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Overproduction of γ-Linolenic and Eicosapentaenoic Acids by Algae[1]

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ABSTRACT

The pharmaceutical interest and limited availability of γ-linolenic acid (GLA) and eicosapentaenoic acid (EPA) prompted the search for genetic means for increasing the production of these fatty acids from algal sources. Cell lines of *Spirulina platensis* and *Porphyridium cruentum* resistant to the growth inhibition of the herbicide Sandoz 9785 were selected by serial transfers of the culture in the presence of increasing concentrations of the herbicide. The resistant cell lines of *S. platensis* overproduced GLA and those of *P. cruentum* overproduced EPA and were stable for at least 50 generations in the absence of the inhibitor.

The recent pharmaceutical interest in the polyunsaturated fatty acids, EPA[2] and GLA (7, 17) and the limitations of their current availability (2, 5) triggered the search for potential new sources for these fatty acids. We (1, 5) have previously demonstrated that the marine red microalga *Porphyridium cruentum* and the cyanobacterium *Spirulina platensis* are among the best producers of EPA and GLA, respectively. Strain selection and manipulation of physiological and environmental conditions brought about an increased content of these polyunsaturated fatty acids (1, 2, 4–6). Yet, the inherent limitation of these approaches prompted us to look for genetic means that could result in even higher contents of EPA and GLA.

A possible approach for increasing the content of particular cell metabolites is the use of inhibitors of specific steps in biosynthetic pathways or analogs of specific products. Generally, such inhibitors and analogs inhibit growth as well. Thus, resistance to the inhibitor could be achieved by overproduction of the inhibited metabolites. It was indeed shown in higher plants that some lines selected for resistance to the growth inhibition are overproducers of the metabolite in question (8, 10, 15). Mutants of *Spirulina* showing elevated production of proline were obtained by selection in the presence of proline analogs (12). Similar results were obtained in the cyanobacterium *Nostoc* (9) and the alga *Nannochloris bacilaris* (13).

Several herbicides of the substituted pyridazinone family were shown to inhibit fatty acid desaturation. Of these, SAN 9785 is the most effective inhibitor of ω3 desaturation (11), and its effect on reduction of 18:3ω3 levels in the glycolipids of higher plants and algae was widely studied. Recently, we (1) found this herbicide to be an effective inhibitor also for Δ6 desaturation of linoleic acid in *Spirulina*. Although SAN 9785 has a certain inhibitory effect on photosynthesis, it was shown that the effect on fatty acid desaturation is a direct inhibition of the desaturase (16).

In the present paper, we describe the successful selection of *P. cruentum* and *S. platensis* cell lines that display stable resistance to the growth inhibition of SAN 9785. These cell lines over produced EPA and GLA, respectively. To the best of our knowledge, this is the first report of a fatty acid overproduction in either higher or lower plants induced by perturbation of fatty acid metabolism.

MATERIALS AND METHODS

Organisms and Culture Conditions

Spirulina platensis strain 2340 was obtained from the University of Texas Culture Collection and was cultivated on Zarrouk's medium at 30°C as previously described (5). *Porphyridium cruentum* strain 1380 1d was obtained from the Goettingen Algal Culture Collection (Goettingen, Germany). Cultures were grown on Jones's medium as previously described (6). Cultures were grown exponentially (with proper dilution) under the appropriate conditions for at least 4 d prior to the onset of the experiment. The specific growth rate was estimated by measurements of Chl concentration and turbidity. Solutions of the herbicides in DMSO were added to exponentially growing cultures. The final concentration of DMSO did not exceed 1%. Cultures of *S. platensis* were cultivated in the presence of 0.2 mM SAN 9785, which inhibited growth by about 80%. After several weeks of growth in the presence of the inhibitor (with occasional dilution with fresh medium containing the required concentration of the herbicide), the growth rate gradually increased, approaching that of the control cells. The concentration of the inhibitor was increased to 0.4 mM and the culture was cultivated as above for several months. *P. cruentum* cultures were similarly treated with an initial inhibitor concentration of 0.08 mM,

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[2] Abbreviations: EPA, eicosapentaenoic acid, 20:5ω3; GLA, 18:3ω6, γ-linolenic acid; SAN 9785, BASF 13-338, 4-chloro-5(dimethylamino)-2-phenyl-3(2H) pyridazinone; SRS-1–3, SAN-resistant lines of *Spirulina*; SRP-6–7, SAN-resistant lines of *Porphyridium*.

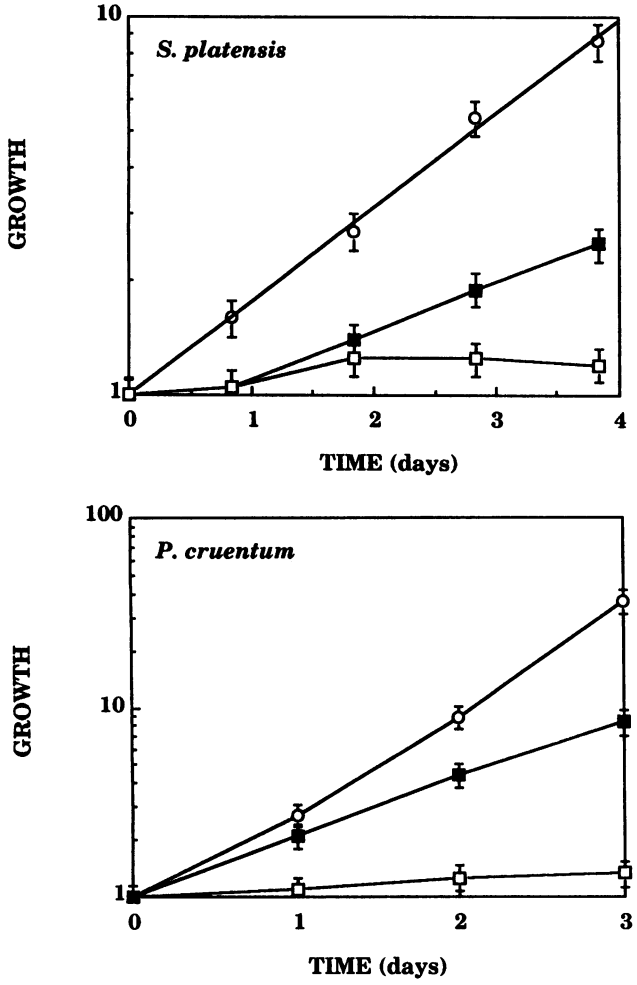

Figure 1. Effect of 0.4 mM SAN 9785 on the growth rate (expressed as the increase in Chl concentration relative to day 0) of *S. platensis* and *P. cruentum* (○, control; □, freshly exposed to the inhibitor; ■, inhibitor resistant culture).

which was gradually increased over a 9-month period, finally reaching 0.4 mM.

Selection of Resistant Lines

S. platensis culture resistant to 0.4 mM herbicide was diluted with inhibitor-containing medium and distributed to 50 test tubes having on the average half a filament per test tube. The test tubes were incubated for a period of several weeks. The test tubes in which growth occurred were rescued. Cell lines of *P. cruentum* resistant to 0.4 mM of the herbicide were obtained by screening of the resistant suspension on agar plate containing 0.4 mM of the inhibitor.

Fatty Acid Analysis

Freeze-dried samples of biomass were transmethylated with MeOH-acetyl chloride as previously described (2). Heptadecanoic acid was added as an internal standard. Gas chromatographic analysis was performed on a Supelcowax 10 fused silica capillary column (30 m × 0.32 mm) at 200°C (injector and flame ionization detector temperatures 230°C, split ratio 1:100) and integrated with an HP 3396A integrator. Fatty acid methyl esters were identified by cochromatography with authentic standards (Sigma) and by GC-MS. Fatty acid contents were determined by comparing their peak areas with that of the internal standard.

RESULTS AND DISCUSSION

It is reasonable to assume that the growth inhibition incurred by these herbicides emanates to a certain extent from their effect on fatty acid desaturation, particularly the transformation of linoleic acid. Linoleic acid is further desaturated in *S. platensis* to GLA and in *P. cruentum* to α-linolenic acid, which is in all likelihood a precursor of EPA. Thus, we hypothesized that overproducers of GLA and EPA may be found among SAN 9785-resistant cell lines of *S. platensis* and *P. cruentum*, respectively. Clones of these algae that are re-

Table I. *Fatty Acid Composition of S. platensis Cell Lines Resistant to SAN 9785*

The values presented are means ± SD ($n = 4$). A t test was used to determine significantly different values of 18:3 and TFA at $P < 0.025$.

Culture	Fatty Acid Composition						Fatty Acid Content	
	16:0	16:1	18:0	18:1	18:2 ω6	18:3 ω6	TFA[a]	18:3
	% of total fatty acids						% of dry wt	
WT[b]	42.11 ± 0.82	5.66 ± 0.91	0.90 ± 0.05	2.06 ± 0.45	25.52 ± 0.46	21.57 ± 0.47	4.09 ± 0.42	0.88 ± 0.07
WT[c]	42.16 ± 0.93	6.29 ± 0.16	0.85 ± 0.08	2.77 ± 0.10	25.91 ± 0.47	20.21 ± 0.41	4.30 ± 0.14	0.87 ± 0.05
SRS-1[d]	43.34 ± 1.29	4.44 ± 1.89	0.70 ± 0.07	2.39 ± 0.87	24.48 ± 0.76	23.13 ± 1.18[e]	5.55 ± 0.35[e]	1.28 ± 0.13[e]
SRS-3	42.80 ± 1.34	5.16 ± 0.22	0.84 ± 0.34	2.66 ± 1.11	24.07 ± 0.27	23.36 ± 1.21[e]	4.93 ± 0.24[e]	1.15 ± 0.09[e]
SRS-1h	40.69 ± 1.67	4.95 ± 0.39	0.67 ± 0.04	2.58 ± 0.42	25.09 ± 0.53	23.57 ± 0.52[e]	6.07 ± 0.18[e]	1.43 ± 0.05[e]

[a] Total fatty acids. [b] Wild type. [c] Wild type culture subjected to 0.4 mM SAN 9785 for 4 d and brought back to inhibitor-free medium, similar to the resistant culture. [d] Resistant cell lines selected by filament isolation from the resistant culture and cultivated on inhibitor-free medium for 11 d. [e] Significantly different from wild type. Lipid transmethylation and fatty acid analyses were performed as previously reported (3).

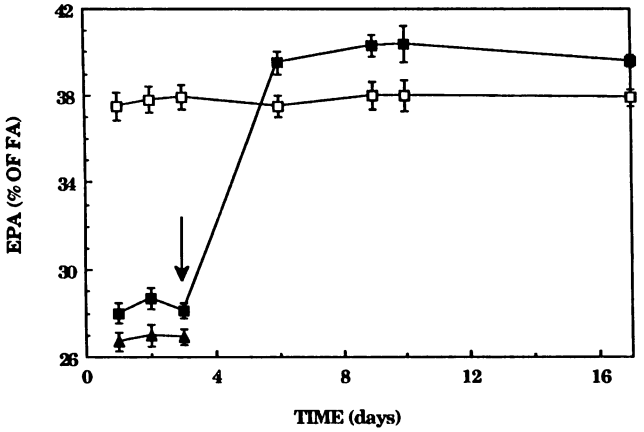

Figure 2. EPA content (% of fatty acids) of *P. cruentum* wild type grown with (▲) or without (□) the herbicide SAN 9785 and of a resistant cell line grown continuously in the presence of the herbicide (■) and shifted to a herbicide-free medium (arrow). Due to dependence of the fatty acid composition on the Chl level (5), the fatty acid composition was measured at a cell concentration corresponding to 4 mg/L Chl.

sistant to the growth inhibition of SAN 9785 were indeed selected from the wild type (Fig. 1).

Cell lines resistant to 0.4 mM SAN 9785 were obtained by a stepwise increase of the herbicide concentration in the growth medium. When cultivated in the presence of this concentration, the resistant cultures could grow, although at a reduced growth rate, whereas the wild type collapsed after a few days (Fig. 1). When brought back to inhibitor-free medium, their respective growth rates were back to normal (data not shown). The resistance was maintained even after 50 generations in an inhibitor-free medium, indicating a genetic change.

Relative to the wild type, the resistance in *Spirulina* was associated, as predicted, with elevated levels of GLA both in the presence (data not shown) and in the absence (Table I) of the inhibitor. In inhibitor-free medium, GLA increased from 21.6% (of total fatty acids) in the wild type to 23.1, 23.4, and 23.6% in the resistant isolates SRS-1, SRS-3, and SRS-1h, respectively. The proportions of 18:0, 18:1, and 18:2 decreased compared with the wild type (Table I). The three isolates had an improved fatty acid content (percent of dry weight) of 5.55, 4.93, and 6.07%, respectively, compared with 4.09% in the wild type. The increase in GLA content on a dry weight basis from 0.88% in the wild type to 1.28, 1.15, and 1.43% in SRS-1, SRS-3, and SRS-1h, respectively, became even more apparent. It was possible that the GLA overproduction obtained by transferring to inhibitor-free medium was merely an overshoot resulting from the absence of the inhibition. To overrule such a possibility, a wild-type culture was subjected to the inhibitor for 4 d and then brought back to inhibitor-free medium. Both its fatty acid composition and content were not significantly different from that of a wild type cultivated on control medium (Table I).

The culture of *P. cruentum* resistant to SAN 9785 displayed, in the presence of the inhibitor, EPA levels higher than those of freshly exposed cultures of the wild type (28.0 and 26.5% of fatty acids, respectively) (Fig. 2). Furthermore, elevated EPA levels were obtained when the resistant cultures were shifted to herbicide-free medium, where it reached 40.4% as compared with 36.8% in the wild type. The increase in EPA resulted primarily from a decrease in 16:0. The enhanced EPA level under inhibitor-free conditions was sustained for at least 2 weeks (Fig. 2). By plating on agar, six colonies were selected, three of which displayed even higher proportions of EPA and a higher fatty acid content, resulting in a 27% enhancement of the EPA content. Strains SPR-6 and SPR-7 showed the highest EPA proportions, each attaining a level of ~41% (of total fatty acids). On a dry weight basis, the fatty acid content of each of these lines increased to 5.6% (of dry weight) compared with 4.8% in the wild type, resulting in an EPA content of 2.3% as compared with 1.8% in the wild type (Table II).

It is difficult at present to point at the mechanism(s) of resistance to the herbicide. Murphy *et al.* (11) have shown that the uptake of SAN 9785 varies in various plants. They have further shown that the herbicide was rapidly metabolized in pea but only gradually in cucumber and ryegrass (11). Other possibilities could involve the modification of the target enzyme to reduce its affinity to the inhibitor, or an increase in the level of the relevant enzyme (14). The increase of GLA in *S. platensis* and EPA in *P. cruentum* may be attributed to

Table II. *Fatty Acid Composition of P. cruentum Cell Lines Resistant to SAN 9785*

The values presented are means ± SD ($n = 3$). A t test was used to determine significantly different values of EPA and TFA at $P < 0.05$.

Culture	Fatty Acid Composition											Fatty Acid Content	
	16:0	16:1	16:3	18:0	18:1 ω9	18:2 ω6	18:3 ω6	20:2 ω6	20:3 ω6	20:4 ω6	EPA	TFA[a]	EPA
	% of total fatty acids											% of dry wt	
WT[b]	30.3 ±0.62	5.20 ±0.34	0.62 ±0.06	0.44 ±0.02	0.53 ±0.04	5.44 ±0.11	0.89 ±0.13	0.54 ±0.15	0.68 ±0.07	16.1 ±0.40	38.2 ±0.75	4.78 ±0.17	1.83 ±0.04
SRP-6[c]	29.2 ±1.5	4.70 ±0.07	0.62 ±0.08	0.47 ±0.07	0.53 ±0.04	5.31 ±0.24	0.92 ±0.15	0.63 ±0.14	0.37 ±0.01	15.4 ±0.10	41.1 ±0.39[d]	5.64 ±0.67[d]	2.32 ±0.39[d]
SRP-7[c]	29.5 ±1.2	4.54 ±0.30	0.60 ±0.06	0.46 ±0.10	0.47 ±0.06	5.34 ±0.23	0.90 ±0.04	0.47 ±0.02	0.59 ±0.16	15.5 ±0.45	40.9 ±1.1[d]	5.62 ±0.67[d]	2.31 ±0.36[d]

[a] Total fatty acids. [b] Wild type. [c] Resistant cell lines selected by agar plating of the resistant culture. [d] Significantly different from wild type.

one of the last two mechanisms. Thus, overproduction could be a means for counteracting the effect of the herbicide.

The data presented in this communication indicate that the hypothesis suggesting the use of inhibitors of fatty acid desaturation as means for obtaining fatty acid overproduction was apparently correct. We are aware that further exploitation of this approach could be hampered by the reduced specificity of the herbicide. Thus, more specific inhibitors, such as transition stage analogs, are being sought. We anticipate that further selection of GLA and EPA overproducing strains would make the production of these fatty acids from algal sources feasible.

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