Carbon and electron flow in *Clostridium butyricum* grown in chemostat culture on glycerol and on glucose

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The metabolism of *Clostridium butyricum* DSM 5431 was studied in chemostat culture under carbon limitation using either glucose or glycerol. On glycerol, the enzymes glycerol dehydrogenase, diol dehydratase and 1,3-propanediol (1,3-PD) dehydrogenase constitute the branch point that partitions the carbon flux between the competing pathways, i.e. formation of either 1,3-PD or acetate and butyrate. The increasing levels of these enzyme activities with increasing dilution rates (*D*) explained the constant proportion of glycerol conversion into 1,3-PD. The production of acetate or butyrate constitutes another important branch point and when *D* increased (i) large amounts of intracellular acetyl-CoA accumulated, (ii) the carbon flux switched from butyric acid to acetic acid, (iii) the specific activity of thiolase was not affected, suggesting this enzyme may be the bottleneck for carbon flux to butyrate biosynthesis providing an explanation for the accumulation of large amounts of intracellular acetyl-CoA, and (iv) high levels of NADH were found in the cell. Oxidation of NADH by 1,3-PD dehydrogenase was linked to the production of 3-hydroxypropionaldehyde (3-HPA) by glycerol dehydratase. The fact that high intracellular concentrations of NADH were found means that diol dehydratase activity is the rate-limiting step in 1,3-PD formation, avoiding the accumulation of 3-HPA which is a very toxic compound. The specific rate of glucose catabolism ($q_{\text{glucose}} = 11.1 \text{ mmol h}^{-1} \text{ g}^{-1}$) was around four times lower than the specific rate of glycerol catabolism ($q_{\text{glycerol}} = 57.4 \text{ mmol h}^{-1} \text{ g}^{-1}$). On glucose-grown cells, reducing equivalents which are released in the glycolytic pathway were reoxidized by the butyric pathway and the low specific formation rate of butyric acid led to an increase in the intracellular level of acetyl-CoA and NADH. Carbon flow was higher on glycerol due to the reoxidation of NADH by both butyric and PD pathways.

Keywords: *Clostridium butyricum*, glycerol catabolism, glucose catabolism, carbon flow, electron flow

INTRODUCTION

Over recent years there has been an increase in research and development efforts involving clostridial fermentation, namely the fermentation of glucose by butyric acid bacteria (Woods, 1993). Clostridia are able to ferment the carbon source to a variety of end-products by means of branched fermentation pathways. Generally, the different branches of fermentation are not equivalent with respect to the generation of energy or reducing equivalents, and

the amount of reduced versus neutral and oxidized products has the potential for a great deal of natural variation. It was shown (Jungermann *et al.*, 1973; Petitdemange *et al.*, 1976) that the reduced electron carrier ferredoxin (fd) plays a pivotal role in electron distribution in the cell in that it can either transfer electrons via hydrogenase to generate hydrogen or transfer electrons to the pyridine nucleotides via the appropriate fd oxidoreductase. The activities of three enzymes, NADH-fd oxidoreductase, NADPH-fd oxidoreductase and hydrogenase are responsible for controlling the electron flow, which in turn determines the carbon flow within the cell.

Clostridium butyricum is known as a classical acid producer

Abbreviations: DHA, dihydroxyacetone; fd, ferredoxin; 3-HPA, 3-hydroxypropionaldehyde; PD, propanediol.

Fig. 1. Schematic diagram depicting the catabolism of glucose and glycerol by *C. butyricum*. 1, Diol dehydratase; 2, 1,3-PD dehydrogenase; 3, glycerol dehydrogenase; 4, DHA kinase; 5, glyceraldehyde-3-P dehydrogenase; 6, phosphoroclastic reaction; 7, NADH-fd oxidoreductase; 8, hydrogenase; 9, thiolase; 10, butyrate kinase; 11, acetate kinase. $q_{\text{glycerol}} = q_{\text{PD}} + q_{\text{pyruvate}}$; $q_{\text{pyruvate1}} = 2q_{\text{glucose}}$; $q_{\text{pyruvate2}} = q_{\text{glycerol}} - q_{\text{PD}}$.

and usually ferments carbohydrates to butyrate, acetate, CO₂ and molecular hydrogen. Usually about 2 mol CO₂ and 2.5 mol H₂ are produced per mol hexose catabolized (Jungermann *et al.*, 1973). The production of acetate or butyrate constitutes an important branch point (Fig. 1). *C. butyricum* is also able to catabolize glycerol and an additional branch point of fermentation exists (Fig. 1): glycerol can be either oxidized to dihydroxyacetone (DHA) and subsequently phosphorylated to yield DHAP, or it can be dehydrated to 3-hydroxypropionaldehyde (3-HPA). The latter compound is then reduced to 1,3-propanediol (1,3-PD). Fermentative utilization of glycerol, a more reduced substrate than glucose, requires the disposal of the two extra hydrogen atoms; this could be accomplished by stimulating H₂ production. Surprisingly, the reducing power is not used to form H₂, but rather a more reduced compound, 1,3-PD. The concentrations of the fermentation end-products have been extensively studied in *C. butyricum* (Biebl, 1991; Biebl *et al.*, 1992; Günzel, 1991; Günzel *et al.*, 1991; Heyndrickx *et al.*, 1991b); in all fermentations the yield of 1,3-PD from glycerol was higher than expected from NADH pro-

duction by acid formation. In contrast, H₂ production was lower than expected, if it is assumed that one mol H₂ is released per mol acetyl-CoA formed. So nothing is known about how the oxidation/reduction state is balanced during glycerol catabolism. Also, very little is known regarding the regulation of the acetate/butyrate ratio when *C. butyricum* is growing on glucose, except that the excess H₂ is formed by NADH-fd reductase activity to balance the amount of acetate made (Jungermann *et al.*, 1973).

From the scheme shown in Fig. 1, it is interesting to note that when glycerol and glucose are used as carbon sources for the growth of *C. butyricum*, the carbon metabolism pathway from glyceraldehyde-3-P to pyruvate is the same but there is a different distribution of the electron flow since during glucose fermentation more H₂ is produced than CO₂ (Jungermann *et al.*, 1973), whereas glycerol fermentation yields considerably less H₂ than CO₂ (Biebl *et al.*, 1992). This is why in this paper, we deal with the metabolic aspect of *C. butyricum* when grown on glucose or glycerol, using continuous culture, an obvious tool to unravel metabolic regulation. Furthermore, to understand the observed changes in metabolism in the steady-state chemostat culture, the level of the key enzyme activities and the NAD⁺, NADH and acetyl-CoA pools were analysed. *C. butyricum* DSM 5431 was chosen for this study because it has been used in several investigations relating to the various events which take place during glycerol metabolism (Biebl, 1991; Biebl *et al.*, 1992; Günzel, 1991; Günzel *et al.*, 1991).

METHODS

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

Organism and medium. *C. butyricum* DSM 5431 was used. The spores of the strain were stored at 4 °C in RCM (Reinforced Clostridial Medium purchased from Oxoid). *Clostridium pasteurianum* ATCC 6013 was grown in the same medium as *C. butyricum* but with glucose as carbon source.

The preculture medium contained the following components (l distilled H₂O)⁻¹: glycerol, 20 g; K₂HPO₄, 1.0 g; KH₂PO₄, 0.5 g; (NH₄)₂SO₄, 2.0 g; MgSO₄·7H₂O, 0.2 g; CaCl₂·2H₂O, 15 mg; FeSO₄·7H₂O, 5 mg; CaCO₃, 2.0 g; yeast extract, 1.0 g; trace element solution SL7 (Biebl & Pfennig, 1982), 2 ml. The growth temperature was 32 °C.

Fermentation modes. Continuous cultures were carried out aseptically in a 2 l bioreactor (LSL-Biolafitte, 2 l growth vessel with a 1 l working volume). The temperature was set at 32 °C and pH kept at 7 with 2 M KOH. Anaerobic conditions were maintained by sparging with nitrogen. Agitation was kept constant at 100 r.p.m. The culture volume was kept constant at 1 l by automatic regulation of the culture level. The bioreactor was inoculated (10%, v/v) with exponential preculture. The culture was grown in batches for 12 h before the medium flow was started at a low dilution rate (*D*). Two substrates were used in this study, glycerol at a concentration of 10 g l⁻¹ and glucose at 1, 3 and 10 g l⁻¹.

Analytical methods. Glycerol was determined enzymically via glycerol kinase, pyruvate kinase and L-lactate dehydrogenase

using the Boehringer test kit and instructions. Glucose was assayed with hexokinase and glucose-6-phosphate dehydrogenase using the Boehringer diagnostic kit. Concentrations of 1,3-PD, acetic and butyric acids were determined by GC (Intersmat IGC 121 FL, equipped with a flame ionization detector). Separation took place in a glass column (2 m × 2 mm i.d.) packed with chromosorb 101/80–100 mesh. Nitrogen was used as the carrier gas with n-butanol as the internal standard and the temperature of the column was 170 °C. Data were analysed with an Intersmat ICR IB Integrator.

The cell concentrations were estimated by cell dry weight measurements using a predetermined correlation between A_{650} (Shimadzu UV160A double beam spectrophotometer) and cell dry weight.

Preparation of cell free extracts. Cells were centrifuged at 12000 *g* for 15 min; after washing with Tris buffer (50 mM Tris/HCl, 2.0 mM DTT, 0.1 mM MnSO₄, pH 7.4) cells were resuspended in this buffer. The cells were sonicated at 2 °C four times for 20 s at a frequency of 20 kcycles s⁻¹, separated by 60 s (MSE, 150 watt ultrasonic disintegrator). The supernatant was collected from the cell lysate following centrifugation at 12000 *g* for 20 min at 4 °C. At each step, extracts were maintained under anaerobic conditions. To measure hydrogenase activity, cell-free extract was prepared as described but without washing.

Protein concentrations of cell extracts were determined according to the Bradford (1976) method, using crystalline bovine serum albumin as standard.

Enzyme assays.

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

fd-NAD⁺ reductase (EC 1.18.1.3) was assayed with pyruvate as the reductant of fd and under a CO atmosphere according to Petitdemange *et al.* (1976), modified by the addition of 1 mM purified fd (Sigma).

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

Glycerol dehydrogenase (EC 1.1.1.6) and **1,3-PD dehydrogenase (EC 1.1.1.202)** activities were measured by the linear increase in A_{340} produced by addition of cell extracts (Ruch *et al.*, 1974). The assays contained the following components: 100 mM glycerol or 1,3-PD, 2 mM NAD⁺, 30 mM ammonium sulfate and 100 mM potassium carbonate buffer (pH 9.0).

Diol dehydratase (PD dehydratase, EC 4.2.1.28) activity was determined by the 3-methyl-2-benzothiazolinone hydrazone (MBTH) method according to Toraya *et al.* (1977). The assays contained the following components: 0.2 M 1,2 PD, 0.05 M KCl, 0.035 M potassium phosphate buffer (pH 7.0), 15 μM adenosylcobalamin and an appropriate amount of cell extract in a total volume of 1 ml. After incubation at 37 °C for 10 min, the enzyme reaction was stopped by adding 1 ml 0.1 M potassium citrate buffer (pH 3.6) and 0.5 ml 0.1 % MBTH hydrochloride. After 15 min at 37 °C, 1 ml water was added and the amount of propionaldehyde was determined from A_{305} . The apparent molar extinction coefficient at 305 nm for the coloured product formed from propionaldehyde in the MBTH method was $13.3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

Acetate kinase (EC 2.7.2.1) and **butyrate kinase (EC 2.7.2.7)**. The enzymic activities were determined by the analytical procedure for acetyl-phosphate (Lipmann & Tuttle, 1945). Enzyme

activities were assayed in a volume of 0.8 ml containing (mmol): Tris/HCl buffer (pH 7.5), 40; potassium acetate or potassium butyrate, 160; manganese sulfate, 2.4; ATP, 4. The reaction was initiated by addition of crude extract. After 5 min at 37 °C, 0.2 ml hydroxylamine (2.1 M, pH 6.4, freshly neutralized) was added. After 5 min at room temperature, the reaction was stopped by addition of 0.2 ml TCA (15 %, w/v), 0.2 ml HCl (4 M) and 0.2 ml FeCl₃ (5 %, w/v, in 0.1 M HCl). The amounts of acetyl hydroxamate or butyryl hydroxamate formed were determined at 540 nm using a standard curve obtained with purified acetate kinase, since acetyl hydroxamate and butyryl hydroxamate have identical extinction coefficients (Lipmann & Tuttle, 1945).

Phosphoroclastic activities. Assays were based on ability to catalyse formation of acetyl phosphate in pyruvate oxidation as described by Mortenson *et al.* (1963), modified as follows: 25 mM potassium phosphate buffer (pH 6.5), 1 mM sodium pyruvate, 0.05 mM CoA and 1 mM methyl viologen.

Thiolase. The condensation reaction of two molecules of acetyl-CoA forming acetoacetyl-CoA and CoASH was followed by measuring the oxidation of NADH, coupled with the reduction of acetoacetyl-CoA by β-hydroxyacyl-CoA dehydrogenase (Lynen *et al.*, 1952). With the regenerating system, acetyl-phosphate and phosphotransacetylase plus CoASH, activities were determined as described by Berndt & Schlegel (1975). A cuvette contained: 100 mM Tris/HCl buffer, pH 8.2, 0.63 mM CoASH, 20 mM acetyl-phosphate and 5 μl phosphotransacetylase (2 mg ml⁻¹). The mixture was incubated for 10 min, then the reaction was started by adding 0.30 mM NADH, 5 μl β-hydroxyacyl-CoA dehydrogenase (2 mg ml⁻¹) and 10 μl cell extract.

Determination of NAD⁺ and NADH pools. Levels of NAD⁺, NADH and acetyl-CoA were measured after extraction of a culture broth sample, neutralization and filtration. Samples of 4 ml were taken quickly from the bioreactor with sterile syringes and immediately put into tubes containing the extractant, without separating the cells from the medium.

NAD⁺ was extracted with HCl (NADH and NADPH were degraded) and NADH was extracted with KOH (NAD⁺ and NADP⁺ were degraded) as described by Wimpenny & Firth (1972). Before assays for NADH, this coenzyme was converted to NAD⁺ with D-lactate dehydrogenase (EC 1.1.1.28) (Klingenberg, 1965). NAD⁺ was assayed with a NAD(H)-specific alcohol dehydrogenase (EC 1.1.1.1) (Klingenberg, 1965; Vasconcelos *et al.*, 1994) and the resulting NADH was determined by fluorometry (Hitachi, model F-2000) and by spectrophotometry.

Determination of intracellular acetyl-CoA. Acetyl-CoA was extracted with perchloric acid as described by Decker (1965). The level of acetyl-CoA was determined by a method described by Tubbs & Garland (1969) using citrate synthase (EC 4.1.3.7) and malate dehydrogenase (EC 1.1.1.37). The citrate synthase consumes oxaloacetate and acetyl-CoA, and so displaces the equilibrium of the reaction catalysed by malate dehydrogenase; thus acetyl-CoA removal causes the formation of NADH₂, which can be measured spectrophotometrically.

All enzyme assays, except acetate and butyrate kinase, were performed under anaerobic conditions. Each enzyme assay was done in duplicate on three extracts. One unit of enzyme activity is defined as the amount of enzyme that catalyses the conversion of 1 μmol substrate min⁻¹.

Calculations. The main products of glycerol fermentation by *C. butyricum* were 1,3-PD, butyrate and acetate (Biebl *et al.*, 1991) (see Fig. 1). The minimum or maximum amount of NADH

formed can be calculated according to the equations of glycerol conversion to acetate and butyrate:

NADH is formed by glycerol dehydrogenase and glyceraldehyde-3-P dehydrogenase. ATP is formed by phosphoglycerate kinase, pyruvate kinase and acetate kinase, whereas DHA kinase uses one ATP.

If reduced fd is not reoxidized by hydrogenase but is entirely reoxidized by the fd-NAD⁺ reductase according to:

the conversion of glycerol to acetate becomes:

The minimum amount of NADH formed via the acetate pathway is 2 mol and the maximum NADH is 3 mol, hence the corresponding production of 1,3-PD must be between 2 and 3 mol.

Conversion of glycerol to butyrate can be written as:

On the assumption that the reduced fd is entirely reoxidized by the fd-NAD⁺ reductase activity rather than being released as molecular H₂ via hydrogenase, the conversion of glycerol to butyrate becomes:

Four moles of NADH results from the production of 2 mol by glycerol dehydrogenase and 2 mol by glyceraldehyde-3-P dehydrogenase, whereas 2 mol are used by 3-hydroxybutyryl-CoA dehydrogenase and butyryl-CoA dehydrogenase. The minimum amount of NADH formed via the butyrate pathway is 2 mol and the maximum NADH is 4 mol, hence the corresponding production of 1,3-PD must be between 2 and 4 mol.

In glucose catabolism, acetate formation can be written:

ATP is formed by phosphoglycerate kinase, pyruvate kinase and acetate kinase. The minimum amount of NADH formed via the acetate pathway is 2 mol NADH produced by glyceraldehyde-3-P dehydrogenase and the maximum amount is 4 mol on the assumption that the reduced fd is entirely reoxidized by the fd-NAD⁺ reductase activity.

Glucose conversion to butyrate can be written:

ATP is formed by phosphoglycerate kinase, pyruvate kinase and butyrate kinase. The formation of butyrate is redox neutral since the 2 mol NADH produced by glyceraldehyde-3-P dehydrogenase are used in butyrate biosynthesis, but the maximum NADH is 2 mol if the reduced fd is entirely reoxidized by the fd-NAD⁺ reductase.

The specific rate of pyruvate formation with glycerol as substrate was calculated as follows:

$$q_{\text{glycerol}} - q_{\text{PD}} = q_{\text{pyruvate}} = q_{\text{glyceraldehyde}}$$

where q_{glycerol} , q_{PD} , q_{pyruvate} and $q_{\text{glyceraldehyde}}$ are the specific rates

of glycerol used, 1,3-PD formation, pyruvate formation and glyceraldehyde formation, respectively, all in units of [mmol (g dry wt cells)⁻¹ h⁻¹].

The specific rate of NADH production with glycerol as substrate by the fd-NAD⁺ reductase activity ($q_{\text{fd-NAD}}$) is the difference between the specific rate of NADH consumption (q_{NADHcons}) to form end-products and the specific rate of NADH production ($q_{\text{NADHproduct}}$) during substrate conversion to pyruvate.

$$q_{\text{fd-NAD}} = q_{\text{NADHcons}} - q_{\text{NADHproduct}}$$

$$q_{\text{NADHcons}} = q_{\text{PD}} + 2q_{\text{butyrate}}$$

$$q_{\text{NADHproduct}} = 2q_{\text{pyruvate}}$$

With glucose as substrate, the specific rate calculation becomes:

$$q_{\text{NADH-fd}} = q_{\text{pyruvate}} - q_{\text{butyrate}} = q_{\text{excessNADH}}$$

where q_{glucose} is the specific rate of glucose used in units of [mmol (g dry wt cells)⁻¹ h⁻¹].

RESULTS

Carbon flow in *C. butyricum* grown in continuous culture under glycerol limitation

Data on the effect of D on growth and product formation with *C. butyricum* are compiled in Table 1. No significant concentration of residual glycerol was observed at D values of up to 0.26 h⁻¹. The dry weight was almost constant between 0.04 h⁻¹ and 0.26 h⁻¹ and decreased markedly at 0.30 h⁻¹ corresponding to glycerol accumulation.

Whatever the D value, conversion of glycerol into 1,3-PD was between 0.57 and 0.60 [mol PD (mol glycerol)⁻¹] showing that the carbon flux distribution through diol dehydratase and 1,3-PD dehydrogenase was balanced by the flux through glycerol dehydrogenase and DHA kinase. These facts were confirmed by study of the specific rates of glycerol utilization and product formation. Fig. 2 shows the linearity of the specific rates of glycerol utilization and 1,3-PD and pyruvate formation as a function of D . Whereas at low D values, the cells produced more butyrate than acetate, at high D values, i.e. high carbon flux, a switch was observed and the cells produced more acetate than butyrate (Fig. 3).

Below a D of 0.15 h⁻¹, i.e. $q_{\text{pyruvate}} = 11.9$ mmol h⁻¹ g⁻¹, the specific rate of butyrate formation increased more rapidly than the specific rate of acetate formation (Fig. 3). Between 0.15 and 0.26 h⁻¹ there is an increase of the specific rate of acetate formation concomitant with a decrease of the specific rate of butyrate formation. The fact that the quantities of 1,3-PD formed were greater than the quantities of NADH generated (between 0.04 h and 0.26 h⁻¹, Table 1) in the catabolic reactions from glycerol to pyruvate suggested the existence of another source of NADH. Part of the reduced fd was reoxidized by the fd-NAD⁺ reductase; the specific rate of NADH generation by this activity increased from 0.8 to 4.5 mmol h⁻¹ g⁻¹ in parallel with an increase in growth rate from 0.04 to 0.26 h⁻¹. At a D of 0.30 h⁻¹, due to the

Table 1. Effect of *D* on growth and product formation by *C. butyricum* DSM 5431 grown under glycerol- or glucose-limited chemostat cultures

Data in parentheses are SD values from four determinations. NA, not applicable.

Input	<i>D</i> (h ⁻¹)	Biomass (g l ⁻¹)	Substrate used (mM)	Products (mmol l ⁻¹)			Ac/But	Conversion [mol PD (mol glycerol) ⁻¹]	Carbon recovery (%)	NADH (mmol)		NADH recovery (%)
				Acetate	Butyrate	1,3-PD				min	max	
Glycerol (108.5 mM)	0.04	0.64 (0.07)	108 (13.1)	5.3 (0.6)	21.1 (1.2)	62 (3.0)	0.25	0.57	101	52.8	100.3	61
	0.08	0.64 (0.08)	108 (12.3)	5.1 (0.5)	20.4 (2.1)	64.6 (3.5)	0.25	0.60	102	51	96.9	66.6
	0.15	0.57 (0.06)	108 (12.2)	5.4 (0.5)	19.2 (0.9)	62.8 (2.9)	0.28	0.58	98.7	49.2	93	67.5
	0.26	0.60 (0.07)	107.4 (10.6)	7.9 (0.9)	15.4 (1.4)	64.8 (3.5)	0.51	0.60	96.4	46.6	85.3	75.9
	0.30	0.33 (0.02)	63.1 (5.2)	13.8 (1.2)	3.7 (0.8)	36.6 (1.5)	3.73	0.58	91.6	35	56.2	65.1
Glucose (5.55 mM)	0.08	0.18 (0.11)	5.4 (0.6)	4.8 (1.3)	2.7 (1.8)	NA	1.8	NA	91	9.6	24.6	22
	0.30	0.19 (0.02)	5.2 (0.4)	5.9 (0.9)	3.6 (0.7)	NA	1.6	NA	94.6	11.8	19	30.5

Fig. 2. Influence of *D* on specific rates of glycerol utilization and PD production in a continuous culture of *C. butyricum*. The glycerol input concentration was 108 mM. □, q_{glycerol} ; ●, q_{PD} ; △, $q_{\text{glyceraldehyde}} = q_{\text{pyruvate}}$. **Fig. 3.** Influence of the specific rates of pyruvate production in a continuous culture of *C. butyricum* on specific rates of acetate and butyrate production. The glycerol input concentration was 108 mM. □, q_{acetate} ; ■, q_{butyrate} .

limitation of the butyrate pathway, the NADH produced by glycerol dehydrogenase and glyceraldehyde-3-P dehydrogenase was sufficient for the formation of 1,3-PD.

Carbon flow in *C. butyricum* grown in continuous culture with glucose

Cultures were grown in a chemostat at glucose concentrations of 5.55, 16.6 and 55.5 mM (Table 2). Whereas with a glycerol-limited medium a rate of glycerol consumption of 46.5 mmol h⁻¹ g⁻¹ was obtained without residual glycerol (Fig. 2, *D* = 0.26 h⁻¹), a specific rate as low as 2.1 mmol glucose g⁻¹ h⁻¹ led to residual glucose (Table 2). With a glucose input of 55.5 mM only 55 and 26% was used at *D* = 0.08 and 0.30 h⁻¹, respectively.

Fig. 1 compares glycerol and glucose as carbon sources for growth. They show a number of differences in catabolism but have in common the pathway from

glyceraldehyde-3-P to pyruvate. The lower rate of glucose catabolism ($q_{\text{glucose}} = 11.1 \text{ mmol h}^{-1} \text{ g}^{-1}$, Table 2) compared to that of glycerol catabolism ($q_{\text{glycerol}} = 57.4 \text{ mmol h}^{-1} \text{ g}^{-1}$, Fig. 2) cannot be explained by a change in the activity of this pathway which remained almost the same for culture on glycerol ($q_{\text{pyruvate}} = 24.2 \text{ mmol h}^{-1} \text{ g}^{-1}$, Fig. 2) or on glucose ($q_{\text{pyruvate}} = 22.2 \text{ mmol h}^{-1} \text{ g}^{-1}$, Table 2).

Glucose-grown cultures produced butyrate and acetate. The difference between the specific production rate of NADH and the specific consumption rate indicates an excess of NADH which was reoxidized by the NADH-fd reductase and the reduced fd reoxidized by hydrogenase. The specific rate of NADH oxidation by the NADH-fd reductase increases in parallel with the specific rates of substrate consumption.

Table 2. Catabolic pathway fluxes of glucose chemostat

Pathway flux values are expressed in $\text{mmol h}^{-1} \text{g}^{-1}$.

Glucose (mM)	D (h^{-1})	Substrate used (mM)	Biomass (g l^{-1})	q_{glucose}	q_{pyruvate}	q_{acetate}	q_{butyrate}	$q_{\text{NADH-fd}}$
5.55	0.08	5.4	0.18	2.4	4.8	2.1	1.2	3.6
	0.30	5.2	0.19	8.2	16.4	9.3	5.7	10.7
16.6	0.08	14.4	0.55	2.1	4.2	1.1	1.5	2.7
	0.30	13.7	0.50	8.2	16.4	8.0	4.0	12.4
55.5	0.08	30.5	0.96	2.5	5.0	1.1	1.8	3.2
	0.30	14.4	0.39	11.1	22.2	6.8	5.2	17.8

Table 3. Effect of D on enzymic activities from cell-free extracts of glycerol-limited steady-state cultures of *C. butyricum* DSM 5431

Data in parentheses are SD values from four determinations. NP, not possible.

Enzyme	Specific activity ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)						
	Glycerol (108.5 mM)					Glucose (5.55 mM)	
	<i>D</i> (h^{-1}):	0.04	0.08	0.15	0.26	0.30	0.08
Glycerol dehydrogenase	1.43 (0.32)	1.61 (0.35)	1.72 (0.34)	1.75 (0.40)	2.41 (0.43)	NP	NP
1,3-PD dehydrogenase	0.58 (0.11)	0.74 (0.18)	0.87 (0.26)	1.07 (0.32)	1.22 (0.19)	NP	NP
Diol dehydratase	0.24 (0.06)	0.27 (0.07)	0.32 (0.08)	0.35 (0.08)	0.39 (0.09)	NP	NP
Thiolase	0.185 (0.027)	0.178 (0.025)	0.177 (0.026)	0.181 (0.032)	0.153 (0.022)	0.169 (0.025)	0.160 (0.022)
Acetate kinase	0.57 (0.17)	0.89 (0.21)	0.72 (0.22)	1.01 (0.17)	9.04 (0.85)	4.78 (0.76)	5.92 (0.83)
Butyrate kinase	0.54 (0.11)	0.79 (0.17)	0.89 (0.21)	1.23 (0.23)	1.20 (0.31)	2.71 (0.41)	3.57 (0.46)
Hydrogenase	0.11 (0.03)	0.03 (0.01)	0.025 (0.012)	0.027 (0.014)	0.037 (0.014)	0.080 (0.023)	0.164 (0.04)
Phosphoroclastic	0.028 (0.011)	0.036 (0.013)	0.04 (0.018)	0.033 (0.011)	0.048 (0.021)	0.091 (0.027)	0.107 (0.034)
fd-NAD reductase	0.0091	0.011	0.0095	0.0128	0.009	0.007	0.007
NADH-fd reductase	0.011	0.0096	0.0083	0.0093	0.008	0.009	0.013

Levels of key metabolic enzyme activities from cell extracts of continuous glycerol-limited steady-state cultures of *C. butyricum*

As seen in Table 3, in the steady-state cultures, *in vitro* glycerol dehydrogenase, 1,3-PD dehydrogenase and diol dehydratase activities were higher under conditions giving higher *in vivo* specific consumption or production rates. This correlation between *in vitro* activities and *in vivo* consumption and formation rates can also be observed in Fig. 4 where these parameters have been plotted against each other for a number of different steady-state conditions. In contrast, whatever the value of D , the specific activities of thiolase were relatively constant. Concomitant with the increase in D , there was an increase of acetate kinase activity which was significantly higher at 0.30 h^{-1} , corresponding to the switch in acetate and

butyrate formation (Fig. 3). From the lowest ($D = 0.04 \text{ h}^{-1}$) to the highest ($D = 0.30 \text{ h}^{-1}$) D values, acetate kinase increased 15.7-fold and butyrate kinase 2.2-fold. All the oxidoreductases exhibited maximum activity with NAD^+ : fd-NAD⁺ reductase, NADH-fd reductase, glycerol-NAD⁺ oxidoreductase and PD-NAD⁺ oxidoreductase activities were 6.1-, 8.8-, 7.6- and 6.2-fold higher, respectively, than with NADP^+ . The values of the phosphoroclastic reaction were fairly constant whereas hydrogenase activity was significantly higher at the low D of 0.04 h^{-1} , and decreased about 3.5-fold at 0.30 h^{-1} . The electrons which are ultimately transferred to NAD^+ by the fd-NAD⁺ reductase are obtained from the oxidation of pyruvate via the endogenous pyruvate dehydrogenase, hence the fd-NAD⁺ reductase activity was dependent on the phosphoroclastic reaction and is in the same range of activity, i.e. in the range of nmol. fd-NAD⁺ and

Fig. 4. *In vivo* specific carbon fluxes as a function of *in vitro* specific enzyme activities in a continuous culture of *C. butyricum*. The glycerol input concentration was 108 mM. (a) *In vivo* specific carbon fluxes through the glyceraldehyde-3-P path versus specific glycerol dehydrogenase activity. (b,c) *In vivo* specific carbon fluxes through the 1,3-PD pathway versus specific diol dehydratase activity (b) and versus 1,3-PD dehydrogenase activity (c).

NADH-fd reductase activities were detected with almost the same ratio at all *D* values.

Levels of key metabolic enzyme activities from cell extracts of continuous glucose-limited steady-state cultures of *C. butyricum*

No activity of glycerol dehydrogenase, 1,3-PD dehydrogenase and diol dehydratase was detected in cell-free extracts of glucose-grown cells. Thiolase activity was similar to that found in extracts of cells grown on glycerol.

The level of this enzyme is low; for comparison, in *C. pasteurianum* ATCC 6013, thiolase activity determined in the same physiological direction was around $1 \mu\text{mol min}^{-1} \text{mg}^{-1}$, i.e. sixfold higher. High levels of acetate kinase were found in cell-free extracts of glucose-grown cells. Butyrate kinase, hydrogenase and phosphoclastic activities were increased in glucose-grown cells compared to glycerol-grown cells. Nevertheless, the hydrogenase activity of *C. butyricum* DSM 5431 is weak, for example we found a specific activity around $7.4 \mu\text{mol min}^{-1} \text{mg}^{-1}$, i.e. 11.5-fold higher, in cell-free extracts of *C. pasteurianum* ATCC 6013. The specific activities of fd-NAD⁺ reductase and NADH-fd reductase were in the same range for cells grown either on glucose or on glycerol.

Intracellular acetyl-CoA, NAD⁺ and NADH pools of continuous steady-state cultures of *C. butyricum*

The levels of NAD⁺, NADH and acetyl-CoA for the steady-states on glycerol and on glucose are shown in Table 4. In glycerol-grown cells the concentrations of acetyl-CoA, NADH and total NAD⁺ + NADH increased sharply with the increase in *D* and a switch in the NAD⁺/NADH ratios was observed above a *D* of 0.15 h^{-1} . In glucose-grown cells, the levels of NADH were always higher than the NAD⁺ levels and the concentrations of the three compounds were at or well above the levels found in glycerol-grown cultures. Fluorometric and colorimetric methods for NAD⁺ and NADH assay gave similar results.

DISCUSSION

Our results show clearly that growing *C. butyricum* in continuous culture at different growth rates and under glycerol limitation did not affect the conversion of glycerol into 1,3-PD. This differs dramatically from *C. acetobutylicum*, another producer of reduced compounds, where low *D* values were associated with a rise in solvent formation and high *D* values with the accumulation of acetic and butyric acids (Bahl & Gottschalk, 1985; Fick *et al.*, 1985). The relatively constant value of the $q_{\text{PD}}/q_{\text{glyceraldehyde}}$ ratio in the fermentation of glycerol suggests the presence of regulatory mechanisms that precisely partition the flow between the competing pathways. The enzymes glycerol dehydrogenase, diol dehydratase and 1,3-PD dehydrogenase constitute a branch point and we found increasing levels of these enzyme activities with increasing *D*, thus contributing to a constant ratio of carbon flux through that branch point (Fig. 2). This allows us to explain the minor changes found for glycerol conversion into 1,3-PD, whatever the value of the *D*. The main observation for high *D* values was the decrease in butyrate production and a concomitant increase in acetate formed (Table 1). These changes in the acetate/butyrate ratio were also observed in *C. butyricum* growing on glycerol (Günzel, 1991) and in *C. tyrobutyricum* (Michel-Savin *et al.*, 1990) and *C. butyricum* growing on glucose (Crabbendam *et al.*, 1985). The production of

Table 4. Nucleotide levels of continuous steady-state *C. butyricum* DSM 5431 with glucose or glycerol as substrate

Data in parentheses are SD values from six determinations from three separated cultures measured either by colorimetric or fluorometric methods.

Substrate	<i>D</i> (h ⁻¹)	Nucleotide concn [$\mu\text{mol (g dry cell wt)}^{-1}$]				
		NAD ⁺	NADH	NAD ⁺ + NADH	NADH/NAD ⁺	Acetyl-CoA
Glycerol (108.5 mM)	0.04	7.5 (0.3)	3.90 (0.2)	11.4	0.52	12.1 (0.8)
	0.08	12.1 (0.7)	7.5 (0.5)	19.6	0.62	11.0 (0.7)
	0.15	14.5 (1.0)	8.2 (0.6)	22.7	0.56	15.3 (0.9)
	0.26	8.6 (0.6)	15.9 (0.9)	24.5	1.84	17.2 (1.2)
	0.30	15.3 (0.9)	24.5 (2.2)	39.8	1.60	24.1 (1.9)
Glucose (5.55 mM)	0.08	12.2 (0.8)	20.8 (1.2)	33.0	1.70	24.1 (1.2)
	0.30	12.2 (0.7)	25.3 (1.3)	37.5	2.06	38.8 (2.3)
Glucose (16.5 mM)	0.08	12.4 (0.8)	25.2 (1.2)	37.6	2.00	15.2 (0.9)
	0.30	12.8 (0.7)	28.4 (1.4)	41.2	2.20	33.4 (2.0)
Glucose (55.5 mM)	0.08	17.1 (1.0)	20.3 (1.2)	37.4	1.18	23.1 (1.1)
	0.30	11.1 (0.6)	27.4 (1.6)	38.5	2.45	32.3 (1.9)

acetate or butyrate constitutes another important branch point and when the carbon flux switches from butyric acid to acetic acid formation we observed a corresponding shift in the relative activities of acetate and butyrate kinase, whereas the specific activities of thiolase were not affected by changes in *D*. Glucose- or glycerol-grown cells have in common the pathway from glyceraldehyde-3-P to pyruvate and whatever the carbon source, q_{pyruvate} increased as *D* increased producing large amounts of acetyl-CoA. The intracellular concentration of acetyl-CoA increased from 12.1 $\mu\text{mol g}^{-1}$ at *D* = 0.04 h⁻¹ to 24.1 $\mu\text{mol g}^{-1}$ at *D* = 0.30 h⁻¹ when glycerol was the carbon source. The shift in carbon flux took place at a *D* of roughly 0.26 h⁻¹, when 17.2 $\mu\text{mol g}^{-1}$ of acetyl-CoA was produced.

The thiolase that converts acetyl-CoA to acetoacetyl-CoA may be the bottleneck for carbon flux to butyrate biosynthesis since our results show that its level did not change significantly with *D*. This suggests that the acetate pathway is an important pathway of overflow metabolism when the specific rate of acetyl-CoA formation increases above a critical threshold. For a q_{pyruvate} which increased 6.6-fold from *D* = 0.04 h⁻¹ to *D* = 0.26 h⁻¹, the intracellular NADH increased fourfold, whereas between *D* = 0.26 and 0.30 h⁻¹ the intracellular concentration increased less than twofold. This can be explained by the fact that between *D* = 0.04 and 0.26 h⁻¹, the formation of both PD and butyrate regenerates NAD⁺, whereas above *D* = 0.26 h⁻¹, NADH was used principally (high level of intracellular NADH) for PD formation. Considering that oxidation of NADH by 1,3-PD dehydrogenase was linked to the production of 3-HPA by diol dehydratase, the fact that the intracellular concentrations of NADH were extremely high demonstrates that diol dehydratase activity was the rate-limiting step in 1,3-PD formation; in that

way 3-HPA does not accumulate. This limitation was maintained whatever the rate of carbon flux since the activities of all three enzymes (glycerol dehydrogenase, 1,3-PD dehydrogenase and glycerol dehydratase) were regulated in similar ways (Fig. 4). We suggest that the high concentration of NADH in the cell prevents the accumulation of 3-HPA which is a very reactive and toxic compound that has been shown to be excreted by *Lactobacillus reuteri* and termed reuterin (Axelsson *et al.*, 1989; Chung *et al.*, 1989). It is a bioactive substance which at low concentrations inhibits the growth of Gram-positive and -negative bacteria and lower eukaryotic organisms.

A number of investigators have noted the more reduced state of glycerol in comparison to glucose and suggested that glycerol could stimulate hydrogen production (Heyndrickx *et al.*, 1991a, Dabrock *et al.*, 1992). In fact, growth of *C. butyricum* on glycerol induced the four enzymes of the *dba* regulon (Heyndrickx *et al.*, 1991b, Tong *et al.*, 1991) and regenerated the excess reducing equivalents via the butyrate pathway and principally via the highly active 1,3-PD dehydrogenase. In glycerol catabolism, our data indicate that the NADH produced by glycerol dehydrogenase and glyceraldehyde-3-P dehydrogenase is not sufficient for 1,3-PD formation and that part of the reduced fd produced by the pyruvate-fd oxidoreductase is reoxidized by fd-NAD⁺ reductase activity to produce NADH.

The reduction of NAD⁺ by reduced fd and the reductase occurred via the endogenous pyruvate dehydrogenase and hence the fd-NAD⁺ reductase activities were dependent on the phosphoroclastic reaction. In cell-free extracts of glycerol-limited cultures, these two reactions showed similar specific activities; between 0.04 and

0.26 h⁻¹ the phosphoroclastic activity was 2.6- to 4.2-fold higher than the fd-NAD⁺ reductase activity. When cells were grown on glycerol, an 80 % decrease of hydrogenase activity was noted compared to growth on glucose at all *D* values. The low level of hydrogenase associated with the fd-NAD⁺ reductase activity contributed to production of NADH. This kind of regulation, a high level of intracellular NADH and a decrease of hydrogenase activity, has been reported elsewhere (Girbal *et al.*, 1995).

In contrast, growth on glucose generates reducing equivalents which are released in excess in the glycolytic pathway and are reoxidized by NADH-fd reductase and higher levels of hydrogenase (Table 3). In glucose-grown cells, the low level of thiolase (Table 3) and the high intracellular concentration of acetyl-CoA and of NADH (Table 4) suggest that the butyric pathway is a bottleneck for the carbon flux and hence is insufficient to regenerate the NADH produced by glycolysis or the acetyl-CoA produced by the phosphoroclastic reaction, and in turn a high level of NADH and acetyl-CoA explain the low amount of glucose used. On the basis of the results presented it is apparent that *C. butyricum* DSM 5431 is not adapted to grow at high glucose concentrations since the low specific formation rate of butyric acid leads to increased levels of acetyl-CoA and NADH. This high level of NADH was reported to inhibit glyceraldehyde-3-P dehydrogenase in *C. acetobutylicum* (Girbal & Soucaille, 1994) but surprisingly, the specific rates of pyruvate formation were almost the same either on glucose or glycerol whatever the NADH/NAD⁺ ratio. We can explain a higher carbon flow on glycerol by the fact that NADH is reoxidized by both butyric and PD pathways. Nevertheless, a high flux of glycerol also led to a high level of NADH due to the limited production of 3-HPA by diol dehydratase. The variation of NAD⁺ and NADH content with *D* values has already been reported in a *Pseudomonas* sp. (Matin & Gottschalk, 1976). Because the reoxidation of NADH by 1,3-PD and butyrate biosynthesis are limiting steps, catabolism of the carbon substrate may be facilitated by a concomitant increase in NAD⁺ biosynthesis which would tend to maintain the NADH/NAD⁺ ratios. Usually intracellular NAD⁺ plus NADH concentrations in clostridia are around 7–14 µmol (g dry cell wt)⁻¹ (Meyer & Papoutsakis, 1989; Girbal & Soucaille, 1994); nevertheless values as high as 39.8 µmol (g dry cell wt)⁻¹ were reported in *C. kluyveri* (Decker & Pfitzer, 1972). Glycerol dehydratase is rather difficult to measure and no detailed investigations of this enzyme have been reported in clostridia. From our results it is apparent that the glycerol dehydratase plays a key function in glycerol catabolism and remains to be more fully studied.

ACKNOWLEDGEMENTS

The authors wish to thank Dr H. Biebl and Ms A. Reimann for helpful discussions and are grateful to E. Guedon and E. Spiesser for skilful co-operation in part of the experiments. This work was supported by the commission of the European communities AAIR programme: Contract no. AIR 2-CT93-0825 (DG 12 SSMA).

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Received 28 July 1995; revised 20 December 1995; accepted 10 January 1996.