Carbon Flux Distribution and Kinetics of Cellulose Fermentation in Steady-State Continuous Cultures of Clostridium cellulolyticum on a Chemically Defined Medium

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The metabolic characteristics of *Clostridium cellulolyticum*, a mesophilic cellulolytic nonruminal bacterium, were investigated and characterized kinetically for the fermentation of cellulose by using chemostat culture analysis. Since with *C. cellulolyticum* (i) the ATP/ADP ratio is lower than 1, (ii) the production of lactate at low specific growth rate (μ) is low, and (iii) there is a decrease of the NADH/NAD⁺ ratio as the dilution rate (D) increases in carbon-limited conditions, the chemostats used were cellulose-limited continuously fed cultures. Under all conditions, ethanol and acetate were the main end products of catabolism. There was no shift from an acetate-ethanol fermentation to a lactate-ethanol fermentation as previously observed on cellubiose as μ increased (E. Guedon, S. Payot, M. Desvaux, and H. Petitdemange, J. Bacteriol. 181:3262–3269, 1999). The acetate/ethanol ratio was always higher than 1 but decreased with D. On cellulose, glucose 6-phosphate and glucose 1-phosphate are important branch points since the longer the soluble β-glucan uptake is, the more glucose 1-phosphate will be generated. The proportion of carbon flowing toward phosphoglucomutase remained constant (around 59%), while the carbon surplus was dissipated through exopolysaccharide and glycogen synthesis. The percentage of carbon metabolized via pyruvate-ferredoxin oxidoreductase decreased with *D. Acetyl* coenzyme A was mainly directed toward the acetate formation pathway, which represented a minimum of 27.1% of the carbon substrate. Yet the proportion of carbon directed through biosynthesis (i.e., biomass, extracellular proteins, and free amino acids) and ethanol increased with *D*, reaching 73.7 and 16.8%, respectively, at 0.083 h⁻¹. Lactate and extracellular pyruvate remained low, representing up to 1.5 and 0.2%, respectively, of the original carbon uptake. The true growth yield obtained on cellulose was higher, [50.5 g of cells (mol of hexose eq)⁻¹] than on cellubiose, a soluble cellodextrin [36.2 g of cells (mol of hexose eq)⁻¹]. The rate of cellulose utilization depended on the solid retention time and was first order, with a rate constant of 0.05 h⁻¹. Compared to cellubiose, substrate hydrolysis by cellulose when bacteria are grown on cellulose fibers introduces an extra means for regulation of the entering carbon flow. This led to a lower μ, and so metabolism was not as distorted as previously observed with a soluble substrate. From these results, *C. cellulolyticum* appeared well adapted and even restricted to a cellulolytic lifestyle.

Cellulose is of cardinal importance in the global carbon cycle: it accumulates in the environment due to its durable nature (5), and the main final products released during its fermentation are CH₄ and CO₂ (76). Bacteria are the major agents of cellulose digestion by clostridia (27, 40). The cellulose degradation process which occurs through cellulases is the subject of considerable research, while few studies have focused on the metabolic aspects of cellulase action (6). Genes encoding cellulases as well as the mechanism of cellulosome assembly as previously observed on cellobiose (34). The cellulose degradation process which occurs through cellulase is the subject of considerable research, while few studies have focused on the metabolic aspects of cellulase action (6). Genes encoding cellulases as well as the mechanism of cellulosome assembly (4, 6). The multienzymatic complexes found at the surface of the cells are responsible for the adhesion of bacteria to cellulose fibers and allow a very efficient synergism of action of the different enzyme components (8). Genes encoding cellulosomes as well as the mechanism of action of the cellulosomes are the subject of considerable research, while few studies have focused on the metabolic aspects of cellulose digestion by clostridia (27, 40).

Recent characterization of the carbohydrate catabolism of *Clostridium cellulolyticum*, a nonruminal mesophilic bacterium able to degrade crystalline cellulose, showed that (i) better control of catabolism occurred on a mineral salt-based medium (24, 48), (ii) carbon-limited and carbon-sufficient chemostats displayed major differences in regulatory responses of the carbon flux (25), and (iii) in nitrogen-limited conditions, glucose 6-phosphate (G6P) and glucose 1-phosphate (G1P) branch points play an important role in carbon flux divergence (22). These investigations, however, were performed with cellubiose, which is one of the soluble cellodextrins released during cellulolysis (56). In such investigations, the use of soluble sugars obviated the bacterial metabolic analysis on cellulose that was assumed difficult to undertake. Metabolic regulation processes found using cellubiose could differ or even be distorted from those with insoluble substrates.

While the first studies of cellulose focused mainly on *C. cellulolyticum* behavior, such as colonization or degradation with an insoluble substrate (19–21), recent investigations of cellulose fermentation in batch culture (12) have indicated that (i) metabolite yields depend strongly on the initial cellulose concentration and (ii) early growth arrest is linked to pyruvate overflow as in cellubiose batch culture (23).

In the last decade, efficient continuous-culture devices for

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growth on insoluble compounds have been developed (30, 31, 33, 37, 46, 63, 74) and used mainly to estimate the kinetics of cellulose degradation or colonization by various bacteria (1, 43, 58, 59, 71). Continuous culture is also a particularly useful and powerful tool for analyzing the physiology of microorganisms (42, 64).

The aim of this study was to investigate the carbon flow distribution and degradative characteristics of *C. cellulolyticum* when grown in mineral salt-based medium with cellulose, its natural substrate, in chemostat culture.

**MATERIALS AND METHODS**

**Chemicals.** All chemicals were of highest-purity analytical grade. Unless mentioned otherwise, commercial reagents, enzymes, and coenzymes were obtained from Sigma Chemical Co., St. Louis, Mo. All gases used were purchased from Air Liquide, Paris, France.

**Organism and medium.** *C. cellulolyticum* ATCC 35319 was originally isolated from decayed grass (52). Stocks of spores, stored at 4°C, were transferred to broth sample by HClO₄, using a rapid extraction system (24).

**Analytical procedures.** Biomass was estimated by bacterial protein measurement (46) using the Bradford dye method (10) as previously described (12).

**Cellulose concentration** was determined as described by Huang and Forsberg (29), using a washing procedure (69) and quantification by the phenol-sulfuric acid method (13) as already reported (12).

**The relative crystallinity index of the cellulose was determined by the procedure of Shi and Weimer (59).**

**Hydrogen and carbon dioxide** were analyzed on a gas chromatography unit as previously described (24).

**Extracellular proteins and amino acids** were assayed as previously reported (22–25), using the Bradford dye method (10) and the procedure of Mokrasch (41), respectively.

**Glucose** was assayed enzymatically, using glucose oxidase and peroxidase with o-dianisidine as a chromophore.

**Soluble cellodextrins** were quantitatively assayed by high-performance liquid chromatography (HPLC) using refractive index detector and qualitatively using thin-layer chromatography (TLC) as already described (22).

**Glycogen determination** were performed using amyloglucosidase (EC 3.2.1.3) according to the procedure of Matheron et al. (38) as previously indicated (22).

Acetate, ethanol, lactate, and succinate were estimated by using the appropriate enzyme kits (Boehringer Mannheim, Meylan, France).

Extracellular pyruvate was assayed enzymatically by fluorometric detection of NADH as previously described (24).

**Enzyme assays.** Cells were centrifuged (12,000 × g, 15 min, 4°C), and pellets were rapidly frozen with liquid nitrogen and stored at −80°C. Cells were resuspended in Tris-HCl buffer (50 mM, 2 mM dithiothreitol [DTT] [pH 7.4]) and then sonicated four times for 20 s each with a break of 60 s at a frequency of 20 kilocycles s⁻¹. The supernatant was collected from the cell lysate following centrifugation (12,000 × g, 20 min, 4°C). The protein content of extracts was determined by the method of Bradford (10), using crystalline bovine serum albumin as the standard. Anaerobic conditions were maintained throughout the entire procedure, and all manipulations were performed under oxygen-free nitrogen atmosphere. All enzyme assays were performed at 34°C. Specific activity was determined in a range where linearity with protein concentration was established. For calculation of enzymatic activity, the molar extinction coefficients used for 5,5’-dithiobis-(2-nitrobenzoic acid), methyl viologen, and NAD(P)H were 13.6 (15), 7.71 (50), and 6.22 mM⁻¹ cm⁻¹ (55), respectively.

The phosphoglucomutase (PGM; EC 5.4.2.2) assay was based on the method of Lowry and Passonneau (36). The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 20 mM DTT, 10 mM MgCl₂, 1 mM AMP, 1 mM NAD⁺, 2 mM G1P, 3 mM glucose 1.6-diphosphate, and 4 U glucose-6-phosphate dehydrogenase (EC 1.1.1.49).

Glyceroldehyde 3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) activity was determined by the method of Ferdinand (16).

Pyruvate-ferridoxin oxidoreductase (PFO; EC 1.2.7.1) was assayed as described by Meinecke et al. (39). The reaction mixture contained 25 mM potassium phosphate buffer (pH 7.2), 0.14 mM NADH, and 2 U acetyl-CoA. The assay was carried out anaerobically at 34°C for 10 min.

**Acetate kinase (AK; EC 2.7.2.1) activity** was determined by coupling hexokinase (EC 2.7.1.1) and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and by following the NADP-dependent oxidation of G6P to 6-phosphogluconate at 340 nm (32).

Acetate dehydrogenase (AADH; EC 1.2.1.10) was assayed as described by Durre et al. (14). The reaction mixture contained 0.1 M Tris-HCl (pH 7.2), 2 mM DTT, 72 mM semicarbazide, 0.2 mM NADH, and 0.6 mM acetyl-CoA.

**Acetate kinase** was determined by the method of Ferdinand (16).

**Acetate dehydrogenase** was determined by coupling hexokinase and glucose 6-phosphate dehydrogenase complex from pig heart to initiate CoA consumption. After complete depletion of the CoA in the extract, 2 U of citrate synthase (EC 4.1.3.7) and 4 mM oxaloacetate were added to measure the acetyl-CoA concentration.

Fluorimetric determination of G1P and G6P was performed on the basis of the G6P assay described above, using glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and G6P (EC 5.4.2.2) as previously described (22).

Calculation. The metabolic pathways and equations for cellulose fermentation by *C. cellulolyticum*, expressed as n hexose equivalents corresponding to n glucose residues of the cellulose chain, were previously reported (12).

\[ q_{\text{cellulose}} \text{is the specific rate of hexose residue fermented in millimoles per gram of cells per h; } q_{\text{acetate}}, q_{\text{ethanol}} \text{and } q_{\text{lactate}} \text{are the specific rates of product formation.} \]
formation in millimoles per gram of cells per hour. \( q_{\text{extracellular pyruvate}} \) is the specific rate of extracellular pyruvate formation in micromoles per gram of cells per hour. \( q_{\text{pyruvate}} \) is the specific rate of pyruvate used in millimoles per gram of cells per hour, determined as follows: \( q_{\text{pyruvate}} = q_{\text{acetate}} + q_{\text{lactate}} + q_{\text{extracellular pyruvate}} \times \text{NADH produced} \) and \( q_{\text{NADH consumed}} \) the specific rate of NADH production and NADH consumption, respectively, in millimoles per gram of cells per hour. \( \text{NADH consumed} \) was calculated as follows: \( q_{\text{NADH produced}} = q_{\text{pyruvate}} + q_{\text{lactate}} + q_{\text{acetate}} \). The energetic charge and oxidation/reduction (O/R) index were calculated according to Gottschalk (26).

The catabolic reduction charge (CRC) and anabolic reduction charge (ARC) were calculated as follows: CRC = NADH/(NAD(NADH + NAD\(^+\)) and ARC = NADPH/(NADPH + NADP\(^+\)).

The molar growth yield \( (Y_{\text{X}}) \) was expressed in grams of cells per mole of hexose equivalents fermented. The Pirt plot was used for determination of maximum yield and maintenance coefficient with the following equation (53): \( 1/Y_{\text{X}} = 1/sY_{\text{max}} + m \cdot t_g \), where \( Y_{\text{max}} \) is the true yield (in the absence of maintenance requirements), \( s \) the observed yield, \( m \) is the maintenance coefficient, and \( t_g \) is the retention time (i.e., \( t_g = 1/D \), where \( D \) is the dilution rate).

The first-order rate constant of cellulose removal was determined using the model equation (47) \( \ln S_0 = k \cdot t + x \), where \( S_0 \) is the concentration of cellulose in the feed reservoir, \( S_t \) is the concentration of cellulose in the culture vessel, \( k \) corresponds to the rate constant of cellulose degradation, and \( x \) is equal to 1 since it corresponds to the y intercept (i.e., \( t_g = 0 \) and thus \( S_0S_x = 1 \)).

**Mapping of the carbon flow.** Distribution of the carbon flow was determined by adapting the model developed by Holms (28) to C. cellulolyticum metabolism. In the steady state, the flux through each enzyme of the known metabolic pathway was determined as specified in Table 1. Carbon fluxes were expressed in millimoles per gram of cells per hour.

It was assumed that the intracellular \( \beta \)-glucan composed of \( n \) hexose residues was catabolized according to the model proposed by Stobro (62) as previously described (12). If \( n = 2 \), then \( \beta \)-glucan \( + P_1 \rightarrow G1P + \beta \)-glucan \( (n - 1) \), through cellobextrin and cellobiose phosphorylase. If \( n = 1 \), then glucose \( + \text{ATP} \rightarrow \text{G6P} + \text{ADP} \) via glucokinase. As a result, the entering carbon flow directed toward G6P was \( \frac{1}{2} q_{\text{cellulose}} \) and that toward G1P was \( \frac{1}{2} q_{\text{cellulose}} \). For example, if \( n = 1 \) (glucose) for the entering carbohydrate, then \( \frac{1}{2} q_{\text{cellulose}} \) to G6P is equal to \( q_{\text{cellulose}} \) and that toward G1P is \( \frac{1}{2} q_{\text{cellulose}} \). When Glc1P, then the \( \frac{1}{2} q_{\text{cellulose}} \) toward G6P equals to \( q_{\text{cellulose}} \) and that toward G1P is \( \frac{1}{2} q_{\text{cellulose}} \) since 1 glucose and 1 G1P are formed. If \( n = 3 \) (cellotriose) for the entering soluble biopolymer, then the \( \frac{1}{2} q_{\text{cellulose}} \) toward G6P is equal to \( \frac{1}{2} q_{\text{cellulose}} \) and that toward G1P is \( \frac{1}{2} q_{\text{cellulose}} \) since 1 glucose and 2 G1P are formed. For soluble \( \beta \)-glucans (\( n \)), \( 1 \leq n \leq 7 \), average carbon flows directed toward G6P of 0.37 \( q_{\text{cellulose}} \) and toward G1P of 0.63 \( q_{\text{cellulose}} \) were calculated.

The turnover of a pool per hour was calculated from the specific rate and pool size expressed in moles or in carbon equivalents (22). It corresponded to the rate of input or output divided by the pool size, which is then the number of times the pool turns over every hour.

**RESULTS**

**Cellulose digestion and biomass formation.** Preliminary results in chemostats indicated that with between 2 and 6 g of cellulose liter\(^{-1}\) and at a dilution rate of 0.025 h\(^{-1}\), biomass formation increased proportionately to cellulose concentration (data not shown). C. cellulolyticum was then grown with 3.7 g of cellulose liter\(^{-1}\) at different steady-state combinations of \( D \)—equal to the microbial specific growth rate (\( \mu \))—which ranged from 0.014 to 0.083 h\(^{-1}\) (Table 2); steady-state growth on cellulose was attained neither at \( D < 0.01 \) h\(^{-1}\) nor at \( D > 0.09 \) h\(^{-1}\) since washout then occurred. The latter value corroborated the maximum growth rate \( (\mu_{\text{max}}) \) of 0.087 h\(^{-1}\) (i.e., a generation time of 8 h) estimated by following growth with the rate of tritiated thymidine incorporation into DNA during the exponential phase of batch growth on cellulose (20). Cell density was maximum at low \( D \), i.e., 0.207 g liter\(^{-1}\) at 0.014 h\(^{-1}\); but slowly decreased with increasing \( D \) to reach 0.154 g liter\(^{-1}\) at 0.083 h\(^{-1}\) (Table 2). In all of the runs, microscopic examinations of the cultures revealed that almost all of the cellulose fibers were colonized by bacteria; the few particles that were not were most likely those that had been recently introduced into the bioreactor (74). Unattached cells were mostly observable under conditions of low \( D \).
TABLE 2. Fermentation parameters from continuous steady-state cultures of *C. cellulo-
lyticum*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0.014</th>
<th>0.027</th>
<th>0.046</th>
<th>0.064</th>
<th>0.076</th>
<th>0.083</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomass (g liter(^{-1}))</td>
<td>0.207</td>
<td>0.196</td>
<td>0.185</td>
<td>0.172</td>
<td>0.154</td>
<td>0.136</td>
</tr>
<tr>
<td>Consumed cellulose (mmol of hexose eq)</td>
<td>17.59</td>
<td>16.79</td>
<td>15.86</td>
<td>14.96</td>
<td>13.96</td>
<td>12.96</td>
</tr>
<tr>
<td>t(_R) (h)</td>
<td>1.19</td>
<td>1.57</td>
<td>2.14</td>
<td>2.71</td>
<td>3.19</td>
<td>3.65</td>
</tr>
<tr>
<td>Glycerogen (mg (g of cells)(^{-1}))</td>
<td>0.90</td>
<td>0.49</td>
<td>0.36</td>
<td>0.26</td>
<td>0.19</td>
<td>0.15</td>
</tr>
<tr>
<td>Protein (mg liter(^{-1}))</td>
<td>138.9</td>
<td>138.9</td>
<td>138.9</td>
<td>138.9</td>
<td>138.9</td>
<td>138.9</td>
</tr>
<tr>
<td>Free amino acids (mg liter(^{-1}))</td>
<td>11.8</td>
<td>11.8</td>
<td>11.8</td>
<td>11.8</td>
<td>11.8</td>
<td>11.8</td>
</tr>
<tr>
<td>Y(_{X/S}) (g of cells per mol of hexose eq)</td>
<td>2.07</td>
<td>2.07</td>
<td>2.07</td>
<td>2.07</td>
<td>2.07</td>
<td>2.07</td>
</tr>
<tr>
<td>Carbon recovery (%)</td>
<td>96.5</td>
<td>98.3</td>
<td>97.1</td>
<td>97.2</td>
<td>97.4</td>
<td>97.6</td>
</tr>
</tbody>
</table>

* Where indicated, values are averages of samples at steady-state ± standard deviations. All other values were determined with an average accuracy of ±10%.

In continuous culture at 3.7 g of cellulose liter\(^{-1}\), *C. cellulo-
lyticum* always left some undigested cellulose (Fig. 1). The longer was the solid retention time (t\(_R\)), the higher was the percentage of cellulose degradation: from 0.014 h\(^{-1}\) (i.e., t\(_R\) = 71.4 h) to 0.083 h\(^{-1}\) (i.e., t\(_R\) = 12.0 h), the percentages of digested cellulose at steady state were 75.0 and 20.9, respectively (Fig. 1). Regardless of D, glucose or cellodextrins could not be detected in the supernatant using enzymatic, HPLC, and TLC techniques, and so cellulose hydrolysis did not yield a significant pool of soluble sugars. Crystallinity measurements showed that the relative crystallinity index of cellulose in chemostat at 0.014 h\(^{-1}\) was 88.8, compared to 89.6 for the original cellulose MN301. Thus, even at long t\(_R\), the residual cellulose was not enriched in its crystalline content during the fermentation. Plots of S\(_N\)/S\(_0\) versus t\(_R\) were linear, and so cellulose digestion follows first-order kinetics where linear regression of the data (r\(^2\) = 0.976) gives a first-order rate constant for cellulose removal of 0.05 h\(^{-1}\) (Fig. 1).

The observed cell yields increased with increasing D and varied from 11.8 to 32.8 g of cells per mol of hexose eq consumed (Table 2). From the observed growth yield, which is affected by microbial endogenous metabolism and maintenance energy requirements, the true growth yield was calculated. A Pirt plot (r\(^2\) = 0.987) allowed determining the maintenance coefficient and Y\(_{X/S}\), which were estimated at 0.9 mmol of hexose eq/g of cells/h and 50.5 g of biomass/mol of hexose eq consumed, respectively.

**Metabolite production.** Acetate, ethanol, lactate, H\(_2\), and CO\(_2\) were the primary metabolic end products, and no succinate accumulation was observed (Table 2). The percentage of carbon flow toward fermentative metabolites, given by the ratio q\(_{pyruvate}/q_{cellulose}\), indicated that 67.0 to 81.5% of the consumed cellulose was converted to extracellular pyruvate, lactate, CO\(_2\), acetate, and ethanol. The remaining carbon flow was oriented toward amino acid, protein, biomass, and exopolysaccharide formation. Exopolysaccharides could not be measured as previously described (48) because of significant inter-
progressive shift toward ethanol production with increasing carbon flow was for acetate and ethanol production, with a polymerization, i.e., the number of glucose residues inside the cells.

The carbon balance, calculated by taking into account amino acids, proteins, fermentative end products, and biomass, was then in a range between 95.8 and 98.7%.

Microscopic examination. The carbon balance, calculated by taking into account amino acids, proteins, fermentative end products, and biomass, was then in a range between 95.8 and 98.7%.

The synthesis of biomass—with an elemental composition of C₆H₆O₆ (24)—from cellulose digestion can be represented by the overall scheme C₆H₁₀O₅ + 1.5 NH₃ → 1.5 C₆H₁₂O₆ + 2.4 H₂O. Since no available hydrogen atoms were used to equilibrate the equation, this meant that when bacteria grow on cellulose, reducing equivalents NAD(P)H are well balanced by biomass synthesis. It was assumed that CO₂ production through general decarboxylation enzymes and CO₂ fixation in biomass gave an value of almost nil (24, 44, 45).

Energetic and redox balance. The stoichiometry of ATP generated over hexose equivalents fermented, i.e., ATP-Eff, was higher at low D since it was 2.72 and declined to 2.07 with the highest D value (Table 3) due to the decrease of the percentage of acetate production (Table 2). As D increased, qₐₐₜₑₑₚ increased from 3.24 to 5.25 mmol (g of cells)⁻¹ h⁻¹ (Table 3). The apparent energetic yield increased with increasing D as well (Table 3). The low Yₐₜₑₑₚ obtained at low μ reflected an expenditure of energy due to the more pronounced maintenance requirement at low μ. The Pirt plot (r² = 0.984) permitted determining a true energetic yield (Yₐₜₑₑₚ) of 30.3 g of cells (mol of ATP)⁻¹ and a maintenance energy (mₐₜₑₑₚ) estimated at 2.9 mmol of ATP (g of cells)⁻¹ h⁻¹.

Whatever the μ, the pool of ATP and ADP rose while AMP remained quite constant at ca. 0.10 μmol (g of cells)⁻¹. The ratio ATP/ADP fluctuated between 0.37 and 0.46, while a mean value of 0.63 was obtained for the adenylate energy charge.

From the known catabolic pathways which produced and consumed reducing equivalents, the coenzyme balance could be calculated. qₐₐₜₑₑₚ increased with increasing D, from 1.94 to 3.39 mmol (g of cells)⁻¹ h⁻¹, as did qₐₐₜₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑᵣ the number of glucose residues inside the biopolymer. From cellulose degradation, it was assumed that soluble cellodextrins with a degree of polymerization n between 1 and 7 could potentially be incorporated and fermented by the cells (12, 51, 56). This corresponds to an average formula of hexose equivalents from glucose to cellulose-204, utilized by bacteria of C₆H₁₂O₅.

FIG. 2. Influence of D on qacetate (○), qethanol (■), and qlactate (▲).
Results obtained at $D = 0.014$ to $0.083$ h$^{-1}$.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Results obtained at $D (h^{-1})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH [$\mu$mol (g of cells)$^{-1}$]</td>
<td>$3.29 \pm 0.05$ to $4.14 \pm 0.09$</td>
</tr>
<tr>
<td>NADPH [$\mu$mol (g of cells)$^{-1}$]</td>
<td>$7.47 \pm 0.16$ to $8.04 \pm 0.02$</td>
</tr>
<tr>
<td>NADP [$\mu$mol (g of cells)$^{-1}$]</td>
<td>$0.74 \pm 0.03$ to $0.99 \pm 0.04$</td>
</tr>
<tr>
<td>G6P [$\mu$mol (g of cells)$^{-1}$]</td>
<td>$5.20 \pm 0.27$ to $14.32 \pm 0.02$</td>
</tr>
<tr>
<td>6PG [$\mu$mol (g of cells)$^{-1}$]</td>
<td>$1.71 \pm 0.08$ to $1.97 \pm 0.01$</td>
</tr>
<tr>
<td>HCO$_3$-$[$ meq of C (g of cells)$^{-1}$]</td>
<td>$0.98 \pm 0.09$ to $1.56 \pm 0.05$</td>
</tr>
<tr>
<td>O/R index</td>
<td>0.99 to 0.97</td>
</tr>
</tbody>
</table>

Concerning the phosphopyridine nucleotides involved in biosynthesis pathways, the reduced form was in excess since the NADPH pool was hardly detectable (Table 4). As a result, the ARC was constant and equal to 1.00, meaning that NADPH was largely available for biosynthesis reaction.

**Kinetic analysis of cellulose fermentation.** The effects of $D$ on cellulose consumption and product formation are summarized in Fig. 3. The rate of cellulose consumption varied from 5.14 to 15.20 meq of C $(g$ of cells)$^{-1}$ h$^{-1}$ with increasing $D$.

Carbon conversion to biomass, extracellular proteins, and free amino acids increased from 1.03 to 4.14 meq of C $(g$ of cells)$^{-1}$ h$^{-1}$ (Fig. 3) and reached 27.3% of the original carbon uptake at $D = 0.083$ h$^{-1}$. Most of the carbon used for these biosyntheses, however, was converted to biomass since regardless of $D$, both extracellular proteins and free amino acids represented a constant proportion of the original carbon, around 7.5%.

The cellulose catabolism leading to pyruvate from which fermentation end products were formed—i.e., acetate, CO$_2$, extracellular pyruvate, ethanol, and lactate—increased from 5.82 to 10.18 meq of C $(g$ of cells)$^{-1}$ h$^{-1}$ (Fig. 3) and represented 27.3% of the original carbon uptake at $D = 0.083$ h$^{-1}$.

While through the acetate pathway this percentage represented a constant proportion of the original carbon, around 7.5%, the flux through the acetate pathway decreased from 39.6 to 27.1%, while through the ethanol production route this percentage rose from 13.5 to 16.8. $q_{acetate}$ increased only from 0.11 to 0.13 meq of C $(g$ of cells)$^{-1}$ h$^{-1}$ and represented a small portion of the carbon uptake since it dropped from 1.5 to 0.9%. The specific rate of extracellular pyruvate formation ranged from 0.02 to 0.04 mmol $(g$ of cells)$^{-1}$ h$^{-1}$, and this leak toward the outside of the cells bottomed out at 0.2% of the carbon uptake.

Another part of the entering carbon flow was directed toward exopolysaccharide synthesis (Fig. 3), which increased from 0.25 to 0.63 meq of C $(g$ of cells)$^{-1}$ h$^{-1}$ and represented up to 4.2% of the specific consumption rate of cellulose.

**Intracellular pool of hexose phosphate and CoA derivative.** The G6P pool was fueled by the carbon flow from glucokinase activity and by PGM activity from the G1P pool (Fig. 3). At this metabolic node, the G6P pool decreased with increasing growth rate (Table 5), indicating that the turnover of the pool increased and actually reached 212.3 h$^{-1}$ at $\mu = 0.083$ h$^{-1}$ (Fig. 3). This variation was correlated with the increase of the carbon flow through glycolysis and biosynthesis metabolic pathways with higher $\mu$. The $q_{G6P}$ increased from 6.86 to 14.32 meq of C $(g$ of cells)$^{-1}$ h$^{-1}$ with higher $D$ (Fig. 3); whatever the $D$, it represented a mean of 96.0% of the carbon uptake. The G1P pool rose from 5.71 to 20.12 mmol $(g$ of cells)$^{-1}$ with $D$ (Table 5) and thus corresponded to a decrease in the turnover of the pool (Fig. 3). As a result, the G6P/G1P ratio ranged from 6.48 to 0.56 with increasing $\mu$ (Table 5).
From the G1P junction, the carbon could be either stored as glycogen or converted to exopolysaccharide, or it could be directed more toward glycolysis via PGM (Fig. 3). Whatever the D, no cellotriose could be detected and G1P was metabolized as exopolysaccharides. With increasing D, the carbon flow via PGM increased from 4.21 to 8.70 meq of C (g of cells)\(^{-1}\) h\(^{-1}\), and the percentage of the original carbon flowing through this metabolic pathway remained quite constant, ranging from 59.0 to 57.2%. \(q_{\text{glycogen}}\) increased from 0.03 to 0.24 meq of C (g of cells)\(^{-1}\) h\(^{-1}\) and represented up to 1.6% of the entering carbon flow. The turnover of the glycogen pool remained low since it increased from 0.02 to 0.09 times per hour with the highest D value and was correlated with increasing \(q_{\text{pyruvate}}\) and \(q_{\text{biosynthesis}}\).

The pool of CoA was formed from phosphotransacetylase and acetaldehyde dehydrogenase, and that of acetyl-CoA was calculated as 3.42 to 12.05 mM (Table 5); these data suggest that the PGM reaction was zero-order kinetics with respect to GAPDH level increased 3.3-fold. From 0.014 to 0.083 h\(^{-1}\), PFO increased 1.7-fold, PTA increased 1.4-fold, AK increased 7.1-fold, AADH increased 3.2-fold, and ADH increased 2.9-fold.

When the flux was expressed as micromoles per milligram per hour of protein from previously calculated values (Fig. 3), the ratio R (specific enzyme activity in biomass/metabolic flux) could be calculated (28). For metabolic pathways leading to acetate production via PTA and AK, R varied between 22.1 and 23.9 and from 6.9 to 33.6, respectively; with PFO, R was ca. 10.2. Similarly, R for enzymes of the ethanol pathway ranged from 5.4 to 10.6 and from 14.9 to 18.5 for AADH and ADH, respectively. However, with the metabolic route through PGM, this ratio was constant at only ca. 1.2. This indicated that flux via PGM utilized all of the available activity of the enzyme. The specific activity of PGM increased 2.1-fold from lowest to highest D (Table 6) and was thus correlated with higher specific metabolic rates. These results mean that the enzyme regulates activity in the cell to balance the flux from G1P to G6P whatever the \(\mu\). An apparent \(k_m\) of 0.21 mM for G1P catalyzed by PGM was calculated from an Eadie-Hofstee plot (17) (data not shown). By assuming an internal volume of 1.67 ml (g of cells)\(^{-1}\) (24), the steady-state internal G1P concentration was calculated as 3.42 to 12.05 mM (Table 5); these data suggest that the PGM reaction was zero-order kinetics with respect to catalyzed substrate, which is in good agreement with the analysis of metabolic flux. In contrast, the ratio R for the lactate

---

**TABLE 5. Intracellular metabolite levels of continuous steady-state cultures of C. cellulolyticum**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0.014</th>
<th>0.027</th>
<th>0.035</th>
<th>0.041</th>
<th>0.055</th>
<th>0.064</th>
<th>0.076</th>
<th>0.083</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6P (^{\text{a}}) (\mu\text{mol (g of cells)}^{-1})</td>
<td>36.98 ± 1.14</td>
<td>35.76 ± 0.92</td>
<td>29.98 ± 0.79</td>
<td>26.31 ± 0.73</td>
<td>16.64 ± 0.58</td>
<td>14.28 ± 0.51</td>
<td>11.29 ± 0.62</td>
<td>11.24 ± 0.55</td>
</tr>
<tr>
<td>G1P (^{\text{a}}) (\mu\text{mol (g of cells)}^{-1})</td>
<td>5.71 ± 0.33</td>
<td>8.01 ± 0.40</td>
<td>9.65 ± 0.32</td>
<td>10.99 ± 0.54</td>
<td>14.16 ± 0.61</td>
<td>15.96 ± 0.57</td>
<td>18.23 ± 0.75</td>
<td>20.12 ± 0.68</td>
</tr>
<tr>
<td>G6P/G1P ratio</td>
<td>6.47</td>
<td>4.46</td>
<td>3.11</td>
<td>2.39</td>
<td>1.89</td>
<td>0.89</td>
<td>0.62</td>
<td>0.57</td>
</tr>
<tr>
<td>Acetyl-CoA (\mu\text{mol (g of cells)}^{-1})</td>
<td>2.51 ± 0.18</td>
<td>2.98 ± 0.21</td>
<td>3.56 ± 0.23</td>
<td>3.81 ± 0.25</td>
<td>5.75 ± 0.39</td>
<td>6.27 ± 0.42</td>
<td>7.06 ± 0.46</td>
<td>8.37 ± 0.56</td>
</tr>
<tr>
<td>CoA (\mu\text{mol (g of cells)}^{-1})</td>
<td>0.48 ± 0.03</td>
<td>0.59 ± 0.04</td>
<td>0.74 ± 0.06</td>
<td>0.87 ± 0.05</td>
<td>1.29 ± 0.09</td>
<td>1.54 ± 0.11</td>
<td>2.12 ± 0.14</td>
<td>2.86 ± 0.19</td>
</tr>
<tr>
<td>Acetyl-CoA/CoA ratio</td>
<td>5.23</td>
<td>5.05</td>
<td>4.81</td>
<td>4.38</td>
<td>4.46</td>
<td>4.07</td>
<td>3.33</td>
<td>2.93</td>
</tr>
</tbody>
</table>

\(^{\text{a}}\) See Table 2, footnote b.

---

**TABLE 6. Specific enzymatic activity and flux relative to available enzyme activity in C. cellulolyticum cell extract at steady-state growth**

<table>
<thead>
<tr>
<th>Enzyme [^{\text{a}}]</th>
<th>0.014</th>
<th>0.035</th>
<th>0.064</th>
<th>0.083</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean SEA (\mu\text{mol min}^{-1}) (mg of protein)(^{-1}) SD</td>
<td>R</td>
<td>Mean SEA (\mu\text{mol min}^{-1}) (mg of protein)(^{-1}) SD</td>
<td>R</td>
<td>Mean SEA (\mu\text{mol min}^{-1}) (mg of protein)(^{-1}) SD</td>
</tr>
<tr>
<td>GADPH (EC 1.2.1.2)</td>
<td>1.13 ± 0.19</td>
<td>2.37 ± 0.28</td>
<td>2.82 ± 0.31</td>
<td>3.81 ± 0.45</td>
</tr>
<tr>
<td>PGM (EC 5.4.2.2)</td>
<td>0.021 ± 0.006</td>
<td>1.3</td>
<td>0.028 ± 0.008</td>
<td>1.2</td>
</tr>
<tr>
<td>LDH (EC 1.1.1.27)</td>
<td>0.13 ± 0.01</td>
<td>150.6</td>
<td>0.26 ± 0.03</td>
<td>298.3</td>
</tr>
<tr>
<td>PFO (EC 1.2.7.1)</td>
<td>0.44 ± 0.04</td>
<td>10.2</td>
<td>0.57 ± 0.06</td>
<td>9.8</td>
</tr>
<tr>
<td>PTA (EC 2.3.1.8)</td>
<td>0.76 ± 0.08</td>
<td>23.9</td>
<td>0.95 ± 0.01</td>
<td>23.2</td>
</tr>
<tr>
<td>AK (EC 2.7.2.1)</td>
<td>0.22 ± 0.03</td>
<td>6.9</td>
<td>0.46 ± 0.04</td>
<td>11.3</td>
</tr>
<tr>
<td>AADH (EC 1.2.1.10)</td>
<td>0.09 ± 0.01</td>
<td>7.9</td>
<td>0.09 ± 0.02</td>
<td>5.4</td>
</tr>
<tr>
<td>ADH (EC 1.1.1.1)</td>
<td>0.17 ± 0.02</td>
<td>15.2</td>
<td>0.25 ± 0.03</td>
<td>14.9</td>
</tr>
</tbody>
</table>

[^{\text{a}}]: SEA, specific activity of enzyme; R, ratio of specific enzymatic activity to metabolic flux through the metabolic pathway (see Results).
formation pathway was always very high, ranging from 150.6 to 434.2. This indicates that the enzyme concentration increases as catabolic carbon flow increases but was not correlated with a proportional rise of the specific production rate of lactate; LDH increased 3.5-fold, from 0.014 to 0.083 h⁻¹ (Table 6), while $q_{\text{lactate}}$ varied only slightly (Fig. 3).

**DISCUSSION**

Based on microscopic observations, cellulose fibers were found to be covered by the microbial cell; such continuous cultures are generally regarded as cellulose limited (59, 71, 74). Yet cells which adhere to cellulose can also be considered to be in substrate-sufficient conditions. In fact, soluble sugars liberated from cellulose are the real substrate for growth and are incorporated by cells as rapidly as they are formed, since no glucose or cellohexitols could be detected in the supernatant. It may therefore be possible that bacteria display a metabolism under or near carbon-sufficient conditions and not carbon-limited conditions as previously suggested by microscopic examination. In cellbiose-sufficient conditions, the ATP/ADP ratios were as high as 7.21 and always higher than 1 (25), while in cellbiose limitation the ATP/ADP ratios ranged between 0.21 and 0.69 (E. Guedon, M. Desvaux, and H. Petitdemange, unpublished data). In the cellulose chemostat cultures carried out, the ATP/ADP ratios ranged from 0.37 to 0.46 and were always lower than 1 as in carbon-limited chemostats. In cellulbiose limitation, few lactate molecules were produced at low $D$, contrary to what was observed in cellbiose-sufficient continuous culture (25). Moreover, on cellulose continuous culture, the ratios NADH/NAD⁺ and $q_{\text{NADH produced}}/q_{\text{NADH used}}$ dropped as $D$ increased, while in cellbiose-sufficient conditions these ratios rose (25). All of these results lead to the conclusion that the cellulose continuous cultures described here were performed under carbon limitation; it can thus be maintained that the chemostats were cellulose-limited continuous cultures.

In such conditions, the cellulose catabolism of *C. cellulolyticum* led to an acetate-ethanol fermentation maximizing ATP production. Concerning the distribution of the fluxes throughout the metabolic network, at least 94.2% of the carbon flow was used for generation of energy and biosynthetic precursors; the remaining carbon was converted to glycogen and exopolysaccharides. The proportion of carbon flow through G6P remained quite constant regardless of $D$ but differed between $q_{\text{biosynthesis}}$ and $q_{\text{pyruvate}}$ as $\mu$ increased; more of the carbon uptake was directed toward biosynthesis pathways at high $D$ values than at lower ones. Regardless of $D$, extracellular proteins and free amino acids represented around 7.5% of the entering carbon. The carbon flow was always directed mainly toward acetate production, which represented a minimum of 27.1% of the original carbon at the highest $\mu$. Yet the carbon from glycolysis directed through biosynthesis and ethanol increased to 27.3 and 16.8%, respectively, at $D = 0.083$ h⁻¹. Theoretical calculation suggest on the one hand that with *Trichoderma reesei* used as a model, optimal growth of a cellulolytic anaerobe requires major allocation of ATP toward cellulase biosynthesis (70) and on the other hand that with low ATP production per mole of hexose equivalent, most of the carbon source is used for the generation of energy (57). From lowest to highest $D$, *C. cellulolyticum* diverted 7.8 to 21.6% of the cellulose to cell carbon, whereas 81.3 to 66.7% was used for ATP production.

On cellulose, G6P-G1P was an important branch point since the longer the soluble β-glucan uptake period is, the more G1P will be generated. The ratio of PGM specific activity to $q_{\text{PGM}}$ (close to 1) reflected the precision of the control exerted by this enzyme on the partition of the flux at this junction (28), where the conversion of G1P to G6P feeds further the Embden-Meyerhof pathway. A high $R$ indicates that the fluxes are determined by the concentration of substrate available in the pool rather than the enzyme activity (28). However, intracellular concentrations of substrates, products, cofactors, or effectors molecules as well as intracellular ionic strength, redox potential, or pH could influence the partition and regulation of flux at each step in the central metabolic pathways (28). In vitro enzyme assays could then differ from in vivo conditions, and the significance of a low $R$ could be more difficult to establish. Control of the PGM pathway by the amount of PGM was reinforced by the finding that $K_m$ was more than 10-fold lower than the lowest intracellular G1P concentration determined. With higher $D$, the flow through glycolysis and biosynthesis increased, as did G6P turnover. The G1P pool, however, increased because the proportion of carbon flow via PGM did not vary as much. G1P was thus directed toward exopolysaccharide formation, which reached a maximum of 4.2% of the original carbon uptake. The proportion of the flow through glycogen synthesis increased as well and represented up to 1.6% of the entering carbon flow. Here, glycogen turnover increased with $D$ but remained low (maximum of 0.09 h⁻¹), while the glycogen pool increased up to 108.7 mg (g of cells)⁻¹ at 0.035 h⁻¹ and then slowly decreased; glycogen biosynthesis could be adjusted as a function of the carbon flow. At the G1P-G6P branch point, the flux partitioning was then stabilized via PGM while the carbon surplus was dissipated by exopolysaccharide and intracellular glycogen synthesis. In fact, the percentage of G1P converted to G6P via PGM remained constant at around 93.7%, whereas the proportion of G1P converted to glycogen increased from 0.7 to 2.6% between $D = 0.014$ and 0.083 h⁻¹. In the same time, exopolysaccharide represented around 5.3% of the G1P produced. These productions seem to buffer the increasing carbon flow from G1P, which could not be metabolized via PGM. On cellulose-limited chemostat cultures, carbon excess at this branch point was limited compared to ammonium-limited chemostat culture performed on cellulbiose (which corresponded to an uncou-
plunging between catabolism and anabolism), where exopolysaccharides and glycogen could represent up to 16.0 and 21.4%, respectively, of the specific rate of carbon consumed and where cellulotriose was detected extracellularly (22). Glycogen was synthesized even in carbon-limited conditions and was present at all dilution rates. Such observations parallel those for *Fibrobacter succinogenes*, for which futile cycling of glycogen was reported (18, 38). These results could suggest that glycogen biosynthesis in cellulolytic bacteria involved in carbon flow regulation (7) rather than in prolonging cell viability by providing energy storage (54) as for sporogenesis (49). Such a cycle could also be considered an energy-wasting reaction, as suggested by use of the term “futile cycle” for glycogen metabolism in *Fibrobacter*; however, here cellulose-fed continuous cultures were energy-limited cultures which do not normally waste ATP (57).

The flow of carbon was facilitated by the increase of specific enzymatic activity as $\mu$ increased. The fact that metabolic fluxes were much less than the available enzyme activities (except for PGM as specified above) indicated that specific enzyme activities were limited by the supply of substrate. The decline of acetyl-CoA turnover was corroborated by the analysis of metabolic flux distribution; the percentage of the carbon fluxes (excluding PFO) was much less than the available enzyme activities (except for PGM as specified above) indicated that specific enzyme activities were limited by the supply of substrate. The regulation of NADH-fd reductase activities which, through the consumption of NADH, direct the fluxes of metabolites in the direction of acetate production was energy-limited cultures which do not normally waste ATP (57).

The growth maintenance coefficient reported in the literature could not be truly comparable since the rate of consumed hexose required for maintenance is related to the metabolic

### Table 7. Kinetic and growth parameters of various species of cellulolytic bacteria determined in continuous cultures

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Substrate</th>
<th>$k$ (h$^{-1}$)</th>
<th>$m$ (mmol of hexose eq [g of cells]$^{-1}$ h$^{-1}$)</th>
<th>$Y_{\text{ATP}}$ (g of cells [mol of hexose eq]$^{-1}$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Clostridium cellulolyticum</em> ATCC 35319</td>
<td>MN301</td>
<td>0.05</td>
<td>0.9</td>
<td>50.5</td>
<td>This study</td>
</tr>
<tr>
<td>C. thermocellum ATCC 27405</td>
<td>Cellulose</td>
<td>0.09</td>
<td>ND</td>
<td>36.2</td>
<td>25</td>
</tr>
<tr>
<td><em>Fibrobacter succinogenes</em> S85</td>
<td>Avicel</td>
<td>0.17$^b$</td>
<td>0.3$^b$</td>
<td>24.3</td>
<td>37</td>
</tr>
<tr>
<td><em>Ruminococcus albus</em> 7</td>
<td>Sigmacell 20</td>
<td>0.09$^c$</td>
<td>0.3$^b$</td>
<td>56.7</td>
<td>73</td>
</tr>
<tr>
<td><em>R. flavefaciens</em> FD1</td>
<td>Avicel</td>
<td>0.05</td>
<td>0.6$^b$</td>
<td>20.1</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>Sigmacell 20</td>
<td>0.11</td>
<td>0.4$^b$</td>
<td>38.9</td>
<td>75</td>
</tr>
</tbody>
</table>

$^a$ $k$ is the rate constant of cellulose degradation; $m$ is the growth maintenance coefficient; $Y_{\text{ATP}}$ is the true growth yield. ND, not determined.

$^b$ Calculated from previously reported data.

$^c$ Average of previously reported data.
pathway which allowed the ATP production (57, 61, 65). Since the catabolic networks vary among the reported bacterial species (Table 7), differences in $m$ values could be attributed to ATP generating a more or less efficient pathway that thus requires more or less hexose equivalents but can generate the same quantity of ATP per gram of cells per hour. Thus, comparison of $m_{ATP}$ between cellulolytic bacteria would be more informative for evaluating the energy maintenance requirement.

The first step in investigating *C. cellulolyticum* metabolism was the use of a chemostat technique which leads to the accumulation of NADH (48). The use of a mineral salt-based medium permitted the induction of different metabolic regulatory responses and better control of the carbon and electron flows (24). Such findings led to the hypothesis of growth adapted to nutrient-poor conditions (23). These studies, however, were performed with soluble sugars, which facilitated study of the bacterial metabolism of cellulolytic bacteria. The next step was therefore investigation of the physiology of this microorganism on an insoluble substrate, more closely related to the natural ecological niche. In the present work with cellulose-limited continuous culture, no accumulation of NADH was observed and pyruvate overflow as high as on cellobiose was observed and pyruvate overflow as high as on cellobiose limited chemostats, some metabolic regulation such as the shift from acetate-ethanol fermentation to lactate-ethanol fermentation at high catabolic rates should be interpreted as a deregulation of the metabolism attributed to the growth of *C. cellulolyticum* on soluble sugars which represent conditions far from the physical nature of the cellulose. In cellulose continuous cultures, compared to celluliose chemostats, a second regulation of the entering carbon was introduced by the depolymerization of the insoluble substrate into soluble sugars readily metabolized by bacteria for growth. This limitation led to a lower maximum specific growth rate reached on cellulose than on celluliose; in turn, pyruvate leakage was limited. The observations with celluliose chemostats should be interpreted as laboratory artifacts due to culture conditions far from those in which this bacterium has evolved in nature and emphasizes that the efficiency of catabolism is related to the degradative property of the bacterial cellulolose. In the course of evolution, the catabolic pathways are optimized as a function of the carbon flow from cellulase activities and are not adapted to higher catabolic rates as in other clostridial bacteria (43). *C. cellulolyticum* appeared, therefore, well adapted and even restricted to a low carbon flow, which is characteristic of growth on its natural substrate, cellulose.

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