Interphylum Protoplast Fusion and Genetic Recombination of the Algae *Porphyridium cruentum* and *Dunaliella* spp.

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True protoplasts of the red alga *Porphyridium cruentum* and the halophilic green algae *Dunaliella bardawil* and *Dunaliella salina* were induced by treatment with pectinase and cellulysin. Protoplasts of *P. cruentum* and the *Dunaliella* spp. were fused by polyethylene glycol treatment and then allowed to regenerate on selection media. Fusants that were essentially *Porphyridium* in nature were generated between *P. cruentum* and *D. bardawil* and *D. salina*; these tolerated a salinity of up to 3.5 M for the former and 2.5 M for the latter, indicating that they were hybrid strains, acquiring the osmotolerance from the *Dunaliella* spp. A *Dunaliella*-like clone was isolated from protoplast fusion between *P. cruentum* and *D. salina*, with altered antibiotic sensitivity. The acquired resistance to penicillin and erythromycin appears to be the result of a genetic transfer from *P. cruentum*.

INTRODUCTION

Using somatic hybridization by protoplast fusion, hybrids have been isolated from auxotrophic mutants of bacteria (Fodor & Alföldi, 1976; Schaeffer *et al.*, 1976), fungi (Ferenczy *et al.*, 1974; Binding & Weber, 1974), yeast (Fournier *et al.*, 1977; Sipiczki & Ferenczy, 1977; Ferenczy & Maraz, 1977; Yamamoto & Fukui, 1977; van Solingen & Plaats, 1977) and plants (Carlson *et al.*, 1972). Somatic fusion between cell-wall mutants of the green alga *Chlamydomonas reinhardtii* has also been achieved (Matagne *et al.*, 1979). There has been no reported success of fusing algal protoplasts either intra- or interspecifically. In theory, fusion of isolated protoplasts allows for hybridization between sexually incompatible microbial species. It was thus of interest to determine whether protoplasts of economically important algae can be induced to fuse to produce a genetically improved strain.

Algae of the genus *Dunaliella* tolerate high salinity by accumulating glycerol intracellularly (Ben-Amotz & Avron, 1978, 1982; Aaronson *et al.*, 1980; Williams *et al.*, 1978). On the other hand, the red alga *Porphyridium cruentum* might prove useful for producing the medically important arachidonic acid (Ahern *et al.*, 1983). Protoplasts of the two algae were induced to fuse by polyethylene glycol. Genetic recombinants of the resulting fusants were analysed.

METHODS

Organisms and growth. *Dunaliella salina* (LB200) and *Porphyridium cruentum* (161) were obtained from the Culture Collection of Algae at the University of Texas at Austin, USA, and *Dunaliella bardawil* (30861) was obtained from the American Type Culture Collection. The algal cultures were maintained photosynthetically by successive aseptic transfer into their respective liquid media, and agar slopes were prepared by adding 1.5% (w/v) agar into the media. *P. cruentum* was grown in chemically defined medium A1 (Jones *et al.*, 1963). The *Dunaliella* spp. were cultured in the chemically defined medium of Ben-Amotz & Avron (1982), designated A2.

Induction of protoplasts. Protoplasts of *P. cruentum* were prepared by a modification of the method of Clement-Métal (1976). Cells in the late exponential phase (5×10^6 cells ml⁻¹) were harvested and washed once with 0.6 M-NaCl. They were resuspended in 0.6 M-NaCl (1 g wet weight per 20 ml) containing 0.4% pectinase (*Aspergillus niger*, Sigma) and 4% (w/v) cellulysin (Calbiochem) and incubated overnight at 37 °C with gentle shaking. The treatment with the digestive enzymes were terminated by centrifugation at 5000 g for 5 min. The protoplasts were

resuspended in A1 medium supplemented with 0.6 M-NaCl. Regeneration of the protoplasts was observed upon plating on normal A1 agar plates (0.46 M-NaCl).

Dunaliella cells in the late exponential phase (2×10^8 cells ml⁻¹) were harvested and washed once with 25 mM-potassium phosphate buffer (pH 6.0). They were resuspended in 0.6 M-sorbitol/0.6 M-mannitol containing 2% (w/v) cellulysin (Calbiochem), and incubated for 1 h at 25 °C with gentle shaking. The cells were then centrifuged for 5 min at 5000 *g* and resuspended in A2 medium containing 0.6 M-sorbitol/0.6 M-mannitol. The cells after incubation were non-motile and round. The protoplasts could regenerate into whole *Dunaliella* cells when plated on A2 agar plates (1.53 M-NaCl).

Polyethylene glycol (PEG) treatment. After 1 h in their respective media, equal volumes of the protoplasts were mixed and 3 ml dispersed and allowed to settle at the bottom of a Petri dish (5 min). PEG solution (3 ml) containing 0.2 M-glucose, 10 mM-CaCl₂, 0.7 mM-KH₂PO₄ and 0.125 mM-PEG (*M_r* 4000), pH 5.8, was added for 30 min, and the protoplasts formed adhesion bodies. Dilution of PEG was initiated by adding, in droplets, 5 ml of the eluting medium, containing (a) 100 mM-glycine, 0.3 M-glucose, pH 10.5; (b) 100 mM-CaCl₂, 0.3 M-glucose. Solutions (a) and (b) were mixed in equal proportions just before use. A further 10 ml eluting medium was added after 10 min. Fusion took place during elution. The fused protoplasts were washed five times with A1 medium containing 0.6 M-NaCl, remaining for 5 min between washings, resuspended in the same medium and shaken overnight at 100 r.p.m. at 25 °C. All solutions used in this fusion procedure were filter-sterilized.

DNAase treatment. DNAase I (Sigma) at a concentration of 3 µg ml⁻¹ was added before the enzyme treatment and was present in all media used subsequently, including the selection media.

Fluorescence microscopy. The autofluorescence of the algae was used to distinguish hybrid protoplasts. *Dunaliella* cells visualized using fluorescence microscopy (Polyvar, Austria) appeared orange, while *P. cruentum* cells were yellow. Hybrid protoplasts could be detected by the presence, within a single cytoplasm, of both types of fluorescence.

Selection of fusants. We determined that *P. cruentum* tolerated salinities up to 1.53 M (Sommerfeld & Nichols, 1970, reported 1.50 M). Cultures with 2 M-NaCl did not grow. On the other hand, *Dunaliella* spp. have been reported to grow at salinities up to 5 M (Ben-Amotz & Avron, 1981; Brown & Borowitzka, 1979). Thus red cells that could tolerate salinities above 1.53 M were selected. Cells after fusion were plated on A1 agar plates containing 2 M-NaCl, as were the cells of the parental *P. cruentum*, the former giving the rate of transfer of the halotolerant character from *Dunaliella* to *P. cruentum*, and the latter the spontaneous mutation rate. Fusants were subsequently grown in A1 medium containing 2 M-NaCl. In only one case were green (*Dunaliella*-like) cells obtained. These were subjected to PAGE analysis and antibiotic sensitivity tests and compared with the parental *Dunaliella* spp.

Antibiotic sensitivity tests. Algal cultures in the exponential phase were spread on their respective agar plates using a cotton wool swab. Antibiotic discs (BBL) were then placed on the agar plate. Clear zones around any disc after incubation for 1 week at 25 °C indicated that the algae were sensitive to that antibiotic. Five antibiotics were tested: chloramphenicol, 30 µg; penicillin, 10 units; tetracycline, 30 µg; streptomycin, 10 µg; erythromycin, 15 µg.

Staining procedures. Protoplasts and whole cells of *P. cruentum* and *Dunaliella* spp. as well as the fusants were suspended for 10 min in a freshly prepared solution of 0.01% crystal violet (Merck) in 0.6 M-NaCl, washed once in 0.05% CuSO₄ prepared in 0.6 M-NaCl, resuspended in 0.6 M-NaCl, and examined under a light microscope.

Transmission electron microscopy. The cell sample (2–4 ml) was fixed for 1 h at room temperature in 3.5% (w/v) glutaraldehyde in buffer (0.4 M-NaCl in 0.1 M-cacodylate buffer at pH 7.4 for *P. cruentum* and the two fusants, and 0.6 M-sorbitol/0.6 M-mannitol in 250 mM-Na₂PO₄/KH₂PO₄ buffer at pH 6.0 for *Dunaliella* spp.), and then post-fixed in 1% (v/v) OsO₄ in the respective buffers for 1 h at room temperature. The fixed material was dehydrated in a graded ethanol series (50, 70 and 90%, v/v, ethanol for 10 min each; 100% ethanol three times for 15 min), infiltrated in 1:1 (v/v) ethanol/low-viscosity epoxy resin (LVER) overnight and finally embedded in a polythene capsule. The embedded material was sectioned, stained with saturated uranyl acetate and lead citrate and observed with the electron microscope (Philips-4007).

PAGE. The protein profiles of the algae were studied using the electrophoresis technique for protein separation (Studier, 1973).

Estimations. DNA was isolated and measured as described by Marmur (1961). Total chlorophyll was determined as described by Jensen (1978). Phycoerythrin was extracted as follows. Algal cells were rinsed and resuspended in 0.06 M-phosphate (Na₂HPO₄ and KH₂PO₄) buffer, pH 6.8. They were then broken by sonication (Branson Sonifier cell disrupter B-30) and deoxycholate was added to a final concentration of 0.5%. Chloroplast membranes and other cell debris were removed by centrifugation at 30000 *g* for 20 min and phycoerythrin was measured in the supernatant at 545 nm, using the absorption coefficient $\epsilon_{\text{cm}}^{1\%} = 87.8$ (Gantt & Lipschultz, 1972; Clement-Métal, 1976). The total carbohydrate content of the algal cells was determined using the phenol method (Dubois *et al.*, 1956; Herbert *et al.*, 1971). Glycerol was determined by treatment with acetylacetone (Ben-Amotz & Avron, 1978). Biomass protein was first solubilized in 1 M-NaOH, and then measured by the biuret method (Lee *et al.*, 1985).

Growth rates in batch culture were determined by measuring the increase in OD₆₄₅ or OD₅₄₅, at regular short time intervals in the exponential growth phase, using the relationship $\mu = \ln 2/t_d$, where t_d is the doubling time (Pirt, 1975).

Fig. 1. Hybrid protoplasts of *P. cruentum* and *D. salina* exhibiting both orange (R) and yellow (Y) autofluorescence within a single cytoplasm. Bar, 10 μ m.

RESULTS

Protoplast fusion

Under light microscopy, cells of the red alga *P. cruentum* were typically round while those of the green algae *Dunaliella* spp. were spindle-shaped with two flagella. Electron microscopy revealed that both algae lacked a visible cell wall. Cells of *P. cruentum* were surrounded by a thick layer of polysaccharide sheath with a fine fibrillar texture.

After enzyme treatment, about 67% of the *Dunaliella* cells became rounded and burst in water, therefore being true protoplasts. Since the *P. cruentum* cells were already round, and enzyme-treated cells did not burst readily but released phycoerythrin in water, a differential staining method was developed to show that the cells had been converted to protoplasts. Normal cells retained their red colour when stained with crystal violet and CuSO_4 while protoplasts from which the polysaccharide capsule had been removed appeared green: the frequency of protoplast formation was about 72%.

When protoplasts of *Dunaliella* and *P. cruentum* were mixed in PEG and CaCl_2 solutions, fusion occurred separately among the *Dunaliella* cells and the *P. cruentum* cells, and also between *Dunaliella* and *P. cruentum* cells. Hybrid protoplasts, which exhibited both yellow and orange fluorescence (see Methods) (Fig. 1), were observed at a frequency of 10^{-5} of the total protoplasts present. The frequency of *Porphyridium*-like cells tolerating 2 M-NaCl estimated by plating was lower (10^{-6}).

Genetic recombination

Fusants were designated Pb and Ps, indicating fusions between *P. cruentum* and *D. bardawil* or *D. salina*, respectively. Altogether, 54 isolates of halotolerant Pb and 32 isolates of halotolerant Ps were obtained. The fastest-growing halotolerant Pb and Ps isolates were selected for further study. The growth rates of the fusants were slower than that of the parental *P. cruentum* at a salinity of 0.5–1.0 M but they were more osmotolerant, withstanding up to 3.5 M-NaCl (Pb) and 2.5 M-NaCl (Ps) (Fig. 2). At the extreme salinities, Pb and Ps grew very slowly or not at all, but the cells remained intact for at least one month. This indicated osmotolerance of the fusants to these high salinities although cell growth was inhibited. In some cases, lysis of cells was observed at the extreme salinities, resulting in negative 'growth' rates (Fig. 2). The osmotolerance of the fusants was probably dependent on the physiological state of the culture inocula.

Morphologically, the cells of Pb and Ps resembled those of *P. cruentum* under the electron microscope and were devoid of any visible cell wall. However, the sheath of *P. cruentum* appeared to be a relatively thick layer with a fine fibrillar texture (Fig. 3a), while the capsule of Ps appeared to consist of concentric rings of such sheath material (Fig. 3b). To our knowledge,

Fig. 2. Specific growth rates of *P. cruentum* (□), *Porphyridium*-like hybrid of *P. cruentum* and *D. bardawil* (Pb) (●) and *Porphyridium*-like hybrid of *P. cruentum* and *D. salina* (Ps) (△) measured at various NaCl concentrations. Negative 'growth' rates are equivalent to specific death rates due to lysis of cells in media of high salinity.

Table 1. A comparison of the macromolecular contents of exponential phase cultures of parental and fusant cells in their respective media containing 0.46 M-NaCl

The values represent the mean of three experiments.

	<i>P. cruentum</i>	Pb	Ps	<i>D. bardawil</i>	<i>D. salina</i>
DNA ($10^{13} \times$ g per cell)	4.9	7.0	4.3	11.1	12.8
Glycerol [mg (g dry wt) $^{-1}$]	50.5	35.8	67.6	65.3	74.8
Protein [mg (g dry wt) $^{-1}$]	91.7	64.2	121.4	138.2	115.8
Carbohydrate [mg (g dry wt) $^{-1}$]	45.9	15.1	40.4	29.7	16.0
Phycoerythrin/chlorophyll ratio	9.16	8.90	10.69	—	—

such a concentric nature of the polysaccharide cell covering has never been documented for *P. cruentum* (Brody & Vatter, 1959; Gantt & Conti, 1965, 1966), and it appears to be a unique feature of the fusant Ps. *Dunaliella* cells were never found to possess a polysaccharide capsule.

A comparison of the different macromolecular contents of parental and fusant cells is shown in Table 1. While Ps resembled *P. cruentum* in DNA and carbohydrate contents, its glycerol and protein contents were closer to those of *D. salina*. The DNA content of Pb was intermediate between that of the parental strains while the contents of glycerol, protein and carbohydrate were decreased. The phycoerythrin to chlorophyll *a* ratios of Pb and Ps were, however, similar to that of *P. cruentum* (Table 1).

Of the two crosses described above, only that between *P. cruentum* and *D. salina* yielded green colonies on the 2 M-NaCl agar plates (with a frequency of 10^{-5} – 10^{-6}) and with altered antibiotic sensitivity. These cells were pooled together, cultured in A2 medium plus 1.5 M-NaCl, and their antibiotic sensitivity profile determined. *P. cruentum* was resistant to all five antibiotics tested, while *D. salina* was resistant only to streptomycin; the green cells that arose from the cross, designated DSC, had acquired additional resistance to erythromycin and penicillin. The protein profiles of *D. salina* and DSC were compared by PAGE. The two profiles were generally similar except for a band of about 30 kDa, which was consistently absent in DSC (not shown), indicating that there was indeed a difference in the cell components of the two algal strains. This protein band was also absent in *P. cruentum*.

When cells of *P. cruentum* were fused together in a separate experiment, no clones resistant to 2 M-NaCl could be generated, from the 1×10^8 cells plated.

Fig. 3. (a) Electron micrograph of *P. cruentum*. The cell is devoid of cell wall. (b) Electron micrograph of Ps (a hybrid between *P. cruentum* and *D. salina*). Note the concentric nature of the polysaccharide sheath and the absence of cell wall. Bars, 500 nm.

DISCUSSION

Protoplasts of bacteria and fungi are relatively easy to generate, and fusions, both intra- and interspecific, have often been described. On the other hand, generation of algal protoplasts has been less well studied, and fusion of algal protoplasts has not previously been reported. Although Matagne *et al.* (1979) reported somatic fusion between cell-wall mutants of *Chlamydomonas reinhardtii*, these differ from true protoplasts in that they do not burst in hypotonic medium or even in distilled water. The protoplasts we prepared from algae of the genera *Dunaliella* and *Porphyridium*, however, can be considered as true protoplasts since in addition to lacking a cell wall they either burst or release phycobiliproteins in water. Staining with crystal violet and CuSO_4 confirmed the protoplast nature of the *P. cruentum* preparation and is more conclusive than Clement-Métal's (1976) method of detecting phycoerythrin release in acetate buffer. In addition, the use of autofluorescence provides an easy means of detecting hybrid protoplasts. The isolation of stable hybrids possessing combined characteristics of the parents following

PEG-induced hybridization of *P. cruentum* with *D. bardawil* or *D. salina* is the first report of a fusion between algae belonging to two different phyla.

Although the hybrid DSC resembled *D. salina* in morphology, being spindle-shaped and flagellated, its acquired resistance to penicillin and erythromycin appeared to be the result of a genetic transfer from *P. cruentum* since the latter was resistant to all the antibiotics tested. The difference in the protein profiles between DSC and *D. salina* is further evidence that there is indeed a change at the genomic level.

Similarly, the increased osmotolerance of Pb and Ps indicates that these are hybrid strains, acquiring the osmotolerance from the *Dunaliella* spp. When cells of *P. cruentum* were fused together in a separate experiment, no clones resistant to 2 M-NaCl could be generated, thus eliminating the possibility of mutation. Also the presence of DNAase in the medium and the observation of both types of fluorescence within a single cytoplasm rule out transformation. In addition, the concentric nature of the polysaccharide capsule of Ps could be interpreted as an indication of an altered genotype. All of these observations point to the occurrence of protoplast fusion and genetic recombination.

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