Metabolism of cellobiose by *Clostridium cellulolyticum* growing in continuous culture: evidence for decreased NADH reoxidation as a factor limiting growth

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Previous results indicated that molar growth yields are reduced when *Clostridium cellulolyticum* is cultured in media containing cellobiose concentrations greater than 1 g l⁻¹. Continuous cultures were examined to determine the physiological basis of these poor growth yields. Acetate was the main product of *C. cellulolyticum* metabolism, whereas the production of reduced compounds such as ethanol or lactate was low. Such patterns of product formation were accompanied by a 12-fold increase in intracellular NADH concentration when the cellobiose flow was increased. Catabolic enzymic activities were measured *in vitro*. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), acetate kinase and phosphoroclastic activities were found at similar levels as in cells metabolizing higher substrate concentrations. In contrast, lactate dehydrogenase activity was low and correlated with the rate of lactate production. Furthermore, an inhibition of GAPDH activity by high NADH/NAD⁺ ratios was established. These results suggested that a decreased NADH reoxidation could be responsible for limiting *C. cellulolyticum* growth. Lactate and ethanol production were not sufficient to balance out the NADH produced in the GAPDH step of glycolysis. One consequence of poor NADH reoxidation would be an increase in intracellular concentration of NADH, which in turn could inhibit GAPDH activity.

Keywords: *Clostridium cellulolyticum*, continuous culture, cellobiose, NADH/NAD⁺ ratio, growth inhibition

INTRODUCTION

Cellulose is the most abundant biopolymer on earth and is the chief component of plant biomass. A community of physiologically diverse micro-organisms is responsible for the anaerobic degradation of cellulose (Beguín & Aubert, 1994). In most natural environments, mesophilic cellulolytic clostridia play a major role in cellulose decomposition (Leschine, 1995). Fundamental studies of cellulolytic clostridia were developed about 20 years ago and were devoted essentially to cellulase systems including the well-characterized cellulosome of *Clostridium thermocellum* (Beguín & Aubert, 1994). The cellulosome, which is cell-associated, exhibits multiple functions, including both cell adhesion and hydrolysis of

lignocellulosic compounds (see Felix & Ljungdahl, 1993; Bayer *et al.*, 1996 for reviews). The cellulosome is considered to mediate the cohesive association of cellulases and xylanases and to promote their synergistic action, avoiding the production of free extracellular enzymes. It is clear that the efficiency of cellulose hydrolysis correlates with the population of cellulolytic bacteria and the tight adhesion of these bacteria to their substrate (Weimer, 1996). Bacterial growth on cellulose is rather difficult to follow (Gelhaye *et al.*, 1993b) and most studies that focused on carbon metabolism in these bacteria were performed with cellobiose, the major end-product of the degradation process, which is taken up and assimilated by the cells (Ng & Zeikus, 1982; Giallo *et al.*, 1983; Strobel, 1995; Strobel *et al.*, 1995).

Clostridium cellulolyticum ATCC 35319 was isolated from decayed grass (Petitdemange *et al.*, 1984) and its growth cycle in contact with cellulose was described by

Abbreviations: fd, ferredoxin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Gelhay *et al.* (1993a, b). As found with other cellulolytic clostridia, due to early inhibition of metabolism and growth, *C. cellulolyticum* classically uses low quantities of sugar (Giallo *et al.*, 1983; Cailliez *et al.*, 1992). A mean value of 250 mg dry weight cells l⁻¹ was found for cultures grown with a cellobiose concentration of 2 g l⁻¹; above this value, no further increase in biomass occurred. In comparison with other clostridia such as *C. butyricum* or *C. acetobutylicum*, these values are low; biomasses of 3–6 g l⁻¹ are usually obtained and 30–100 g l⁻¹ carbohydrate catabolized (Vasconcelos *et al.*, 1994; Abbad-Andaloussi *et al.*, 1995). No increase in growth yield was observed by increasing the concentration of either yeast extract or Casamino acids, and growth limitation was not the result of low pH due to acid formation (Giallo *et al.*, 1983). Furthermore, end products have no inhibitory effect when added at concentrations equal to the final titres (Giallo *et al.*, 1983).

The purpose of this research was to identify the factors responsible for this growth limitation and to examine the metabolism of *C. cellulolyticum* ATCC 35319 with cellobiose as growth substrate in continuous culture.

METHODS

Chemicals. All chemicals were reagent grade. Enzymes and coenzymes were obtained from Sigma. All gases used were purchased from Air Liquide.

Organism. *C. cellulolyticum* ATCC 35319, a mesophilic cellulolytic bacterium isolated from decayed grass (Petitdemange *et al.*, 1984), was used.

Medium and culture conditions. The bacterium was grown anaerobically on a complex medium (CM3) (pH 7.8) containing (l⁻¹): KH₂PO₄, 1.4 g; K₂HPO₄·3H₂O, 2.9 g; (NH₄)₂SO₄, 1.3 g; MgCl₂·6H₂O, 0.1 g; CaCl₂·2H₂O, 0.02 g; yeast extract, 5 g; 5% (w/v) FeSO₄ in 0.1 M H₂SO₄, 25 µl; cysteine hydrochloride, 1 g; and 0.2% (w/v) resazurin, 0.5 ml.

The bacterium was routinely transferred into a medium containing 7.5 g l⁻¹ cellulose MN 300 (Serva Feinbiochemica). Routine transfers were also carried out in 9 ml of medium supplemented with cellobiose (ICN Biochemicals) to a final concentration of 3 g l⁻¹.

The medium was reduced by the technique of Hungate (1969) and was dispensed under a constant flow of N₂ in Hungate tubes (Bellco Glass) or 'penicillin flasks' before autoclaving for 20 min at 120 °C.

The anaerobic culture technique used was that proposed by Hungate (1969) as modified by Bryant (1972). Bacteria were grown at 34 °C without shaking except in fermenter cultures.

Growth measurement. Growth was measured by reading optical density at 600 nm with a Beckman model 34 spectrophotometer. It was established that an optical density of 1 at 600 nm corresponded to a bacterial dry weight of 0.5 g l⁻¹ (Gehin *et al.*, 1995).

Fermentation modes. For continuous culture experiments, CM3 medium was again used. It was prepared as follows: a solution containing 8.5 l distilled water, KH₂PO₄, K₂HPO₄, (NH₄)₂SO₄, MgCl₂, cysteine hydrochloride and resazurin was

autoclaved in a 10 l glass flask. Yeast extract, CaCl₂, FeSO₄ and cellobiose were autoclaved separately and aseptically transferred with a peristaltic pump to the solution prepared as described earlier.

Continuous culture was carried out in a 2 l fermenter (LSL BioLafitte) with a 1.5 l working volume maintained by overflow through a sidearm in the fermenter jar. The temperature was controlled at 34 °C and medium pH was adjusted to 7.2 with 1 M NaOH. The feed flask and fermenter were maintained under filter-sterilized nitrogen gas and the fermenter was gently stirred (50 r.p.m.). The bioreactor was inoculated (10%, v/v) with an exponential-phase preculture. The culture was grown in batch for 15 h before the medium flow was started. A period of three residence times was generally found to be sufficient to achieve steady-state values of the parameters measured. At least three samples were taken for each set of steady-state data points reported.

Analytical methods. Reducing sugars were determined by the method of Miller (1959) using cellobiose as the standard.

Ethanol, L-lactate and acetate were determined spectrophotometrically with enzyme kits (Boehringer Mannheim).

Preparation of cell-free extracts. Cells were centrifuged at 12000 g for 20 min. After washing with Tris buffer (50 mM Tris/HCl, 2 mM DTT, pH 7.4), cells were resuspended in this buffer. The cells were then sonicated four times for 20 s at a frequency of 20 kcycles s⁻¹, separated by 60 s (MSE 150 W ultrasonic disintegrator). The supernatant was collected from the cell lysate following centrifugation at 12000 g for 20 min at 4 °C. At each step, extracts were maintained under anaerobic conditions. Protein concentrations of cell extracts were determined according to the method of Bradford (1976) using crystalline BSA as the standard.

Enzyme assays

Ferredoxin (fd)–NAD(P)⁺ reductase [EC 1.18.1.3 (EC 1.18.1.2)] was assayed with pyruvate as the reductant of fd and under a carbon monoxide atmosphere according to Petitdemange *et al.* (1976), modified by the addition of 1 mM purified fd.

NADH–fd reductase was measured by metronidazole reduction according to the method of Blusson *et al.* (1981).

Hydrogenase (EC 1.18.99.1) in the direction of methyl viologen reduction was assayed as described by Junelles *et al.* (1988) with the following modifications: 60 mM potassium phosphate buffer (pH 7.2), 20 mM methyl viologen, 140 mM β-mercaptoethanol and incubation under a hydrogen atmosphere were used.

Phosphoroclastic activities. Assays were based on formation of acetyl phosphate during the oxidation of pyruvate, as described by Mortenson *et al.* (1963), with the following modifications: 25 mM potassium phosphate buffer (pH 6.5), 1 mM sodium pyruvate, 0.05 mM coenzyme A and 1 mM methyl viologen were used.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (EC 1.2.1.12) activity was followed by the increase in A₃₄₀ according to the method of Ferdinand (1964).

Acetate kinase (EC 2.7.2.1) activity was determined by the analysis of acetylphosphate (Lipmann & Tuttle, 1945). The reaction volume was 0.4 ml and the reaction mixture contained: Tris/HCl (pH 7.5), 100 mM; potassium acetate, 40 mM; manganese sulfate, 6 mM; ATP, 10 mM. The reac-

tion was initiated by addition of cell-free extract. After 5 min at 37 °C, 0.2 ml of hydroxylamine (700 mM), pH 6.4 (freshly neutralized), was added. After 5 min at room temperature, the reaction was stopped by addition of 0.2 ml trichloroacetic acid (15%, v/v), 0.2 ml HCl (4 M) and 0.2 ml FeCl₃ (5% w/v in 0.1 M HCl). The amount of acetyl hydroxamate formed was determined by absorbance at 540 nm after centrifugation of the samples for 5 min at 12000 g.

Alcohol dehydrogenase (EC 1.1.1.1) activity was determined by following NAD(P)H-dependent acetaldehyde reduction at 340 nm. The assay mixture contained 100 mM potassium phosphate buffer (pH 7.4), 0.2 mM NAD(P)H, 2 mM DTT and 40 mM acetaldehyde (Lamed & Zeikus, 1980).

Lactate dehydrogenase (EC 1.1.1.27) activity was measured by following at 340 nm the NAD(P)H-dependent reduction of pyruvate to lactate (Abbe *et al.*, 1982). The assay mixture contained 20 mM potassium phosphate buffer (pH 7.4), 0.4 mM NAD(P)H, 1 mM fructose 1,6-bisphosphate and 20 mM pyruvate.

Extraction of nucleotides. NADH, NADPH, NAD⁺, NADP⁺, ATP and ADP levels were measured after extraction of culture broth samples. Samples of 10 ml were rapidly removed from the bioreactor with sterile syringes and immediately put into tubes containing extractant. Cells were not separated from the medium except for the NAD⁺ or NADP⁺ assays. NAD⁺, NADP⁺, ATP and ADP were extracted with HCl (NADH and NADPH were degraded), NADH and NADPH were extracted with KOH (NAD⁺ and NADP⁺ were degraded), as described by Wimpenny & Firth (1972). For NAD⁺ and NADP⁺ determinations, cells were centrifuged and resuspended in deionized water to avoid medium interference during the fluorimetric assays. NADH was measured using a lactate dehydrogenase assay (Klingenberg, 1965), in which the decrease in fluorescence (Hitachi model F-2000 fluorometer) was proportional to the quantity of NADH. NAD⁺ and NADP⁺ were assayed with a NAD(H)-specific alcohol dehydrogenase and a glucose-6-phosphate dehydrogenase (EC 1.1.1.49), respectively (Klingenberg, 1965; Vasconcelos *et al.*, 1994) and the resulting NADH or NADPH was determined by fluorimetry. NADPH was converted to NADP⁺ using glutamate dehydrogenase (EC 1.4.1.4) (Klingenberg, 1965; Wimpenny & Firth, 1972) and NADP⁺ was then determined as described before.

For ADP determinations, the ADP was converted to ATP by creatine phosphokinase (EC 2.7.3.2) in a reaction mixture containing 2 ml supernatant, 7 mM phosphocreatine in Tris/HCl buffer (0.1 M, pH 7.4), 0.4 mM MgSO₄ and 4 U creatine phosphokinase from rabbit muscle maintained for 20 min at 30 °C. The reaction was stopped by addition of 6 M HCl (pH 2.0) and the mixture centrifuged for 10 min at 12000 g before neutralization with 10 M NaOH and storage of the supernatant at -80 °C. ATP was determined by bioluminescence using the luciferin-luciferase system (Microbial Biomass Reagent kit; Lumac). Experiments were made to verify complete ADP to ATP conversion under the conditions tested. ADP was calculated by subtracting the ATP values from the ADP plus ATP assay values and taking into account volume variations.

Measurement of glycogen and exopolysaccharides. Glycogen was assayed by the method of Robson *et al.* (1974).

Exopolysaccharides were separated from cells by centrifugation at 36000 g for 40 min in the presence of a chelating agent (10 mM tetrasodium EDTA) and 100 mM NaCl. Super-

Fig. 1. Schematic diagram depicting the catabolism of cellobiose by *C. cellulolyticum*. 1, GAPDH; 2, lactate dehydrogenase; 3, phosphoroclastic reaction; 4, hydrogenase; 5, NADH-fd oxidoreductase; 6, alcohol dehydrogenase; 7, acetate kinase.

natant (25 ml) was added to propan-2-ol (75 ml). The precipitate was collected by centrifugation, dried for 24 h at 45 °C and resuspended in distilled water. Polysaccharides were assayed by the method of Dubois *et al.* (1956).

Calculations. The main products of cellobiose fermentation by *C. cellulolyticum* were acetate, ethanol, lactate, H₂ and CO₂ (Giallo *et al.*, 1983) (see Fig. 1).

If one assumes, as did Strobel (1995) and Strobel *et al.* (1995), who demonstrated in a cellulolytic bacterium, *C. thermocellum*, that the transport of cellodextrin, cellobiose and glucose requires an ATP molecule and that intracellular cellobiose is converted to glucose 1-phosphate and glucose by cellobiose phosphorylase (EC 2.4.1.20) (Schimz *et al.*, 1983; Thurston *et al.*, 1993; Wells *et al.*, 1995), then the conversion of cellobiose to acetate can be written as:

The conversion of cellobiose to ethanol can be written as:

and the conversion of cellobiose to lactate as:

NADH is formed by GAPDH, and ATP is formed by phosphoglycerate kinase, pyruvate kinase and acetate kinase.

The specific rate of NADH production and NADH consumption were calculated as follows:

$$q_{\text{NADH produced}} = q_{\text{ethanol}} + q_{\text{acetate}} + q_{\text{lactate}}$$

$$q_{\text{NADH consumed}} = 2q_{\text{ethanol}} + q_{\text{lactate}}$$

where q_{acetate} , q_{ethanol} and q_{lactate} are the specific rates of product formation.

In the following sections, $q_{\text{cellobiose}}$ is the specific rate of cellobiose used in $\text{mmol (g dry wt cells)}^{-1} \text{h}^{-1}$ and $q_{\text{pyruvate}} = q_{\text{lactate}} + q_{\text{acetate}} + q_{\text{ethanol}}$.

RESULTS

Continuous culture on cellobiose as a function of dilution rate

Fig. 2 illustrates the utilization of cellobiose and the steady-state biomass at various dilution rates (D). At a cellobiose concentration in the feed medium of 2.34 mM and over a wide range of low dilution rates (from 0.015 to 0.080 h^{-1}), the residual cellobiose concentrations were low; above 0.080 h^{-1} , the concentration increased and reached that in the inflow of the nutrition solution. These data are typical of a continuous culture carried out under carbon limitation. Continuous cultures were

Fig. 2. Residual cellobiose concentration (□) and steady-state biomass concentration (●) as functions of the dilution rate. The cellobiose input concentration was 2.34 mM. Results presented are means $\pm$ SD calculated from three different experiments.

Fig. 3. Specific production rates of pyruvate (Δ), acetate (■), lactate (○) and ethanol (▲) as functions of the specific cellobiose uptake rate in cellobiose-limited continuous culture. Results presented are means $\pm$ SD of three independent determinations from different cultures.

shown to be carbon-limited when the cellobiose concentration in the feed medium was less than 2.9 mM. Based on a plot of $1/D$ versus $1/Y_{\text{ATP}}$, the maintenance cost was estimated to be 0.93 $\text{mmol ATP g}^{-1} \text{h}^{-1}$ (data not shown); this cost may be responsible for the decrease in biomass observed at low dilution rates.

End-product formation

The main product of *C. cellulolyticum* cellobiose metabolism was acetate at all the dilution rates tested. Acetate production reached a mean of 60% of the carbon flowing to products; the other 40% was distributed between lactate (30%) and ethanol (10%). The relationship of these distributions is shown in Fig. 3. Acetate formation was proportional to carbon flow in the first part of the curve, and decreased as carbon flow increased. Pyruvate formation showed the same pattern in relation to carbon flow. It can be concluded that above a critical carbon flow of 0.66 $\text{mmol cellobiose (g dry wt cells)}^{-1} \text{h}^{-1}$, part of the flow did not proceed to the end of the glycolytic pathway, since the q_{pyruvate} decreased while the $q_{\text{cellobiose}}$ increased. These data are consistent with cellobiose conversion to polymers (see below). Lactate formation was seen only when the carbon flow was greater than a $q_{\text{cellobiose}}$ value of 0.27 $\text{mmol cellobiose (g dry wt cells)}^{-1} \text{h}^{-1}$. However, less lactate was produced than acetate. Its production paralleled acetate formation until it reached a maximal value at a $q_{\text{cellobiose}}$ of 0.5 $\text{mmol cellobiose (g dry wt cells)}^{-1} \text{h}^{-1}$, and decreased at a $q_{\text{cellobiose}}$ greater than 0.68 $\text{mmol cellobiose (g dry wt cells)}^{-1} \text{h}^{-1}$. Ethanol production was always low and was not affected by high carbon flow.

Table 1. Fermentation parameters for continuous steady-state cultures of *C. cellulolyticum*

Values for biomass and residual cellobiose concentrations are means ($\pm$ SD) of three different experiments. All other values are also means and had an SE of $\pm 10\%$.

<i>D</i> (h ⁻¹) ...	0.015	0.030	0.030	0.054	0.068	0.075	0.080	0.085
Biomass concn (g l ⁻¹)	0.15 (0.01)	0.16 (0.01)	0.61 (0.07)	0.21 (0.02)	0.22 (0.02)	0.32 (0.04)	0.23 (0.02)	0.27 (0.02)
Fed substrate concn (mM)	2.34	2.34	16.67	2.34	2.34	5.85	2.92	2.34
Residual cellobiose concn (mM)	0.38 (0.15)	0.05 (0.03)	4.97 (0.60)	0.44 (0.19)	0.24 (0.10)	2.95 (0.30)	1.02 (0.10)	0.38 (0.11)
Molar growth yield [g cells (mol hexose) ⁻¹]	38	35	26	55	51	55	60	55
<i>q</i> _{cellobiose} [mmol (g cells) ⁻¹ h ⁻¹]	0.196	0.429	0.575	0.488	0.649	0.680	0.661	0.617
Product yield [mol (mol substrate) ⁻¹]								
Acetate	2.70	2.56	1.26	1.80	2.20	1.62	2.37	1.90
Lactate	0.13	0.32	0.61	1.03	0.75	0.96	0.83	0.50
Ethanol	0.58	0.65	0.22	0.34	0.39	0.36	0.31	0.45
<i>Y</i> _{ATP} [g cells (mol ATP) ⁻¹]	12.5	11.5	15.5	22.2	18.9	24.2	20.6	29.0
<i>q</i> _{NADH produced} [mmol (g cells) ⁻¹ h ⁻¹]	0.65	1.34	1.21	1.56	2.17	2.00	2.31	1.77
<i>q</i> _{NADH used} [mmol (g cells) ⁻¹ h ⁻¹]	0.24	0.39	0.61	0.84	1.01	1.15	0.93	0.87
<i>q</i> _{NADH produced} / <i>q</i> _{NADH used}	2.70	3.40	1.98	1.85	2.14	1.73	2.48	2.03
Carbon recovery (%) [*]	85.2	87.7	52.0	79.2	83.5	73.5	87.7	71.2

^{*} Carbon recovery was calculated as a function of the concentrations (mol l⁻¹) of ethanol (E), acetic acid (A), lactic acid (L), feed or initial substrate (*S*₀) and effluent or final substrate (*S*_e) concentrations by using the following equation:

$$\text{carbon recovery} = [3 \times (E + L + A)] / [12 \times (S_0 - S_e)]$$

assuming stoichiometric production of CO₂ with acetic acid and ethanol. These values did not include cell carbon.

Fermentation parameters

Table 1 presents different parameters determined during continuous culture of *C. cellulolyticum*.

Maximal carbon flow supported by *C. cellulolyticum* was 0.68 mmol cellobiose (g dry wt cells)⁻¹ h⁻¹ [equivalent to 11.3 nmol cellobiose min⁻¹ (mg cells)⁻¹ or 22 nmol cellobiose min⁻¹ (mg protein)⁻¹]. This value is in the same range reported for *C. thermocellum* [18 nmol cellobiose min⁻¹ (mg cells)⁻¹; Ng & Zeikus, 1982] or *Clostridium thermohydrosulfuricum* [24 nmol cellobiose min⁻¹ (mg cells)⁻¹; Ng & Zeikus, 1982]. Higher values have been reported for another cellulolytic bacterium, *Fibrobacter succinogenes* [76 nmol min⁻¹ (mg protein)⁻¹; Wells *et al.*, 1995] and for a rumen bacterium, *Prevotella ruminicola* [175 nmol min⁻¹ (mg cells)⁻¹; Lou *et al.*, 1996].

The highest molar growth yield obtained in this work was 60 g cells (mol hexose)⁻¹ [0.2 g protein (g carbohydrate)⁻¹]. This value is higher than those reported for *C. thermocellum* [38 g (mol hexose)⁻¹] and *C. thermohydrosulfuricum* [19 g (mol hexose)⁻¹; Ng & Zeikus, 1982] but in the same range as that of *P. ruminicola* [0.19 g protein (g carbohydrate)⁻¹]. In batch cultures a

molar growth yield of 40 g cells (mol hexose)⁻¹ was reported (Giallo *et al.*, 1983).

A mean value of 19 g cells (mol ATP)⁻¹ was obtained except at low dilution rates where a lower value was found. This decrease reflected an expenditure of energy due to the maintenance energy being more pronounced when the growth rate is low. In batch cultures of *C. cellulolyticum* a lower value was reported for *Y*_{ATP} [15 g cells (mol ATP)⁻¹] (Giallo *et al.*, 1983).

These high values of *Y*_{ATP} are consistent with the fact that the high level of acetate production was accompanied by the highest level of ATP formation. However, this pattern of product formation (i.e. the high level of acetate production and the low level of lactate and ethanol production) coincided with an excess of NADH since the ratio *q*_{NADH produced}/*q*_{NADH used} was always greater than one, ranging from 1.73 to 3.40 (Table 1).

Carbon recoveries obtained for continuous culture of *C. cellulolyticum* were between 71 and 87% (with one exception, 52%), consistent with values found generally for cellulolytic bacteria (Lamed & Zeikus, 1980; Giallo *et al.*, 1983). They did not include cells since it is difficult to estimate the part of cellobiose devoted to biomass

Table 2. Nucleotide levels in continuous steady-state cultures of *C. cellulolyticum*

Dilution rates and cellobiose input concentrations for experiments I–IV were 0.030 h⁻¹ and 2.34 mM, 0.030 h⁻¹ and 16.67 mM, 0.080 h⁻¹ and 2.92 mM, and 0.075 h⁻¹ and 5.85 mM, respectively. Values are means ($\pm$ SD) calculated from triplicate experiments.

Nucleotide	Concentration [μ mol (g dry cell mass) ⁻¹]			
	Expt I	Expt II	Expt III	Expt IV
NAD ⁺	0.5 (0.1)	1.0 (0.1)	0.7 (0.1)	0.9 (0.2)
NADP ⁺	0.3 (0.1)	0.8 (0.1)	0.6 (0.1)	0.3 (0.1)
NADH	3.0 (0.5)	15.9 (3.0)	40.0 (6.0)	37.5 (3.5)
NADPH	< 0.2*	< 0.2*	< 0.2*	< 0.2*
ATP	1.0 (0.1)	1.8 (0.2)	1.9 (0.2)	1.8 (0.2)
ADP	5.7 (0.3)	4.2 (0.4)	3.0 (0.2)	4.1 (0.2)

* Values less than detection level.

production on complex medium. Lowest values were found for chemostats conducted on 5.85 mM cellobiose at 0.075 h⁻¹ or on 2.34 mM cellobiose at 0.085 h⁻¹. Assays for glycogen synthesis revealed a significantly higher glycogen storage in cells from these chemostats [75 and 80 mg glucose eq. (g cells)⁻¹, respectively versus 5 mg glucose eq. (g cells)⁻¹ on 2.34 mM cellobiose at 0.030 h⁻¹] (data not shown). However, a glycogen storage of 60 mg glucose eq. (g cells)⁻¹ did not account completely for the loss in carbon recovery (only 52 %) observed for cells from chemostats conducted on 16.67 mM cellobiose at 0.030 h⁻¹. Microscopic observation revealed a polysaccharidic network where cells were embedded which should account for the low values observed in carbon recovery. Several procedures have been tested to measure these exopolysaccharides. No one gave satisfactory results. An interference, probably due to a component of the complex CM3 medium used, must be responsible for the results obtained (high background and non-reproducibility). Furthermore, filtration or dialysis of the samples did not remove this interference.

Nucleotide levels

These were determined in four cases: at low or high dilution rate under carbon-limitation (experiments I and III, respectively, Table 2) or carbon-excess (experiments II and IV, respectively, Table 2). Whereas the total pool size of NADPH and NADP⁺ of cells grown with different carbon flows was quite constant, the NADH concentration dramatically increased with the carbon flow. Increasing cellobiose concentration at $\mu = 0.030$ h⁻¹ led to an increase in NADH concentration; starting from usual values such as 3.0 μ mol (g cells)⁻¹ [experiment I, Table 2, $q_{\text{cellobiose}} = 0.429$ mmol cellobiose (g dry wt cells)⁻¹ h⁻¹], it reached values of 15.9 μ mol (g cells)⁻¹ [experiment II, Table 2, $q_{\text{cellobiose}} = 0.575$ mmol cellobiose (g dry wt cells)⁻¹ h⁻¹]. Similar values [40 and 37.5 μ mol (g cells)⁻¹] were obtained at 0.080 h⁻¹ [experiment III, $q_{\text{cellobiose}} = 0.661$ mmol cellobiose (g dry

wt cells)⁻¹ h⁻¹] and 0.075 h⁻¹ [experiment IV, $q_{\text{cellobiose}} = 0.680$ mmol cellobiose (g dry wt cells)⁻¹ h⁻¹] when cellobiose concentration was increased from 2.92 mM to 5.85 mM, indicating the saturation of the phenomenon observed (i.e. an increase of the NADH concentration with the specific rate of cellobiose consumption).

In spite of the fact that acetate formation was associated with ATP production, no significant variation was noted in the total pool size of ATP and ADP.

Enzymic activities

In the four cases described previously, enzymic activities were determined from cell extracts (Table 3). GAPDH and acetate kinase were found to be in the same range as activities previously described for bacteria metabolizing high concentrations of carbohydrate (Andersch *et al.*, 1983; Hüsemann & Papoutsakis, 1989; Girbal *et al.*, 1995a; Abbad-Andaloussi *et al.*, 1996). Lactate dehydrogenase and ethanol dehydrogenase exhibited almost no activity with NADP⁺. Lactate dehydrogenase activity was shown to be low but correlated with the rate of lactate formation. By comparison, activities reported for other bacteria were 3- to 10-fold higher (Lamed & Zeikus, 1980; Lovitt *et al.*, 1988). By contrast, alcohol dehydrogenase activity determined *in vitro* was not affected by carbon flow; values reported were lower than those described for *C. thermocellum* or *C. thermohydrosulfuricum* [2 and 0.4 μ mol min⁻¹ (mg protein)⁻¹, respectively] (Lamed & Zeikus, 1980; Lovitt *et al.*, 1988). In these conditions, the low lactate and ethanol production must be balanced by H₂ gas formation via the NAD(P)H-fd oxidoreductases, the physiological role of which could be to regenerate the reducing equivalents (Thauer *et al.*, 1971). fd-NADP⁺ reductase activities were much higher than the fd-NAD⁺ reductase activities, which were hardly detectable. In contrast, high NADH-fd reductase activities [0.074 μ mol min⁻¹ (mg protein)⁻¹] were found for cells that exhibit a high

Table 3. Enzymic activities from cell extracts of continuous cultures of *C. cellulolyticum*

Dilution rates and cellobiose input concentrations for experiments I–IV were 0.030 h⁻¹ and 2.34 mM, 0.030 h⁻¹ and 16.67 mM, 0.080 h⁻¹ and 2.92 mM, and 0.075 h⁻¹ and 5.85 mM, respectively. Values are means ($\pm$ SD) calculated from triplicate experiments.

Enzyme	Specific activity [$\mu\text{mol min}^{-1}$ (mg protein ⁻¹)]			
	Expt I	Expt II	Expt III	Expt IV
GAPDH	3.9 (0.5)	5.9 (0.2)	3.4 (0.5)	3.7 (0.2)
Phosphoroclastic activity	0.092 (0.008)	0.024 (0.001)	0.031 (0.003)	0.024 (0.001)
Hydrogenase	0.85 (0.19)	2.00 (0.30)	0.25 (0.05)	0.29 (0.05)
Lactate dehydrogenase	0.038 (0.008)	0.014 (0.002)	0.110 (0.020)	0.180 (0.020)
Acetate kinase	2.6 (0.2)	5.7 (0.4)	2.4 (0.3)	1.6 (0.1)
Alcohol dehydrogenase	0.22 (0.01)	0.20 (0.03)	0.28 (0.02)	0.34 (0.08)
fd-NADP reductase	0.003 (0.001)	0.004 (0.001)	0.027 (0.001)	0.025 (0.005)
NADH-fd reductase	0.015 (0.002)	0.050 (0.010)	0.074 (0.015)	0.074 (0.018)

NADH content and data on NADH-fd reductase activities showed a direct correlation between the specific activities, which increased from 0.015 to 0.074 $\mu\text{mol min}^{-1}$ (mg protein)⁻¹, and the NADH content of the cell. NADH-fd reductase activity was higher than that described for *C. acetobutylicum*, *Clostridium tyrobutyricum* or *C. thermohydrosulfuricum* [25, 30 and 12 nmol min⁻¹ (mg protein)⁻¹, respectively] but lower than the value reported for *Clostridium pasteurianum* [171 nmol min⁻¹ (mg protein)⁻¹] (Petitdemange *et al.*, 1976; Lovitt *et al.*, 1988). The function of pyridine nucleotide oxidoreductases depends on both the bacterial species and strain and the specific growth conditions of a given strain (Petitdemange *et al.*, 1976; Girbal *et al.*, 1995a). With a hardly detectable fd-NAD⁺ reductase activity and a high NADH-fd reductase activity, the enzyme can function in NADH oxidation and H₂ production via the hydrogenase. Hydrogenase activities were found to be higher at low dilution rates (experiments I and II, Table 3) and significantly higher in carbon excess at low dilution rate (experiment II) than under carbon limitation or excess at high dilution rates (experiments III and IV). However, it remained weak when compared with activities described for other bacteria [for example, 37 $\mu\text{mol min}^{-1}$ (mg protein)⁻¹ for *C. thermohydrosulfuricum* (Lovitt *et al.*, 1988) or 74 $\mu\text{mol min}^{-1}$ (mg protein)⁻¹ for *C. thermocellum* AS 39 (Lamed & Zeikus, 1980)] even at high dilution rates where a high activity would be required to regenerate NADH via the NADH-fd reductase activity. Because the NADH-fd reductase activity is interconnected with hydrogenase, the decrease in hydrogenase activity could affect the efficiency of this NAD⁺ regenerating system and lead to an increase in the NADH pool. Phosphoroclastic activity was significantly lower under carbon saturation conditions and at high dilution rates corresponding to the higher detected intracellular NADH concentrations. This could be a way for the cells to limit acetate production and to make easier the reoxidation of NADH via lactate production.

Fig. 4. Influence of the NADH/NAD⁺ ratio on GAPDH activity determined *in vitro*. Data points indicate residual activity in the presence of the given NADH/NAD⁺ ratio. Control (100%) activity was 1.2 $\mu\text{mol min}^{-1}$ (mg protein)⁻¹. The concentration of NAD⁺ was 0.1 mM.

The effect of NADH on GAPDH activity was determined to explain how high cellular NADH/NAD⁺ ratios inhibited catabolism. In these experiments, arsenate was added to the GAPDH assay system since this competes with phosphate and forms an unstable intermediate which rapidly decomposes. 1,3-Biphosphoglycerate never accumulates and a significant reverse reaction is not possible (Krebs, 1955; Krebs *et al.*, 1969; Blusson *et al.*, 1981; Hyun *et al.*, 1983). Under these assay conditions, it is possible to observe the effect of NADH as an effector rather than a substrate for the reverse reaction. The results (Fig. 4) show that, as the level of NADH was increased, the rate of NAD⁺ reduction by GAPDH activity was significantly inhibited. These data indicate that GAPDH activity is significantly inhibited when the NADH/NAD⁺ ratio is greater than 1.

DISCUSSION

Cellobiose metabolism by *C. cellulolyticum* has thus far been studied only in batch cultures (Giallo *et al.*, 1983; Gehin *et al.*, 1995). This cellulolytic bacterium is able to metabolize completely only a small quantity of soluble carbohydrate (3 g l^{-1}). The molar growth yields were reduced when the initial cellobiose concentration exceeded 2 g l^{-1} ; sugars were consumed without an increase in cell density (Giallo *et al.*, 1983). To determine the physiological basis for growth limitation in *C. cellulolyticum*, we have now grown the cells in continuous culture with cellobiose as substrate.

The results presented in this paper suggest that growth of *C. cellulolyticum* is limited due to a low rate of NADH reoxidation leading to an intracellular accumulation of the reduced nucleotide. As described previously for batch cultures (Giallo *et al.*, 1983), acetate is the main product of *C. cellulolyticum* cellobiose metabolism in continuous culture. Acetate formation was found to increase with increasing carbon flow, leading to high ATP production and to an insufficient rate of NADH regeneration. Production of reduced compounds such as ethanol and lactate was not sufficient to compensate for the slow rate of NADH reoxidation. An alternative method for NAD^+ regeneration from NADH in clostridia is by means of the NADH-fd oxidoreductase, which catalyses the electron flow from NADH ($E'_0 = -320 \text{ mV}$) via fd to protons ($E'_0 = -420 \text{ mV}$). This reaction is thermodynamically unfavourable under standard conditions (Jungermann *et al.*, 1973) and needs interspecies H_2 transfer to be efficient (Wolin & Miller, 1988; Morvan *et al.*, 1996). The low rate of reoxidation of the excess reducing equivalents produced during glycolysis resulted in an approximatively 12-fold increase in the intracellular NADH concentration. Similar NADH accumulation has been reported previously in *C. acetobutylicum* (sevenfold) (Girbal *et al.*, 1995b) and in *C. butyricum* (eightfold) (Abbad-Andaloussi *et al.*, 1996). Our results demonstrate that the high NADH/ NAD^+ ratio inhibits the *in vitro* activity of GAPDH, suggesting a possible limitation of the carbon flow as described previously in several other clostridia (Lovitt *et al.*, 1988; Girbal & Soucaille, 1994; Girbal *et al.*, 1995b). However, the high *in vitro* GAPDH activity could be sufficient to ensure the carbon flow measured in cellobiose-saturated conditions [$q_{\text{cellobiose}} = 0.680 \text{ mmol cellobiose (g dry wt cells)}^{-1} \text{ h}^{-1}$ (Table 1), corresponding to a GAPDH specific activity of $3.7 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ (Table 3). The value of q_{pyruvate} expressed in $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ was 0.073 i.e. 2% of the *in vitro* detected GAPDH activity, a value obtained at high NADH/ NAD^+ ratio (Fig. 4)]. These data suggest that *C. cellulolyticum* is not able to metabolize high quantities of soluble sugars under these growth conditions.

Our data demonstrated that, at least on complex medium in continuous culture, *C. cellulolyticum* is not able to cope with carbon overflow by, for example, inducing lactate production. *C. cellulolyticum* and many other clostridia show lactate dehydrogenase activity,

but lactic acid is not normally a major end product (Russell *et al.*, 1996). Furthermore, many clostridia, such as *C. acetobutylicum*, are able to induce metabolic shift to produce solvents (ethanol, butanol or acetone). This metabolic shift is associated with high intracellular ATP and NAD(P)H contents. In particular, solvent production in *C. acetobutylicum* is induced under conditions of low ATP requirement, high ATP availability and high intracellular NADH content (see Girbal *et al.*, 1995b for a review). Our results show that under similar conditions, *C. cellulolyticum* is not able to induce metabolic shift to produce reduced compounds such as ethanol.

These results described in this paper concerning carbon metabolism by a mesophilic cellulolytic clostridium have to be considered in relation to the natural environment of the bacterium. In previous studies, we have shown that growth of *C. cellulolyticum* occurs as the cells adhere to cellulose fibres (Gelhay *et al.*, 1993a, b). In this context, the cellulose is usually coated by other polymers, predominantly xylan and lignin, which hinder cellulolysis. Our results suggest that catabolism in *C. cellulolyticum* is adapted to low carbon flows, consistent with the notion that high concentrations of soluble sugars, such as 2 or 3 g l^{-1} , are probably not found in natural ecosystems. Furthermore, in many ecosystems where lignocellulose compounds are degraded, interspecies hydrogen transfer occurs and the H_2 produced by cellulolytic bacteria, such as *C. cellulolyticum*, is used immediately by methanogens or other H_2 -using species (Wolin & Miller, 1988; Morvan *et al.*, 1996). Given that H_2 does not accumulate, the formation of H_2 from NADH would not be inhibited and the bacteria would not need to produce reduced compounds (e.g. ethanol) to reoxidize the NADH produced during glycolysis. Thus, we demonstrate that in pure culture *C. cellulolyticum*, producing mainly acetate, CO_2 and H_2 , is not adapted to high carbon flow catabolism.

To the best of our knowledge, this study is the first on cellobiose catabolism in free-living mesophilic cellulolytic clostridia. We showed that the catabolic enzymic activities detected *in vitro* were high compared to the low carbon flow measured *in vivo* in *C. cellulolyticum*. Indeed, the GAPDH, phosphoroclastic and acetate kinase activities detected *in vitro* are in the same range as values reported for clostridia such as *C. acetobutylicum* (Girbal *et al.*, 1995a) or *C. butyricum* (Abbad-Andaloussi *et al.*, 1996) metabolizing high concentrations of carbohydrate. Since, in any ecosystem, a cellulolytic microbe exists in proximity to other microorganisms, these high enzymic activities could be a way for the cellulolytic clostridium to seize the small amounts of carbohydrates provided by cellulose degradation and so to compete efficiently with non-cellulolytic bacteria.

ACKNOWLEDGEMENTS

This work was supported by the commission of European Communities FAIR programme [contract no. CT 95-091 (DG 12 SSMA)].

The authors thank Michel Quittelier for his technical assistance.

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Received 13 June 1997; revised 6 October 1997; accepted 7 October 1997.