ABSTRACT. Addition of caffeine to the recovering medium after mutagenesis of *Zymomonas mobilis* by N-methyl-N-nitrosoguanidine increased 4-fold the number of auxotrophic mutants obtained. Moreover, while the mutants isolated without caffeine survived only a few repeated serial transfers on minimal medium supplemented with the required growth factor, 40% of those obtained in the presence of caffeine were stable.

Induced mutation is still a valuable tool for improving the performance of microorganisms in biological processes. *Zymomonas mobilis* is an anaerobic Gram-negative bacterium reported to be a promising alternative for the industrial production of ethanol (Swings and De Ley 1977; Rogers et al. 1979, 1982; Baratti and Bu'Lock 1986; Rodriguez and Callieri 1986; Abate et al. 1989) and levan (Dawes et al. 1966; Swings and De Ley 1977; Yoshida et al. 1990). Moreover, *Z. mobilis* produces antimicrobial agents (Gonçalves de Lima et al. 1968, 1970, 1972) and it was also tested as a host for the expression of heterologous proteins (Uhlenbusch et al. 1991; Sprenger et al. 1993). In this context the possibility to obtain stable mutants with improved properties, such as ethanol tolerance and flocculation in the case of industrial production of ethanol, would be of interest. However, although several agents, such as N-methyl-N-nitrosoguanidine (NTG) and UV light, exert a marked mutagenic effect on *Z. mobilis* (Doele et al. 1993), the resulting mutants showed a high phenotypic instability (Sprenger et al. 1993).

In this paper we report the preparation of stable auxotrophic mutants of *Z. mobilis* by treatment with NTG and caffeine.

**MATERIALS AND METHODS**

*Bacterial strains and growth conditions.* *Zymomonas mobilis* strain ATCC 10988 was grown at 30°C in a complex medium (CM) containing in g/L: yeast extract 10, glucose 30, KH₂PO₄ 1, MgSO₄·7H₂O 1, (NH₄)₂SO₄ 1. Minimal medium (MM): biotin 0.01, D-pantothenic acid (semicalcium salt) 0.01, glucose 30, KH₂PO₄ 1, MgSO₄·7H₂O 1, (NH₄)₂SO₄ 1.

*Mutagenic treatment.* The concentration of NTG used for the mutagenic treatment reduced the population viability by 99.2%. Caffeine was used in the concentration range at which no effect on the viability of the cells was observed, viz. 1 and 5 g/L for solid and liquid media, respectively.

The microorganism was cultured in CM. When mid-to-late exponential phase of growth was reached, the following mutagenic conditions were tested:

(a) Addition of NTG, dissolved in water–ethanol (4:1) to a final concentration of 0.8 g/L. After 3 h of incubation (about two duplication times) the cells were harvested by centrifugation, washed twice with saline-phosphate buffer of pH 7.2 (Skotnicki et al. 1980), incubated for 3 h at 30°C in liquid CM, and plated on CM to allow segregation and phenotypic expression (Goodman et al. 1982).

(b) The same treatment as in (a) but adding 5 g/L of caffeine, dissolved in water, to the liquid CM and 1 g/L to solid CM for plating. After further 3 h the cells were collected, washed as indicated in (a), incubated for 3 h in liquid CM containing 5 g/L caffeine and plated on CM containing 1 g/L of caffeine.

*Isolation and characterization of mutants.* The auxotrophy of isolated which grew on CM but not on MM was determined by replica plating using MM containing the substance to be tested. When assaying the isolates obtained applying the mutagenic treatment (b) 1 g/L of caffeine was also added to the medium.

*Determination of the stability of mutants.* It was determined by growing them in liquid caffeine-free CM at 30°C for 2 d. This was repeated several times, using a portion of a growing culture as
inoculum for the next one. Samples of each culture were serially diluted in sterile water and survivors determined by using poured plates of CM and MM incubated at 30 °C for 2 and 5 d, respectively.

RESULTS AND DISCUSSION

The mutants obtained by treatment with NTG, with or without the addition of caffeine, were identified as thr-, leu-, asp-, pro-, trp-, cys-, gly-, ser-, arg-, ile-, his-, lys- and phe-. Some of the isolates could not be identified on the plates with growth factor pools and were assumed to have multiple mutations.

As shown in Table I the mutants obtained by treatment with NTG in the absence of caffeine, became nonviable after 4 serial transfers to MM supplemented with the required growth factor although they could grow on CM. It was also clear that caffeine included in the mutagenesis procedure led to an enhanced yield of mutants and to a marked positive effect on the stability of auxotrophy. The stability of the mutants was further investigated by repeated serial transfers to CM, without caffeine, which led to a 60 % of reversion and nonviability. The remaining mutants were maintained by monthly transfers to MM supplemented with the required growth factor, no reversion being observed up to date.

Table I. Effect of caffeine on the induction and stability of Zymomonas mobilis mutants

<table>
<thead>
<tr>
<th>Mutagenic treatment</th>
<th>Number of transfers</th>
<th>Auxotrophs recovery, %</th>
<th>Mutagenic treatment</th>
<th>Number of transfers</th>
<th>Auxotrophs recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTG</td>
<td>1</td>
<td>14</td>
<td>NTG + caffeine</td>
<td>1</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4</td>
<td></td>
<td>2</td>
<td>43</td>
</tr>
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<td></td>
<td>3</td>
<td>2</td>
<td></td>
<td>3</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>&gt;1</td>
<td></td>
<td>4</td>
<td>23</td>
</tr>
</tbody>
</table>

The percentage of auxotrophs recovering during serial transfers on minimal medium supplemented with the required growth factor is shown.

At the concentration used caffeine is not mutagenic but might improve the mechanisms of repair. At high concentrations of mutagenic agents, constitutive repair mechanisms, such as excision, are overloaded (Shetty et al. 1986). Therefore, under these critical circumstances the presence of caffeine might contribute to the induction of error-prone enzymes belonging to the SOS system which are able to repair damaged DNA in the absence of DNA template. The improvement of mutagenesis by the addition of caffeine was also reported for Schizosaccharomyces pombe (Clark 1973) and Lactobacillus delbrueckii ATCC 9649 (Demirci and Pometto 1992) although other mutagens than NTG were used.

The preparation and long-term maintenance of mutants of Z. mobilis may contribute to a wider use of this microorganism in industrial processes and genetic studies.

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