (pancreatic).

RESULTS

Polyacrylamide gels of ^{32}P-labeled *Porphyridium* RNA show four well-resolved species of approximate molecular weights 1.3, 1.1, 0.68, and 0.55 $\times$ 10^6, which we here term 28S, 23S, 18S, and 16S rRNA, respectively. 23S and 16S rRNA together comprise about 35% of the total labeled material. Species of similar molecular weight and even greater relative abundance from *P. aerugineum* were designated chloroplast rRNAs by Howland and Ramus (18). Methods for isolation of *Porphyridium* chloroplasts are not available (E. Gantt, personal communication), and we have perforce assumed that the 16S species we obtain derives primarily from chloroplasts and is not significantly (<20%) contaminated by RNA of mitochondrial origin. This same assumption is explicit or implicit in most recent studies of algal rRNAs (18–21) and is justified in the present case by the facts that (*i*) very few minor oligonucleotides (present in less than 0.5 copy per molecule of 16S rRNA) were found on primary fingerprints, and (*ii*) this 16S species shows significant homology with 16S rRNA from purified *Euglena* chloroplasts (see below and ref. 15), and little or no homology with mitochondrial RNAs of this organism (C. R. Woese, personal communication). It should be realized that even contamination as high as 20% would not interfere with our analysis, since oligonucleotides present in significantly less than unimolar amounts (of which there were very few) were systematically excluded from the catalog.

Electrophoretic fingerprints of oligonucleotides released by T1 ribonuclease digestion of 18S and 16S rRNAs are shown in Fig. 1. The sequences of oligonucleotides of chain length (N) five or greater contained in each spot of the primary 16S fingerprint were determined by appropriate secondary and tertiary nuclease treatment and are listed in Table 1. This table also indicates, for each *Porphyridium* 16S rRNA oligonucleotide, the presence or absence of identical sequences in the 16S rRNAs of three prokaryotes [*Bacillus subtilis* (C. R. Woese and M. L. Sogin, personal communication), *Escherichia coli* (13), and the unicelluar blue-green alga *Anacystis nidulans* (14)] and in the 18S rRNA of *Porphyridium*. The 10 to 12 oligonucleotides from *Porphyridium* 18S rRNA listed as shared with *Porphyridium* 16S rRNA comprise at most 12% of the total oligonucleotides ($N \geq 5$) present on primary fingerprints of this cytoplasmic species. The sequence of some 60 additional oligonucleotides ($N \geq 5$) from this molecule has been determined, but there is not space to indicate these sequences here. Preliminary characterization

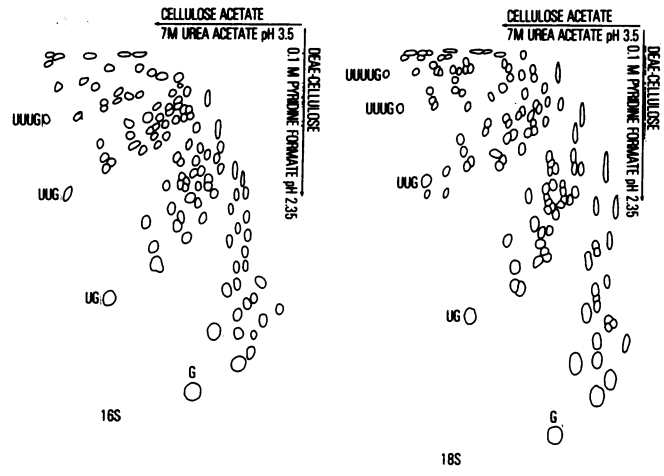

FIG. 1. Primary electrophoretic fingerprints of T1 ribonuclease digests of 16S and 18S rRNAs of *Porphyridium*.

(principally by pancreatic RNase digestion) of the remaining 30 to 40 oligonucleotides of the 18S species is adequate to insure that none is coincident in sequence with any *Porphyridium* 16S rRNA oligonucleotide.

Table 1 further indicates, at the end of each group of oligonucleotides of size N, the total number (k_P) of oligonucleotides of this size class common to *Porphyridium* 16S rRNA and each of the four rRNAs to which it is compared. The significance of such a number of coincident sequences can be assessed by substituting for the n_P *Porphyridium* N-mers, sets of n_P N-mers of random (unrelated) sequence. The probability (P) of obtaining the observed or a greater number of coincidences ($k \geq k_P$) with such sets of random N-mers is given (22) by

$$\sum_{k=k_P}^{n_P} P(k) = \sum_{k=k_P}^{n_P} \binom{n_x}{k}\binom{N' - n_x}{n_P - k} \div \binom{N'}{n_P},$$

where n_x is the total number of N-mers in the rRNA (prokaryotic 16S or cytoplasmic 18S) to which comparison is made and N' is the total number of possible guanylate-ending sequences of size N (or 3^{N-1}). These values of P are shown in Table 1 along with the modes and means of the probability functions. The mode is the most probable number of coincident sequences, k, in such comparisons between sets of n_p random N-mers and each of the sets of n_x N-mers derived from the four prokaryotic or cytoplasmic molecules. The mean is the average of k values for an infinite number of comparisons of this type, and is given by $(n_p \cdot n_x) \div N'$.

The data indicate strong sequence homology between *Porphyridium* 16S rRNA and each of the prokaryotic rRNAs considered here. The number of coincident sequences in each oligonucleotide size class exceeds that expected for two RNAs of unrelated (random) sequence, and the probability of obtaining the observed number of coincidences between unrelated RNAs is in most cases less than one in 10^2, and in several cases less than one in 10^5. By these criteria, the evolutionary distance separating *Porphyridium* 16S rRNA and prokaryotic 16S rRNAs is no greater than that which separates the prokaryotic RNA species themselves. Surprisingly, *Porphyridium* shares more 16S oligonucleotides of size 7 through 12 with *A. nidulans* (11 or 12 oligonucleotides) and *B. subtilis* (12 oligonucleotides) than either of these latter shares with *E. coli* (9 and 11 oligonucleotides, respectively).

TABLE 1. *Sequences of oligonucleotides from Porphyridium 16S rRNA*

Porphyridium 16S rRNA oligonucleotide sequence[a]	Presence of identical sequence in: *A. nidulans* 16S	*B. subtilis* 16S	*E. coli* 16S	*Porphyridium* 18S
Pentamers ($N = 5$)				
CC*CCG	+	+	+	−
CCCAG	+	+	+	−
C*AACG	+	+	+	−
CAACG	+	+	+	−
ACACG	+	+	+	−
AACAG	−	+	−	+
AAACG	+	−	+	+
AAAAG	−	−	−	−
UCCCG	+	+	+	−
CUCAG	+	+	+	?[d]
CAUCG	−	−	−	−
(U,C)AAG[b](2)[c]	+	?[d]	+	?[d]
CAAUG	+	+	−	−
AUCAG	+	+	+	+
ACUAG	+	−	−	−
AAUCG	+	+	+	−
AACUG	−	−	+	−
UAAAG (2)[c]	+	+	+	−
AAUAG	−	+	−	−
AAAUG (3)[c]	+	+	+	−
CCUUG	+	+	+	+
CUCUG	−	+	−	+
CUUAG	−	−	−	+
UUCAG	+	−	−	−
UACUG	−	−	−	−
CUAUG	−	−	−	−
UUAAG (2)[c]	+	+	+	−
UAAUG	−	−	+	−
AUUAG (2)[c]	+	+	+	−
AAUUG	+	+	+	+
UUCUG	−	−	−	+
1 unsequenced				
(1) Total observed coincident pentamers:	20	19–20[e]	19	8–10[e]
(2) For 'random' pentamers,[f] *P*:	3×10^{-5}	8×10^{-5}	2×10^{-3}	0.6
mode:	11	11	12	9
mean:	11	11	12	9
Hexamers ($N = 6$)				
CACAAG	+	+	+	−
AAACAG	−	−	−	−
C_{1-2}CUAAG	−	−	−	−
CUAACG	−	+	+	−
UAAACG	+	+	+	−
ACACUG	−	−	−	−
AAUAAG	+	−	−	−
UUCCCG	+	+	+	−
UACCUG	+	−	−	−
UAAUCG	+	+	+	−
CAUUAG	−	+	−	−
AUCCUG	+	+	−	+
UUAAAG	−	−	−	−
AAUUCG	−	−	−	−
AAUCUG	+	−	−	+
AACUUG	−	+	−	−
(C,U)UUCG	−	−	−	−
UCUUAG	−	−	−	−
AUAUUG	−	−	−	−
UUUUAG	−	−	−	−
(1) Total observed coincident hexamers:	8	8	5	2
(2) For 'random' hexamers,[f] *P*:	1×10^{-5}	1×10^{-5}	2×10^{-3}	0.6
mode:	1	1	1	2
mean:	1.5	1.5	1.7	1.9
Heptamers ($N = 7$)				
CCCAAAG	−	−	−	−
AACAAAG	−	+	−	−
C_{0-1}CCUCAG	−	−	−	−
CUA,CA,CG	−	−	−	−
UAACACG	−	+	−	−
U*AACAAG	+	+	+	−
AAACUCG	−	−	−	−
AAUACAG	−	−	−	−
AAACAUG	−	−	−	−
UAUCCCG	−	−	−	−
CCUAUAG	−	−	−	−
UAAUACG	+	+	+	−
AUACUAG	−	−	−	−
CCUUUAG	−	−	−	−
UUAUCCG	+	−	−	−
UCUAAUG[g]	−	−	−	−
A(U,U,C)AUG[g]	−	−	?[d]	−
UUU(C,U)AG	−	−	?[d]	−
(C,U)UUUAG	−	−	−	−
UUUUCUG	−	−	−	−
(1) Total observed coincident heptamers:	3	4	2–4[e]	0
(2) For 'random' heptamers,[f] *P*:	1×10^{-3}	3×10^{-4}	7×10^{-3}	1
mode:	0	0	0	0
mean:	0.3	0.4	0.4	0.3
Octamers ($N = 8$)				
AA(CA,CCA)G[b]	+	+	−	−
AAACACCG	+	−	−	−
CCA,CA,CUG[b]	+	+	+	−
CAAUACCG	−	−	−	−
CCUACAUG	−	−	−	−
CUACAAUG	−	+	+	−
$C_{0-1}AAAC_{2-1}UUG$[g]	−	−	−	−
AAUCACUG	−	−	−	−

TABLE 1. (*Continued*)

Porphyridium 16S rRNA oligonucleotide sequence[a]	Presence of identical sequence in: *A. nidulans* 16S	*B. subtilis* 16S	*E. coli* 16S	*Porphyridium* 18S
ACUAAUCG[g]	–	–	–	–
CUCUACUG	–	–	–	–
UUUAAUUG[h]	–	–	–	–
1 unsequenced[i]				
(1) Total observed coincident octamers:	3	3	2	0
(2) For 'random' octamers,[f]				
P:	1×10^{-5}	1×10^{-5}	1×10^{-3}	1
Mode:	0	0	0	0
Mean:	6×10^{-2}	6×10^{-2}	6×10^{-2}	7×10^{-2}
Nonamers ($N = 9$)				
CUCACCAAG	–	+	–	–
UACACACCG	+	+	+	–
CUAACUCCG	–	–	+	–
ACUCCUACG	+	+	+	–
$C_{0-1}AAUC_{2-1}UCAG$[g]	?[d]	–	–	–
ACUCUAAAG	–	–	–	–
AAAACUUCG	–	–	–	–
AAUUUUCCG	–	–	–	–
3–4 unsequenced[i]				
(1) Total observed coincident nonamers:	2–3[e]	3	3	0
(2) For 'random' nonamers,[f]				
P:	2×10^{-4}	3×10^{-6}	1×10^{-6}	1
Mode	0	0	0	0
Mean:	2×10^{-2}	3×10^{-2}	2×10^{-2}	3×10^{-2}
Decamers ($N = 10$)				
AAACUCAAAG	+	+	–	–
$C_{0-1}CCCCUUACG$[j]	–	–	?[d]	–
CUCAACCCUG	–	–	–	–
AAACUACUAG	–	–	–	–
AU,AC,AUCAUG	–	–	–	–
(1) Total observed coincident decamers,	1	1	0–1[e]	0
(2) For 'random' decamers,[f]				
P:	1×10^{-3}	1×10^{-3}	1	1
Mode:	0	0	0	0
Mean:	1×10^{-3}	1×10^{-3}	5×10^{-4}	2×10^{-3}
Undecamers and larger ($N \geq 11$)				
AACCUUACCAG[g]	+	+	–	–
$C_{0-1}AUAACAACUAG$	–	–	–	–
CUUAA,CA, CAUG[h]	+	–	–	–
CCAUCAUUAAG	–	–	–	–
(U_{1-2},U,U,C) UUCAUG	–	–	–	–
UUACUCUAA-CCG[g]	–	–	–	–
2–3 unsequenced				
(1) Total observed coincident undecamers:	2	1	0	0
(2) For random undecamers,[f]				
P:	3×10^{-8}	3×10^{-4}	1	1
Mode:	0	0	0	0
Mean:	3×10^{-4}	3×10^{-4}	4×10^{-4}	2×10^{-4}

SUMMARY

Sum of observed coincidences,[e]				
$N = 5$–12	39–40	39–40	31–34	10–12
$N = 6$–12	19–20	20	12–15	2
$N = 7$–12	11–12	12	7–10	0
$N = 8$–12	8–9	8	5–6	0
$N = 9$–12	5–6	5	3–4	0
Sum of mode values for 'random' oligonucleotides,				
$N = 5$–12	12	12	13	11
$N = 6$–12	1	1	1	2
$N = 7$–12	0	0	0	0
$N = 8$–12	0	0	0	0
$N = 9$–12	0	0	0	0

[a] For brevity, oligonucleotides X-Y-Zp, etc. are indicated XYZ, etc. Asterisk indicates modified residue. Modified positions in CC*CCG and G*CCG were identified by L. Zablen (unpublished).

[b] Sequence is ambiguous. In all but four cases of ambiguous sequence none of the possible alternative sequences is coincident with known prokaryotic or 18S sequences. In these four cases, coincidence has been assumed and is, at least for AA(CA,CCA)G, CCA,CA,CUG, and CUUAA,CA,CAUG, likely.

[c] Oligonucleotide is present in multiple copies (number of copies in parentheses). Scored here only as single coincidence. Note that Zablen *et al.* score such events as multiple coincidences (15).

[d] Sequence may be present.

[e] Where range is indicated, lower number is certain coincidences; higher includes oligonucleotides designated "?".

[f] Values of P, mode, and mean calculated for lowest k_P, highest n_P, and n_x (least favorable case, see *text*).

[g] Sequence tentative, further confirmation desirable.

[h] The three prokaryotic rRNAs do show UUUAAUUCG.

[i] Poorly characterized, but secondary and/or tertiary digestion indicate no coincidence with known prokaryotic 16S or cytoplasmic 18S oligonucleotides in same position on primary fingerprints.

[j] *E. coli* has CCCCUUACG.

TABLE 2. *Presence of "conserved prokaryotic sequences" in Porphyridium 16S and 18S rRNA*[a]

Conserved sequence	Presence in 16S	Presence in 18S	Conserved sequence	Presence in 16S	Presence in 18S
G*CCG	+	−	CACAAG	+	−
CC*CCG	+	−	UAAACG	+	−
CCCAG	+	−	AAUACG	−	+
C*AACG	+	−	AAACUG	−	+
CAACG	+	−	UUCCCG	+	−
ACACG	+	−	UAAUCG	+	−
UCCCG	+	−	U*AACAAG	+	−
CUCAG	+	?[b]	CAACUCG	−	−
UAACG	−	+	UAAUACG	+	−
AUCAG	+	+	CCA,CA,CUG[c]	+	−
AAUCG	+	−	CUA,CA,CACG[c]	−	−
UAAAG	+	−	UACACACCG	+	−
AAAUG	+	−	ACUCCUACG	+	−
CCUUG	+	+	UUUAAUUCG	−	−
UUAAG	+	−			
AUUAG	+	−	Total present:	25	6–7
AAUUG	+	+			

[a] Conserved sequences defined as present in *A. nidulans*, *B. subtilis*, and *E. coli*. Only pentamers and larger considered, except for the tetramer G*CCG.

[b] Presence uncertain.

[c] See footnote[b], Table 1.

Of all pairwise combinations, that of *Porphyridium* and *E. coli* 16S rRNAs shows least (although still highly significant) sequence homology.

At the same time (Table 1), there is no substantial homology between *Porphyridium* 16S rRNA and the 18S species of this same organism. These two molecules have only 8 to 10 pentamers and 2 hexamers in common, while 9 coincident pentamers and 2 coincident hexamers could reasonably be expected from RNAs of this size and unrelated sequence. There is, in fact, little homology between *Porphyridium* 18S rRNA and any of the prokaryotic 16S molecules discussed here. This is most evident from Table 2, in which we indicate the presence or absence of "conserved prokaryotic sequences" in *Porphyridium* 18S and 16S rRNAs. These 31 sequences ($N \geq 5$) are defined as those found in each of the three prokaryotic (bacterial or blue-green algal) rRNAs. Their retention by three such diverse species should argue their functional importance and, indeed, 25 of these sequences are also present in *Porphyridium* 16S rRNA. Nevertheless, *Porphyridium* 18S rRNA shows at most 7 such oligonucleotides. Thus, either the evolutionary divergence of 16S and 18S rRNA molecules is a very ancient one, or significant selection pressure has been exerted *against* retention of sequence homology between these molecules.

DISCUSSION

These data seem most consistent with the hypothesis that red algal chloroplasts evolved from endosymbiotic prokaryotes (1–4). They cannot, unfortunately, exclude the possibility that nuclear and chloroplast rRNA genes both evolved within a common cytoplasm, from a single, originally "prokaryotic" genome (7, 8). Subsequent evolution of nuclear genes must then have been much more rapid than that of their organellar homologs. It is difficult to visualize experiments with existing organisms that can distinguish between these alternatives.

Neither hypothesis predicts our finding of as extensive sequence homology between red algal chloroplast 16S rRNA and the 16S rRNA of a bacterium (*B. subtilis*) as between the former and a blue-green algal 16S molecule. Morphological and biochemical arguments for specific evolutionary affinity between blue-green algae and red algal chloroplasts are strong (3, 4). We are currently assembling oligonucleotide catalogs for several additional blue-green algae in a search for 16S rRNAs showing specific sequence homology with the *Porphyridium* chloroplast species.

Elsewhere in this issue, Zablen *et al.* (15) present results of a comparable analysis of *Euglena* chloroplast 16S rRNA. This molecule is also clearly "prokaryotic," and similarly shows homology with 16S rRNAs of the Bacillaceae. It shares with *Porphyridium* 16S rRNA some large oligonucleotides not found in the three prokaryotes for which data are presented here (C-C-U-A-A-G, U-U-A-A-A-G, A-A-A-C-U-C-G, U-A-U-C-C-C-G, C-A-A-U-A-C-C-G, C-C-U-A-C-A-U-G, and A-A-U-U-U-U-C-C-G). Nevertheless, the total number of coincident pentamer and higher sequences (37 to 39, comprising 18 or 19 pentamers, 5 or 6 hexamers, 5 or 6 heptamers, 4 octamers, 2 nonamers, 1 decamer, and 2 undecamers) is not substantially greater than that observed between *Porphyridium* chloroplast 16S rRNA and *B. subtilis* or *A. nidulans* rRNAs. We find this easiest to reconcile with hypotheses that contend that chloroplasts of the several major eukaryotic algal groups derive from two (or more) independent lines of prokaryotic photosynthetic endosymbionts (2–4), although Zablen *et al.* (15) interpret these data somewhat differently.

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