Minimal Medium for Isolation of Auxotrophic Zymomonas Mutants

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A minimal medium which allowed the sustained, rapid growth of Zymomonas mobilis and the isolation of a range of auxotrophic mutants was developed.

The bacterium Zymomonas mobilis is of interest because it has the capacity to produce ethanol at a specific rate considerably higher than that at which ethanol is produced by yeasts (9). It has recently been shown that mutagenesis can be used to improve some properties such as ethanol tolerance and flocculence (10) and that some R plasmids can be transferred into or between strains of Z. mobilis (12). However, further genetic analysis of this bacterium would be facilitated by the availability of auxotrophic mutants, the isolation of which has been prevented mainly by the lack of a suitable minimal medium for Zymomonas spp.

Although several minimal media have been described for Zymomonas spp. (2, 3, 13), we did not find them to be satisfactory for repeated serial cultivation. We tried to grow Z. mobilis on various mineral media which are standard for other gram-negative organisms such as Escherichia coli (8), Pseudomonas spp. (5, 16), Rhizobium spp. (11), and even Thiobacillus spp. (15). None of these minimal media sustained growth of Z. mobilis for more than one or two subcultures in liquid media or on solid media. Initial growth was presumably due to the carry-over of nutrients, as found by Swings and DeLey (14) for Z. mobilis on the minimal medium of Kluvyer and Hoppenbrouwers (7). Over 200 combinations of mineral salts and vitamins were tested as growth-optimizing factors for several Z. mobilis strains. The factors tested included calcium pantothenate, thiamine, biotin, riboflavin, nicotinic acid, inositol, and pyridoxine at different concentrations and in various combinations. The common inorganic salts required for bacterial growth, also at different concentrations and in various combinations, were tested in conjunction with these growth factors. Agar from Difco Laboratories was found to give 100% colony-forming efficiency, whereas several cheaper brands of agar gave poor results. The optimization of medium composition was necessary for Z. mobilis auxotroph isolation since previously described media (14) either gave poor growth and did not sustain growth on serial subculturing or contained many amino acids, salts, and vitamins (3). We report here the composition of a basal medium devised to give sustained, rapid Z. mobilis growth over many subculturings and 100% colony-forming efficiency and the procedure for isolation of auxotrophic mutants. The basal medium (BM) contained the following (grams per liter): K2HPO4, 1; KH2PO4, 1; NaCl, 0.5; and (NH4)2SO4, 1 (final pH, 6). After the BM was autoclaved and had cooled to below 55°C, the following filter-sterilized solutions (milligrams per liter [final concentration]) were added: MgSO4·7H2O, 200; CaCl2·2H2O, 200; Na2MoO4·2H2O, 25; and FeSO4·7H2O, 25. We also added 1 ml of a filter-sterilized vitamin solution which contained the following (milligrams per liter): calcium pantothenate, 5; thiamine hydrochloride, 1; pyridoxine hydrochloride, 1; biotin, 1; and nicotinic acid, 1. Glucose was added to a final concentration of 2% (wt/vol). The medium was solidified with 15 g of agar (Difco) per liter. The composition of the rich liquid medium (RM) used contained the following (grams per liter): glucose, 20; yeast extract (Oxoid Ltd.), 10; and KH2PO4, 2 (12).

The strains of Z. mobilis used in this study were ZM4, AgI1, and ZM1 (12). All cultures in liquid media were grown without shaking in screw-capped tubes or bottles at 30°C.

The increases in the size of colonies of Z. mobilis ZM4 and two auxotrophs on RM and BM plates at 30°C are shown in Table 1.

Auxotrophic mutants of strain ZM4 were induced by treatment with N-methyl-N′-nitro-N-nitrosoguanidine (NTG), which was dissolved in water and then added immediately to log-phase cells in RM. The mixture was incubated at 30°C for 30 or 60 min. The cells were then washed twice in BM or saline phosphate buffer (12) before being plated directly onto RM or resuspended in liquid RM to allow segregation and phenotypic expression to occur during growth. This method gave a much higher level of auxotrophs than that achieved when NTG was added to the cells in buffer. The percent viability of
TABLE 1. Rate of Z. mobilis colony development

<table>
<thead>
<tr>
<th>Growth time and medium</th>
<th>Colony size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ZM4</td>
</tr>
<tr>
<td>3 Days</td>
<td></td>
</tr>
<tr>
<td>BM</td>
<td>0.5</td>
</tr>
<tr>
<td>RM</td>
<td>1.5-2</td>
</tr>
<tr>
<td>5 Days</td>
<td></td>
</tr>
<tr>
<td>BM</td>
<td>1</td>
</tr>
<tr>
<td>RM</td>
<td>2-2.5</td>
</tr>
<tr>
<td>7 Days</td>
<td></td>
</tr>
<tr>
<td>BM</td>
<td>2</td>
</tr>
<tr>
<td>RM</td>
<td>3</td>
</tr>
</tbody>
</table>

* BM was supplemented with cysteine or adenine plus arginine for growth of the appropriate auxotrophs.

About 100 colonies of each type were measured. Mean measurements are given.

strain ZM4 cells after treatment with three concentrations of NTG is shown in Table 2.

After replica plating, colonies which could grow on RM but not BM were purified and identified by the method of Davis et al. (6), i.e., by further replication of the colonies on BM plates containing a series of growth factor pools.

Doubly marked mutants were produced by repeated NTG treatment.

Treatment with 25 μg of NTG per ml for 30 min followed by growth in RM for 2 h to allow expression was sufficient to induce a high percentage of auxotrophs. Higher levels of NTG often induced multiple mutations. Although the percent recovery of auxotrophs was very high after treatment with 100 μg of NTG per ml—up to 83% (Table 2)—about one-third of the auxotrophs could not be identified on the plates with growth factor pools and were assumed to have multiple mutations. A similar effect of high NTG levels was found by Adelberg et al. (1). Furthermore, if the mutated strain ZM4 culture was grown to stationary phase in RM, the recovery of auxotrophs was only about 3%. This was most probably due to the growth rate of auxotrophs being lower than that of prototrophs in liquid medium. On solid media, a greater prototroph growth rate was indicated by a more rapid increase in colony diameter than for auxotrophs (Table 1).

Thus, the method of achieving a good percentage of Zymomonas mutants is somewhat different from the standard procedure (8): NTG is added to a log-phase culture in RM rather than to cells in buffer, and after mutagenesis, the cells are grown in RM for only a few hours, rather than overnight, to allow expression.

The distribution of auxotrophic markers isolated was not completely random. In six mutagenesis experiments with strain ZM4, 67 mutants were identified; of these, 15 required adenine, and 41 required cysteine. Of the mutants requiring cysteine, 38 were able to grow on BM supplemented with either cysteine or thiamine, and 3 grew only with cysteine. Certain E. coli and Neurospora crassa mutants which require the thiazole moiety of thiamine and can utilize cysteine in place of thiazole have been isolated (4). Of the other mutants isolated, three required tryptophan, five required methionine, two required arginine, and one required tyrosine. Doubly marked mutants required adenine and methionine, adenine and arginine, adenine and histidine, cysteine and methionine, cysteine and arginine, and cysteine and adenine.

We isolated auxotrophic mutants of strains ZM1 and Ag11, using the same medium. Of the strain ZM1 mutants isolated, three required adenine, two required cysteine, two required leucine, one each required methionine, proline, histidine, or arginine, and one had an early block in the aromatic pathway and thus required tryptophan, phenylalanine, and tyrosine. Of the strain Ag11 mutants isolated, two each required adenine, methionine, or tryptophan, and one required cysteine.

Few genetic studies of Zymomonas spp. have been reported (10, 12, 13). A low frequency of chromosome transfer of the rifampin resistance marker between Z. mobilis strains has been achieved by using the chromosome-mobilizing plasmid R68.45 (12). However, chromosome transfer and linkage will be better established when auxotrophic markers are used. Preliminary experiments with strain ZM4 have shown that when plasmid R68.45 is used, an adenine-requiring marker is transferred at a rate of 9 × 10⁻⁵ per recipient cell, and a tryptophan-requiring marker is transferred at a rate of 2 × 10⁻⁴ per

TABLE 2. Percent viability in and auxotroph recovery from strain ZM4 cultures after NTG mutagenesis

<table>
<thead>
<tr>
<th>NTG concn (μg/ml)</th>
<th>Mutagenesis time (min)</th>
<th>Treatment after mutagenesis</th>
<th>% Viability</th>
<th>% Auxotroph recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>30</td>
<td>DP</td>
<td>33</td>
<td>0.2</td>
</tr>
<tr>
<td>25</td>
<td>60</td>
<td>DP</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>50</td>
<td>30</td>
<td>DP</td>
<td>12</td>
<td>NT*</td>
</tr>
<tr>
<td>50</td>
<td>60</td>
<td>DP</td>
<td>2</td>
<td>NT</td>
</tr>
<tr>
<td>100</td>
<td>30</td>
<td>DP</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>100</td>
<td>60</td>
<td>DP</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>100</td>
<td>60</td>
<td>2 h of growth</td>
<td>80</td>
<td>83</td>
</tr>
</tbody>
</table>

* DP, Direct plating.

† NT, Not tested.
recipient cell (A. Goodman, unpublished data). It will now be possible to obtain for Zymomonas spp. chromosomal linkage data such as those achieved for the related genera Pseudomonas and Rhizobium.

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LITERATURE CITED