Isolation of *Cyanidium caldarium* and *Porphyridium purpureum* DNA Fragments Capable of Autonomous Replication in Yeast

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DNA fragments from the unicellular red algae *Cyanidium caldarium* and *Porphyridium purpureum* were inserted at various sites of plasmid pLG4 (pBR325 + a HindIII fragment bearing the yeast arg4 gene) with the purpose of isolating sequences supporting autonomous replication of plasmids in *Saccharomyces cerevisiae*. Plasmid pools were prepared in *Escherichia coli* then used to transform the arg4 yeast strain X3656-7 D to prototrophy. The presence of free plasmids in the yeast transformants was demonstrated by Southern blotting hybridization between yeast DNA and 32P-labelled pBR325 and by the transformation of *E. coli* argH with DNA from yeast transformants. Hybrid plasmids recovered from Arg+ bacterial transformants transformed yeast at high frequency. They contained AvaI fragments of *C. caldarium* total DNA, EcoRI fragments of *P. purpureum* satellite DNA and one BamHI fragment of *P. purpureum* main DNA. These new plasmids have unique restriction sites which make them convenient vectors for cloning in yeast and possibly in algae and other plants.

**INTRODUCTION**

Red algae are a distinctive algal group. They share the accessory photosynthetic pigment phycocyanin with cyanobacteria but have typical eukaryotic cell structure. The systematic position of *Porphyridium purpureum* among the Rhodophyceae is clear but the position of *Cyanidium caldarium* has been much debated. Chapman (1974) considered this thermophilic and acidophilic alga as the most primitive Rhodophyte; Seckback & Fredrick (1981) proposed that it represents the most probable link between cyanobacteria and typical Rhodophytes.

Algae of the genus *Porphyridium* produce considerable amounts of intracellular carrageenan-type polysaccharides (Pircival & Foyle, 1979) which can be used for their gelling properties in various industrial products. Several laboratories are working on the production of these compounds by algae immobilized in polyurethane matrices (Gudin & Thomas, 1981). Release of considerable amounts of organic compounds has also been observed for *C. caldarium* (Belly et al., 1973). Owing to their possible economic interest there is a need to develop methods such as genetic transformation to improve different properties of these algal species.

Very little work has been devoted previously to the DNA of these algae. As pointed out by Barnes *et al.* (1982), 'it would be of considerable evolutionary interest to learn more about their nuclear organization'. The DNA of *P. cruentum* (= *purpureum*) was purified and analysed by Charles (1977), who found two DNA species with buoyant densities of 1.716 g cm⁻³ (main DNA) and 1.694 g cm⁻³ (satellite DNA, representing about 25% of the total DNA). Barnes *et al.* (1982) investigated the structure of *P. purpureum* chromatin and demonstrated that it is not fundamentally different from that of higher eukaryotes.

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**Abbreviations**: ARS, autonomous replication sequence; CTAB, cetyltrimethylammonium bromide.
The techniques of gene cloning now make it possible to investigate the structure and function of the genetic material in these organisms. In addition, the small size of their genomes makes them favourable for the construction of complete gene banks. Results obtained with yeast show that transformation is efficient only when plasmids contain fragments of the yeast 2 \( \mu \)m plasmid or autonomous replication sequences (ARSs). ARSs functioning in yeast have been found in nuclear and mitochondrial DNA of various eukaryotic organisms and in chloroplast DNA of the green alga *Chlamydomonas reinhardtii* (Loppes & Denis, 1983; Vallet et al., 1984) and, among higher plants, in tobacco (Uchimiya et al., 1983) and petunia (Overbeeke et al., 1984).

In the experiments reported here, we purified the DNA of *P. purpureum* and *C. caldarium*. Plasmid pools containing DNA fragments from these algae were prepared in *Escherichia coli* and used to select sequences allowing autonomous plasmid replication in yeast. The results demonstrate the occurrence of such sequences in the DNA of *P. purpureum* and *C. caldarium*. These DNA fragments may be used in the future for the construction of vectors able to transform these algae.

**METHODS**

*Strains and plasmids.* *Escherichia coli* C600 \( \Delta argBH \) (non-lac) \( argT \) \( thi-1 \) \( m^{r}c \) (from M. Crabeel, Laboratorium voor Mikrobiologie, Vrije Universiteit, Brussels) and *Saccharomyces cerevisiae* X3656-7 D a leu1 ade6 ura1 arg4-1 thr1 (from L. Hsiao, University of California, Santa Barbara, USA) were used. Plasmid pLG4 (Loppes & Denis, 1983) is a derivative of pBR325 (Bolivar, 1978) containing a 3 kb *HindIII* fragment bearing the yeast *arg4* gene (Clarke & Carbon, 1978; M. Crabeel, personal communication) and cannot replicate autonomously in yeast. *Porphyridium purpureum* (Bory) Drew and Ross (= *P. cruentum* Nägeli) and *Cyanidium caldarium* (Tilden) Geitler were obtained from the Culture Centre of Algae and Protozoa, Cambridge, UK (strains 1380/1a and 1355/1, respectively).

*Media and growth conditions.* Media used for *E. coli* and yeast were as described by Loppes & Denis (1983). *P. purpureum* was grown at 25°C in the artificial sea water medium of Jones et al. (1963) under continuous light (8000 lx) and aeration. *C. caldarium* was grown at 37°C in the medium of Allen (1959) as modified by Ascione et al. (1966), except that the trace element solution was replaced by that prepared according to Hutner et al. (1950). Media for *C. caldarium* were adjusted to pH 2.0 by the addition of 10 \( \mu \)l-H\(_2\)SO\(_4\) after autoclaving. The cultures were grown in continuous light (8000 lx) with agitation and bubbling with sterile air.

*DNA preparations.* *P. purpureum* was grown to late exponential phase (about 5 \( \times \) 10\(^6\) cells ml\(^{-1}\)) which was harvested by centrifugation (5000 g, 15 min) and washed twice with fresh culture medium to eliminate excreted polysaccharides. The cells were ground in a mortar with quartz sand in the presence of liquid nitrogen. The cell powder was thawed in buffer A (0.1 M NaCl, 5 mM-EDTA, 20 mM-Tris/HCl, pH 8.0) containing 2% (w/v) polysaccharides. The cell suspensions were treated with 2% (w/v) CTAB at low salt concentration (0.2 M-NaCl) at 25°C, conditions in which DNA is precipitated. The presence of polysaccharides in the supernatant was ascertained by their precipitation with ethanol and by their positive response in the test of Dubois et al. (1956). After CTAB treatment, the pellet was rinsed several times with 0.1 M-sodium acetate in 70% (v/v) ethanol, dried, dissolved in TE then purified by CsCl density gradient centrifugation.

*DNA manipulations and transformation procedures.* Restriction and ligation of DNA, electrophoresis, blotting and DNA-DNA hybridizations, and transformation of *E. coli* and yeast were all done as detailed by Loppes & Denis (1983).

**RESULTS**

*DNA isolation and characterization.*

The analysis of DNA by CsCl density gradient centrifugation revealed the presence of two DNA species in both *P. purpureum* and *C. caldarium*. In *P. purpureum*, satellite DNA
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Fig. 1. Electrophoresis of C. caldarium total DNA (1) and P. purpureum satellite DNA (2) restricted with EcoRI.

(representing about 23% of the total DNA, density 1.692 g cm⁻³) could be separated from main DNA (density 1.716 g cm⁻³) by two successive centrifugations, in agreement with the results of Charles (1977).

The presence of discrete bands in P. purpureum satellite DNA restricted by endonucleases indicated that this DNA is quite repetitive: it probably corresponds to chloroplast DNA. Its size was about 100 kb as calculated by summing the lengths of fragments obtained with BamHI (9 fragments: 101-4 kb) or EcoRI (19 fragments: 101-4 kb) (Fig. 1).

In C. caldarium, the separation of the DNA species was never complete because they have very similar densities (1.700 and 1.692 g cm⁻³). Discrete bands superimposed on a smear were found in all preparations after restriction by endonucleases (Fig. 1). The cloning experiments with C. caldarium DNA were done with a mixture of both DNA species (total DNA). In both organisms, main DNA is most likely of nuclear origin.

Insertion of DNA fragments in pLG4

Plasmid pLG4 does not replicate autonomously in yeast and accordingly transforms it at very low frequency. Insertion in pLG4 of DNA fragments bearing sequences promoting autonomous plasmid replication in yeast will result in the ability of this plasmid to transform yeast at high frequency. Preliminary experiments showed that AvaI, BamHI and EcoRI were suitable for inserting DNA fragments into pLG4.

Main and satellite DNA from P. purpureum and total DNA from C. caldarium were separately restricted by the endonucleases listed in Table 1 and ligated to pLG4 restricted by the corresponding enzymes. Restriction and ligation were assayed by electrophoresis. The mixtures were used to transform E. coli C600 to ampicillin resistance. The percentage of hybrid plasmids was unknown in AvaI samples, because insertions at that site do not inactivate any known gene in the pLG4 plasmid. In each series, Amp⁸ clones were pooled and used to prepare plasmids for yeast transformation.
Table 1. Summary of experiments designed for isolating \textit{C. caldarium} and \textit{P. purpureum} DNA fragments promoting high frequency of transformation in yeast (strain X3656-7 D)

<table>
<thead>
<tr>
<th>Source and type of DNA</th>
<th>Enzyme used for cloning</th>
<th>Gene inactivated by cloning</th>
<th>Size of restriction fragments (kb)</th>
<th>No. of Amp(^R) E. coli clones in the pool</th>
<th>Percentage of hybrid plasmids in the pool</th>
<th>No. of Arg(^+) yeast clones isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{C. caldarium}</td>
<td>Total AveI</td>
<td>—</td>
<td>0-5-15</td>
<td>29000</td>
<td>Unknown</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Total BamHI</td>
<td>Tet(^R)</td>
<td>0-5-20</td>
<td>16000</td>
<td>19.6</td>
<td>3</td>
</tr>
<tr>
<td>\textit{P. purpureum}</td>
<td>Main AveI</td>
<td>—</td>
<td>0-5-9</td>
<td>9000</td>
<td>Unknown</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Main BamHI</td>
<td>Tet(^R)</td>
<td>0-5-30</td>
<td>23000</td>
<td>9.2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Satellite EcoRI</td>
<td>Cam(^R)</td>
<td>0-5-20</td>
<td>10500</td>
<td>24</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 2. Derivatives of \textit{pLG4} carrying ARSs from \textit{C. caldarium} and \textit{P. purpureum} DNA, the transformation frequency of X3656-7 D yeast with these plasmids and mitotic stability of yeast transformants

<table>
<thead>
<tr>
<th>Origin of DNA</th>
<th>Enzyme used for cloning</th>
<th>Plasmid</th>
<th>Insert(^*) (kb)</th>
<th>No. of yeast transformants per (\mu)g of DNA</th>
<th>Percentage of yeast Arg(^+) cells after 10 generations</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{C. caldarium} (total)</td>
<td>AveI</td>
<td>pCc4-1</td>
<td>1.6</td>
<td>3900</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pCc4-2</td>
<td>1.6 + 0.8</td>
<td>5700</td>
<td>0.1</td>
</tr>
<tr>
<td>\textit{P. purpureum} (main)</td>
<td>BamHI</td>
<td>pPcn-1</td>
<td>4.2</td>
<td>7400</td>
<td>0.5</td>
</tr>
<tr>
<td>\textit{P. purpureum} (satellite)</td>
<td>EcoRI</td>
<td>pPcs-3</td>
<td>1.65</td>
<td>12900</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pPcs-7</td>
<td>2.5 + 1.05</td>
<td>7200</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pPcs-9</td>
<td>4.8</td>
<td>6900</td>
<td>0.5</td>
</tr>
</tbody>
</table>

\(^*\) Only the fragments shown in \textbf{bold} type bear ARSs.

Selection and study of ARSs of algal origin

Arg\(^+\) yeast transformants were obtained with plasmids extracted from the \textit{C. caldarium} AveI and BamHI pools and from \textit{P. purpureum} BamHI and EcoRI pools. No transformants were obtained with \textit{pLG4} alone or with plasmids from the \textit{P. purpureum} AveI pool (Table 1). Yeast clones were purified and propagated on selective medium and then assayed for their stability under non-selective conditions. They were grown for about 10 generations in YEPD medium before plating onto YEPD and minimal medium; comparison of these plates allowed the determination of the percentage of Arg\(^+\) cells present in the cultures (Table 2). In all transformant cell lines 0-5\% or less of the cells were still Arg\(^+\), which means that the plasmids were lost quite rapidly during growth in complete medium.

The DNA of yeast transformants was isolated by small-scale extraction (Crabeel \textit{et al.}, 1981) and used for hybridization with \(^{32}\)P-labelled \textit{pBR325} and for \textit{E. coli} transformation. Free plasmids hybridizing with the probe were detected in yeast transformants (Fig. 2), indicating that they are able to replicate autonomously in yeast. Minor bands hybridizing weakly with the probe were present in the control: their origin is unknown.

Amp\(^R\) Arg\(^+\) \textit{E. coli} transformants were recovered after transformation with a number of yeast DNA preparations. Plasmids extracted from bacterial clones (Table 2) transformed yeast at a high frequency (about 10000 transformants of strain X3656-7 D were obtained per \(\mu\)g of control plasmid \textit{pGT30} bearing the yeast ARS2 sequence).

Plasmids were restricted with the endonuclease used for cloning and the sizes of the inserts were estimated after electrophoresis (Fig. 3, Table 2). The ARS sequence isolated from \textit{C. caldarium} was located in the 1-6 kb fragment common to both pCc4-1 and pCc4-2. Cutting the pPcs-7 plasmid with \textit{EcoRI} and then ligating the mixture allowed us to isolate plasmids bearing either insert. Only the plasmid (called pPcs-7-1) having the 2-5 kb insert was able to transform yeast efficiently.
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Fig. 2. Hybridization of 32P-labelled pBR325 with DNA extracted from yeast transformed with pPcs-3 (1), pPcs-7 (2), pPcs-9 (3), pPcn-1 (4), pCc4-1 (5), and pCc4-2 (6). 7, Non-transformed host X3656-7 D. a and b show the positions of nuclear and 2 µm DNA respectively.

Fig. 3. Electrophoresis of plasmids bearing ARSs of algal origin: pPcs-3 (1), pPcs-7 (2) and pPcs-9 (3) restricted by EcoRI; pCc4-1 (6) and pCc4-2 (7) restricted by Arai. Plasmid pLG4 restricted by Arai (5) and DNA molecular weight markers (4) are also shown.

DISCUSSION

The present work describes the isolation from the DNA of two red algae (Porphyridium purpureum and Cyanidium caldarium) of fragments promoting high frequency transformation of yeast. This property is dependent on the ability of the selected plasmids to replicate autonomously in yeast. Free plasmids hybridizing to pBR325 and transforming E. coli to arginine prototrophy were indeed found in DNA preparations from yeast transformants. In addition, yeast transformants were mitotically unstable as is the rule for plasmids bearing ARSs from various sources (Stinchcomb et al., 1980).

The origin (nuclear, chloroplastic, mitochondrial) of the sequence found in C. caldarium DNA is unknown because the cloning experiments were done with a mixture of the different classes of DNA. On the other hand, with P. purpureum, we have been able to isolate one ARS from main DNA and three ARSs from satellite DNA. By virtue of its properties (low density, 1.692 g cm⁻³;
size, about 100 kb) this satellite DNA can reasonably be assigned to chloroplast DNA, although we have no direct evidence supporting this interpretation. Accordingly, P. purpureum chloroplast DNA should contain at least three ARSs. These results are reminiscent of those obtained with Chlamydomonas chloroplast DNA (190 kb) in which six ARSs have been detected (Loppes & Denis, 1983; Vallet et al., 1984). It would be of interest to check for structural homology among ARSs isolated from these two organisms and from Chlamydomonas. It is noteworthy that only one ARS was isolated from the plasmid pool prepared with main (most probably nuclear) P. purpureum DNA. This suggests that the frequency of ARSs in P. purpureum nuclear DNA is low as in Chlamydomonas nuclear DNA (Loppes & Denis, 1983).

ARSs functioning in yeast have been found so far in the DNA of many eukaryotes including man (Glineur & Burny, 1984). The present work shows that ARSs are present in the DNA of C. caldarium and P. purpureum, which are considered as very primitive photosynthetic eukaryotic cells. Accordingly, from this point of view, red algae are not fundamentally more primitive than other eukaryotes.

The question of whether the ARSs found during this study actually initiate replication of algal DNA in vivo will remain unsolved until it is possible to re-introduce the DNA sequences into the algae. In this respect, our work represents a first step in the construction of vectors to be used for the transformation of C. caldarium and P. purpureum themselves. Insertion of selectable genes (resistance to certain antibiotics, for example) in these plasmids will provide possible tools for introducing DNA into those algae which could become for the first time open to genetic investigations.

Finally, as Chlamydomonas is now transformable (Rochaix & van Dillewijn, 1982; Rochaix et al., 1984), experiments are in progress to determine whether the ARSs selected in yeast from C. caldarium and P. purpureum DNA are functional in Chlamydomonas, i.e. whether they support autonomous replication of plasmids in the transformed alga.

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