J. Microbiol. Biotechnol. (2004), **14**(6), 1200–1210

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Mapping of Carbon Flow Distribution in the Central Metabolic Pathways of *Clostridium cellulolyticum*: Direct Comparison of Bacterial Metabolism with a Soluble versus an Insoluble Carbon Source

DESVAUX, MICKAËL*

The University of Birmingham, Institute for Biomedical Research, Vincent Drive, Edgbaston, Birmingham B15 2TT, United Kingdom

Received: January 29, 2004
Accepted: June 20, 2004

Abstract Metabolic flux analysis was established by adapting previous stoichiometric model developed during growth with cellulose to cell grown with cellobiose for further direct comparison of the bacterial metabolism. In carbon limitation with cellobiose, a shift from acetate-ethanol fermentation to ethanol-lactate fermentation is observed and the pyruvate overflow is much higher than with cellulose. In nitrogen limitation with cellobiose, the cellodextrin and exopolysaccharide overflows are much higher than on cellulose. In carbon and nitrogen saturation with cellobiose, the cellodextrin, exopolysaccharide, and free amino acids overflows reach the highest levels observed but all remain limited on cellulose. By completely shunting the cellulosome, the use of cellobiose allows to reach much higher carbon consumption rates which, in return, highlights the metabolic limitation of *C. cellulolyticum*. Therefore, the physical nature of the carbon source has a profound impact on the metabolism of *C. cellulolyticum* and most probably of other cellulolytic bacteria. For cellulolytic bacteria, the use of soluble carbon substrate must carefully be taken into consideration for the interpretation of results. Direct comparison of metabolic flux analysis from cellobiose and cellulose revealed the importance of cellulosome, phosphoglucomutase and pyruvate-ferredoxin oxidoreductase in the distribution of carbon flow in the central metabolism. In the light of these findings, future directions for improvement of cellulose catabolism by this bacterium are discussed.

Key words: Metabolic flux analysis, cellulolytic clostridia, bacterial metabolism, cellulose, cellobiose, continuous culture

The biological degradation of cellulose is of key importance from both ecological and biotechnological point of views; it actively participates in the global carbon cycling [2, 23, 39] and it is a promising approach for the generation of alternative energy sources such as hydrogen, methane, or biofuel [1, 19, 24, 25, 31].

Clostridium cellulolyticum is a nonruminal cellulolytic mesophilic bacterium isolated from decayed grass and capable of digesting efficiently crystalline cellulose [34]. The cellulose degradation is mediated *via* the cellulosome which is an extracellular multienzymatic complex found at the bacterial cell surface and containing various cellulases organized around a scaffolding protein called CipC [3]. The cellulosome allows both bacterial adhesion to cellulose fibers and synergism of action of the cellulases, which results in a very efficient microbial of crystalline cellulose [4].

The first studies of *C. cellulolyticum* metabolism were performed in batch cultures [14]. Finer metabolic investigations were later performed in stable physiological conditions using chemostat technique [32]. Growth on complex and mineral-salt based media showed difference in metabolic regulatory response, leading to the hypothesis of a bacterial growth adapted to oligotrophic conditions [17, 18]. In the past two decades, the development of an efficient continuous culture device for growth on insoluble substrates [38] allows to consider metabolic study of *C. cellulolyticum* with cellulose instead of cellobiose, one of the soluble cellodextrins released during cellulolysis. Metabolic flux analysis (MFA), which corresponds to a description of the metabolic events which converts feedstocks to biomass and byproducts [20, 35], allowed to reveal the regulation and distribution of the carbon fluxes inside and outside of the bacterial cell in various growth conditions.

The aim of the present work was to map the carbon flow of *C. cellulolyticum* when grown on a soluble glucide, cellobiose, in conditions of carbon limitation and saturation [16- 18], by adapting the stoichiometric model previously developed from experiments with cellulose [8, 10, 12] to further compare and highlight the influence of the carbon

*Corresponding author
Phone: 44-121-415-8693; Fax: 44-121-414-3599;
E-mail: m.desvaux@bham.ac.uk

substrate on bacterial metabolism. Practically, this will permit to draw future directions for the improvement of cellulose catabolism by this bacterium.

MATERIALS AND METHODS

Organism and Growth

C. cellulolyticum ATCC 35319 was cultivated in chemostats on a defined medium and fermentation was monitored as previously described [8, 10, 12, 16-18]. The temperature was maintained at 34°C and the pH was controlled at 7.2. With cellulose, the chemostat system used was a segmented gas/liquid continuous culture device as described by Weimer *et al.* [38] with some modifications [8].

Analytical Procedures

Bacterial biomass was estimated by cell dry weight measurement and using a mean biomass formula of $C_4H_7O_2N$ [17]. On cellulose, biomass was estimated by bacterial protein measurement as previously described [9]. Cellulose concentration was determined as previously described using the method of Huang and Forsberg [21], the washing procedure was used by the method of Updegraff [37], and quantification was followed by phenol-sulfuric acid method [13]. Equivalent anhydroglucose was used for calculation. In cellobiose chemostats, cellobiose was determined colorimetrically using dinitrosalicylic reagent [27]. Ammonia was assayed by the method of Chaney and Marbach [6]. Acetate, lactate, and ethanol were estimated using the appropriate enzyme kits as previously described [32]. Hydrogen and carbon dioxide were analyzed on a gas chromatography unit as previously described [17]. Extracellular proteins and amino acids were assayed as previously reported [8, 17, 32], using the Bradford dye method [5] and the procedure of Mokrasch [29], respectively. An average extracellular protein formula of $C_{16}H_{25}O_9N_6$ and an elemental amino acid composition of $C_5H_{10}O_{2.5}N$ were further used for the conversion into milliequivalent of carbon [17]. Glycogen determination was performed according to the procedure of Matheron *et al.* [26] as previously indicated [16]. Exopolysaccharides were

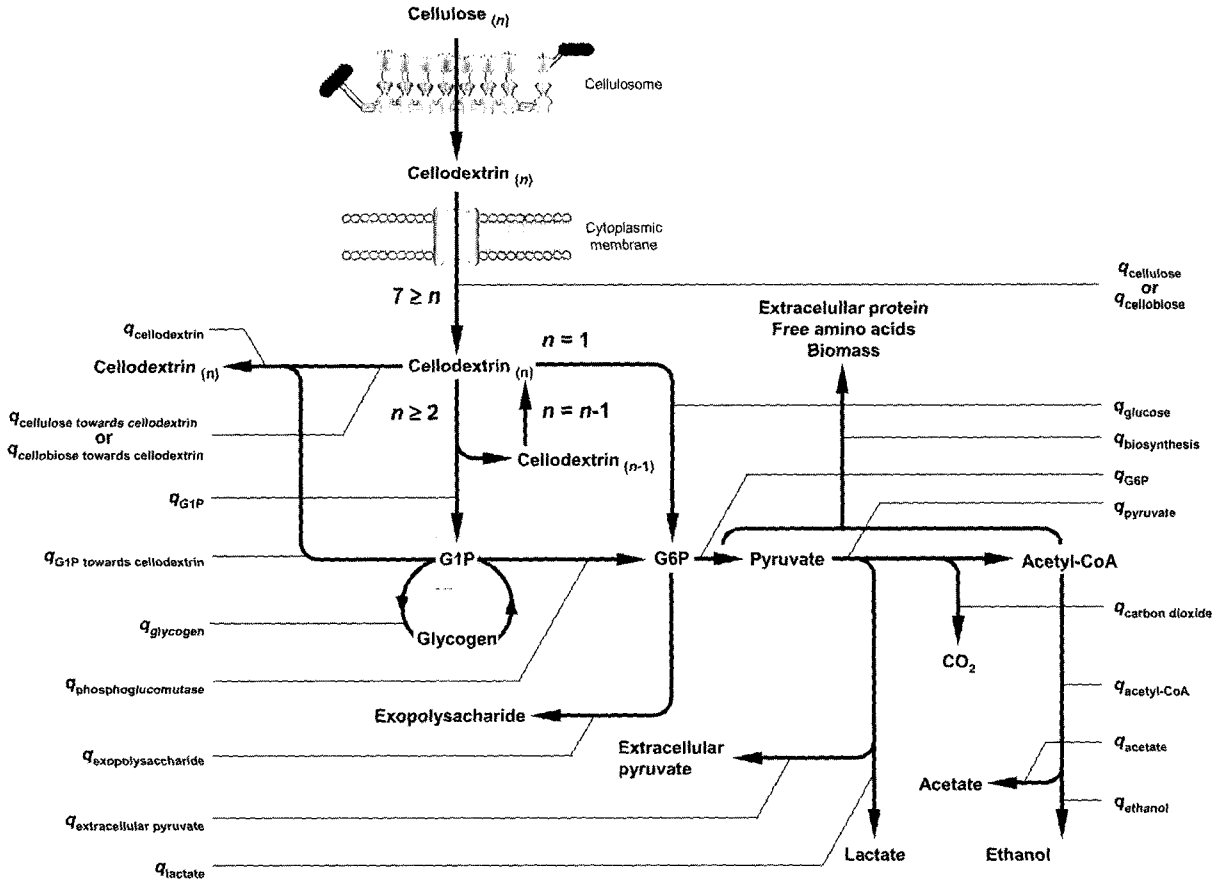

Fig. 1. Carbon flow distribution within the central metabolic pathways of *C. cellulolyticum*. *n* corresponds to hexose equivalents, i.e. the number of glucose residues in the cellulose chain.

Only soluble cellodextrins and glucose are incorporated by the bacterial cell, i.e. celloheptaose (n=7), cellohexaose (n=6), cellopentaose (n=5), cellotetraose (n=4), cellotriose (n=3), cellobiose (n=2), and glucose (n=1).

determined by the phenol-sulfuric acid method using glucose as a standard as described previously [32]. Soluble cellodextrins were quantitatively assayed by high-performance liquid chromatography (HPLC) as previously described [8]. Glucose was assayed enzymatically, using glucose oxidase and peroxidase with *o*-dianisidine as a chromophore [8]. Extracellular pyruvate was assayed enzymatically by fluorometric detection of NADH [17]. NAD^+ and NADH were assayed by fluorimetric measurements as previously described [17]. ATP and ADP were measured using a luciferin-luciferase luminescence system [16].

Calculations and Mapping of the Carbon Flow

The distribution of the carbon flow within the central metabolic pathways of *C. cellulolyticum* when grown on cellulose was depicted in Fig. 1. Glucose and water-soluble cellodextrins, with degrees of polymerization (n) between 2 and 7 [33], could potentially be incorporated by bacteria *via* ABC transporters [8, 9, 36]. It was assumed that the intracellular cellodextrin composed of n hexose residues was catabolized according to the model proposed by Strobel *et al.* [36] as previously described [8, 9]. If $n \geq 2$, then $\text{cellodextrin}_{(n)}+\text{Pi} \rightarrow \text{glucose 1-phosphate}+\text{cellodextrin}_{(n-1)}$ through cellodextrin phosphorylase (EC 2.4.1.49) and cellobiose phosphorylase (EC 2.4.1.20) activities. If $n=1$, then glucose+ATP→glucose 6-phosphate+ADP, *via* glucokinase (EC 2.7.1.1). As a result, the entering carbon flow directed toward glucose is $1/n \times q_{\text{cellulose}}$ and that toward G1P is $(n-1)n \times q_{\text{cellulose}}$ [8]. For soluble cellodextrins, where $1 \leq n \leq 7$, average carbon flows directed toward G1P of $0.63 \times q_{\text{cellulose}}$ and toward glucose of $0.37 \times q_{\text{cellulose}}$ were calculated [8]. For cellobiose, i.e. $n=2$, q_{glucose} is $1/2 \times q_{\text{cellobiose}}$ and q_{G1P} is $1/2 \times q_{\text{cellobiose}}$. For conversion to biomass, carbon source is taken by the cell and fed into the central metabolic pathways. For stoichiometric modeling of *C. cellulolyticum* metabolism [8, 10-12], the calculation of carbon flow through the known metabolic pathways was performed as follows:

On cellobiose:

$$q_{\text{cellobiose}}=(C_{\text{cellobiose}}/X)\times D$$
$$q_{\text{cellodextrin}}=(C_{\text{cellotriose}}/X)\times D$$
$$q_{\text{Glucose}}=1/2\times q_{\text{cellobiose}}$$

On cellulose:

$$q_{\text{cellulose}}=(C_{\text{cellulose}}/X)\times D$$
$$q_{\text{cellodextrin}}=(C_{\text{cellobiose}}+C_{\text{cellotriose}}/X)\times D$$
$$q_{\text{Glucose}}=0.37\times q_{\text{cellulose}}$$

$$q_{\text{lactate}}=(C_{\text{lactate}}/X)\times D$$
$$q_{\text{acetate}}=(C_{\text{acetate}}/X)\times D$$
$$q_{\text{ethanol}}=(C_{\text{ethanol}}/X)\times D$$
$$q_{\text{extracellular pyruvate}}=(C_{\text{extracellular pyruvate}}/X)\times D$$
$$q_{\text{glycogen}}=(C_{\text{glycogen}}/X)\times D$$

Table 1. Carbon fluxes in continuous steady-state cultures of *C. cellulolyticum* in cellobiose-limited condition (2 g l^{-1}).

Carbon flow*	Results obtained at D (h^{-1}) value of:							
	0.033		0.053		0.085		0.120	
	Flux†	%‡	Flux	%	Flux	%	Flux	%
$q_{\text{cellobiose}}$	10.75	100.0	16.08	100.0	18.66	100.0	22.30	100.0
q_{G1P}	5.37	50.0	8.04	50.0	9.33	50.0	11.15	50.0
$q_{\text{cellodextrin}}$	nd§	nd	nd	nd	nd	nd	nd	nd
$q_{\text{G1P towards cellodextrin}}$	nd	nd	nd	nd	nd	nd	nd	nd
$q_{\text{cellobiose towards cellodextrin}}$	nd	nd	nd	nd	nd	nd	nd	nd
q_{glycogen}	0.05	0.5	0.10	0.6	0.13	0.7	0.12	0.4
$q_{\text{exopolysaccharide}}$	0.03	0.3	0.04	0.2	0.05	0.3	0.10	0.4
q_{glucose}	5.37	50.0	8.04	50.0	9.33	50.0	11.15	50.0
$q_{\text{phosphoglucomutase}}$	5.09	47.4	4.68	29.1	7.71	41.3	8.42	37.8
q_{G6P}	10.46	97.4	12.72	79.1	17.04	91.3	19.57	87.8
$q_{\text{biosynthesis}}$	2.27	21.2	3.77	23.4	5.25	28.2	6.31	28.3
q_{pyruvate}	8.19	76.2	8.95	55.7	11.79	63.2	13.26	59.5
$q_{\text{acetyl-CoA}}$	5.35	49.8	5.82	36.2	7.08	37.9	7.57	33.9
q_{lactate}	0.16	1.5	0.22	1.4	0.75	4.0	1.47	6.6
$q_{\text{extracellular pyruvate}}$	nd	nd	nd	nd	0.42	2.2	0.44	2.0
$q_{\text{carbon dioxide}}$	2.68	24.9	2.91	18.1	3.54	19.0	3.78	17.0
q_{ethanol}	1.61	15.0	2.08	12.9	2.88	15.4	3.20	14.4
q_{acetate}	3.74	34.8	3.74	23.2	4.20	22.5	4.36	19.6
Ratio								
H_2/CO_2	1.69	–	1.37	–	1.33	–	1.29	
NADH/NAD^+	0.33	–	0.39	–	0.41	–	0.29	
ATP/ADP	0.46	–	0.40	–	0.39	–	0.35	

*Carbon flow were calculated as specified in the Materials and Methods section. †Metabolic flux is expressed in meqC (g of cells)$^{-1}$ h^{-1}. ‡The percentage represents the proportion of original entering carbon flow, i.e. $q_{\text{cellobiose}}$, in a particular metabolic pathway. §None determined.

$$q_{biosynthesis}=[(C_{biomass}-C_{glycogen}+C_{amino\ acid}+C_{protein})/X]\times D$$

$$q_{carbon\ dioxide}=1/2\times(q_{acetate}+q_{ethanol})$$

$$q_{pyruvate}=q_{acetate}+q_{ethanol}+q_{lactate}+q_{extracellular\ pyruvate}+q_{carbon\ dioxide}$$

$$q_{acetyl\text{-}CoA}=q_{acetate}+q_{ethanol}$$

$$q_{glucose\ 6\text{-}phosphate}=q_{G6P}=q_{biosynthesis}+q_{pyruvate}$$

$$q_{phosphoglucomutase}=q_{PGM}=q_{G6P}-q_{glucose}$$

$$q_{glucose\ 1\text{-}phosphate}=q_{G1P}=1/2\times q_{cellobiose} \qquad q_{G1P}=0.63\times q_{cellulose}$$

$$q_{exopolysaccharide}=(C_{exopolysaccharide}/X)\times D \qquad q_{exopolysaccharide}=q_{G1P}-q_{PGM}-q_{glycogen}-q_{cellodextrin}$$

$$q_{G1P\ towards\ cellodextrin}=2/3\times q_{cellotriose} \qquad q_{G1P\ towards\ cellodextrin}=(1/2\times q_{cellobiose})+(2/3\times q_{cellotriose})$$

$$q_{cellobiose\ towards\ cellodextrin}=1/3\times q_{cellotriose} \qquad q_{cellulose\ towards\ cellodextrin}=(1/2\times q_{cellobiose})+(1/3\times q_{cellotriose})$$

X is the biomass concentration expressed as g l^{-1}. D is the dilution rate in h^{-1} and, at the steady-state of the system, it corresponds to the specific growth rate (μ) in continuous cultures of the type chemostat [40]. C is the concentration in meqC l^{-1}. The specific metabolic rate (q) was expressed in milliequivalents of carbon (meq) per gram of cells per hour. The proportion of carbon flowing through each of the metabolic pathways was also expressed as a percentage of the original entering carbon flow, i.e. $100\times q/q_{cellobiose}$ or $100\times q/q_{cellulose}$.

RESULTS

MFA of *C. cellulolyticum* growing with cellobiose was performed by adapting the previous equations established when *C. cellulolyticum* is growing with cellulose (see Materials and Methods). Such an approach allows the first direct comparison of the distribution of carbon flow when *C. cellulolyticum* grows in carbon limitation, nitrogen limitation, or both, with cellobiose or cellulose as the sole carbon and energy source in continuous cultures (Tables 1 to 6; Fig. 2). With cellulose, the terms excess and limitation of carbon must be understood differently than with a soluble glucide. In fact, it does not involve the amount of cellulose molecules but the number of accessible surface area on cellulose fibers, which then permits the adhesion of bacterial cells prior to efficient degradation of cellulose by the cellulosome. Moreover, in chemostat cultures, the term "nutrient limitation" means "nutrient-limited growth" and refers to conditions where the specific growth rate (μ) is dictated by substrate concentration [22]. This must not be confused with a nutrient limitation in batch culture, which refers only to the stoichiometric aspects of growth.

C. cellulolyticum Metabolism in Carbon Limitation

In chemostats under carbon limitation, despite increasing $q_{pyruvate}$ and $q_{biosynthesis}$ with increasing μ, the proportion of carbon directed towards these pathways clearly indicates that the carbon flow is rerouted from fermentation end-products, i.e. ethanol, acetate, and lactate, in favor of anabolic pathways as μ increases (Tables 1 and 2). As indicated by q_{G6P} on cellobiose and cellulose, respectively, at least 79.1 and 94.2% of the original carbon is used for generation of energy and biosynthetic precursors. From 0.033 to 0.120 h^{-1} with cellobiose, between 21.2 and 28.3% of the entering carbon is directed towards biosynthesis and 76.2 to 59.5% is used for ATP production (Table 1). From

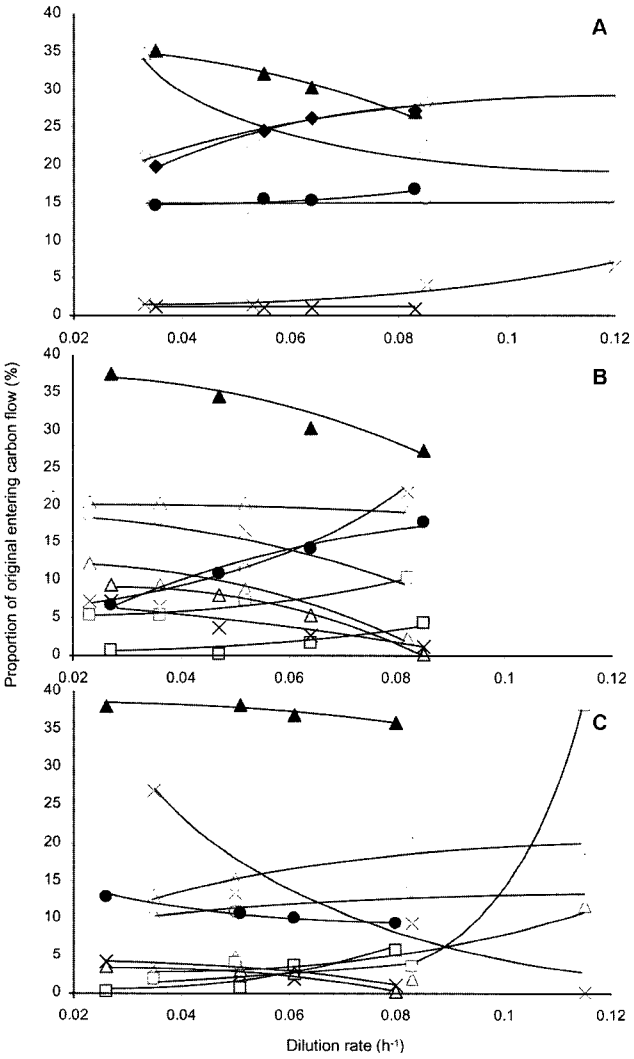

Fig. 2. Influence of the dilution rate (D) on the proportion of original entering carbon flowing through the metabolic pathways. A: Carbon limitation; B: nitrogen limitation; C: carbon and nitrogen saturation. In grey, results from cellobiose continuous cultures. In black, results from cellulose continuous cultures. ◆, Percentage of carbon directed toward biosynthesis; ▲, Percentage of carbon flowing through acetate production; ●, Percentage of carbon flowing through ethanol production; ×, Percentage of carbon flowing through lactate production; △, Percentage of carbon flowing through extracellular cellodextrin formation; □, Percentage of carbon flowing through exopolysaccharide formation.

Table 2. Carbon fluxes in continuous steady-state cultures of *C. cellulolyticum* in cellulose-limited condition (3.7 g l^{-1}).

Carbon flow	Results obtained at D (h^{-1}) value of:							
	0.035		0.055		0.064		0.083	
	Flux	%	Flux	%	Flux	%	Flux	%
$q_{cellulose}$	10.36	100.0	11.75	100.0	12.83	100.0	15.20	100.0
q_{G1P}	6.52	63.0	7.40	63.0	8.08	63.0	9.58	63.0
$q_{cellodextrin}$	nd	nd	nd	nd	nd	nd	nd	nd
$q_{G1P\ towards\ cellodextrin}$	nd	nd	nd	nd	nd	nd	nd	nd
$q_{cellulose\ towards\ cellodextrin}$	nd	nd	nd	nd	nd	nd	nd	nd
$q_{glycogen}$	0.14	1.4	0.20	1.7	0.22	1.7	0.24	1.6
$q_{exopolysaccharide}$	0.30	2.9	0.15	1.3	0.36	2.8	0.63	4.2
$q_{glucose}$	3.83	37.0	4.35	37.0	4.75	37.0	5.62	37.0
$q_{phosphoglucomutase}$	6.09	58.8	7.05	60.0	7.50	58.5	8.70	57.2
q_{G6P}	9.92	95.8	11.40	97.0	12.25	95.5	14.32	94.2
$q_{biosynthesis}$	2.05	19.8	2.88	24.5	3.36	26.2	4.14	27.3
$q_{pyruvate}$	7.86	75.9	8.52	72.5	8.89	69.3	10.18	67.0
$q_{acetyl\text{-}CoA}$	5.15	49.7	5.59	47.6	5.83	45.5	6.67	43.9
$q_{lactate}$	0.12	1.1	0.12	1.0	0.12	1.0	0.13	0.9
$q_{extracellular\ pyruvate}$	0.02	0.2	0.02	0.2	0.02	0.1	0.04	0.2
$q_{carbon\ dioxide}$	2.58	24.9	2.79	23.8	2.92	22.7	3.34	21.9
$q_{ethanol}$	1.51	14.6	1.82	15.5	1.96	15.3	2.55	16.8
$q_{acetate}$	3.64	35.2	3.77	32.1	3.87	30.2	4.12	27.1
Ratio								
H_2/CO_2	1.58		1.38		1.41		1.29	
$NADH/NAD^+$	0.39		0.31		0.33		0.32	
ATP/ADP	0.40		0.39		0.36		0.46	

0.035 to 0.083 h^{-1} with cellulose, 19.8 to 27.3% of carbon is directed towards biosynthesis while 75.9 to 67.0% is used for ATP production (Table 2). However, the carbon flux distribution within the metabolic pathways is different between cellobiose and cellulose (Fig. 2A). In fact, on cellobiose with increasing μ, the percentage of original carbon forming acetate declined, the percentage of carbon towards ethanol remains quite stable, while the proportion of carbons towards lactate production increases (Table 1). With cellobiose or cellulose, the H_2/CO_2 ratio decreases with increasing μ. This indicates that the redox balance is maintained by NADH-ferredoxin (Fd) reductase and hydrogenase activities at low μ, but as μ increases, the contribution of ethanol pathway to the NADH reoxidation is higher. This shift from an acetate-ethanol fermentation to a somewhat ethanol-lactate fermentation is only observed with cellobiose. Therefore, on cellulose, pyruvate-Fd oxidoreductase (PFO) and lactate dehydogenase (LDH) do not compete for carbon flowing from glycolysis. Even with evolving proportion of acetate, ethanol, and lactate with increasing μ, acetate always remains the major fermentation end-product on both cellobiose and cellulose, between 19.6–34.8% and 27.1–35.2% of the original carbon, respectively. With cellobiose, increase of lactate production is correlated to the formation of extracellular pyruvate (Table 1). Such a pyruvate leak indicates that the carbon flowing down glycolysis could not be fully metabolized by enzymes of anabolic and catabolic pathways downstream the pyruvate metabolic node. With cellobiose at μ=0.053 h^{-1}, pyruvate overflow occurs for an entering carbon flow higher than 16.08 meqC (g of cells)$^{-1}$ h^{-1} obtained (Table 1). With cellulose at μ=0.055 h^{-1}, the entering carbon flow is much lower than with cellobiose, i.e. 11.75 meqC (g of cells)$^{-1}$ h^{-1}, and even at the highest μ obtained, $q_{cellulose}$ is only 15.20 meqC (g of cells)$^{-1}$ h^{-1} (Table 2). As a result, lactate production and pyruvate overflow are much more limited with cellulose as a carbon source. For the same specific growth rate, cellobiose allows to reach higher entering carbon flow than cellulose.

C. cellulolyticum Metabolism in Nitrogen Limitation

In chemostats under ammonium-limited condition (Tables 3 and 4), the carbon flow is mainly directed towards acetate production at all μ, i.e. between 20.5 and 19.0% of the entering carbon with cellobiose from μ=0.023 to 0.082 h^{-1}, and from 37.5 to 27.3% on cellulose as μ increases from 0.027 to 0.085 h^{-1} (Tables 3 and 4 and Fig. 2B). While on cellobiose the carbon is rerouted towards lactate production with increasing μ and reaches 21.8% of the original carbon uptake at μ=0.082 h^{-1}, on cellulose the proportion of carbon

Table 3. Carbon fluxes in continuous steady-state cultures of *C. cellulolyticum* in ammonium-limited condition (4.0 mM) with cellobiose as carbon source.

Carbon flow	Results obtained at D (h^{-1}) value of:							
	0.023		0.036		0.052		0.082	
	Flux	%	Flux	%	Flux	%	Flux	%
$q_{cellobiose}$	9.59	100.0	12.24	100.0	17.68	100.0	19.92	100.0
q_{G1P}	4.79	50.0	6.12	50.0	8.84	50.0	9.96	50.0
$q_{cellodextrin}$	1.18	12.3	1.13	9.3	3.96	8.8	0.48	2.4
$q_{G1P\ towards\ cellodextrin}$	0.79	8.2	0.75	6.2	0.47	2.6	0.32	1.6
$q_{cellobiose\ towards\ cellodextrin}$	0.39	4.1	0.38	3.1	0.23	1.3	0.16	0.8
$q_{glycogen}$	0.23	2.5	0.34	2.8	0.38	2.1	0.37	1.9
$q_{exopolysaccharide}$	0.51	5.3	0.65	5.3	1.31	7.4	2.06	10.3
$q_{glucose}$	4.79	50.0	6.12	50.0	8.84	50.0	9.96	50.0
$q_{phosphoglucomutase}$	2.90	30.2	3.38	27.6	5.94	33.6	7.40	37.2
q_{G6P}	7.69	80.2	9.50	77.6	14.78	83.6	17.36	87.2
$q_{biosynthesis}$	1.37	14.2	1.64	13.4	3.08	17.4	3.90	19.6
$q_{pyruvate}$	6.32	66.0	7.85	64.2	11.70	66.2	13.46	67.6
$q_{acetyl\text{-}CoA}$	3.76	39.2	4.71	38.5	5.56	31.5	5.78	29.0
$q_{lactate}$	0.69	7.2	0.79	6.5	2.94	16.6	87.2	21.8
$q_{extracellular\ pyruvate}$	nd	nd	nd	nd	0.42	2.4	0.44	2.2
$q_{carbon\ dioxide}$	1.88	19.6	2.35	19.2	2.78	15.7	2.89	14.5
$q_{ethanol}$	1.79	18.6	2.22	18.1	2.00	11.3	2.00	10.0
$q_{acetate}$	1.97	20.5	2.49	20.3	3.56	20.2	3.78	19.0

increases in favor of ethanol production and reaches 17.7% at μ=0.085 h^{-1} (Fig. 2B). Once soluble cellodextrins enter into the bacterial cell, they are converted into glucose 1-phosphate (G1P) and glucose 6-phosphate (G6P) (Fig. 1). The G6P pool is fuelled by the carbon flowing from glucokinase and PGM activities and is further metabolized by glycolysis. As q_{G6P} increases with μ, it represents a mean of 82.2% of the carbon uptake on cellobiose and 92.3% on cellulose (Tables 3 and 4). G1P pool is issued from cellobiose and cellodextrin phosphorylase activities. The proportion of carbon flowing through phosphoglucomutase (PGM) remains quite constant with increasing μ, around 32.2% on cellobiose and 55.9% on cellulose (Tables 3 and 4). While no cellodextrin could be assayed in the supernatant of carbon-limited chemostats, cellobiose and cellotriose are detected in nitrogen limitation with cellulose. The presence of these cellodextrins is most certainly associated with reversible phosphorylases activity. The cellodextrin, exopolysaccharide, and glycogen biosynthesis pathways balance carbon surplus. Cellodextrins excretion occurs at low μ while exopolysaccharide synthesis occurs essentially at higher μ (Tables 3 and 4). This underlines the importance of G1P-G6P branch point in *C. cellulolyticum* for the distribution of carbon fluxes inside and outside cells. However, this overflow metabolism is clearly lower on cellulose than on cellobiose where cellodextrins represent 12.3% of the entering carbon and exopolysaccharide 10.3% (Tables 3 and 4). Once again, this must be paralleled with specific carbon consumption rates higher with cellobiose than with cellulose. This indicates that the level of cellodextrin and exopolysaccharide production with cellobiose should be interpreted as distortion of bacterial metabolism due to the use of soluble carbon substrate far different from insoluble lignocellulosic compounds. Regardless of μ, most of the carbon is directed towards biosynthesis with cellobiose or cellulose (Tables 3 and 4). However, the presence of free amino acids even in ammonium limitation suggests that the uptake of nutrient and generation of biosynthetic precursors occur faster than their utilization for biomass production. Compared to atmospheric nitrogen, ammonium as primary nitrogen source could also distort the bacterial metabolism [30].

C. cellulolyticum Metabolism in Both Carbon and Nitrogen Saturation

In continuous cultures under sufficient carbon and ammonium conditions with increasing D, the proportion of carbon flowing towards fermentation end-products declined, from 61.8 to 41.4% with cellobiose and from 81.2 to 69.1% with cellulose (Tables 5 and 6). In the same time, the proportion of carbon was enhanced through biosynthesis pathways, from 16.5 to 29.7% with cellobiose and from 16.3 to 23.8% with cellulose. The percentage of carbon directed towards LDH declined with increasing D (Tables 5 and 6; Fig. 2C). At low D, however, the proportion of carbon converted into lactate is much higher with cellobiose; at D=0.035 h^{-1} it reaches 26.8% against 4.2% with cellulose at D=0.026 h^{-1} (Fig. 2C). In all conditions tested, this is

Table 4. Carbon fluxes in continuous steady-state cultures of *C. cellulolyticum* in ammonium-limited condition (4.0 mM) with cellulose as carbon source.

Carbon flow	Results obtained at D (h^{-1}) value of:							
	0.027		0.047		0.064		0.085	
	Flux	%	Flux	%	Flux	%	Flux	%
$q_{cellulose}$	9.50	100.0	12.61	100.0	15.11	100.0	17.39	100.0
q_{G1P}	5.98	68.0	7.94	63.0	9.51	63.0	10.95	63.0
$q_{cellodextrin}$	0.89	9.3	1.04	8.0	0.81	5.4	nd	nd
$q_{G1P\ towards\ cellodextrin}$	0.49	5.2	0.58	4.6	0.46	3.1	nd	nd
$q_{cellulose\ towards\ cellodextrin}$	0.40	4.2	0.43	3.4	0.34	2.3	nd	nd
$q_{glycogen}$	0.15	1.6	0.29	2.3	0.36	2.4	0.43	2.5
$q_{exopolysaccharide}$	0.07	0.7	0.02	0.1	0.26	1.7	0.76	4.4
$q_{glucose}$	3.52	37.0	4.67	37.0	5.60	37.0	6.44	37.0
$q_{phosphoglucomutase}$	5.27	55.5	7.06	56.0	8.43	55.8	9.79	56.3
q_{G6P}	8.79	92.5	11.73	93.0	14.02	92.8	16.20	93.2
$q_{biosynthesis}$	1.73	18.2	2.63	20.9	3.45	22.8	4.26	24.5
$q_{pyruvate}$	7.07	74.4	9.10	72.2	10.58	70.0	11.94	68.6
$q_{acetyl\text{-}CoA}$	4.18	44.0	5.70	45.2	6.74	44.6	7.83	45.0
$q_{lactate}$	0.68	7.2	0.46	3.6	0.40	2.6	0.20	1.1
$q_{extracellular\ pyruvate}$	0.11	1.2	0.09	0.7	0.07	0.4	nd	nd
$q_{carbon\ dioxide}$	2.09	22.0	2.85	22.6	3.37	22.3	3.91	22.5
$q_{ethanol}$	0.63	6.7	1.38	10.9	2.16	14.3	3.08	17.7
$q_{acetate}$	3.55	37.5	4.32	34.4	4.58	30.3	4.75	27.3

Table 5. Carbon fluxes in continuous steady-state cultures of *C. cellulolyticum* in cellobiose-sufficient condition (5 g l^{-1}).

Carbon flow	Results obtained at D (h^{-1}) value of:							
	0.035		0.050		0.083		0.115	
	Flux	%	Flux	%	Flux	%	Flux	%
$q_{cellobiose}$	16.24	100	19.43	100	21.49	100	30.90	100
q_{G1P}	8.12	50	9.71	50	10.75	50	15.45	50
$q_{cellodextrin}$	0.46	2.8	0.96	4.9	0.40	1.9	3.60	11.7
$q_{G1P\ towards\ cellodextrin}$	0.31	1.9	0.64	3.3	0.27	1.2	2.4	7.8
$q_{cellobiose\ towards\ cellodextrin}$	0.15	0.9	0.32	1.6	0.13	0.6	1.20	3.9
$q_{glycogen}$	0.07	0.4	0.09	0.5	0.14	0.6	0.13	0.4
$q_{exopolysaccharide}$	0.30	1.8	0.77	4.0	0.75	3.5	11.76	38.1
$q_{glucose}$	8.12	50.0	9.71	50.0	10.75	50.0	15.45	50.0
$q_{phosphoglucomutase}$	4.59	28.3	4.22	21.7	7.04	32.8	6.52	21.1
q_{G6P}	12.71	78.3	13.93	71.7	17.79	82.8	21.97	71.1
$q_{biosynthesis}$	2.67	16.5	3.70	19.0	4.81	22.4	9.17	29.7
$q_{pyruvate}$	10.03	61.8	10.24	52.7	12.98	60.4	12.80	41.4
$q_{acetyl\text{-}CoA}$	3.74	23.0	5.05	26.0	7.24	33.7	8.50	27.5
$q_{lactate}$	4.34	26.8	2.57	13.3	1.99	9.3	nd	nd
$q_{extracellular\ pyruvate}$	0.09	0.5	0.09	0.4	0.14	0.6	0.05	0.02
$q_{carbon\ dioxide}$	1.87	11.5	13.0	0.09	16.8	0.14	13.8	0.05
$q_{ethanol}$	1.62	10.0	2.10	10.8	2.94	13.7	2.99	9.7
$q_{acetate}$	2.11	13.0	2.95	15.2	4.30	20.0	5.51	17.8
Ratio								
H_2/CO_2	1.03		1.16		1.23		1.56	
$NADH/NAD^+$	0.43		0.55		0.68		1.51	
ATP/ADP	1.97		1.70		4.26		7.21	

Table 6. Carbon fluxes in continuous steady-state cultures of *C. cellulolyticum* in cellulose-sufficient condition (18.1 g·l^{-1}).

Carbon flow	Results obtained at D (h^{-1}) value of:							
	0.026		0.051		0.061		0.080	
	Flux	%	Flux	%	Flux	%	Flux	%
$q_{cellulose}$	9.91	100.0	13.98	100.0	15.20	100.0	17.44	100.0
q_{G1P}	6.24	63.0	8.80	63.0	9.57	63.0	10.98	63.0
$q_{cellodextrin}$	0.34	3.5	0.41	3.0	0.37	2.6	nd	nd
$q_{G1P\ towards\ cellodextrin}$	0.20	2.0	0.24	1.7	0.21	1.5	nd	nd
$q_{cellulose\ towards\ cellodextrin}$	0.15	1.5	0.18	1.3	0.16	1.1	nd	nd
$q_{glycogen}$	0.05	0.5	0.12	0.9	0.22	1.4	0.24	1.4
$q_{exopolysaccharide}$	0.01	0.1	0.10	0.7	0.55	3.6	0.99	5.7
$q_{glucose}$	3.67	37.0	5.18	37.0	5.63	37.0	6.46	37.0
$q_{phosphoglucomutase}$	5.99	60.4	8.34	59.7	8.59	56.5	9.75	55.9
q_{G6P}	9.66	97.5	13.52	96.7	14.22	93.5	16.21	92.9
$q_{biosynthesis}$	1.61	16.3	2.88	20.6	3.22	21.2	4.16	23.8
$q_{pyruvate}$	8.04	81.2	10.64	76.1	11.00	72.3	12.05	69.1
$q_{acetyl\text{-}CoA}$	5.05	50.9	6.82	48.8	7.11	46.8	7.89	45.2
$q_{lactate}$	0.41	4.2	0.35	2.5	0.29	1.9	0.18	1.0
$q_{extracellular\ pyruvate}$	0.06	0.6	0.05	0.4	0.05	0.3	0.04	0.2
$q_{carbon\ dioxide}$	2.52	25.5	3.41	24.4	3.56	23.4	3.95	22.6
$q_{ethanol}$	1.28	12.9	1.50	10.7	1.52	10.0	1.64	9.4
$q_{acetate}$	3.77	38.0	5.33	38.1	5.59	36.8	6.25	35.8
Ratio								
H_2/CO_2	1.43		1.55		1.61		1.67	
$NADH/NAD^+$	0.59		0.63		0.45		0.66	
ATP/ADP	2.01		1.91		1.68		1.51	

only in cellobiose and ammonium saturated conditions at D=0.035 h^{-1} that the production of lactate, or any other fermentations end-products, exceeds the production of acetate (Tables 1 to 6). This is also only in cellobiose and ammonium saturated conditions that the highest carbon consumption rate has been observed with a $q_{cellobiose}$ of 30.90 meqC (g of cells)$^{-1}$ h^{-1} at D=0.115 h^{-1}. Once again, this is clearly associated with a higher carbon consumption rate on cellobiose than on cellulose for same D value. The concomitant decrease of the percentage of carbon flowing through q_{PGM}, q_{G6P}, and $q_{pyruvate}$ in favor of biosynthesis pathways could explain the relative drop in lactate production as D increases (Tables 5 and 6). In contrast to what is observed in carbon limitation with cellobiose or cellulose, lactate-ethanol production is lowered as D rises (Tables 1, 2, 5 and 6; Fig. 2). Thus, hydrogen production *via* NADH-Fd reductase balanced the reorientation of carbon flow through acetate production leading to an increase H_2/CO_2 ratio with increasing D (Tables 5 and 6). In carbon saturation, the electron flow is mainly balanced by the ethanol pathway at low D, and NADH-Fd reductase and hydrogenase at higher D. This regulation of NADH reoxidation into NAD^+ is inverted in carbon limitation (see section *C. cellulolyticum* metabolism in carbon limitation). At D=0.115 h^{-1} with cellobiose, overflows of carbon occur, namely with the production of cellodextrins, exopolysaccharides, and free amino acids, which attain respectively a maximum of 11.7, 38.1, and 15.4% of the original carbon uptake (Table 5). While cellodextrins are undetectable in carbon limitation, they are present in the supernatant of carbon sufficient continuous cultures (Tables 4 to 6). However, the presence of both cellobiose and cellotriose under cellulose sufficient conditions suggests that cellobiose could also be synthesised *de novo* during cell growth on cellobiose. Under cellulose-excess conditions, the exopolysaccharide, cellodextrin, and free amino acids overflows are much more limited and are associated with lower carbon consumption rates than with cellobiose (Tables 5 and 6). Changing from carbon limitation to carbon excess using either cellobiose or cellulose has a profound effect on carbon flow distribution (Tables 1, 2, 5, and 6): (i) lactate production decreases with increasing D, (ii) the proportion of carbon towards exopolysaccharides reaches much higher levels of production.

DISCUSSION

Such modeling of the carbon flow is a powerful tool to identify limiting steps in the central metabolic pathways.

The investigations of *C. cellulolyticum* metabolism, using successively cellobiose and cellulose as the sole carbon and energy source, allow solving of the nature of carbon flow regulation. In fact, it points out (i) the key role of cellulosome in the regulation of entering carbon flow, (ii) the major role of PGM in carbon flow regulation since its controls the entry of G1P towards the central metabolism, and (iii) the importance and sensitivity of the PFO metabolic node in regulation of energetic and electronic fluxes.

While cellobiose is a carbon substrate for bacterial growth, cellulose is only a carbon source. The cellulosomal cellulases permit the cellulose depolymerization resulting in release of glucose and soluble cellodextrins. These soluble glucides are then the true carbon substrates allowing bacterial growth from cellulose. Cellobiose, a soluble cellodextrin, shunts completely the cellulosome. Thus, by reaching much higher carbon consumption rate, the use of cellobiose highlights metabolic limitation of *C. cellulolyticum*. In fact, for the same specific growth rate or *D*, higher carbon consumption rates are systematically obtained with cellobiose than with cellulose. It also demonstrates that growth with cellobiose does not result in the same metabolism than with cellulose contrary to what was originally suggested. The occurrence and level of overflows originally observed with cellobiose must be considered as distortion of bacterial metabolism due to the use of carbon source far more easily available than natural lignocellulosic compounds. On cellulose, these overflows are much more limited and correspond most certainly to physiological regulation; the use of cellobiose pushes *C. cellulolyticum* metabolism to some of its limits. In the course of evolution, the catabolic pathways of *C. cellulolyticum* have been optimized as a function of the carbon flowing from cellulases activities. Compared to other saccharolytic clostridia [28], *C. cellulolyticum* copes with difficulty with high catabolic rates. Therefore *C. cellulolyticum* appears restricted to a cellulolytic lifestyle. However, much remains to be learned about the N_2-fixing ability of and catabolism of lignocellulosic compounds, which are both more closely related to the natural ecosystem of this bacterium. From the assumption that cellobiose is the major soluble cellodextrin released in the course of cellulolysis, this glucide is widely used as a model carbon source for metabolic studies of anaerobic cellulolytic bacteria. However, by comparing directly the distribution of the carbon flow within the central metabolism of *C. cellulolyticum* with cellobiose on one hand and cellulose on the other hand, the present investigation clearly demonstrates the profound influence of soluble or insoluble nature of carbon sources on the metabolism of cellulolytic clostridia. This strongly suggests that the use of cellobiose for metabolic studies of cellulolytic microorganisms must be avoided, or at least carefully taken into consideration for the interpretation of results.

C. cellulolyticum growth inhibition occurs in both cellobiose and cellulose fermentation with concomitant excretion of pyruvate when the carbon source is present in excess. This suggests that the rate of carbon flowing down glycolysis is higher than the rate of enzymatic processing of pyruvate by the anabolic and catabolic pathways. However, biotechnological exploitation of this bacterium will not involve limited substrate concentration but saturated substrate conditions. From the indepth analysis of *C. cellulolyticum* metabolism, metabolic engineering of this microorganism to improve its cellulolytic performance has been undertaken by Guedon *et al.* [15]. A new catabolic pathway, branched on the pyruvate node and constituted of pyruvate decarboxylase and alcohol dehydrogenase, was inserted. While higher production of CO_2, H_2, acetate, and ethanol was achieved, acetate remains the main fermentation end-product. This approach proves that cellulose fermentation by *C. cellulolyticum* can be greatly improved by genetic engineering of its metabolism. Even if it failed to reroute completely the carbon flow towards the formation of ethanol, this recombinant strain is of interest for biotechnological process involving the conversion of lignocellulosic compounds into hydrogen or methane [1, 31].

Practically, MFA allows applied microbiologists to make choices in the future directions to take for the improvement of cellulose catabolism by *C. cellulolyticum*. The direct comparison of MFA from cellobiose and cellulose revealed the importance of three enzymatic systems in the regulation and distribution of carbon flow, i.e. cellulosome, PFO, and PGM. Concerning the cellulosome, MFA demonstrates that the use of pure cellulose at high concentrations results in high carbon flow correlated with early growth arrest. Contrary to some assumptions of the enzymatic paradigm [25], this clearly indicates that cellulose depolymerization is not the limiting step of microbial cellulose digestion by *C. celluloyticum*. The cellulosome is a highly efficient cellulasic system for degradation of crystalline cellulose. As strongly supported by MFA and further demonstrated by metabolic engineering, improvement of cellulolysis by *C. cellulolyticum* must primarily focus on bacterial metabolism rather than catalytic activity of cellulosome. PFO appears the most sensitive metabolic node of *C. cellulolyticum* catabolism as indicated by the presence of pyruvate in the extracellular medium and as demonstrated by metabolic engineering of this metabolic node, which allows to prolong cell growth, and as a consequence, to reach higher cell density, cellulolysis, and final concentrations of catabolites [15]. One of the reasons that can be advanced to explain the incomplete rerouting of carbon flow towards ethanol production arises from MFA of *C. cellulolyticum*. In fact, with cellulose as a carbon source, acetate is always the main fermentation end-product, pointing out the importance of this catabolic pathway in the metabolism of *C. cellulolyticum*. This pathway permits the generation of 2

extra moles of ATP per mole of hexose catabolized and is therefore essential to maintain a correct energetic balance. Thus, if extra generation of ATP is not maintained, the complete rerouting of carbon flow away from the acetate pathway is certainly physiologically impossible. Considering the regulation of carbon, redox, and energetic flows revealed by MFA, an alternative approach would consist in improving carbon flow rate at the pyruvate node by incorporating a PFO enabled to support a high catabolic rate, i.e. from saccharolytic clostridia such as *C. acetobutylicum* or *C. pasteurianum* [7]. Such a strategy would still permit the carbon flow to be directed towards the formation of acetate and ethanol, and maintain a correct energetic and redox balance. Furthermore, from this recombinant strain, other catabolic pathways could be inserted to produce catabolites of industrial interest such as butanol, acetone, or isopropanol. Ultimately, however, the catabolism of *C. cellulolyticum* would reach the limit imposed by PGM, which exerts a strict control of the carbon flow directed towards glycolysis. Also, improvement of the catalytic activity of this metabolic node would most probably result in overflow(s) in the anabolic and/or catabolic pathways downstream. An alternative approach would be the directed evolution of the metabolism in chemostat.

Acknowledgments

I express my gratitude to Professor H. Petitdemange for helpful discussions. I am thankful to Dr. N. J. Parham for correcting the English and for critical reading of the manuscript. This work was supported by the "Conseil Régional de Lorraine" following the attribution of the "1er Prix de la Thèse 2002" to MD.

REFERENCES

1. Angelidaki, I., L. Ellegaard and B. K. Ahring. 2003. Applications of the anaerobic digestion process. *Adv. Biochem. Eng. Biotechnol.* **82:** 1-33.
2. Bayer, E. A. and R. Lamed. 1992. The cellulose paradox: Pollutant par excellence and/or a reclaimable natural resource? *Biodegradation* **3:** 171-88.
3. Bélaïch, J. P., C. Tardif, A. Bélaïch, and C. Gaudin. 1997. The cellulolytic system of *Clostridium cellulolyticum*. *J. Biotechnol.* **57:** 3-14.
4. Boisset, C., H. Chanzy, B. Henrissat, R. Lamed, Y. Shoham, and E. A. Bayer. 1999. Digestion of crystalline cellulose substrates by *Clostridium thermocellum* cellulosome: Structural and morphological aspects. *Biochem. J.* **340:** 829-835.
5. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.
6. Chaney, A. L. and E. P. Marbach. 1962. Modified reagents for determination of urea and ammonia. *Clin. Chem.* **8:** 130-132.
7. Desvaux, M. 2001. La fermentation de la cellulose par *Clostridium cellulolyticum*: Métabolisme modèle d'un *Clostridium* cellulolytique mésophile. PhD thesis, Université Henri Poincaré-Nancy I, Nancy, France.
8. Desvaux, M., E. Guedon, and H. Petitdemange. 2001. Carbon flux distribution and kinetics of cellulose fermentation in steady-state continuous cultures of *Clostridium cellulolyticum* on a chemically defined medium. *J. Bacteriol.* **183:** 119-130.
9. Desvaux, M., E. Guedon and H. Petitdemange. 2000. Cellulose catabolism by *Clostridium cellulolyticum* growing in batch culture on defined medium. *Appl. Environ. Microbiol.* **66:** 2461-2470.
10. Desvaux, M., E. Guedon, and H. Petitdemange. 2001. Kinetics and metabolism of cellulose degradation at high substrate concentrations in steady-state continuous cultures of *Clostridium cellulolyticum* on a chemically defined medium. *Appl. Environ. Microbiol.* **67:** 837-845.
11. Desvaux, M., E. Guedon, and H. Petitdemange. 2001. Metabolic flux in cellulose batch and cellulose-fed continuous cultures of *Clostridium cellulolyticum* in response to acidic environment. *Microbiology* **147:** 1461-1471.
12. Desvaux, M. and H. Petitdemange. 2001. Flux analysis of the metabolism of *Clostridium cellulolyticum* grown in cellulose-fed continuous culture on a chemically defined medium under ammonium-limited conditions. *Appl. Environ. Microbiol.* **67:** 3846-3851.
13. Dubois, M., K. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1951. A colorimetric method for the determination of sugars. *Nature* **168:** 167.
14. Giallo, J., C. Gaudin, and J. P. Bélaïch. 1985. Metabolism and solubilization of cellulose by *Clostridium cellulolyticum* H10. *Appl. Environ. Microbiol.* **49:** 1216-1221.
15. Guedon, E., M. Desvaux, and H. Petitdemange. 2002. Improvement of cellulolytic properties of *Clostridium cellulolyticum* by metabolic engineering. *Appl. Environ. Microbiol.* **68:** 53-58.
16. Guedon, E., M. Desvaux, and H. Petitdemange. 2000. Kinetic analysis of *Clostridium cellulolyticum* carbohydrate metabolism: Importance of glucose 1-phosphate and glucose 6-phosphate branch points for distribution of carbon fluxes inside and outside cells as revealed by steady-state continuous culture. *J. Bacteriol.* **182:** 2010-2017.
17. Guedon, E., S. Payot, M. Desvaux, and H. Petitdemange. 1999. Carbon and electron flow in *Clostridium cellulolyticum* grown in chemostat culture on synthetic medium. *J. Bacteriol.* **181:** 3262-3269.
18. Guedon, E., S. Payot, M. Desvaux, and H. Petitdemange. 2000. Relationships between cellobiose catabolism, enzyme levels, and metabolic intermediates in *Clostridium cellulolyticum* grown in a synthetic medium. *Biotechnol. Bioeng.* **67:** 327-335.

19. Hofman-Bang, J., D. Zheng, P. Westermann, B. K. Ahring, and L. Raskin. 2003. Molecular ecology of anaerobic reactor systems. *Adv. Biochem. Eng. Biotechnol.* **81:** 151-203.
20. Holms, H. 1996. Flux analysis and control of the central metabolic pathways in *Escherichia coli*. *FEMS Microbiol. Reviews* **19:** 85-116.
21. Huang, L. and C. W. Forsberg. 1990. Cellulose digestion and cellulase regulation and distribution in *Fibrobacter succinogenes* subsp. *succinogenes* S85. *Appl. Environ. Microbiol.* **56:** 1221-1228.
22. Kovarova-Kovar, K. and T. Egli. 1998. Growth kinetics of suspended microbial cells: From single-substrate-controlled growth to mixed-substrate kinetics. *Microb. Mol. Biol. Rev.* **62:** 646-666.
23. Leschine, S. B. 1995. Cellulose degradation in anaerobic environments. *Annu. Rev. Microbiol.* **49:** 399-426.
24. Lynd, L. R., J. H. Cushman, R. J. Nichols, and C. E. Wyman. 1991. Fuel ethanol from cellulosic biomass. *Science* **251:** 1318-1323.
25. Lynd, L. R., P. J. Weimer, W. H. van Zyl, and I. S. Pretorius. 2002. Microbial cellulose utilization: Fundamentals and biotechnology. *Microbiol. Mol. Biol. Rev.* **66:** 506-577.
26. Matheron, C., A. M. Delort, G. Gaudet, E. Forano, and T. Liptaj. 1998. ^{13}C and ^{1}H nuclear magnetic resonance study of glycogen futile cycling in strains of the genus *Fibrobacter*. *Appl. Environ. Microbiol.* **64:** 74-81.
27. Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal. Chem.* **31:** 426-428.
28. Mitchell, W. J. 1998. Physiology of carbohydrate to solvent conversion by clostridia. *Adv. Microb. Physiol.* **39:** 31-130.
29. Mokrasch, L. C. 1967. Use of 2,4,6-trinitrobenzenesulfonic acid for the coestimation of amines, amino acids, and proteins in mixtures. *Anal. Biochem.* **18:** 64-71.
30. Monserrate, E., S. B. Leschine, and E. Canale-Parola. 2001. *Clostridium hungatei* sp. nov., a mesophilic, N_2-fixing cellulolytic bacterium isolated from soil. *Int. J. Syst. Microbiol.* **51:** 123-132.
31. Nandi, R. and S. Sengupta. 1998. Microbial production of hydrogen: An overview. *Crit. Rev. Microbiol.* **24:** 61-84.
32. Payot, S., E. Guedon, C. Cailliez, E. Gelhaye, and H. Petitdemange. 1998. Metabolism of cellobiose by *Clostridium cellulolyticum* growing in continuous culture: Evidence for decreased NADH reoxidation as a factor limiting growth. *Microbiology* **144:** 375-384.
33. Pereira, A. N., M. Mobedshashi, and M. R. Ladish. 1988. Preparation of cellodextrins. *Meth. Enzymol.* **160:** 26-45.
34. Petitdemange, E., F. Caillet, and C. Gaudin. 1984. *Clostridium cellulolyticum* sp. nov., a cellulolytic mesophilic species from decayed grass. *Int. J. Syst. Microbiol.* **34:** 155-159.
35. Stephanopoulos, G. 1999. Metabolic fluxes and metabolic engineering. *Metab. Eng.* **1:** 1-11.
36. Strobel, H. J., F. C. Caldwell, and K. A. Dawson. 1995. Carbohydrate transport by the anaerobic thermophile *Clostridium thermocellum* LQRI. *Appl. Environ. Microbiol.* **61:** 4012-4015.
37. Updegraff, D. M. 1969. Semimicro determination of cellulose in biological materials. *Anal. Biochem.* **32:** 420-424.
38. Weimer, P. J., Y. Shi, and C. L. Odt. 1990. A segmented gas/liquid delivery system for continuous culture of microorganisms on insoluble substrates and its use for growth of *Ruminoccus flavefaciens* on cellulose. *Appl. Microbiol. Biotechnol.* **36:** 178-183.
39. Wolin, M. J. and T. L. Miller. 1987. Bioconversion of organic carbon to CH_4 and CO_2. *Geomicrobiol. J.* **5:** 239-259.
40. Zeng, A. P. 1999. *Continuous Culture*, 2nd Ed. American Society for Microbiology, Washington D.C., U.S.A.