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Metabolism of lactose by *Clostridium thermolacticum* growing in continuous culture

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Abstract The objective of the present study was to characterize the metabolism of *Clostridium thermolacticum*, a thermophilic anaerobic bacterium, growing continuously on lactose (10 g l^{-1}) and to determine the enzymes involved in the pathways leading to the formation of the fermentation products. Biomass and metabolites concentration were measured at steady-state for different dilution rates, from 0.013 to 0.19 h^{-1}. Acetate, ethanol, hydrogen and carbon dioxide were produced at all dilution rates, whereas lactate was detected only for dilution rates below 0.06 h^{-1}. The presence of several key enzymes involved in lactose metabolism, including beta-galactosidase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate:ferredoxin oxidoreductase, acetate kinase, ethanol dehydrogenase and lactate dehydrogenase, was demonstrated. Finally, the intracellular level of NADH, NAD^+, ATP and ADP was also measured for different dilution rates. The production of ethanol and lactate appeared to be linked with the re-oxidation of NADH produced during glycolysis, whereas hydrogen produced should come from reduced ferredoxin generated during pyruvate decarboxylation. To produce more hydrogen or more acetate from lactose, it thus appears that an efficient H_2 removal system should be used, based on a physical (membrane) or a biological approach, respectively, by cultivating *C. thermolacticum* with efficient H_2 scavenging and acetate producing microorganisms.

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Introduction

Lactose, the main milk sugar, is available in large amounts in milk and whey permeates rejected by dairy industry. By screening various thermophilic anaerobic bacteria, *Clostridium thermolacticum* (DSM 2910) was found appropriate for the production of acetate from lactose, to be used eventually as calcium magnesium acetate, a non-corrosive salt for roadway de-icing (Talabardon et al. 2000a).

However, when grown in batch culture on lactose, *C. thermolacticum* produces not only acetate, but also ethanol, hydrogen and carbon dioxide as by-products during the exponential phase of growth. Afterwards a shift of metabolism towards lactate production is observed (Talabardon et al. 2000a; Collet et al. 2003). It has also been shown that the overall production of acetate increases when *C. thermolacticum* is cultivated in the presence of hydrogen-scavenging microorganisms (Talabardon et al. 2000b; Collet et al. 2003, 2005).

At the present time however, very little is known about the metabolic processes in *C. thermolacticum*, especially how lactose is metabolized into the different end-products. Metabolic studies have been carried out with other *Clostridia*, including the mesophilic *C. acetobutylicum* (Girbal et al. 1995), *C. cellulolyticum* (Desvaux et al. 2001a), *C. butyricum* (Saint-Amans et al. 2001) and the thermophilic *C. thermohydrosulfuricum* (Lovitt et al. 1988) and *C. thermosuccinogenes* (Sridhar et al. 2000). The fermentation of different substrates by these strains yields similar metabolites, usually acetate, ethanol, lactate, H_2 and CO_2. The enzymes involved in the formation of the end-products have been described for all these bacteria, but no enzymatic study on *Clostridia* able to degrade lactose has been carried out yet. In contrast, the catabolism of lactose by *Lactococcus lactis*

has been reviewed recently (Cocaign-Bousquet et al. 2002). After the cells take up lactose, it is split into glucose and galactose. Once phosphorylated, the sugars enter central metabolism via glucose-6-phosphate, which follows the glycolytic pathway to pyruvate.

The objective of the present study was thus to characterize the metabolism of *C. thermolacticum* growing continuously on lactose and to determine the enzymes involved in the pathways leading to the formation of the fermentation products. Based on previous results obtained under batch conditions, the bacterium was grown on 29 mmol lactose l^{-1} (10 g l^{-1}). The biomass and metabolic pattern were monitored at steady-state for different dilution rates. The presence of several key enzymes involved in the formation of end-products from lactose has been shown, whereas the intracellular concentrations of several important cofactors have been quantified under different conditions.

Materials and methods

Microbial species

The heterofermentative anaerobic bacterium *Clostridium stercorarium* subsp. *thermolacticum* (*C. thermolacticum*) DSM 2910 (Fardeau et al. 2001), formerly *Clostridium thermolacticum* (Le Ruyet et al. 1985), was used in this study. Stock cultures were maintained in minimal medium containing lactose (Talabardon et al. 2000a). The cultures were stored at 4°C and sub-cultured once every month. The purity of the culture was routinely checked by microscopy.

Culture conditions and analytical techniques

The medium was prepared as described by Collet et al. (2004). It contained 29 mmol l^{-1} lactose (Merck), was sterilized by filtration through a cartridge Opticap 4'', 0.22 µm pore size (Millipore) into a sterile 50-litre tank (Blefa), and then stored under nitrogen.

A 2-litre bioreactor (Biolafitte) was used for these studies. It was autoclaved for 100 min at 121°C, prior filling with fresh medium. To initiate anaerobic conditions in the bioreactor, the medium was shortly sparged with N_2. The bioreactor was stirred at 100 rpm, and the temperature was maintained at 58°C. Twenty millilitre of cell suspension in the exponential phase of growth were added as inoculum. The bacteria were grown in batch for 24 h before the medium flow was started. For each dilution rate, the chemostat was allowed to stabilize until biomass, liquid and gaseous metabolites concentration was constant. The culture volume was kept at 1.0 l by automatic regulation; the pH of the medium was monitored online (Mettler-Toledo) and maintained at 7.0 by automatic addition of 2 M NaOH. Samples were withdrawn from the reactor for absorbance readings at 650 nm and HPLC analysis. One unit of A_{650} was found to be equivalent to 0.73 g l^{-1} cell dry weight for *C. thermolacticum*.

Studies for glucose/galactose utilization were performed in duplicate batch cultures, in 590 ml screw-capped serum bottles, with 200 ml of medium and fitted with gas-proof black butyl rubber septa under N_2-CO_2 atmosphere (80:20; 150 kPa) and non-controlled pH conditions, in a constant temperature incubator (58°C, 100 rpm). Twenty millilitre of cell suspension in the exponential phase of growth were added as inoculum to each serum bottle.

Lactose, glucose, galactose, lactate, acetate and ethanol were identified and quantified by HPLC using an ORH-801 column (Interaction) at 60°C and a differential refractometer ERC7517A (ERMA) at 45°C. Elution was done by 5 mmol l^{-1} sulphuric acid at a flow rate of 0.6 ml min^{-1}.

The quantity and quality of gas produced was followed online as described by Collet et al. (2005).

Preparation of cell extracts

Samples for enzyme assays (15 ml) were withdrawn anaerobically into oxygen-free serum tubes and collected by centrifugation (9,000 × *g*, 4°C, 15 min). The pellet was resuspended in 0.75 ml degassed buffer (0.1 M Tris-HCl, 5 mM dithiothreitol (DTT, Fluka), 10% glycerol, pH 7.6). Bacteria were destroyed by an ultrasonic disrupter Vibracell 72434 (Bioblock) at 4°C for four cycles of 30 s, interspaced by cooling period of 2 min, in an anaerobic chamber. Cell debris were removed anaerobically by centrifugation (13,000 × *g*, 4°C, 10 min). The cell-free extract was used for all enzyme assays. Protein concentration was determined using a modified Lowry Protein Assay (Pierce).

Enzymatic assays

Assays were carried out on cell-free extracts. Some enzymatic assays were carried out at 37°C, to preserve the activity of commercial enzymes used in these tests. In such a case, they proved the presence of the enzyme but did not reflect activities that could have been measured at 58°C and could not be compared to in vivo metabolite productivities measured. For enzymatic assays carried out under anaerobic conditions, all reagent solutions were prepared in distilled water, previously boiled and sparged with nitrogen, then kept under N_2 atmosphere. All enzyme activities were determined in the physiological direction. Each enzyme assay was done in duplicate. The concentrations of components in the reaction mixtures (1 ml total volume) are given below.

Beta-galactosidase, modified from Miller (1972): 0.1 M Tris-HCl (pH 7.0), 2 mM DTT, 10 mM KCl, 5 mM $MgCl_2$, cell extract. Start reaction with 100 µl

o-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma) 4 mg ml^{-1}.

Glyceraldehyde-3-phosphate dehydrogenase was coupled with aldolase under anaerobic conditions (Vasconcelos et al. 1994): 0.1 M Tris-HCl (pH 8.1), 2 mM DTT, 5 mM KH_2PO_4, 20 mM KH_2AsO_4, 0.5 mM NAD^+, 2 U aldolase (Sigma), cell extract. Start reaction with 100 µl fructose-1,6-bisphosphate (100 mM). To assess the effect of NADH/NAD^+ ratio, NAD^+ concentration was kept constant.

Pyruvate:ferredoxin oxidoreductase was assayed under anaerobic conditions (Vasconcelos et al. 1994): 0.1 M Tris-acetate (pH 7.8), 4 mM DTT, 5 mM KH_2PO_4, 7 mM KH_2AsO_4, 5 mM sodium pyruvate, 0.1 mM acetyl coenzyme A (Sigma), cell extract. Sparge carbon monoxide, and then start the reaction with 100 µl methylviologen at 20 mM (Sigma).

Lactate dehydrogenase (Sridhar et al. 2000): 0.1 M Tris-HCl (pH 6.5), 1 mM fructose-1,6-bisphosphate (Sigma), 0.25 mM NADH, cell extract. Start reaction with 100 µl sodium pyruvate (100 mM). To assess the effect of the NADH/NAD^+ ratio, NADH concentration was kept constant.

Acetate kinase (Vasconcelos et al. 1994): 0.1 M Tris-HCl (pH 7.2), 2 mM DTT, 3 mM $MgCl_2$, 2 mM glucose, 0.5 mM $NADP^+$ (ICN), 1 U glucose-6-phosphate dehydrogenase (ICN), 1 U hexokinase (ICN), 5 mM acetyl-phosphate (Sigma), cell extract. Start reaction with 100 µl ADP at 10 mM (ICN).

Ethanol dehydrogenase (Sridhar et al. 2000): 0.1 M Tris-HCl (pH 6.5), 2 mM DTT, 0.3 mM NADH, cell extract. Start reaction with 100 µl acetaldehyde (100 mM).

Pyridine nucleotide reduction or oxidation was monitored at 340 nm ($\varepsilon_{340\ nm} = 6.22\ mM^{-1}\ cm^{-1}$), and ONPG hydrolysis into galactose and ONP was measured at 420 nm ($\varepsilon_{420\ nm} = 4.5\ mM^{-1}\ cm^{-1}$). For all enzymes tested, the accuracy was ±5%.

Estimation of cofactors concentration

Samples for NADH and NAD^+ determination (4 ml) or ATP and ADP determination (3 ml) were withdrawn quickly from continuous cultures by a peristaltic pump and flash frozen in liquid nitrogen.

NAD^+ was extracted with 4 M HCl (NADH degraded) and NADH was extracted with 10 M KOH (NAD^+ degraded). Before assay for NADH, this cofactor was converted to NAD^+ with lactate dehydrogenase (ICN). Levels of NAD^+ were determined by fluorimetric enzyme assay, using ethanol dehydrogenase (ICN), with a fluorimeter model F-2000 (Hitachi). Excitation was at 340 nm and emission at 460 nm. ATP and ADP were extracted with KOH. ADP was converted to ATP by creatine phosphokinase (ICN). ATP levels were measured by a luminescence assay using firefly luciferase (Sigma) with a luminometer model 1250 (LKB Wallac, Turku, Finland). A known amount of NAD^+ (ICN), NADH (ICN) or ATP was added to the assay as internal standard. For all cofactors measured, the accuracy was ±10%.

Results

Production of biomass, acetate, ethanol and lactate

Lactose specific consumption rate, metabolite specific productivity and biomass concentration were monitored as a function of dilution rate (D), from 0.013 to 0.19 h^{-1}. Specific productivity and consumption were derived from Eqs. 1–4 presented in Table 1.

As shown in Fig. 1, cell dry weight (CDW) was maximal at low dilution rate: 0.69 g l^{-1} at $D = 0.028\ h^{-1}$. It slowly decreased with increasing dilution rate down to 0.27 g l^{-1} at $D = 0.19\ h^{-1}$. The specific lactose consumption rate increased with dilution rate to a maximum of 2.52 mmol lactose g^{-1} (CDW) h^{-1} at $D = 0.13\ h^{-1}$ and then decreased at higher dilution rate. Acetate specific productivity followed the same tendency: it peaked at $D = 0.13\ h^{-1}$ with 3.16 mmol acetate g^{-1} CDW h^{-1}. For dilution rates $> 0.06\ h^{-1}$, ethanol and acetate were the main products, whereas lactate accumulated significantly at $D < 0.06\ h^{-1}$.

The yield coefficient of biomass increased with increasing dilution rates from 5.4 to 21.1 g CDW per mol lactose (Table 2). The yield coefficient of acetate was between 1.0 and 1.3 mol mol^{-1} lactose. The maximal acetate volumetric productivity obtained was 1.37 mmol l^{-1} h^{-1} at $D = 0.105\ h^{-1}$.

From results shown in Table 2, it came out that ethanol was the main product in the liquid phase for all dilution rates. The average mass fraction of carbon was 0.43 for acetate and 0.57 for ethanol. For all dilution rates tested, the ratio $C_{acetate}/C_{ethanol}$ remained between 0.7 and 0.8. The mass fraction of carbon as lactate increased with the decrease of the dilution rate: at $D = 0.013\ h^{-1}$, 32% of the carbon was recovered as lactate. Consequently, the mass fraction of carbon as ethanol and acetate declined, down to 0.40 and 0.28, respectively. Glucose and galactose were found only at low dilution rates: respectively 0.1 and 0.01 mmol l^{-1} at $D = 0.028\ h^{-1}$ and 0.32 and 0.75 mmol l^{-1} at $D = 0.013\ h^{-1}$.

Table 1 Calculations for analysis of the carbon flow during lactose fermentation by *C. thermolacticum*

Eq.	Specific metabolic flux [mmol (g CDW)$^{-1}$ h^{-1}]	Calculation
1	$q_{lactose}$	$(S/X)D$
2	$q_{acetate}$	$(C_{acetate}/X)D$
3	$q_{ethanol}$	$(C_{ethanol}/X)D$
4	$q_{lactate}$	$(C_{lactate}/X)D$
5	q_{ATP}	2(2) + (3) + (4)
6	$q_{NADH\ produced}$	(2) + (3) + (4)
7	$q_{NADH\ consumed}$	2(3) + (4)
8	$q_{Fd\ produced}$	(2) + (3)

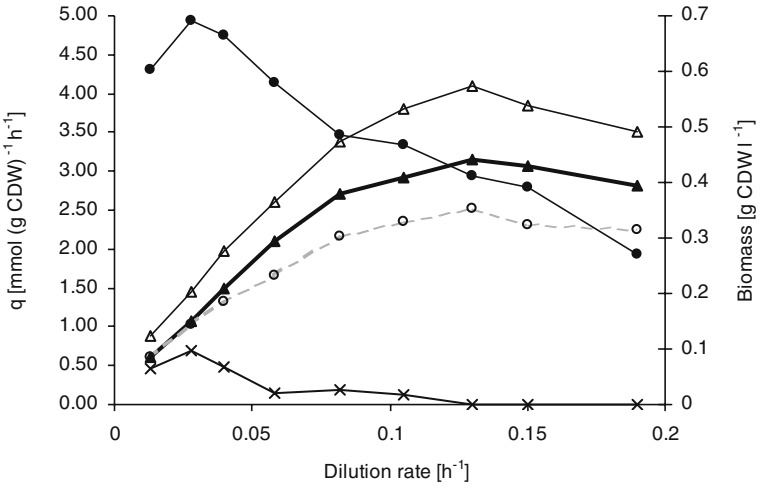

Fig. 1 Metabolite specific productivity, lactose specific consumption rate and biomass cell dry weight (CDW) concentration for continuous cultures of *C. thermolacticum* at steady-state on 29 mmol l^{-1} lactose concentration in the feed, pH 7.0: acetate (*filled triangle*), ethanol (*triangle*), lactate (*cross*), consumed lactose (*circle*), biomass CDW (*filled circle*). Results are presented as mean values from four independent experiments (standard deviation < 17%)

The productivity of hydrogen was maximal for *D* values between 0.04 and 0.10 h^{-1} (Table 2).

In order to check if the bacterium had any preference for one of the monomers derived from lactose hydrolysis, *C. thermolacticum*, pre-cultured on 29 mmol l^{-1} lactose, was grown in batch culture on a mixture of 18 mmol l^{-1} glucose and 18 mmol l^{-1} galactose (Fig. 2). It can be clearly seen that galactose was metabolized preferentially until it was entirely consumed, whereas glucose was only poorly degraded. The pattern of products formed was similar to that obtained previously for batch culture on lactose (Collet et al. 2003): acetate and ethanol were produced in the exponential phase of growth before metabolism was shifted towards lactate production.

Enzymes involved in lactose catabolism

Clostridium thermolacticum degraded lactose into different end-products: acetate, ethanol, lactate, hydrogen and carbon dioxide, whereas some glucose and galactose accumulated at low specific growth rate. Thus, it was assumed that lactose was transported and hydrolyzed into glucose and galactose, further metabolized into pyruvate via Embden-Meyerhof-Parnas (EMP) pathway. Pyruvate should be the branching point towards lactate and acetyl-CoA, whereas acetate and ethanol would be generated from acetyl-CoA (Collet et al. 2003). In this study, one key enzyme from each branch of the possible lactose metabolism was measured. Enzymes to be assayed were selected on the basis of previous studies with other *Clostridia* (Jungermann et al. 1973; Church et al. 1988; Lovitt et al. 1988; Guedon et al. 1999; Sridhar et al. 2000; Saint-Amans et al. 2001).

Most of the tested enzymes had a substantial activity at 37°C (Table 3). The specific activity of the enzymes was similar at the three dilution rates tested, except for lactate dehydrogenase (LDH), whose specific activity increased when dilution rate was decreasing.

Additional tests were carried out at 58°C. The optimal pH for both β-galactosidase and pyruvate: ferredoxin oxidoreductase was between pH 6.6 and 7.2. No

Table 2 Fermentation parameters in continuous cultures of *C. thermolacticum* at steady-state

Parameters	*D* [h^{-1}]								
	0.013	0.028	0.040	0.058	0.082	0.105	0.130	0.150	0.190
$Y_{X/S}$ [g CDW (mol hexose)$^{-1}$]	10.84	13.51	15.14	17.37	18.95	22.33	25.75	32.50	42.19
w(C) acetate [mol mol^{-1}]	0.28	0.30	0.36	0.43	0.42	0.42	0.43	0.44	0.44
w(C) ethanol [mol mol^{-1}]	0.40	0.41	0.47	0.53	0.53	0.55	0.57	0.56	0.56
w(C) lactate [mol mol^{-1}]	0.32	0.29	0.17	0.04	0.05	0.03	0.00	0.00	0.00
ratio $C_{acetate}$ / $C_{ethanol}$	0.68	0.74	0.76	0.81	0.80	0.76	0.77	0.80	0.80
$R_{acetate}$ [mmol l^{-1} h^{-1}]	0.36	0.74	1.00	1.22	1.31	1.37	1.30	1.20	0.76
$Y_{acetate/lactose}$ [mol mol^{-1}]	1.0	1.0	1.1	1.3	1.3	1.2	1.3	1.3	1.3
$Y_{lactate/lactose}$ [mol mol^{-1}]	0.8	0.7	0.4	0.1	0.1	0.1	0.0	0.0	0.0
$R_{carbon\ dioxide}$ [mmol l^{-1} h^{-1}]	1.22	2.60	3.28	3.77	3.94	4.52	4.03	4.05	3.42
$R_{hydrogen}$ [mmol l^{-1} h^{-1}]	0.85	2.18	2.44	2.55	2.46	2.31	2.21	2.10	1.52
Carbon balance [%]	99	98	97	97	98	100	100	102	103
Redox balance [%]	95	95	93	94	95	97	98	101	101

Results are presented as mean values from four independent experiments (standard deviation <17%)
Mass fraction of carbon in acetate: $w(C) = \frac{2c(CH_3COOH)}{2c(CH_3COOH)+2c(C_2H_5OH)+3c(CH_3COCOOH)}$

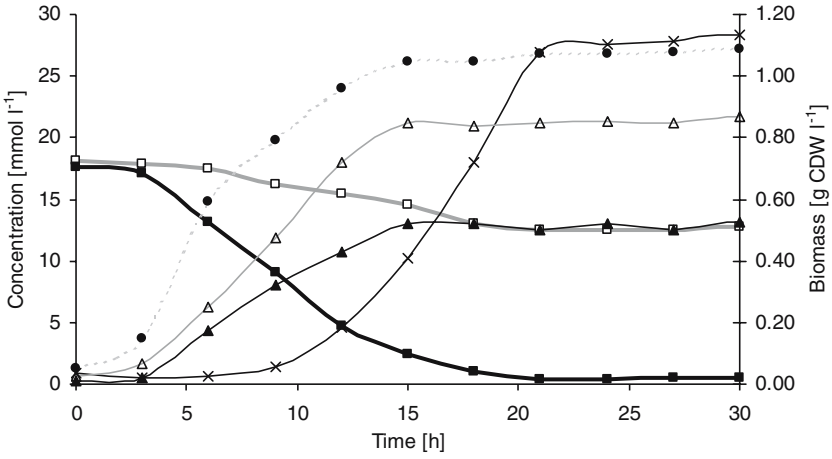

Fig. 2 Biomass and metabolite concentration for batch culture of *C. thermolacticum* on a mixture of 18 mmol l^{-1} glucose and 18 mmol l^{-1} galactose initial concentration, pH non-controlled: glucose (*square*), galactose (*filled square*), acetate (*filled triangle*), ethanol (*triangle*), lactate (*cross*), biomass (*filled circle*). Results are presented as mean values from two independent experiments (error < 15%)

Table 3 Specific enzymatic activities measured at 37°C in *C. thermolacticum* cell extract at steady-state

Enzyme	Specific activity [nkat (mg protein)$^{-1}$][a]		
	$D = 0.013$ h^{-1}	$D = 0.028$ h^{-1}	$D = 0.082$ h^{-1}
Beta-galactosidase	1.7 ± 0.2	1.2 ± 0.2	1.7 ± 0.3
Glyceraldehyde-3P dehydrogenase	8.3 ± 1.3	6.9 ± 1.1	7.4 ± 1.1
Pyruvate:ferredoxin oxidoreductase	7.3 ± 2.5	9.9 ± 1.5	7.8 ± 1.7
Lactate dehydrogenase	1.5 ± 0.0	0.6 ± 0.1	0.4 ± 0.1
Acetate kinase	3.1 ± 0.2	3.5 ± 0.2	3.6 ± 0.3
Ethanol dehydrogenase	0.6 ± 0.1	1.0 ± 0.3	0.7 ± 0.2

Results are the mean of two independent experiments ± error
[a] nkat = 1 nmol s^{-1}

β-galactosidase activity was detected in the culture medium.

The effect of the ratio $NADH/NAD^+$ on the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity was tested. Maximum specific activity was 9.02 nkat mg^{-1} (100%), with 0.25 mM NAD^+ and no NADH in the assay. GAPDH specific activity decreased with increasing ratio $NADH/NAD^+$ down to 3.33 nkat mg^{-1} (37%) at a ratio $NADH/NAD^+ = 0.5$.

The optimal pH for lactate dehydrogenase was found between pH 6.0 and 6.7. The effect of fructose-1,6-bisphosphate (FBP) on the activity of LDH was also studied at 58°C. When 1 mM FBP was added, the specific activity was 3.98 nkat mg^{-1} (100%). The specific activity of the enzyme decreased with decreasing amounts of FBP: 1.51 nkat mg^{-1} (38%) at 0.05 mM FBP and 0.68 nkat mg^{-1} (17%) in the absence of FBP. The effect of the ratio $NADH/NAD^+$ was also studied on LDH at 58°C. Maximum specific activity was 4.14 nkat mg^{-1} (100%), with 0.25 mM NADH, and no NAD^+. The specific activity of LDH decreased with decreasing ratio $NADH/NAD^+$: 2.56 nkat mg^{-1} (62%) for ratio $NADH/NAD^+ = 0.5$ down to 1.24 nkat mg^{-1} (30%) for $NADH/NAD^+ = 0.05$.

Energetic (ATP) and redox (NADH) balance

Two ATP are produced during hexose metabolism through EMP pathway into 2 pyruvate, and one more ATP is generated per acetate produced (Thauer and Kroeger 1984). Given that acetate, ethanol and lactate originated from pyruvate, it was possible to determine the specific pyruvate formation rate ($q_{pyruvate}$) and the associated ATP specific production rate (q_{ATP}), calculated from acetate, ethanol and lactate specific productivities, as shown in Table 1, Eq. 5.

The stoichiometry of ATP generated per hexose equivalent consumed (ATP/hexose) remained stable at 2.1 mol for all dilution rates tested (Table 4). As dilution rate increased, the specific ATP production rate (q_{ATP}) increased from 2.6 to approximately 10 mmol g^{-1} (CDW) h^{-1}. The apparent energetic yield (Y_{ATP}) rose with increasing dilution rate. The pool of ATP and ADP measured in the cells also increased with dilution rate and the ratio ATP/ADP was more than doubled from $D = 0.01$ h^{-1} to $D = 0.08$ h^{-1} (Table 5).

One NADH was generated for each pyruvate produced, one NADH oxidized for each lactate formed and 2 NADH oxidized for each ethanol generated. Ferredoxin was reduced during pyruvate decarboxylation into acetyl-CoA and it could be reoxidised by hydrogen production. Thus, the redox mediators' balance could be estimated from acetate, ethanol and lactate specific productivity as shown in Table 1, Eqs. 6–8.

Both the NADH specific production rate ($q_{NADH\ produced}$) and consumption rate ($q_{NADH\ consumed}$) increased with dilution rate (Table 4). The difference $q_{NADH\ produced} - q_{NADH\ consumed}$ was always negative, indicating that the NADH produced during lactose catabolism was completely reoxidized during ethanol and lactate formation. In order to compensate the deficit in NADH, it is likely that some redox mediators were rerouted from H_2 formation towards NADH production via ferredoxin:oxidoreductases.

The intracellular NAD^+ and NADH concentrations were determined: NADH concentration was very low compared to NAD^+ (Table 5). Even if the ratio

Table 4 Calculations of energetic and redox balance for *C. thermolacticum* at steady state

Parameters	D [h^{-1}]								
	0.013	0.028	0.040	0.058	0.082	0.105	0.130	0.150	0.190
q_{ATP} [mmol (g CDW)$^{-1}$ h^{-1}]	2.6	4.3	5.5	6.9	9.0	9.8	10.4	10.0	9.1
ATP / hexose [mol mol^{-1}]	2.13	2.07	2.07	2.08	2.08	2.07	2.06	2.17	2.03
Y_{ATP} [g CDW mol^{-1}]	5.1	6.5	7.3	8.4	9.1	10.8	12.5	15.0	20.8
$q_{NADH\ prod}$ [mmol (g CDW)$^{-1}$ h^{-1}]	1.96	3.23	3.97	4.84	6.28	6.84	7.26	6.92	6.33
$q_{NADH\ cons}$ [mmol (g CDW)$^{-1}$ h^{-1}]	2.24	3.61	4.45	5.34	6.96	7.74	8.20	7.69	7.04
$q_{NADH\ prod}-q_{NADH\ cons}$	−0.28	−0.38	−0.48	−0.50	−0.68	−0.90	−0.94	−0.77	−0.71
$q_{Fd\ prod}$ [mmol (g CDW)$^{-1}$ h^{-1}]	1.49	2.53	3.48	4.70	6.09	6.72	7.26	6.92	6.33

Values were calculated from the mean of three independent measurements

NADH/NAD^+ increased somewhat with D, it remained small. Therefore, it seemed that NADH was completely reoxidised only by ethanol and lactate formation, and that hydrogen formation was therefore not associated with NADH reoxidation. As a consequence, hydrogen should be formed exclusively during ferredoxin reoxidation.

Discussion

The productivity of acetate and ethanol was directly dependent on bacterial specific growth rate and lactose consumption rate. In contrast, lactate appeared only at dilution rates below 0.06 h^{-1}, when lactose consumption rate was low. This confirmed the results obtained previously in batch cultures (Talabardon et al. 2000a; Collet et al. 2003). The shift of metabolism was however not due to low remaining lactose: when *C. thermolacticum* is growing on low lactose concentration in batch culture, acetate and ethanol are produced, but almost no lactate is formed. Therefore, a growth rate above 0.06 h^{-1} should be maintained to allow acetate production and avoid lactate formation. The metabolism of *C. thermolacticum* was thus linked to the growth rate or phase: acetate and ethanol were strictly growth-dependent, whereas lactate was not. Similar results have been reported for *C. cellulolyticum* growing on cellobiose (Payot et al. 1999) and for *C. thermohydrosulfuricum* on starch (Heitmann et al. 1996).

Table 5 Adenylate and pyridine nucleotide content measured in cells of *C. thermolacticum* in continuous cultures at steady-state

	$D = 0.013$ h^{-1}	$D = 0.028$ h^{-1}	$D = 0.082$ h^{-1}
NAD^+ [µmol (g CDW)$^{-1}$]	17 ± 2	32 ± 1	23 ± 4
NADH [µmol (g CDW)$^{-1}$]	0.5 ± 0.4	3.0 ± 0	3.8 ± 1.6
Ratio NADH/NAD^+	0.03	0.09	0.17
ATP [µmol (g CDW)$^{-1}$]	0.64 ± 0.07	1.22 ± 0.06	2.19 ± 0.22
ADP [µmol (g CDW)$^{-1}$]	5.41 ± 2.35	6.85 ± 1.68	8.14 ± 1.88
Ratio ATP/ADP	0.12	0.18	0.27

Results are the mean of three independent measurements, with standard deviation

The presence of substantial β-galactosidase activity in cell extracts, but not in the culture medium, indicates that lactose was hydrolysed inside the cells. Except at low dilution rate, none of the lactose moieties were released into the medium, suggesting that both were rapidly phosphorylated and metabolized. In other bacteria, it has been observed that one of the monomers, generally galactose, is excreted and thus promotes lactose uptake (Hickey et al. 1986; Hutkins and Ponne 1991; Benthin et al. 1994). *C. thermolacticum* is able to use either glucose or galactose as sole carbon source for growth. However in the presence of a mixture of both substrates, in batch culture, diauxic growth was observed and galactose was preferentially consumed. This observation is uncommon although it has already been reported that *Azotobacter vinelandii* consumes galactose at a higher rate than glucose during the first phase of growth (Wong et al. 1995). In general, bacteria consume glucose preferentially over galactose, e.g. *Klebsiella oxytoca* (Champluvier et al. 1989).

A high activity of GAPDH indicates that EMP pathway with the associated NADH formation was used for both sugars, for galactose probably after its conversion to glucose-6-P via the Leloir pathway. The activity of the enzyme was strongly affected by an increase of the ratio NADH/NAD^+, as reported for *C. thermohydrosulfuricum* (Lovitt et al. 1988), *C. acetobutylicum* (Girbal and Soucaille 1994), *C. cellulolyticum* (Payot et al. 1998) and *L. lactis* (Even et al. 1999). Thus NADH could act as an inhibitor of glycolysis at the level of GAPDH. Intracellular accumulation of NADH, due to inefficient reoxidation, did slow down the metabolic flux through glycolysis. As a consequence, substrate metabolization decreased until NADH was reoxidised via ethanol, lactate or H_2 production.

Lactate was formed from pyruvate by lactate dehydrogenase and associated NADH reoxidation to NAD^+, even if the activity was low when tested at 37°C. It has been reported that this enzyme looses 2/3 of activity when measured at 37°C instead of 58°C for *C. thermosuccinogenes* (Sridhar et al. 2000). LDH in *C. thermolacticum* was also sensitive towards NADH/NAD^+ ratio, as reported for *L. lactis* (Garrigues et al.

1997; Even et al. 1999). Fructose-1,6-bisphosphate, a glycolytic metabolite, did act as an activator of LDH in *C. thermolacticum*, like in other *Clostridia* (Germain et al. 1986; Freier and Gottschalk 1987; Vancanneyt et al. 1990; Sridhar et al. 2000). The accumulation of fructose-1,6-bisphosphate, caused by a metabolic inhibition, an increased intracellular NADH level, or a decreased intracellular pH, should thus promote LDH activation.

The presence of pyruvate:ferredoxin oxidoreductase indicates that pyruvate was decarboxylated into acetyl-CoA, using ferredoxin as a cofactor, like reported for other *Clostridia* (Uyeda and Rabinowitz 1971; Meinecke et al. 1989; Vasconcelos et al. 1994; Sridhar et al. 2000; Desvaux et al. 2001b). The maximal activity was found at neutral pH, like for *C. acetobutylicum* (Meinecke et al. 1989). Thus a decrease in intracellular pH below 7.0 would decrease the activity of this enzyme and cause a decreased metabolic flux to acetate and ethanol routes.

Furthermore, an efficient reoxidation of reduced ferredoxin by H_2 production is necessary for maximum activity of pyruvate:ferredoxin oxidoreductase. Clostridial hydrogenases are generally reduced in vivo by ferredoxin transferring electrons from pyruvate:ferredoxin oxidoreductase (Church et al. 1988; Saint-Amans et al. 2001; Vignais et al. 2001). Even if not determined in the present study, we could assume that it would also be the case for *C. thermolacticum*, since it has been previously reported that 2 to 3 H_2 are produced per lactose consumed (Collet et al. 2004).

The detection of acetate kinase activity in *C. thermolacticum* confirmed the presence of the acetate branch from acetyl-CoA and the supplementary ATP formation associated to this pathway (Winzer et al. 1997; Lin et al. 1998; Sridhar et al. 2000; Desvaux et al. 2001a and b; Saint-Amans et al. 2001).

Based on results from enzymatic tests and metabolites analysis, a scheme for lactose metabolism was proposed (Fig. 3), assuming the use of the Leloir pathway for galactose conversion to glucose-6-P (Chen et al. 2002).

It has been previously suggested that H_2 itself inhibits its production and induces the production of ethanol (Collet et al. 2003). In the present study, the ratio

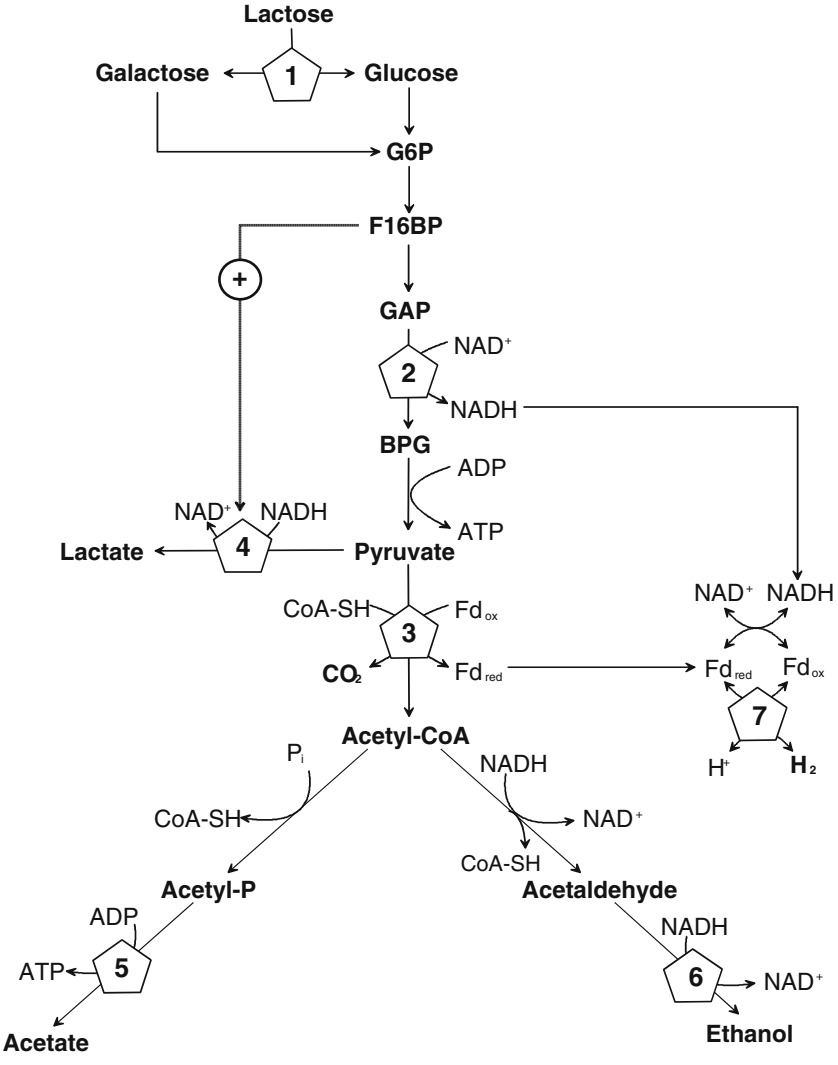

Fig. 3 Proposed metabolic pathways of *C. thermolacticum* with the following enzymes: *1* beta-galactosidase, *2* glyceraldehyde-3P dehydrogenase, *3* pyruvate:ferredoxin oxidoreductase, *4* lactate dehydrogenase, *5* acetate kinase, *6* ethanol dehydrogenase, *7* hydrogenase

$C_{acetate}/C_{ethanol}$ remained constant, close to 0.8, for all dilution rates. Hydrogen inhibition could also be responsible for the low yield coefficient of acetate on lactose. This value was about four times lower than the theoretical yield: two acetate per hexose in other *Clostridia* (Jungermann et al. 1973), thus corresponding to four acetate per lactose. Ethanol formation, responsible for the decrease in acetate formation was a consequence of the inhibition of H_2 production from NADH arising from glycolysis (Thauer and Kroeger 1984).

It thus appears that the production of ethanol in *C. thermolacticum* was the consequence of H_2 accumulation, enabling NADH to be reoxidised without the involvement of NADH-ferredoxin oxidoreductase plus hydrogenase system, which is inhibited by high H_2 partial pressure (Stams 1994). Indeed experiments with *C. thermolacticum* growing on lactose in continuous culture, under efficient continuous removal of H_2, demonstrated that the production of ethanol can be efficiently decreased and acetate productivity significantly increased (Collet et al. 2005).

After pyruvate decarboxylation, the reduction of one acetyl-CoA to one ethanol enabled the re-oxidation of 2 NADH, while one acetate was formed, thus allowing generation of one extra ATP (Fig. 3). The results indicate that the calculated specific NADH production during glycolysis was lower than the specific NADH consumption for ethanol or lactate production, at all dilution rates tested. Intracellular NADH concentration was always at least 5 times lower than NAD^+ concentration. This shows that NADH was fully re-oxidized during ethanol and lactate formation. Apparently no H_2 was formed from NADH, whereas all H_2 produced should come from reduced ferredoxin generated during pyruvate decarboxylation. Therefore, it could be assumed that some reducing equivalents were transferred from ferredoxin to NAD^+ and re-oxidized during ethanol and lactate formation, since this reaction is thermodynamically possible, but not used for H_2 production.

The ATP and ADP pool increased with dilution rate as did the ATP/ADP ratio and the ATP specific production rate. More ATP was formed at higher dilution rates, probably to sustain growth. The average value for Y_{ATP} was 10.6 g CDW per mol ATP and the average value for ATP yield was 2.1 mol ATP per mol hexose equivalent. Similar results have been reported for *Streptococcus faecalis* grown anaerobically in a complex medium containing glucose as energy source (Bauchop and Elsden 1960). Besides, it was observed that the yield coefficient for biomass CDW on hexose equivalents ($Y_{X/S}$) increased with increasing dilution rate. These results were in the same range as results from a continuous culture of *C. cellulolyticum* degrading cellulose (Desvaux et al. 2001a).

A metabolic shift, like the high lactate production observed in this study, has been reported for *L. lactis* (Russel et al. 1996; Garrigues et al. 1997; Cocaign-Bousquet et al. 2002; Melchiorsen et al. 2002), *C. cellulolyticum* (Desvaux et al. 2001a), *C. acetobutulicum* (Girbal et al. 1995) and *C. thermosuccinogenes* (Sridhar and Eiteman 2001). This shift is caused by a decreased intracellular pH, high NADH levels, an inhibition of acetate production or an activation of lactate production.

An acetate accumulation in the medium at low dilution rates would lead to its intracellular accumulation, causing a decreased intracellular pH. It was shown, in this study, that the pH optimum for pyruvate:ferredoxin oxidoreductase was around pH 7.0, whereas it was around pH 6.0–6.5 for lactate dehydrogenase. A decrease of the intracellular pH would thus decrease the activity of pyruvate:ferredoxin oxidoreductase. As a consequence, an accumulation of glycolytic intermediates would occur: among them, fructose-1,6-bisphosphate, an activator of lactate dehydrogenase. A decreased pyruvate:ferredoxin oxidoreductase activity would also decrease acetyl-CoA production, thus lowering acetate and ethanol formation. As a result, NADH reoxidation by acetaldehyde and ethanol dehydrogenase should decrease and lead to NADH accumulation; ATP formation by acetate kinase was also lowered, causing a slow growth rate. NADH and fructose-1,6-bisphosphate accumulation as well as a decrease of intracellular pH would result in the rerouting of the carbon flow from acetate-ethanol fermentation towards the production of lactate.

To produce more hydrogen or more acetate from lactose, it thus appears that an efficient H_2 removal system should be used, based on a physical (membrane) or a biological approach, respectively, by cultivating *C. thermolacticum* with efficient H_2 scavenging and acetate producing microorganisms (Talabardon et al. 2000b; Collet et al. 2005). Alternatively, a genetic improvement would be required to optimize acetate production from lactose.

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