Mutagenesis of the cyanobacterium *Spirulina platensis* by UV and nitrosoguanidine treatment

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1. SUMMARY

The production of *Spirulina platensis* cells resistant to 8-azaguanine or β-(2-thienyl)-DL-alanine following mutagenesis with N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) and UV-irradiation is described. The conditions for the mutagenesis were determined by monitoring cell viability and the appearance of the two types of mutants as a function of the stage of growth of the tricomes and the length and the conditions of the treatment. The optimal conditions for UV and MNNG mutagenesis were found to be 1–3 min irradiation and 30 min incubation with 50 μg MNNG/ml of tricomes derived from cultures entering stationary phase sonicated for 10 s and 5 s respectively. Under these conditions β-(2-thienyl)-DL-alanine-resistant mutants appeared at a frequency $\geq 10^{-4}$ and $\geq 10^{-5}$ following UV- and MNNG-mutagenesis, respectively. Mutants resistant to 8-azaguanine were found at a frequency approx. $10^{-5}$ only after MNNG mutagenesis. A few chlorate-resistant mutants were also obtained following UV treatment.

2. INTRODUCTION

The filamentous cyanobacterium *Spirulina platensis* has been studied primarily for its nutritional properties which give this organism a biotechnological interest [1,2]. Several efforts have been made for its commercial production and to find suitable systems for its cultivation and utilization in human and animal nutrition [3,4]. On the other hand, knowledge of the genetic properties of this organism are limited and concern primarily the identification, cloning, and characterization of some genes having extended homology to those of other cyanobacteria or microorganisms [2,5,6]. Genetic recombination is un-
known for Spirulina and very few mutants of this organism have been described so far [7,8].

Although some classical methods for mutagenesis of filamentous cyanobacteria are known from the literature [9], they are difficult to apply in S. platensis and the only available information concerns the possibility of obtaining mutants of Spirulina by use of N-methyl-N'-nitro-N-nitroso- guanidine (MNNG) [7,8]. For this reason, in the present work, we have carried out a systematic study on the mutagenic effect of MNNG and UV irradiation of S. platensis as a function of the stage of growth of this organism and we have determined the optimal conditions for obtaining a large number of mutants resistant to β-(2-thienyl)-DL-alanine and to 8-azaguanine.

3. MATERIALS AND METHODS

3.1. Bacterial strains and growth conditions

S. platensis C1 (a kind gift of Dr. O. Tiboni, University of Pavia) has been used throughout this study. The composition of the minimal medium (MM) and growth conditions have been described elsewhere [7]. Complete medium (CM) is obtained by adding glucose (final concentration 0.05%), casamino acids (Difco, final concentration 0.015%) and vitamin and nucleic acid solutions [10] at 2.5 μl/ml and 1.5 μl/ml, respectively. For the mutagenesis experiments, 4-L cultures in MM were incubated with constant stirring, applying 12-h cycles of dark and light in order to synchronize the culture [8]. The growth was followed spectrophotometrically measuring the turbidity at 560 nm. The initial inocula had an optical density (OD560) between 0.03 and 0.05.

Plating was done by pouring 5 ml of soft agar (0.5% agar in MM) containing the tricome fragments over standard Petri dishes containing 10 ml 1% agar in MM. Separate ‘colonies’ can be obtained after 15–20 days at 30°C in an illuminated incubator. Plating efficiency can reach 100% when intact tricomes are used.

3.2. Sonication

The tricomes, harvested by filtration on paper discs at different stages of growth, were resuspended in MM at approx. 1 × 10⁶/ml and subjected to sonication at intensity ‘2’ on a Heat System Ultrasonic Model W380-20 sonicator. Sonication was for 5 or 10 s depending on whether the tricomes had to be mutagenized by MNNG or by UV-treatment, respectively.

3.3. Mutagenesis by UV-irradiation

The sonicated tricomes were centrifuged at 1000 × g for 5 min and resuspended in MM at approximately 0.5 × 10⁶ fragments/ml as determined in a Bürker cell-counting chamber. Twenty-ml aliquots were placed in quartz tubes with constant stirring and irradiated for 1–7 min with a UV lamp (GE G15-T8-15 w) at a distance of 10 cm. After incubation in the dark for approx. 12 h, the samples were plated to determine the plating efficiency calculated as: colonies obtained/number of fragments plated × 100. To determine the number of mutants, the samples were transferred to liquid CM for 10 days to allow the expression of possible mutations. The cells were then collected by centrifugation, washed with MM and finally plated on MM (control plates) as well as on three types of selection plates. These consisted of a) MM containing 20 mM β-(2-thienyl)-DL-alanine, b) MM containing 0.4 mM 8-azaguanine, c) nitrate-free MM containing 20 mM arginine and 120 mM KClO₃.

3.4. Mutagenesis by MNNG

The sonicated fragments were resuspended at 0.5 × 10⁶ fragments/ml in 50 mM sodium phosphate buffer (pH 7.5) and divided into two aliquots, one of which received MNNG (50 μg/ml). After 30 min at 30°C in a rotating water bath (200 rpm), the MNNG was removed by washing the cells three times with cold MM. The subsequent steps were the same as those described above.

4. RESULTS AND DISCUSSION

Since S. platensis consists of tricomes having a multicellular organization, ideally every mutagenic treatment should be carried out on single cells, preferably derived from synchronized tri-
comes. To approach as much as possible these conditions we have used *S. platensis* cultures subjected to light-synchronization [8] from the time of inoculation and we have subjected the tricomes to ultrasonic fragmentation with a procedure which yields fragments consisting of 1–5 cells. Since sonication affects the viability of the fragments and perhaps the capacity of the cells to divide, in preliminary experiments, we have looked for the best conditions to obtain a good fragmentation of the tricomes coupled with the best plating efficiency. As seen from Table 1, the plating efficiency varies depending on the conditions of the treatment: in fact it increases sharply with the age of the culture and, as expected, decreases with the length of the sonication.

To assess the efficiency of the mutageneses, spontaneous and induced mutants resistant to one of three different substances (i.e. β-(2-thienyl)-dl-alanine, 8-azaguanine and potassium chlorate) were selected before and after UV or nitrosoguanidine treatment. The frequency of appearance of spontaneous mutants resistant to β-(2-thienyl)-dl-alanine and 8-azaguanine is approximately $3 \times 10^{-7}$ and $6 \times 10^{-7}$, respectively (see also Fig. 2), but it is not always possible to determine directly when starting from young cultures since the number of starting cells is too low. No spontaneous mutants resistant to KClO$_3$ have ever been found.

Resistance to amino acid analogues (e.g. β-(2-thienyl)-dl-alanine) have been studied in *Anabena*, *Anacystis nidulans* and other cyanophycea in addition to *S. platensis* [7,11]. The acquired resistance can be explained by hypothesizing that mutants are impermeable to the analogues or have developed a mechanism which either enhances the incorporation of the normal amino acid in place of the analogue or alters the biosynthetic pathway leading to an overproduction of the normal amino acid. Chlorate-resistant mutants, on the other hand, have been found in some microorganisms (e.g. *Aspergillus nidulans*, *Escherichia coli*, etc.) but never in cyanobacteria. In general, the mechanisms of chlorate-resistance are different in different cases and in part unknown. Since chlorate can be a substrate for nitrate reductase, which can transform it into products toxic to the organism, the chlorate-resistance is in general attributed to a lack or a reduction of the activity of this enzyme in the mutant cells [12].

Finally, 8-azaguanine-resistant mutants have been identified and characterized in *Salmonella typhimurium*. The resistance has been linked to mutations of components of the purine biosynthetic pathway such as the nucleoside diphosphokinase (*ndk* gene), to hypoxanthine and guanine-xanthine phosphoribosyltransferase or to the development of permeability barriers [13–16].

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**Table 1**

Survival of *S. platensis* after sonication of tricomes as a function of the growth phase

<table>
<thead>
<tr>
<th>A. Tricomes sonication - 5 s</th>
<th>0.4</th>
<th>0.9</th>
<th>1.5</th>
<th>1.9</th>
<th>2.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD$_{560}$nm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plating efficiency (%)</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Exp.</td>
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</tr>
<tr>
<td>I</td>
<td>47.0</td>
<td>57.0</td>
<td>62.0</td>
<td>60.0</td>
<td>74.0</td>
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<tr>
<td>II</td>
<td>48.3</td>
<td>57.3</td>
<td>58.9</td>
<td>59.0</td>
<td>80.0</td>
</tr>
<tr>
<td>III</td>
<td>47.5</td>
<td>56.9</td>
<td>59.2</td>
<td>60.8</td>
<td>81.0</td>
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<table>
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<tr>
<th>B. Tricomes sonication - 10 s</th>
<th>0.3</th>
<th>0.7</th>
<th>1.0</th>
<th>1.4</th>
<th>1.8</th>
<th>2.0</th>
</tr>
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<tbody>
<tr>
<td>OD$_{560}$nm</td>
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<td>Plating efficiency (%)</td>
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<td></td>
<td>Exp.</td>
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<tr>
<td>I</td>
<td>16.0</td>
<td>27.0</td>
<td>30.6</td>
<td>38.0</td>
<td>49.0</td>
<td>49.3</td>
</tr>
<tr>
<td>II</td>
<td>16.2</td>
<td>28.1</td>
<td>31.2</td>
<td>39.1</td>
<td>51.3</td>
<td>51.9</td>
</tr>
<tr>
<td>III</td>
<td>16.4</td>
<td>27.6</td>
<td>30.8</td>
<td>38.6</td>
<td>49.9</td>
<td>50.7</td>
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The effect of exposure to UV irradiation on cell viability (Fig. 1A) and on the appearance of mutants resistant to β-(2-thienyl)-dl-alanine, 8-azaguanine and KClO₃ was studied as a function of the stage of growth of the culture and of the length of the UV treatment. As seen from Fig. 1B, the mutation for β-(2-thienyl)-dl-alanine-resistance was detected only upon irradiation of cells derived from medium-late log phase and with the exception of one case (5 min exposure of cells derived from cultures with OD₅₆₀ = 1), the frequency of the mutation increases with increasing dosage of irradiation. Mutants were also obtained after 7 min of UV exposure, but in this case the rate of survival is so low that the data cannot be regarded as meaningful for a quantitative determination of the rate of mutation. Remarkably, no 8-azaguanine-resistant mutants were ever found following UV mutagenesis, under any of the conditions tested. Finally, KClO₃-resistant mutants appeared at a frequency of $5 \times 10^{-6}$ but only within a very narrow range of conditions, e.g. upon 3 min UV treatment of cells derived from cultures harvested at OD₅₆₀ = 1 (data not shown).

Preliminary experiments obtained during the setting up of the method for treatment with MNNG had established that the best combination of sonication time, dosage and length of exposure to the mutagen was 5 s, 50 μg/ml in phosphate buffer and 30 min. These conditions result in a level of cell survival which is far higher than that reported to obtain mutants (0.1–1%) [7].

Under our conditions we obtained β-(2-thienyl)-dl-alanine-resistant mutants in every phase of growth with a maximum in the late-log phase while 8-azaguanine-resistant mutants were found only in medium-late log phase (Fig. 2). No chlorate-resistant mutants were ever detected after MNNG treatment.

In conclusion, in the present paper we have investigated the optimal conditions to obtain mu-

Fig. 1. Growth phase and dosage dependence of the effect of UV irradiation on the cell viability and the appearance of β-(2-thienyl)-dl-alanine resistant mutants of Spirulina platensis. (○) growth curve of the culture from which the tricomes were derived and subjected to 10 s sonication before mutagenic treatment with UV light for 1 (•), 3 (▲) or 5 (■) min. Panel A, cell viability. Panel B, frequency of appearance of β-(2-thienyl)-dl-alanine resistant colonies. In all cases spontaneous mutants < 0.5 × 10⁻⁶.
tants of the cyanobacterium *S. platensis* by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) treatment and UV irradiation. Our data have shown that several parameters are critical in determining whether and to which extent mutagenesis would occur. Thus, in addition to the synchronization of the culture and to the length of the sonication which precedes the actual mutagenic treatment, we found that the stage of growth of the trichomes and the length and the conditions of the treatment are essential in determining the efficiency of the mutagenesis in that they affect both viability and mutability of the cells. Thus, the survival after various exposure times decreases (as expected) with increasing dosage but in different ways depending on the stage of growth of the culture. In general, the survival capacity increases with the age of the culture until late log. Then it drastically decreases.

As to the type of mutagenic treatment, our data allow us to conclude that both experimental protocols for mutagenesis can be suitable and effective but that the effectiveness of the two mutagens depends strongly on the age of the culture and the type of mutations. Thus, *β*-(2-thienyl)-*D*L-alanine-resistant mutants appear at every stage of growth after treatment with MNNG while those resistant to 8-azaguanine were obtained only upon MNNG treatment of cells taken from a given stage of growth and not at all after UV irradiation. Cells treated by UV irradiation, on the other hand, display a high rate of mutation to *β*-(2-thienyl)-*D*L-alanine resistance and, under specific conditions, to KCIO₃ resistance.

Finally, the availability of at least three types of selectable markers and the likelihood of obtaining more mutants applying the mutagenesis protocols defined here should facilitate the task of setting up an experimental system to investigate genetic recombination in *S. platensis*.

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