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In vivo ^{13}C NMR study of glucose and cellobiose metabolism by four cellulolytic strains of the genus *Fibrobacter*

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Abstract

The metabolism of glucose and cellobiose, products of cellulose hydrolysis, was investigated in four cellulolytic strains of the genus *Fibrobacter*: *Fibrobacter succinogenes* S85, 095, HM2 and *Fibrobacter intestinalis* NR9. *In vivo* ^{13}C nuclear magnetic resonance was used to quantify the relative contribution of glucose and cellobiose to metabolite production, glycogen storage and cellodextrins synthesis in these four strains. The same features were found in all four strains of the genus *Fibrobacter* metabolizing simultaneously glucose and cellobiose: i) differential metabolism of glucose and cellobiose; glucose seems preferentially used for glycogen storage and energy production, while part of cellobiose seems to be diverted from glycolysis, ii) synthesis of cellodextrins, mainly from cellobiose not entering into glycolysis, iii) accumulation of glucose 6-phosphate, iv) simultaneous presence of cellobiose phosphorylase and cellobiase activities.

Although genetically diverse, the *Fibrobacter* genus appears to possess a marked homogeneity in its carbon metabolism.

Introduction

Microbial cellulases and hemicellulases are widely used in different industries, but also in biological treatment of fibrous feeds in the non-ruminant livestock industry. The increasing concern about environmental issues has also led to the use of these enzymes in the treatment of domestic wastes. Thus, in the aim of anaerobic waste water treatment, many researchers have focussed on selection and characterization of strains able to convert lignocellulosic residues.

Ruminants are efficient animals to digest lignocellulosic substrates, due to their complex ruminal flora. Up to 50% of the organic matter in straws can be degraded by the ovine and caprine. *Fibrobacter succinogenes*, a strictly anaerobic bacterium from the rumen, has large potentials in degradation of lignocellulosic residues. In the rumen, this bacterium becomes predominant among the cellulolytic bacteria when ruminants are fed with poor diet, ie. highly lignified material (Bryant & Burkey 1953). Pure cultures of *F. succinogenes* digest more cellulose from intact forages than other cellulolytic rumen bacterial species (Dehority 1993). The same result was obtained *in vivo* with wheat straw (Fonty et al. 1988). The enzymatic machinery of *F. succinogenes* allows to explain these specific performances. This bacterium degrades cellulose by a very efficient complex cellulolytic system (Forsberg et al. 1994; Chesson & Forsberg 1997). Cellulose is depolymerized at the bacterial surface by different cellulases and the released cellodextrins are hydrolysed into glucose and cellobiose in the periplasm (Huang & Forsberg 1987). It produces ferrulic acid esterase, acetylxylane esterase, and arabinofuranosidase (McDermid et al. 1990) that are necessary to cleave the ester bonds linking hemicelluloses to lignin or to debranch xylanes. Several different xylanases and

an α-glucuronidase complete the cellulolytic system (Malburg et al. 1993; Smith & Forsberg 1991).

To improve the efficiency of *Fibrobacter* to degrade lignocellulosic residues, it is necessary to know how this bacterium converts cellulose to metabolites. Recently, Maglione et al. (1997) studied kinetics of cellulose digestion by *F. succinogenes* S85 and concluded that the cellulase activity of this strain was directly linked to cellobiose metabolism.

Glucose and cellobiose, final products of cellulose hydrolysis are taken up and metabolized by the cells to succinate, acetate and small amount of formate (Miller 1978; Gaudet et al. 1992; Matheron et al. 1996, 1997, 1998). The growth of *F. succinogenes* S85 cells is similar with glucose or cellobiose as substrate (Franklund & Glass 1987; Gaudet & Cheng 1990) and when both sugars are provided to the cells, they are simultaneously metabolized (Matheron et al. 1996). They are transported across the cytoplasmic membrane through independent constitutive permeases, which are sodium-dependent (Chow & Russel 1992; Maas & Glass 1991; Franklund & Glass 1987). When the extracellular sugar concentration is high, part of the sugar is stored as glycogen (Gaudet et al. 1992, Matheron et al. 1998) or is released as cellodextrins into the external medium (Matheron et al. 1996, Wells et al. 1995). Using *in vivo* ^{13}C-NMR, the metabolism of [1-^{13}C] glucose in the presence of cellobiose was investigated previously in *F. succinogenes* S85 and a differential metabolism of these substrates was shown, though they were metabolized simultaneously (Matheron et al. 1996)

Strain S85 was originally isolated from the bovine rumen (Bryant & Doetsch 1954) and has been maintained as a pure culture in laboratory ever since. As it was suggested that when wild strains of *F. succinogenes* are grown under laboratory conditions, physiological changes occur, and variants or mutants are selected (Van Glyswyk et al. 1986; Stewart et al. 1981), we investigated glucose and cellobiose metabolism in other strains of the genus *Fibrobacter.* We studied 3 different strains of *F. succinogenes* (HM2, 095 and S85) and one strain of the second known species of the genus *Fibrobacter* : *F. intestinalis* (NR9) (Amann et al. 1992; Montgomery et al. 1988). Strain 095 was chosen because it has been subcultured little since its isolation. These four strains show very similar cellulolytic and xylanolytic activities (unpublished results). The aim of this work was to study their sugar metabolism in order to compare their potentials towards highly lignified cellulolytic residues. *In vivo* ^{13}C NMR was used to quantify the relative contribution of glucose and cellobiose to metabolites production, glycogen storage and cellodextrin synthesis in these four strains.

Materials and methods

Bacterial strains and culture conditions

The strains used were *Fibrobacter succinogenes* S85 (ATCC 19169), the type strain of this species, isolated from the bovine rumen; 095, isolated from the bovine rumen (obtained from K-J Cheng, Lethbridge, Alberta, Canada) and classified in the same group as *F. succinogenes* S85 (Matheron et al. 1998); HM2 (ATCC 43856), isolated from the sheep rumen, and belonging to a different group of the species (Amann et al. 1992); and *F. intestinalis* NR9 (ATCC 43854), the type strain of the second known species of the *Fibrobacter* genus, isolated from the rat caecum (Montgomery et al. 1988). Bacteria were grown in a medium containing 40% rumen fluid (Halliwell & Bryant 1963) and 3 g.L^{-1} cellobiose.

NMR spectroscopy

For *in vivo* experiments, cells were prepared as described by Matheron et al. (1996). Cells harvested in the late log phase were centrifuged (6000 $\times$ g, 10 min, 4 °C) and resuspended in a reduced 50 mM potassium phosphate-0.4% Na_2CO_3-0.05% cysteine buffer (pH 7.3). Cell suspensions (5 mg protein.ml^{-1}) were incubated with different substrates at 37 °C either in the spectrometer (*in vivo* NMR) or in a water bath.

Each experiment was carried out at least 3 times, using 3 different cultures for each strain.

We checked that no cell lysis occured during the incubations by measuring the activity of L-glutamate dehydrogenase (as described by Aghajanian et al. 1995), a cytoplasmic marker, in the supernatant and in cell extracts.

NMR spectra were recorded on a Bruker MSL 300 spectrometer operating at 75.4 MHz for ^{13}C. The ^{2}H resonance of D_2O (10%) was used to lock the field and for shimming.

In vivo ^{13}C NMR experiments were performed at 37 °C as previously described, using a 10 mm diameter probe (Matheron et al. 1996). ^{1}H were decoupled using a Waltz 16 program to avoid sample heating.

Metabolite assays. Protein concentration was determined by the Bradford method (Bradford 1976), using bovine serum albumin as standard.

Succinate, acetate, formate and glucose were assayed using a Boehringer kit.

Glucose 6-phosphate was determined as glucose except that hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) was omitted. We checked by adding a known amount of glucose to the samples that glucose was not converted to glucose 6-phosphate when commercial hexokinase was omited. This means that *Fibrobacter* hexokinase was not active under these conditions.

Glucose 1-phosphate was determined as for glucose 6-phosphate except that 3.2 U phosphoglucomutase (α-D-Glucose 1,6-phosphomutase, EC 5.4.2.2, from rabbit muscle) was added.

For glycogen determination, cells were harvested by centrifugation (15000 $\times$ g, 15 min, 4 °C) and the pellets were suspended in 0.25% SDS (sodium dodecyl sulfate). The suspension was then diluted 10 times in 50 mM potassium phosphate buffer (pH 4.5) and incubated with 80 mg/mL amyloglucosidase (1,4,α-D-glucan glucohydrolase; EC 3.2.1.3, from *Rhizopus* genus mold) for 60 min at 55 °C. Samples were centrifuged (15000 $\times$ g, 5 min) and glucose was assayed in the supernatant.

Cellobiose phosphorylase and cellobiase activities

Cell extracts: cells were collected by spinning (6000 $\times$ g, 12 min, 4 °C) and washed twice anaerobically in a sterile reduced buffer (pH 7.5) containing 50 mM triethanolamine, 0.4% Na_2CO_3, 5 mM $MgCl_2$, 0.05% cysteine and 2 mM dithiothreitol. The cells were concentrated 25 times and disrupted by sonication with a Branson Sonifier Cell disruptor B15 (ten treatments of 10 s each) in an ice bath. After centrifugation (20 000 $\times$ g, 30 min, 5 °C) in CO_2-filled centrifuge tubes, supernatants were immediately used for enzymatic assays.

Cellobiase and cellobiose phosphorylase activities were spectrophotometrically assayed by measuring glucose, glucose 1-phosphate or glucose 6-phosphate appearance, by NADP-linked reactions. Glucose 1-phosphate or glucose 6-phosphate were measured as follows: cell extracts (90μg protein/mL), sterile 17 mM TEA buffer (pH 7.5), 1U glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate: NADP 1-oxidoreductase, EC 1.1.1.49), 1 mM NADP, 0.1 M potassium phosphate buffer (pH 7.3) and 1.7 mM $MgCl_2$ were directly mixed in micro-cuvettes. The cuvettes were warmed up for 5 min at 37 °C to eliminate traces of glucose 6-phosphate; the reaction was then started by adding 5 mM cellobiose. Assays supplemented with phosphoglucomutase (α-D-glucose 1,6-phosphomutase, EC 5.4.2.2, from rabbit muscle) gave the same results because of the phosphoglucomutase activity in cell extracts of *Fibrobacter.* Control experiments without inorganic phosphate or without cellobiose were carried out as well.

Glucose released by cellobiase from cellobiose in the absence of potassium phosphate, was assayed as described above for glucose 1-phosphate, but 2U hexokinase and 3.5 mM ATP were added.

Chemicals. [1-^{13}C] glucose (99% labeled) was purchased from Eurisotop (France). All enzymes and chemicals were purchased from Sigma or Boehringer.

Results

1. Simultaneous metabolism of glucose and cellobiose

NMR experiments

In this work we studied the influence of cellobiose on the utilization of [1-^{13}C] glucose by four strains of the genus *Fibrobacter* : *F. succinogenes* S85, HM2, 095 and *F. intestinalis* NR9. We previously checked that the four strains were able to grow on both glucose and cellobiose which are the final metabolites of cellulose degradation. Similar growth rate and yield were obtained on both sugars (not shown).

Kinetics of [1-^{13}C] glucose utilization were monitored by *in vivo* NMR spectroscopy with resting cells harvested in late-log phase. After washing, the cells (5 mg protein/ML) were incubated at 37 °C in the presence of 32 mM [1-^{13}C] glucose, 32 mM [1-^{13}C] glucose and 64 mM unlabelled glucose or 32 mM [1-^{13}C] glucose and 32 mM unlabelled cellobiose. The presence of 64 mM unlabelled glucose is equivalent to the presence of 32 mM unlabelled cellobiose. In Figure 1 examples of ^{13}C NMR spectra recorded at the end of simultaneous degradation of [1-^{13}C] glucose and [1-^{12}C] cellobiose for each of the strains are presented.

For all the strains studied and for the 3 types of incubations, the same signals were present in the spectra. They correspond to the two glucose anomers [1-^{13}C β], (96.4 ppm) and [1-^{13}C α], (92.6 ppm) and to [1-^{13}C] glycogen, (100.1 ppm). [6-^{13}C] Glycogen, (61.0

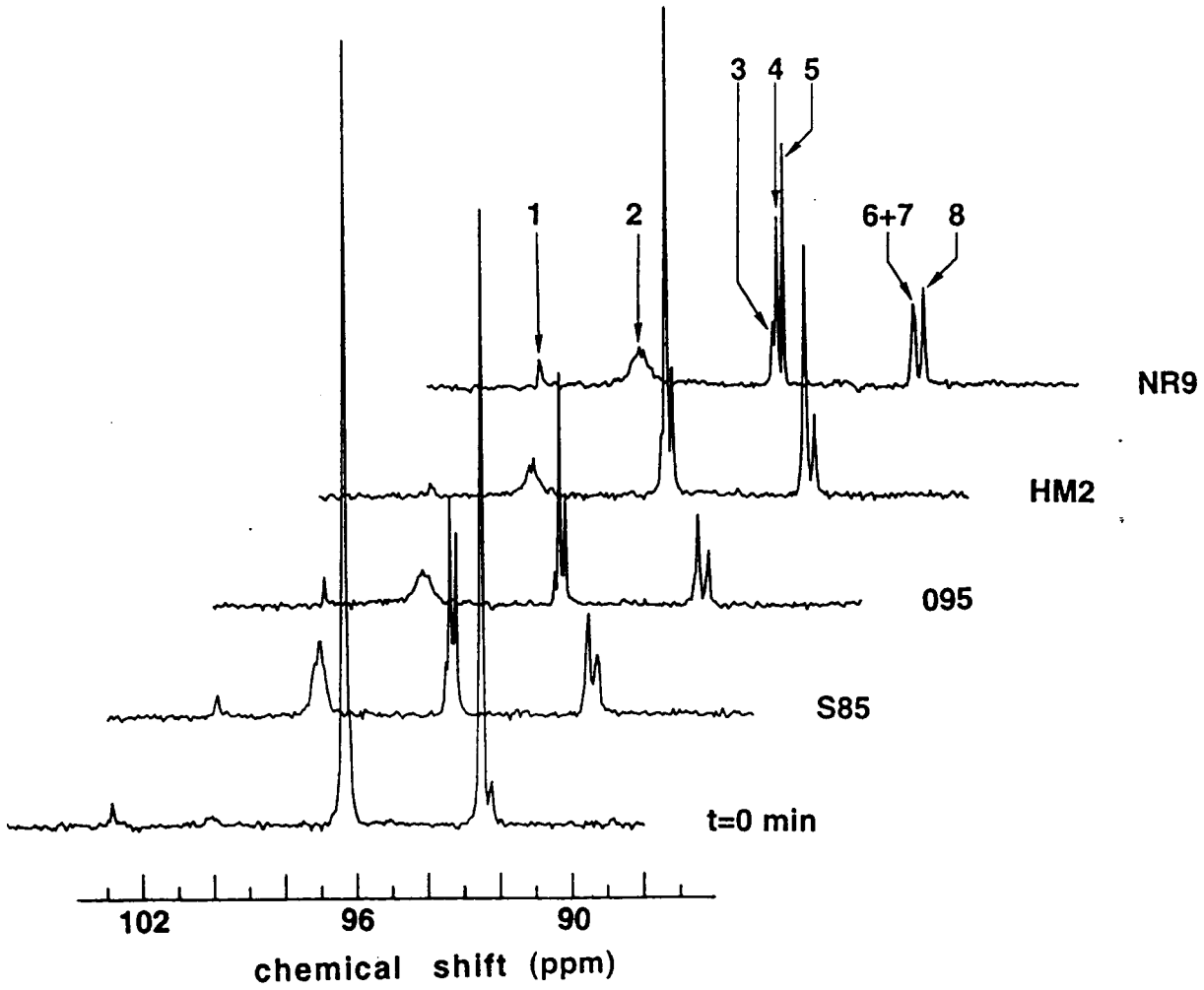

Figure 1. Spectra of *in vivo* kinetics of [1-^{13}C] glucose and cellobiose utilization by resting cells of *Fibrobacter* strains: *F. succinogenes* S85, O95, HM2 and *F. intestinalis* NR9. [1-^{13}C] glucose (32 mM) and cellobiose (32 mM) were added at zero time to suspension of resting cells (5 mg protein/mL). Proton decoupled ^{13}C-NMR spectra were collected every 4.5 min; spectra shown were recorded 27 min after glucose and cellobiose addition. 1: [1-^{13}C] cellobiose and cellodextrines, 2: [1-^{13}C] glycogen, 3 and 6: β and α[1-^{13}C] cellobiose, 4 and 7: β and α [1-^{13}C] glucose, 5 and 8: β and α [1-^{13}C] glucose 6-phosphate.

ppm), [2-^{13}C] succinate (34.5 ppm) and [2-^{13}C] acetate (23.7 ppm) resonances were also detected (not shown) as previously observed with the strain S85 (Matheron et al. 1996).

Additional signals were observed in the incubations of cells with both [1-^{13}C] glucose and cellobiose (Figure 1). These resonances at 96.3, 92.4, 63.5 ppm were assigned to [1-^{13}C] α and β anomers and [6-^{13}C] glucose 6-phosphate respectively, as confirmed by addition of glucose 6-phosphate in extracts. The [6-^{13}C] resonance of glucose 6-phosphate resulted from reversion of glycolysis and isomerization at the triose-phosphate level. Other resonances at 102.8 ppm and 96.5 ppm corresponded to [1-^{13}C] engaged into the β-1,4 linkage of residual cellobiose or cellodextrins, and to the reducing ends of β [1-^{13}C] cellodextrins and cellobiose respectively (the signals of the C1α of the reducing ends are overlapping the signal of the α [1-^{13}C]glucose).

These results show that the strains 095, HM2 and NR9 produce the same metabolites as the strain S85, especially when they are incubated in the presence of both glucose and cellobiose. In particular, they produce cellodextrins and glucose 6-phosphate in large amounts (Matheron et al. 1996).

The relative integrals of [1-^{13}C] glycogen and [2-^{13}C] succinate measured during the 3 different incubations for the strains 095, HM2 and NR9 are presented in the Figures 2A, 2B and 2C respectively.

During the incubation of the cells with the mixture of 32 mM [1-^{13}C] glucose and 64 mM [1-^{12}C] glucose the reductions of incorporation of the ^{13}C1 of [1-^{13}C] glucose in the [1-^{13}C] glycogen and [2-^{13}C] succinate were about 66% (Table 1, incubation 3) for all the strains. These results are consistent with the theoretical isotopic dilution due to the presence of unlabelled glucose.

In the presence of cellobiose, a reduction in the utilization of [1-^{13}C] glucose was observed (not shown). Under this condition (Table 1, incubation 2), the ^{13}C enrichment of the C2 succinate was reduced by 33% for the strain *F. succinogenes* 095, while it was reduced by 46 and 42% for the strains *F. intestinalis* NR9 and *F. succinogenes* HM2, respectively. The isotopic dilutions of the C1 glycogen were about 33%, except for the strain NR9 for which the dilution was 41%.

Enzymatic assays

Parallel to the NMR experiments the same incubations were performed in a water bath at 37 °C, and the concentrations of succinate and acetate, glycogen and glucose 6-phosphate were enzymatically assayed. The results obtained during the 3 types of incubations for the strains 095, HM2 and NR9 are presented in Figures 3A, 3B and 3C, respectively. In the 3 types of incubation, the 3 strains produced similar amount of succinate, acetate and glycogen. The results were similar to those previously obtained with strain S85 (Matheron et al. 1996).

These results confirm that the decrease of the integrals of [2-^{13}C]succinate and [1-^{13}C]glycogen resonances measured during the incubations in the presence of cellobiose and [1-^{13}C]glucose (see Figure 2) was due to an isotopic dilution and not to slowing down of the metabolism.

The results presented in Table 2 indicate that the strains 095 and NR9 of the genus *Fibrobacter* metabolized glucose at the same rate as that measured for the strain S85 (Matheron et al. 1996). On the contrary, the rate of glucose consumption of the strain HM2 was increased by 36% compared to the other strains. In the presence of cellobiose the rates of glucose degradation were decreased by about 50% for the four strains under study. Altogether these results suggest that cellobiose was used by the strains 095, NR9 and HM2 in a similar way as described for strain S85.

The accumulation of glucose 6-phosphate in the cell suspension, evidenced by ^{13}C NMR when the cells were incubated in the presence of both glucose and cellobiose, was confirmed by enzymatic assays for the 3 strains under study. Glucose 6-phosphate concentrations were 4.5; 5 and 2.5 mM when cells metabolized glucose only, and were increased to 7; 8 and 4.8 mM when the cells of strains 095, NR9 and HM2 were simultaneously metabolizing glucose and cellobiose, respectively. The concentrations of glucose 6-phosphate in the cell suspensions were high and varied from one strain to the other. However they were clearly increased by a factor 2 when cellobiose was added. These results are similar to those obtained for the strain S85 (Matheron et al. 1996). The intracellular concentration of glucose 6-phosphate was found to be around 10 mM. This indicated that most of the glucose 6-phosphate accumulated in the presence of cellobiose was excreted out of the cells.

2. Cellobiose cleavage

We previously showed that two enzymes were responsible for cellobiose cleavage in *Fibrobacter succinogenes* S85: a cellobiose phosphorylase and a cellobiase (Matheron et al. 1996). As NMR experiments have shown (Figure 1) that the strains HM2, 095 and NR9 were able to degrade cellobiose and produce cellodextrins in a similar way as strain S85, the presence of cellobiose phosphorylase and cellobiase was investigated in these 3 strains (Table 3). All the strains under study possessed the two enzyme activities, and the cellobiase activity was always much higher than the cellobiose-phosphorylase activity. The values of the activities varied from one cell extraction to the other, as shown by the magnitude of the standard error values (Table 3). This could be explained by different efficiency of the extraction procedure, or by sensitivity of the enzymes to this procedure.

Discussion and Conclusions

In this work we have studied the metabolism of glucose and cellobiose by 3 strains of *Fibrobacter* (095, HM2 and NR9) in order to compare their ability to metabolize the products of cellulose degradation. The use of *in vivo* ^{13}C NMR and complementary enzymatic assays allowed us to demonstrate the differential metabolism of these two sugars (Figure 4), that are transported across the cytoplasmic membrane

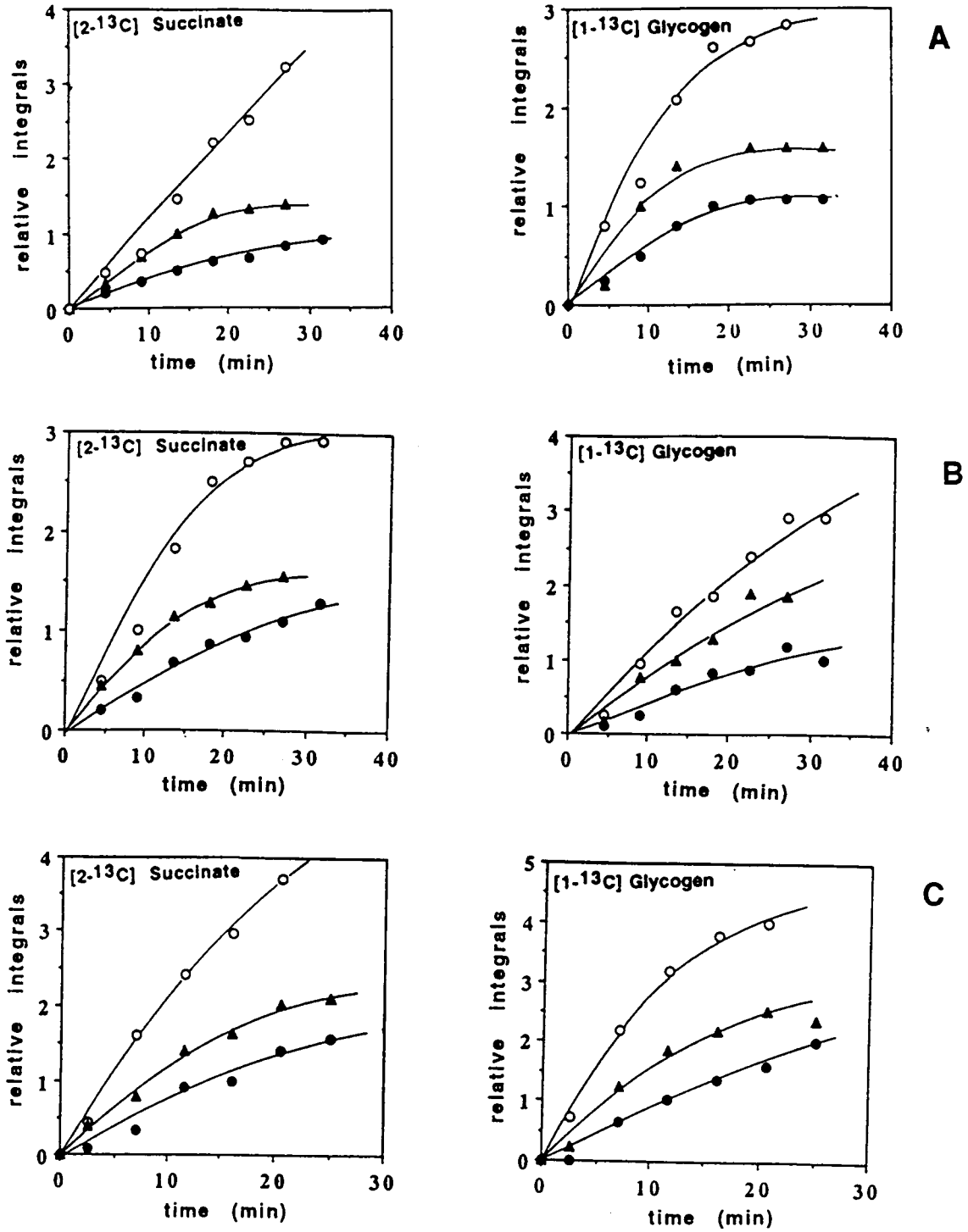

Figure 2. Time dependent changes of signal integrals of [2-^{13}C] succinate and [1-^{13}C] glycogen during [1-^{13}C] glucose utilization (○), supplemented with either 64 mM glucose (●) or 32 mM cellobiose (▲), by resting cells of *Fibrobacter* strains: *F. succinogenes* O95 (A), HM2 (B) and *F. intestinalis* NR9 (C). Integrals were measured on ^{13}C-NMR spectra, experimental conditions as in Figure 1. Relative integrals correspond to the ratio of the integral of [2-^{13}C] succinate or [1-^{13}C] glycogen to the integral the ^{13}C resonance of a benzene capillary fixed in the center of the 10 mm tube.

Table 1. Isotopic dilutions of succinate and glycogen produced by resting cells of *Fibrobacter succinogenes* 095, HM2, S85 and *Fibrobacter intestinalis* NR9 incubated 10 min with 32 mM [1-^{13}C] glucose added with 32 mM cellobiose (2) or 64 mM glucose (3). Isotopic dilutions are relative to the area of the ^{13}C signal of succinate and glycogen produced during a control incubation with 32 mM of [1-^{13}C] glucose alone. Experimental conditions as in Figure 2

Incubations	Isotopic dilutions (%)							
	095		HM2		S85		NR9	
	Succ.	Glyc.	Succ.	Glyc.	Succ.	Glyc.	Succ.	Glyc.
2	33	32	42	35	33	33	46	41
3	66	62	68	65	66	62	66	69

Table 2. Rate of glucose utilization by resting cells of *Fibrobacter succinogenes* 095, HM2, S85 and *Fibrobacter intestinalis* NR9 incubated 10 min with 32 mM [1-^{13}C] glucose alone (1) or supplemented with 32 mM cellobiose (2) or 64 mM glucose (3). Experimental conditions as in Figure 3

Incubation	Rate of glucose utilization (μmol/(mg protein min))			
	095	HM2	S85	NR9
1	0.24	0.37	0.22	0.24
2	0.11	0.20	0.10	0.11
3	0.24	0.37	0.22	0.24

through independent constitutive transporters, which are sodium-dependent (Chow & Russel 1992, Maas & Glass 1991, Franklund & Glass 1987).

The isotopic dilutions of C2 succinate and C1 glycogen measured under the 3 experimental conditions tested clearly show that glucose and cellobiose are not equivalent for *Fibrobacter* metabolism. Glucose seems to be preferentially used for glycogen storage and energy production, while part of cellobiose seems to be diverted from glycolysis. The detection of cellodextrins signals in ^{13}C NMR spectra suggests that the cellobiose which does not enter the glycolysis is used to synthesize cellodextrins. The same phenomenon was observed for strain S85 (Matheron et al. 1996). Wells & Russel (1994) also showed cellodextrins synthesis from cellobiose in *F. succinogenes* S85 cells. Wells et al. (1995) suggested that this bacterium could excrete cellodextrins in order to feed other rumen microbes, including planktonic *F. succinogenes* cells.

The activity of both cellobiose phosphorylase and cellobiase in the 3 strains of the *Fibrobacter* genus was also demonstrated. Under physiological conditions, it is difficult to know which enzyme is responsible for the synthesis of cellodextrins. However, from an energetic point of view, it would be more useful for the cell to synthesize cellodextrins via reverse action of the β-glucosidase, while phosphorylase would cleave cellobiose. As this enzyme keeps the energy of the glycosidic bond via the synthesis of one molecule of glucose 1-phosphate, only one ATP molecule is needed for the phosphorylation of the remaining glucose unit. On the contrary, two ATP molecules are necessary for the phosphorylation of two glucose units resulting from cellobiose cleavage. Study of the location of these two enzymes within the cell and their purification would allow to characterize them better and to understand the respective role of these two enzymes in *Fibrobacter*.

The presence of cellobiose phosphorylase and cellobiase activities were found in other ruminal strains, *F. succinogenes* S85 (Matheron et al. 1996) and *Prevotella ruminicola* (Lou et al. 1996). Similarly *Streptococcus bovis* metabolizes maltose by a maltose phosphorylase and a maltase (Martin & Russel 1987). In *P. ruminicola*, the cellobiase activity was 3 times higher than the cellobiose phosphorylase activity (Lou et al. 1996). We also found that cellobiase had a higher activity in the *Fibrobacter* strains, under the conditions tested. Simultaneous activity of a cellobiose phosphorylase and a cellobiase was also found in non-ruminal cellulolytic bacteria such as *Clostridium thermocellum* (Katayeva et al. 1992). It could thus be a common property of efficient cellulolytic microorganisms.

In vivo ^{13}C NMR experiments showed the accumulation of glucose 6-phosphate when the strains 095, NR9, HM2 of *Fibrobacter* were incubated simultaneously with glucose and cellobiose. This accumulation was confirmed by enzymatic assays. This original feature was first observed for the strain *F. succinogenes* S85 (Matheron et al. 1996). Most of glucose 6-phosphate was excreted in the extracellular medium,

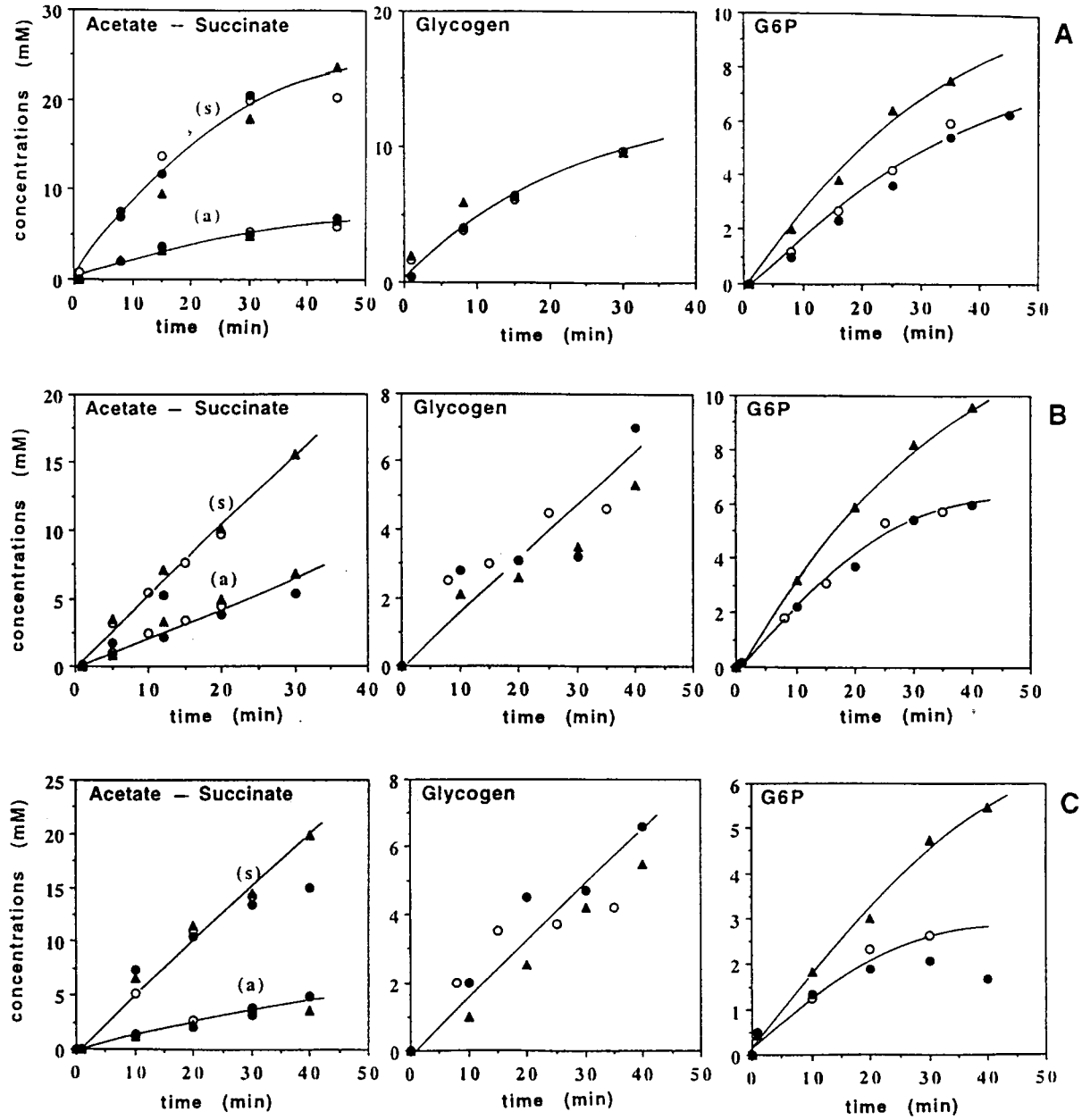

Figure 3. Time dependent changes of acetate (a) and succinate (s), glycogen and glucose 6-phosphate during [1-^{13}C] glucose utilization (○), supplemented with either 64 mM glucose (●) or 32 mM cellobiose (▲), by resting cells of *Fibrobacter* strains: *F. succinogenes* O95 (A), HM2 (B) and *F. intestinalis* NR9 (C). Metabolite concentrations were measured by enzymatic assays; experimental conditions as in Figure 1.

probably by a specific permease, but the mechanism involved in this excretion is not known. Wild-type *E. coli* cells were shown to be able to secrete glucose 6-phosphate when an uncoupler of oxidative phosphorylation was added (Van der Zee et al. 1996).

The precise role of glucose 6-phosphate in *Fibrobacter* is not known. In eukaryotic cells it can regulate the synthesis and degradation of glycogen (Bloch et al. 1994, Villar-Palasi 1991, Fernandez-Novell et al. 1992, François & Hers 1988). However, it is unlikely that glucose 6-phosphate could regulate glycogen metabolism in *Fibrobacter* genus as no modification of glycogen storage was observed in the presence of high concentrations of this metabolite. An

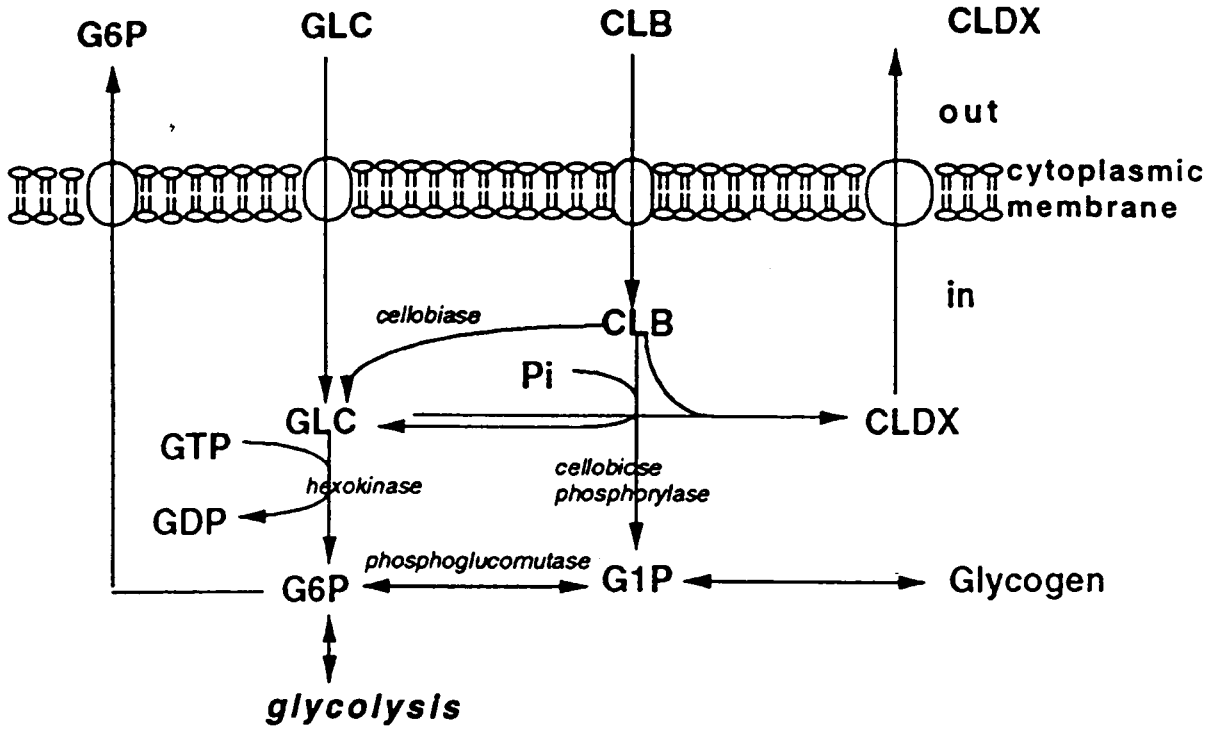

Figure 4. Scheme of simultaneous metabolism of glucose and cellobiose by *Fibrobacter* strains. G6P: glucose 6-phosphate, G1P: glucose 1-phosphate, CLB: cellobiose; CLDX: cellodextrins; GLC: glucose; GTP and GDP: Guanosine tri or di phosphate.

Table 3. Cellobiase and cellobiose phosphorylase activities measured in enzymatic extracts of *Fibrobacter succinogenes* 095, HM2, S85 and *Fibrobacter intestinalis* NR9. Extracts were incubated at 37 °C in the presence of 1 mM NADP, 3.2 U/mL of phosphoglucomutase and 1 U/mL of G6P dehydrogenase. For cellobiase assays, 2 U/mL hexokinase and 3.5 mM ATP were added, while 0.1 M Pi were added for cellobiose phosphorylase assays

	Specific activities[a]			
Activity	095	HM2	S85	NR9
Cellobiase	63 ± 16	40 ± 6	53 ± 10	137 ± 56
Cellobiose phosphorylase	9 ± 5	4 ± 1	12 ± 6	2 ± 1

[a] Specific activities are expressed as nmol of cellobiose cleaved/(mg protein.min) and are the mean values of three assays.

accumulation of glucose 6-phosphate also has been reported in *Leuconostoc oenos* under anaerobiosis (Veiga-Da-Cunha et al. 1993), but the function of this intermediate was not established. In strains of *Bacillus* genus, glucose 6-phosphate regulates the utilization of xylose as an anti-inducer of *xyl*-operon transcription since it can compete with the *xyl*-repressor (Dahl et al. 1995).

The present study has shown that cellobiose metabolism of 3 strains of *F. succinogenes*, the type strain S85, strain 095 classified in the same group (Matheron et al. 1998), strain HM2 belonging to another group (Amann et al. 1992), and the type strain of *F. intestinalis*, NR9, was very similar. The same features were found: differential metabolism of glucose and cellobiose, synthesis of cellodextrins, accumulation and excretion of glucose 6-phosphate, simultaneous presence of cellobiose phosphorylase and cellobiase activities.

In a previous work we showed that these 4 strains produced the same amounts of metabolites (succinate, acetate and formate) in the same ratios, and via the

same metabolic pathways (Matheron et al. 1998). The ^{1}H and ^{13}C NMR experiments showed an equivalent reversal of glycolysis for the 4 strains. In addition, all the strains were able to store glycogen throughout the growth phase with the same constant glycogen/protein ratio during the exponential growth phase. Simultaneous storage and degradation of glycogen were previously found in resting cells of these strains showing that this unusual feature is a common property of the *Fibrobacter* genus (Matheron et al. 1998). Also, other unusual features were previously found in strains representative of the two species, such as the production of a GTP-dependent hexokinase (Glass & Sherwood 1994) or the activity of a pentose-phosphate phosphoketolase (Matheron et al. 1997).

Although strains S85 and 095 were not closely related to strains HM2 and NR9, and strain 095 was not much subcultured compared with S85, the physiological properties tested were similar for all 4 strains. These results indicate that these phenotypic characteristics are an intrinsic property of the *Fibrobacter* genus. Although genetically diverse, the *Fibrobacter* genus appears to possess a marked homogeneity in its carbon metabolism.

Several strains of *F. succinogenes* showed the most extensive solubilization attained by pure cultures of rumen bacteria, especially when tested against poorly degradable substrates such as cereal straws (Dehority 1993). The study of the metabolism of glucose and cellobiose by the four strains of *Fibrobacter* suggested that different strains of this genus may have similar potentials in degrading and metabolizing lignocellulose residues. In the future, the degradation of ^{13}C enriched pure cellulose and plants will be investigated using NMR. This will allow to monitor the metabolism of this predominant rumen cellulolytic species under conditions closer to natural ones, and particularly of cells adherent to their substrate.

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