Isolation of *Clostridium thermocellum* Auxotrophs

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Spontaneous and UV irradiation-induced auxotrophic mutants of *Clostridium thermocellum*, an anaerobic cellulosolytic thermophile, were isolated after penicillin enrichment in a chemically defined medium.

The conversion of biomass to fuels by microbial fermentation offers the potential of solving two of today's important problems: waste accumulation and exhaustion of fossil fuels. Microorganisms with the capabilities of converting biomass components such as cellulose and hemicellulose to chemicals and fuels in a single step are of particular interest. One such microorganism is *Clostridium thermocellum*, a thermophilic anaerobe which degrades cellulose to ethanol and organic acids (11). For efficient industrial use, the cellulosolytic capacity of this strain must be improved by genetic means. Presently, little is known about the genetics of *C. thermocellum* and other related clostridia in spite of their potential utility in fermentation technology. Genetically marked strains required for the development of genetic recombination systems have been lacking. This report describes the isolation of auxotrophic mutants of *C. thermocellum* for the first time.

Johnson et al. (5) described a chemically defined medium (MJ) for *C. thermocellum*. This medium, containing 10 mg of cellulose (Cb) per ml, gives plate counts similar to those obtained with the complex medium CM4/Cb (4). This recovery allowed us to begin the search for auxotrophs.

The strain used was ATCC 27405. Cultures grown in the complex medium CM4/Cb to the mid-exponential phase were irradiated at 245 nm with a General Electric type G875 lamp in doses ranging from 4,000 to 5,000 ergs/mm² under aerobic conditions; growth in the dark was then allowed to proceed for seven generations. The cultures were centrifuged, washed once, and suspended in 10 volumes of MJ/Cb medium. After growth initiation, penicillin G (100 U/ml; Sigma Chemical Co.) was added, and the incubation was allowed to proceed for 6 h. Upon removal of the antibiotic by centrifugation, the cells were allowed to grow in complex medium CM4/Cb; this growth was followed by a second selection with penicillin (100 U/ml) and D-cycloserine (50 µg/ml; Sigma). The cultures were plated on CM4/Cb agar and, after growth, were replica plated onto MJ/Cb and CM4/Cb agars. The colonies that did not grow on MJ/Cb agar were tested on MJ/Cb agar supplemented with pools of different amino acids, purine and pyrimidine bases, and vitamins, as described by Davis et al. (1). One colony of each phenotype was verified and preserved. All plating was done in an anaerobic hood of controlled atmosphere (90% N₂, 50% CO₂, 5% H₂).

To detect the presence of spontaneous mutants, we performed penicillin selection in minimal medium, using nonmutagenized cultures. In this way, we isolated three mutant strains: BM23 Leu−, BM11 Ade−, and BM31 Ade− (Table 1). The two Ade− mutants were independently isolated.

When cultures were mutagenized by UV light and penicillin selection was performed in MJ/Cb medium supplemented with adenosine and L-leucine (MJAL), an isoleucine auxotroph, BM74, was obtained (Table 1). We used MJAL to eliminate the spontaneous mutants that could interfere with the detection of the UV-induced auxotrophs. No spontaneous Ile− mutants were isolated after penicillin selection in MJAL.

The only auxotroph in which we observed spontaneous reversion to prototrophy was BM11. The absence of reversion suggests that the other three genetic lesions are not point mutations.

We have shown (R. F. Gomez, B. Snedecor, and B. S. Mendez, Dev. Ind. Microbiol., in press) that UV and gamma irradiation produces an increase in *C. thermocellum* mutants resistant to 5-fluorouracil and rifampin. In the present work, we induced auxotrophy with UV irradiation, but in three experiments in which gamma irradiation was used as a mutagenic agent and penicillin selection was used in MJAL, we did not detect any auxotrophs.

It is possible that during the penicillin enrich-

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ment, cross feeding allowed the elimination of auxotrophs. We tested the effectiveness of the penicillin enrichment on mixed cultures containing both the wild-type strain and either BM31 or BM23. The proportion of auxotrophs was enhanced from the original 1% to 84% of the total population. We concluded that under our experimental conditions, cross feeding, if it existed, was not allowing enough growth of auxotrophs to cause them to be killed in significant numbers by penicillin.

We have shown above that when the spontaneous Ade" and Leu" mutants were excluded by penicillin selection in supplemented minimal medium, it was possible to detect an auxotrophic mutant induced by UV mutagenesis. However, in two other experiments carried out under the same conditions that led us to the isolation of BM74, no auxotrophs were detected. The difficulty in obtaining auxotrophic mutants was probably not due to the inability of the mutant allele to segregate. The known molecular weight of the bacterial genome ranges from 0.44 x 10^9 (Mycoplasma arthritidis) (7) to 3.5 x 10^9 (Serratia marcescens) (3). Using the diphenylamine method (2), we found the DNA content per cell for C. thermocellum to be 61% that of Escherichia coli (molecular weight, 2.53 x 10^9 [3]). Such a value precludes the possibility of there being a large number of genomes per cell, unless the C. thermocellum genome is unexpectedly small.

The apparently limited degree of induced mutagenesis in C. thermocellum remains to be explained. It may be that an error-prone repair system is absent or not very effective in this strain or that, in the aerobic conditions in which the mutagenesis was performed, it is uninduced. Recent work has pointed out that repair system activities are affected by the presence of O2. Jones and Woods (6) found evidence of an O2 inhibition of a recA-type repair system in Bacteroides fragilis. Rambler and Margulis (8) showed that under anaerobiosis, facultative anaerobes have a reduced photoreactivation activity after UV damage.

The number of auxotrophic mutants and other genetically marked strains in obligate anaerobes is very small (9, 10). The experimental procedure described in this paper should help the development of better mutagenic treatments for this species. The availability of these mutants will allow further studies on the recombination systems of C. thermocellum and eventually other related anaerobes.

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LITERATURE CITED


### TABLE 1. Auxotrophs of C. thermocellum ATCC 27405

<table>
<thead>
<tr>
<th>Mutagen</th>
<th>Auxotroph fraction (×10⁶)</th>
<th>C. thermocellum strain</th>
<th>Phenotype</th>
<th>CFU/ml&lt;sup&gt;a&lt;/sup&gt; on MJ/Cb agar</th>
<th>CFU/ml&lt;sup&gt;b&lt;/sup&gt; on MJ/Cb agar + requirement&lt;sup&gt;c&lt;/sup&gt;</th>
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<tr>
<td>None</td>
<td>13</td>
<td>BM23</td>
<td>Leu&quot;</td>
<td>&lt;2.0 × 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>2.4 × 10&lt;sup&gt;2&lt;/sup&gt;</td>
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<tr>
<td>None</td>
<td>33</td>
<td>BM11</td>
<td>Ade&quot;</td>
<td>2.2 × 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.7 × 10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>None</td>
<td>62</td>
<td>BM31</td>
<td>Ade&quot;</td>
<td>&lt;2.0 × 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>4.0 × 10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>UV</td>
<td>2.4</td>
<td>BM74</td>
<td>Ile&quot;</td>
<td>&lt;2.0 × 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>2.8 × 10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Calculated as follows, after penicillin selection: number of auxotrophic colony-forming units (CFU) / total number of auxotrophic-plus-prototrophic CFU.

<sup>b</sup> Average of four plates for purified strain.

<sup>c</sup> L-Leucine, adenosine, or L-isoleucine.