Mutation of *Clostridium thermocellum* in the presence of certain carbon sources

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1. SUMMARY

When a cellobiose-grown inoculum of *Clostridium thermocellum* was transferred to either glucose or fructose as the sole carbon source, growth occurred only after a long lag of 180–200 h. We established that sugar uptake and phosphorylation were not limiting growth nor was the lag period the time taken for a physiological adaptation process or for the growth of a mutant carried over in the cellobiose-grown inoculum. It became apparent that a mutation was occurring during the lag period in response to the selection pressure exerted by the presence of glucose or fructose as the sole carbon source. Once growth occurred on glucose and fructose, the cells could be transferred to cellobiose and back to glucose or fructose without exhibiting the long lag period. The change was stable over several transfers in the respective sugars.

2. INTRODUCTION

*Clostridium thermocellum* is a thermophilic, anaerobic and cellulolytic bacterium that has been studied extensively for the ability to produce ethanol from biomass. The cellulase complex of this organism and the genes coding for the cellulases have been the subject of considerable research in recent years [1–3]. *C. thermocellum* has been reported to utilize several carbon sources including cellulose, cellobiose, glucose, fructose, mannitol, sorbitol, and xylene; cellobiose is its favored carbon source. While its ability to ferment cellulose and cellobiose remains undisputed, the utilization of the other sugars has been controversial. For example, McBee [4] reported growth on xylose, but not on fructose, mannitol, and sorbitol. Ng et al. [7] could not obtain growth on glucose or xylose. Patni and Alexander [5,6] obtained growth on glucose, fructose, and mannitol. Shinmyo et al., [8], Wiegel and Ljungdahl [9] and Hernandez [10] observed growth on glucose in complex medium after a lag of about 30 h. More recently, Johnson et al. [11] reported that *C. thermocellum* grew on glucose, fructose and sorbitol in chemically defined medium but only after a lag of about 100 h. We have confirmed the finding of...
Johnson et al. [11], although we have observed even longer lags in a somewhat different medium. The present medium had 8-fold more iron. Furthermore, in some of the experiments of Johnson et al. [11], the carbon source concentration was twice that used in the present study. When a cellobiose-grown *Clostridium thermocellum* was transferred to medium containing either glucose or fructose as the sole carbon source, no growth occurred for 180–200 h. At the end of this time, rapid growth ensued and the final cell densities achieved were comparable to those reached in the cellobiose medium. Subsequent transfers into medium with glucose or fructose from glucose or fructose inocula respectively, resulted in extensive growth of the culture by 24 h.

We observed [12] with in vivo $^{13}$C-NMR techniques that cellobiose-grown cells are able to take up glucose and fructose from the medium. Furthermore, $^{31}$P-NMR measurements of cell extracts from cellobiose-grown cells fed glucose and fructose revealed that the sugars underwent phosphorylation. The rates of uptake and phosphorylation were comparable to those in cells that were readily growing on glucose and fructose. These data are presented in a separate publication [12]. It is clear that the initial lack of growth in media with glucose or fructose is not due to problems with uptake or phosphorylation of the sugars. We speculated that the long lag period might be due to the time needed for (i) a physiological adaptation process, (ii) growth of a mutant cell carried over from the cellobiose inoculum, or (iii) a genetic change(s) in the cells during the growth lag, allowing growth on glucose or fructose. In this paper, we describe experiments showing that the third possibility is most likely the cause of the long lag phase.

3. MATERIALS AND METHODS

The strain used was *Clostridium thermocellum* ATCC 27405. The minimal medium was that of Venkateswaran and Demain [13]. This medium is a modification of the MJ medium of Johnson et al. [11]; it contains 8 times as much FeSO$_4$ as the MJ medium. The carbon source for growth (5.0 g/l) is specified in the text for each experiment.

For viable counts, *C. thermocellum* was grown in Hungate tubes in minimal medium with cellobiose. Cells in the exponential phase ($A_{660} = 0.5–0.7$) were diluted in minimal medium without sugar and various dilutions ranging from undiluted to $10^6$ dilution were added in 0.1 ml volumes to petri plates that had been left overnight in the anaerobic chamber. Minimal agar medium (25 ml) containing the different sugars that had been sterilized separately was then added to each plate and the plates were shaken gently and allowed to solidify. Cellobiose-grown cells were plated on medium with cellobiose, glucose and fructose, and as a negative control, with xylose. As positive controls, glucose-grown cells and fructose-grown cells were plated on medium with glucose and fructose respectively. All plates were subsequently incubated at 60°C inside the anaerobic chamber. The plates were observed for the appearance of colonies every day for up to three weeks.

4. RESULTS

As expected, *C. thermocellum* grew readily on cellobiose in minimal medium, the culture achieving its maximum cell density ($A_{660} = 0.9–1.0$) within 18–20 h. When such a culture was inoculated at 1% (v/v) into medium containing either glucose or fructose as carbon source, there was a long lag of 180–200 h, during which time very little growth (0.03 mg DCW/ml) occurred. This is longer than the 90–110 h lag observed by Johnson et al. [11] in a somewhat different medium, who noted that the maximum cell density in the case of fructose or glucose was 50% or less than the cell density reached with cellobiose as carbon source. At the end of the 180–200 h lag period, the maximum cell density reached on glucose or fructose was equivalent to that achieved on cellobiose.

Cells that had grown after the long lag on fructose or glucose were transferred into medium with the same carbon source. This time, the cells reached their maximum cell density within 24 h. The growth curves for cells grown on cellobiose and transferred to cellobiose, fructose and glucose
are shown in Fig. 1 and those for cells growing on the three different sugars are shown in Fig. 2. The growth rates were comparable on the three carbon sources (Table 1).

Table 1  
Growth rate and maximum dry cell weight of cells grown on different carbon sources  

<table>
<thead>
<tr>
<th>Carbon source for preparation of inoculum</th>
<th>Carbon source for growth</th>
<th>Growth rate (h⁻¹)</th>
<th>Maximum DCW (g/L) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellobiose</td>
<td>Cellobiose</td>
<td>0.36</td>
<td>0.60</td>
</tr>
<tr>
<td>Glucose</td>
<td>Glucose</td>
<td>0.40</td>
<td>0.74</td>
</tr>
<tr>
<td>Fructose</td>
<td>Fructose</td>
<td>0.33</td>
<td>0.74</td>
</tr>
</tbody>
</table>

* At the end of 20 h.

Table 2  
Number of colonies obtained when cellobiose-grown cells were transferred to plates with cellobiose, glucose, fructose or xylose as sole carbon source  

<table>
<thead>
<tr>
<th>Carbon source in plates</th>
<th>Time of appearance of colonies</th>
<th>Dilutions</th>
<th>10⁻⁰</th>
<th>10⁻¹</th>
<th>10⁻²</th>
<th>10⁻³</th>
<th>10⁻⁴</th>
<th>10⁻⁵</th>
<th>10⁻⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellobiose</td>
<td>30 h</td>
<td>TNTC *</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>220 h</td>
<td>55</td>
<td>43</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fructose</td>
<td>200 h</td>
<td>62</td>
<td>47</td>
<td>17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xylose</td>
<td>(Observed 20 d)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* TNTC indicates colonies too numerous to count.  
** The numbers in parentheses are the mutant frequencies in each case.
had taken place during the initial long lag period when the cells were first transferred into these sugars. To confirm that a mutation(s) is indeed occurring during the lag on the sugars, viable count experiments were done.

With cellubiose as carbon source, colonies appeared within 30 h on plates from all dilutions, the number of colonies being 34 in the plate with the 10^6 dilution and 330 in the plate with the 10^5 dilution (Table 2). Similarly, on plates to which glucose- or fructose-adapted cells had been added to glucose or fructose plates respectively, growth occurred within 30 h at the dilution commensurate with the number of viable cells present in the inoculum (data not shown). In contrast to the above, no colonies were observed at 30 h on plates in which cellubiose-grown cells had been plated on medium with glucose or fructose as carbon source. In both cases, there was no residual growth during the lag and colonies appeared only after 180 to 200 h. Even after this time, there were no colonies on plates with dilutions greater than the 10^3 dilution. There were about 60 colonies on both the glucose and fructose plates at the 10^0 dilution; about 45 colonies in plates with the 10^1 dilution, about 20 colonies on the plates with the 10^2 dilution and about 8 colonies on the plates with the 10^3 dilution (Table 2). No growth was observed, even at the end of 20 days, in any of the dilutions on plates with xylose as carbon source.

5. DISCUSSION

The fact that no colonies were seen at 30 h on glucose or fructose plates from a cellubiose-grown inoculum strongly indicates that there were no preformed mutants in the inoculum. Thus, the mutation must occur during growth on the plates. The mutation(s) takes as long as 150–170 h to occur (the same as required in liquid medium) and ultimately results in appearance of colonies at 180–200 h. As mentioned earlier, once cells have grown on medium with glucose or fructose, they retain the ability to grow on these two sugars as well as the ability to grow on cellubiose. This is suggestive of a stable mutational event having taken place. The possibility that we are observing a regulatory phenomenon leading to a stable alternative phenotype such as phase variation or phage immunity has not been disproven.

It is also evident that there was not a slow physiological adaptation to growth on glucose and fructose because colonies do not appear gradually between the periods of 30 h and 180 h, but suddenly appear at 180 h on both glucose and fructose plates. Secondly, if the phenomenon were an adaptation process, one would expect nearly the same number of colonies to appear on the glucose and fructose plates as on the plates with cellubiose, but they would take a longer time to appear. As seen in Table 2, this was not found to be the case. There were many fewer colonies on the glucose and fructose plates than on the cellubiose plates. Finally, the ability of the colonies on glucose and fructose to be able to grow on cellubiose and back again on glucose or fructose without a long lag period is an indication of the change being genetic and not a physiological adaptation.

The fact that cellubiose-grown C. thermocellum could not grow on medium with glucose or fructose without the lag period and that growth occurred in these substrates reproducibly at the end of about 180 h suggests that the mutational event is in response to the selection pressure exerted by the presence of glucose or fructose as the sole carbon source.

The frequency with which the mutants appeared on the plates with glucose and fructose as carbon source are shown in parentheses in Table 2. The mutants appear at frequencies (about 2 × 10^-4) that are much higher than that accounted for by spontaneous mutation which in most bacteria is at the rate of 1 cell in 10^8. There have been recent reports of cells being able to mutate at a considerably higher rate in response to selective pressure, most notably in the presence of a carbon source it normally cannot utilize. For example, a lacZ deletion strain of Escherichia coli which cannot utilize lactose can call upon a cryptic gene to hydrolyze the sugar. This would require at least two mutations, each of which normally occurs at a frequency of less than 10^-8. Yet it was found that this same strain grown to stationarity phase on a
MacConkey agar plate with lactose could produce Lac\(^+\) papillae in about two weeks \[14\]. No papillae were observed in plates without lactose. The conclusion was that cells can 'sense' the need for mutation and do so at high frequencies and in response to selection pressure. Hall \[15\] also observed that in *E. coli* K12 strain \(\chi^{3421!D}\), two mutations had to occur for the cell to be able to utilize salicin and this improbable event occurred only on media containing salicin. Other examples of adaptive or directed mutations induced by substrates at low growth rates or in the absence of growth have been recently summarized by Davis \[16\] and a related phenomenon was described by Wright \[17\] many years ago.

Although one might expect the number of mutants that appear to be proportional to the number of cells that were plated, this was not the case. Table 2 shows that about 60 colonies occurred in the undiluted samples, about 45 in the \(10^1\) dilutions, about 20 in the \(10^2\) dilutions, 5–10 in the \(10^3\) dilutions and none in the \(10^4\) dilutions. The data suggest that perhaps a 'population effect' restricts the number of mutants that can arise when a large number of cells are plated and that as the dilutions increase, the proportion of mutants observed is higher. A similar phenomenon was reported by Spiegelman et al. \[18\] in *Saccharomyces cerevisiae*. They found that the expression of a positive phenotype (growth on galactose) was inhibited by the presence of large numbers of the negative phenotype and that the percentage of positive colonies dropped sharply in heavily seeded plates.

There has been speculation on the mechanism(s) responsible for mutations that occur at high frequencies in response to selection pressure. In one such hypothesis, the cell would produce a highly variable set of mRNA molecules and reverse-transcribe the one that made the best protein, or in a less efficient process, the cell would produce reverse transcripts at random and one that carried the variant sequence allowing for growth would eventually get incorporated into the genome during recombination \[14\]. Whatever the mechanism, it is apparent that a mutation does occur in response to selection pressure, resulting in growth of *C. thermocellum* on glucose and fructose. Future work will be addressing the identity of the gene and gene product modified by this mutation.

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**REFERENCES**