Anaerobic pathways of glycerol dissimilation by *Enterobacter agglomerans* CNCM 1210: limitations and regulations

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Continuous cultures of *Enterobacter agglomerans* CNCM 1210 were performed under regulated pH conditions (pH 7.0) with glycerol or glucose (20 g l⁻¹) as carbon source. Cultures grown on glucose produced mainly acetate, ethanol and formate. In contrast, 1,3-propanediol (PPD) was the main product with glycerol. The carbon flow distribution at branching metabolic points was investigated. Higher PPD yields with increased dilution rate were correlated with an important increase in the relative ratio of glycerol dehydratase to glycerol dehydrogenase. Determination of intracellular triose-phosphate and fructose 1,6-biphosphate concentrations demonstrated that glyceraldehyde-3-phosphate dehydrogenase is the limiting step in glycerol dissimilation. At the pyruvate branching point, pyruvate dehydrogenase (PDH) activity was systematically detected. The pyruvate flow shifted to PDH is suspected to represent up to 22% of the acetyl-CoA formed. In addition, this enzyme pattern combined with the enhanced *in vivo* lactate dehydrogenase activity at high growth rates, was correlated with a decrease in the pyruvate formate-lyase activity. A regulation of this latter enzyme by the accumulation of triose-phosphate is suspected.

Keywords: *Enterobacter agglomerans*, glycerol, carbon flow distribution and regulation, reducing equivalent flow, 1,3-propanediol

INTRODUCTION

Due to the scheduled expansion of vegetable oil processing activities (biodiesel), abundant glycerol sources are expected to be generated (Eggersdorfer *et al.*, 1992; Chowdhury & Fouhy, 1993). For this reason, microbial conversion of glycerol to various compounds has been investigated recently and particularly focused upon 1,3-propanediol (PPD) production (Günzel *et al.*, 1991; Biebl *et al.*, 1992; Petitdemange *et al.*, 1995). This hallmark product is of interest in the synthesis of polyesters because of the outstanding properties it confers to the material in terms of mechanical resistance (Elm *et al.*, 1980) and biodegradability (Witt *et al.*, 1994).

Abbreviations: ADH, alcohol dehydrogenase; DA, dehydratase; DH, dehydrogenase; DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; FBP, fructose 1,6-biphosphate; G3P, glycerol 3-phosphate; GAP, glyceraldehyde 3-phosphate; 3-HPA, 3-hydroxypropionaldehyde; KPB, potassium phosphate buffer; LDH, lactate dehydrogenase; PDH, pyruvate dehydrogenase; PFL, pyruvate formate-lyase; PPD, 1,3-propanediol.

The fermentation of glycerol to PPD has been mainly studied with micro-organisms such as *Klebsiella pneumoniae* (Ogbe Solomon *et al.*, 1994; Zeng *et al.*, 1993; Streekstra *et al.*, 1987), *Citrobacter freundii* (Homann *et al.*, 1990; Boenigk *et al.*, 1993) and *Clostridium butyricum* (Abbad-Andaloussi *et al.*, 1996; Saint Amans, 1994; Forsberg, 1987). *Enterobacter agglomerans* is able to convert glycerol to PPD as the major product and to acetic, formic, lactic and succinic acids and ethanol as by-products (Barbirato *et al.*, 1995). The metabolic flexibility of this strain was demonstrated by varying the initial glycerol content during batch fermentation regulated at pH 7: the higher the initial substrate concentration, the higher the PPD conversion yield. However, increasing glycerol content, and thus glycerol uptake rate, resulted in the accumulation of an intermediate metabolite. Identified as 3-hydroxypropionaldehyde (3-HPA), it caused cessation of both growth and product formation (Barbirato *et al.*, 1996a, b).

In the present study, we investigated the metabolic

behaviour of *E. agglomerans* growing anaerobically in a continuous culture with glycerol or glucose as sole carbon source. On the one hand, this was accomplished by following the carbon flow distribution at several important branching points, as well as the regeneration of reducing equivalents. On the other hand, the activities of key enzymes involved in the dissimilation of glycerol and nucleotide pools were measured. Effects of a high specific rate of glycerol dissimilation on the metabolic pathway used are discussed.

METHODS

Bacterial strain and growth conditions. The bacterial strain *Enterobacter agglomerans* CNCM 1210 (Collection Nationale de Cultures de Microorganismmes, Institut Pasteur, Paris, France) was grown in carbon-source-limited, anaerobic continuous cultures. Feed medium contained (per litre deionized water): 20 g glycerol or glucose as carbon source, 1 g K_2HPO_4 , 0.5 g KH_2PO_4 , 2 g $(NH_4)_2SO_4$, 0.4 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 4 mg $CoCl_2$, 2 g yeast extract (Biokar diagnostic), 0.5 g bactopectone (Difco), 0.3 g bacteriological meat extract (Biokar) and 1 ml of a mineral solution containing (per litre deionized water): 5 g EDTA, 2 g $FeSO_4 \cdot 7H_2O$, 1 g $MnCl_2 \cdot 4H_2O$, 0.2 g $CuCl_2 \cdot 2H_2O$, 0.2 g H_3BO_3 , 30 mg $Na_2MoO_4 \cdot 2H_2O$, 30 mg $NiCl_2 \cdot 2H_2O$ and 0.1 g $ZnSO_4 \cdot 7H_2O$. Bottles (10 or 20 litres) containing the feed medium were degassed for 20–24 h under a sterile nitrogen flux after being autoclaved (30 min at 120 °C). Experiments were carried out in 1 litre glass reactors with a constant liquid volume of 0.8 l, that were sparged daily with sterile nitrogen and constantly maintained under a low pressure. The reactors were equipped with pH regulation (2300 Ingold transmitter controlling 5 M NaOH addition) and magnetic stirring. Temperature was maintained at 30 °C by a thermostatted water circulation system.

Analysis. Biomass concentration was determined by measurements of cell dry weight using a predetermined correlation between OD_{620} (Uvikon 930 spectrophotometer) and cell dry weight. Fermentation products were quantified by HPLC. Fermenter gas effluents were analysed by gas chromatography. All the operating conditions have been described previously (Barbirato *et al.*, 1995).

Preparation of cells and cell-free extracts. Cells (25–30 mg dry wt) were harvested from steady-state chemostat cultures, transferred to a nitrogen-purged tube and centrifuged immediately for 8 min at 8000 g and 4 °C (J2-MC, Beckmann). The supernatant was discarded, and the cell pellet was stored under a nitrogen atmosphere at –80 °C until the preparation of cell-free extracts.

Cell-free extract preparation conditions depended on the enzyme activities. For alcohol dehydrogenase, aldehyde dehydrogenase, phosphotransacetylase, formate dehydrogenase and pyruvate formate-lyase, all manipulations were performed in an anaerobic workstation (Model bubble system, La Cahlène) maintained under a gaseous mixture (80% N_2 /10% CO_2 /10% H_2) and with oxygen-free solutions. Activities of these enzymes were greatly enhanced under these conditions in comparison to those measured under aerobic conditions. After slow thawing, the harvested cells were washed with 5 ml 100 mM oxygen-free potassium phosphate buffer (KPB) at pH 7.4 and centrifuged for 5 min at 4000 g. The cell pellet was then resuspended in 3 ml 100 mM KPB (pH 7.4) con-

taining 2 mM dithiothreitol (DTT). Cell suspensions were sonicated in conical tubes at 4 °C with an ultrasonic disintegrator (Vibracell 72434, Bioblock) for 3 cycles of 30 s with 4 min cooling intervals. Cell debris was removed by centrifugation at 13000 g for 4 min (Hettich Microliter Centrifugal, Bioblock).

For glycerol dehydrogenase, glycerol-3-phosphate dehydrogenase, dihydroxyacetone kinase, glycerol kinase, lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and acetate kinase, cell-free extracts were prepared under aerobic conditions. The harvested cells were washed with 5 ml 100 mM KPB (pH 7.5). After centrifugation for 12 min at 12000 g and 4 °C, the washed pellet was resuspended in 3 ml of the same buffer. Cells were then disrupted at 4 °C with a mechanical cell breaker with 3 g glass beads (0.1 µm) for 8 cycles of 1 min with 4 min cooling intervals. Using these conditions, protein extraction was as efficient as using the ultrasonic disintegrator without affecting enzyme activities. Cell debris and glass beads were removed by centrifugation at 15000 g for 15 min.

For the activities of 1,3-propanediol dehydrogenase, pyruvate dehydrogenase, ferricyanide-NADH oxidoreductase and pyruvate kinase, the same operating conditions were used with 100 mM MOPS (pH 7.5) instead of KPB buffer.

Finally, for glycerol dehydratase, an oxygen-free 100 mM glycine/KOH buffer (pH 8.1) containing 250 mM KCl and 1% (w/v) glycerol was used for washing and disrupting steps. Cells were disrupted with the mechanical cell breaker because this method allowed a higher recovery of this enzyme activity in comparison with the ultrasonic disintegrator treatment. Anaerobic conditions were maintained using a nitrogen flux at each step of the cell-free extract preparation.

Cell-free extracts were stored at 4 °C until the assay procedure.

Enzyme assays. Enzyme activities were systematically determined in their physiological direction at 30 °C. All reagent solutions were prepared in deionized water and degassed if necessary. A Uvikon 930 spectrophotometer (Kontron) was used for the enzyme assays performed under aerobic conditions. A Lambda 3 spectrophotometer (Perkin Elmer) was employed for the assays carried out in the anaerobic workstation. One unit of enzyme activity is defined as the amount of enzyme that catalyses the conversion of 1 µmol substrate min^{-1} .

Glycerol dehydrogenase (EC 1.1.1.6) activity (Glyc DH) was measured by following the glycerol-dependent formation of FADH at 340 nm (Ruch *et al.*, 1974). The assay mixture contained 100 mM potassium bicarbonate (pH 9.0), 2 mM FAD, 30 mM $(NH_4)_2SO_4$ and 200 mM glycerol ($\epsilon_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$).

Glycerol-3-phosphate dehydrogenase (EC 1.1.99.5) (G3P DH) was measured by following the G3P-dependent formation of NADH. The assay system contained 50 mM potassium bicarbonate (pH 9.0), 0.5 mM NAD and 5 mM G3P.

Dihydroxyacetone (DHA) kinase (EC 2.7.1.29) activity was followed in a coupled system in which NADH-dependent reduction of the reaction product, dihydroxyacetone phosphate (DHAP), to G3P was measured in a modified assay based on that described by Johnson *et al.* (1984). The assay mixture contained 50 mM potassium bicarbonate (pH 9.0), 2.5 mM ATP, 0.4 mM NADH, 15 mM $MgCl_2$, 2 mM DTT, 18 U glycerophosphate dehydrogenase from rabbit muscle

and 10 mM DHA. To prevent any secondary reaction (with Glyc DH), 15 mM α,α' -dipyridyl was systematically added to the assay mixture (Lin & Magasanik, 1960).

Glycerol kinase (EC 2.7.1.30) activity was measured by the procedure adopted from Kremer & Hansen (1987). The assay mixture contained 100 mM potassium bicarbonate (pH 9.0), 2.5 mM ATP, 0.4 mM NADH, 15 mM MgCl_2 , 10 U G3P DH from rabbit muscle and 20 mM glycerol.

1,3-Propanediol dehydrogenase (EC 1.1.1.202) activity (PPD DH) was determined at 340 nm by measuring the initial rate of 3-hydroxypropionaldehyde (3-HPA) -dependent NADH decrease. The reaction mixture contained 200 mM KPB (pH 7.8), 0.4 mM NADH, 0.4 mM MnCl_2 and 3 mM 3-HPA.

Coenzyme B_{12} -dependent glycerol dehydratase (EC 4.2.1.30) activity (Glyc DA) was determined by the 3-methyl-2-benzothiazolinone hydrazone (MBTH) method described by Toraya *et al.* (1977). It is based on the ability of the reaction product (3-HPA) to react with MBTH generating hydrazone derivatives which are spectrophotometrically quantifiable. The assay system, containing oxygen-free solutions, consisted of 5 mM KPB (pH 9.0), 50 mM KCl, 25 mM MnCl_2 , 15 mM $(\text{NH}_4)_2\text{SO}_4$, 25 mM glycerol and 0.5 mM ATP as enzyme activator (Honda *et al.*, 1980). After 10 min incubation at 37 °C, 12 μM coenzyme B_{12} was added to the assay mixture and the enzyme reaction was stopped at 0, 1 and 2 min respectively by transferring 200 μl of the assay mixture into a mixture containing 0.5 ml 0.1% (w/v) MBTH and 1 ml 100 mM potassium citrate (pH 3.6). After 15 min incubation at 37 °C, the 3-HPA formed was calculated from the absorbance at 305 nm relative to a standard curve.

Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) activity (GAP DH) was estimated by a procedure adopted from Lovitt *et al.* (1988). The assay system contained 100 mM Tricine/NaOH buffer (pH 8.1), 10 mM KH_2AsO_4 , 1 mM NAD, 7.5 mM K_2HPO_4 , 2 mM DTT, 4 U aldolase from baker's yeast and 4 mM fructose-1,6-bisphosphate (FBP).

Pyruvate kinase (EC 2.7.1.40) activity was determined by following the NADH-dependent reduction of the reaction product (pyruvate) to lactate at 340 nm (Hess & Wieker, 1974). The reaction mixture consisted of 100 mM triethanolamine (pH 7.6), 0.2 mM NADH, 5 mM ADP, 10 mM KCl, 2 mM CoCl_2 , 2 mM MnCl_2 , 25 U lactate dehydrogenase (LDH) from rabbit muscle and 10 mM phosphoenolpyruvate (PEP).

Pyruvate dehydrogenase complex (EC 1.2.2.2) activity (PDH) was measured by following, at 430 nm, the NADH-dependent reduction of ferricyanide to ferrocyanide through the NADH-ferricyanide oxidoreductase naturally present in cell-free extracts (Carlsson *et al.*, 1985). The assay mixture contained 50 mM Tricine/KOH (pH 7.0), 0.3 mM thiamine pyrophosphate (TPP), 0.1 mM CoASH, 1 mM NAD, 1 mM potassium ferricyanide, 25 mM Na_2HPO_4 (pH 7.0), 30 mM MgCl_2 and 20 mM pyruvate. Activity of NADH-ferricyanide oxidoreductase was measured by following the reduction of ferricyanide in a reaction mixture containing 100 mM Tricine/KOH (pH 7.0), 50 mM Na_2HPO_4 (pH 7.0), 1 mM potassium ferricyanide and 0.4 mM NADH. This latter activity was systematically at least 200-fold higher than PDH activity ($\epsilon_{430} = 1.03 \text{ mM}^{-1} \text{ cm}^{-1}$).

Pyruvate formate-lyase (EC 2.3.1.54) activity (PFL) was determined with a procedure adopted from Abbe *et al.* (1982). The assay mixture contained 100 mM KPB (pH 7.4), 1 mM

NAD, 2 mM DTT, 0.1 mM CoASH, 14 U malic dehydrogenase from bovine heart, 10 U citrate synthase from porcine heart, 20 mM pyruvate and 6 mM malate.

Formate dehydrogenase activity (EC 1.2.1.2) was assayed in a reaction mixture, adopted from Snoep *et al.* (1990), containing 100 mM KPB, 1 mM NAD and 100 mM formate.

Lactate dehydrogenase (EC 1.1.2.3) activity (LDH) was measured by following the NADH dependent reduction of pyruvate into lactate at 340 nm (Abbe *et al.*, 1982). The assay mixture contained 20 mM KPB (pH 7.4), 0.4 mM NADH, 1 mM FBP and 20 mM pyruvate.

Phosphotransacetylase (EC 2.3.1.8) activity was determined by following, at 405 nm, the reaction between the coenzyme A liberated and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (Andersch *et al.*, 1983). The reaction system contained 100 mM KPB (pH 7.4), 0.2 mM DTNB and 0.3 mM acetyl-CoA ($\epsilon_{405} = 13.6 \text{ mM}^{-1} \text{ cm}^{-1}$).

Acetate kinase (EC 2.7.2.1) activity was measured according to the assay adopted from Lamed & Zeikus (1980). The reaction mixture contained 80 mM Tris/HCl buffer (pH 7.2), 2 mM ADP, 1 mM NADP, 1 mM DTT, 15 mM MgCl_2 , 20 mM glucose, 2 U hexokinase from baker's yeast, 2 U glucose-6-phosphate dehydrogenase from baker's yeast and 10 mM acetylphosphate.

Aldehyde dehydrogenase (EC 1.2.1.10) was assayed by following the NADH-dependent reduction of acetyl-CoA (Dürre *et al.*, 1987). The assay system consisted of 100 mM potassium bicarbonate (pH 9.0), 0.2 mM NADH, 2 mM DTT, 40 mM semicarbazide and 0.6 mM acetyl-CoA.

Alcohol dehydrogenase (EC 1.1.1.1) activity (ADH) was determined by following the NADH-dependent reduction of acetaldehyde at 340 nm. The assay mixture contained 100 mM KPB (pH 7.4), 0.2 mM NADH, 2 mM DTT and 40 mM acetaldehyde (Lamed & Zeikus, 1980).

Protein concentration was determined by use of the Coomassie Brilliant Blue G250 protein assay reagent supplied commercially by Bio-Rad with bovine serum albumin as standard.

Nucleotide extraction. NADH, NAD, ATP and ADP levels were measured after extraction of a culture broth sample as described by Vasconcelos *et al.* (1994). Samples collected were immediately transferred (in less than 5 s) into the extraction solution (NaOH or HCl). NADH was extracted at pH 12.3 with 10 M KOH. After a 10 min incubation at 30 °C, the alkaline solution was centrifuged for 8 min at 5000 *g* and 4 °C and the supernatant was collected. NAD, ATP and ADP were extracted at pH 1.3 with 6 M HCl by incubation for 10 min at 50 °C. After centrifugation for 8 min at 5000 *g* and 4 °C, a third of the supernatant was collected and stored for ATP determination. The second third was progressively neutralized with 2 M NaOH for NAD assay. For the ADP determination, ADP was converted to ATP by creatine phosphokinase in a reaction mixture containing the last third of the supernatant, 50 mM glycine buffer (pH 9.0), 0.4 mM MgSO_4 and 7 U creatine phosphokinase from rabbit muscle maintained for 20 min at 30 °C. The reaction was stopped by addition of HCl (pH 2.0) and the mixture centrifuged. Samples were stored at -80 °C until the assay.

Nucleotide pool assays. Nucleotide pools were measured by use of a Hitachi fluorimeter (model F-2000) according to procedures adopted from Le Bloas (1992). The wavelength of the fluorescence excitation and emission were, respectively,

340 and 460 nm. Nucleotide concentrations were deduced from a calibration curve of pure commercial NADH in a concentration range 0.1–7 μM . Dilutions were performed in 80 mM triethanolamine buffer (pH 7.6), 0.8 mM EDTA and 3 mM MgSO_4 .

NADH concentration was determined by measuring the fluorescence decrease linked to the NADH-dependent conversion of pyruvate into lactate. The reaction mixture contained 100 mM triethanolamine (pH 7.6), 3 mM MgSO_4 , 0.8 mM EDTA, 4 mM pyruvate and 25 U LDH from rabbit muscle.

NAD pools were estimated by quantifying the increase in fluorescence following the oxidation of ethanol. The assay system contained 150 mM pyrophosphate buffer (pH 8.8), 0.8 g semicarbazide l^{-1} , 10 mM ethanol and 27 U ADH from baker's yeast.

ATP concentration was determined by a coupled system in which decrease in NADH was followed. The assay mixture contained 100 mM triethanolamine (pH 7.6), 5 μM NADH, 3 mM MgSO_4 , 0.8 mM EDTA, 2 mM 3-phosphoglycerate, 9 U G3P DH from rabbit muscle and 25 U 3-phosphoglyceric phosphokinase from baker's yeast.

Intermediate metabolite assays. Triose phosphates, GAP and dihydroxyacetone phosphate (DHAP), and FBP were extracted at acid pH as described for NAD and stored at -80°C until assay. Assays were performed with an SLM-Aminco photon counting spectrofluorimeter (model 8000 C). Excitation and emission wavelengths were as described for the nucleotide pools. A calibration curve was created with pure commercial NADH. The reaction mixture contained 50 mM MES (pH 6.5), 50 mM KCl, 5 mM MgCl_2 , 0.05 mM Na_2CO_3 and 12.5 μM NADH and extract. DHAP concentration was deduced from the variation of NADH emission after addition of 4.2 U G3P DH to the assay mixture; the concentration of GAP was determined after addition of 100 U triose phosphate isomerase and FBP content was deduced from the variation of the NADH emission after addition of 2.5 U aldolase.

Transmembrane pH determination. The ΔpH was assayed by measuring the accumulation of [^{14}C]benzoate by the oil centrifugation technique (Loubière *et al.*, 1992). After concentration of the fermentation sample by centrifugation (8000 g for 8 min) and suspension of the pellet in 0.5 ml supernatant, the cell suspension was incubated for 10 min at 30°C with 3.3 μM [^{14}C]benzoate (14.1×10^8 Bq mmol^{-1}) and centrifuged (14000 g for 15 min at room temperature) in the presence of 0.6 ml of a mixture of miscible immersion oils containing 7% (w/w) from Merck and 93% (w/w) from Carlo Erba. Supernatant fractions were collected and transferred into vials containing 10 ml of Ready Protein⁺ scintillation cocktail (Beckman). Radioactivity content was measured using a Packard liquid scintillation analyser. The remaining supernatant and the oil fraction were removed, and the wall of the centrifuge tube was carefully wiped clean. Five-hundred microlitres of 0.5 M NaOH was added to the cell pellet. After homogenization, the suspension was incubated for lysis at room temperature for 30 min and transferred into counting vials containing 10 ml scintillation cocktail. The intracellular benzoate concentration was calculated together with the internal volume.

For the measurement of intracellular volume, the same amount of cell suspension was used. Incubation of the cell suspension was performed with 0.74×10^6 Bq $^3\text{H}_2\text{O}$ (0.37×10^8 Bq ml^{-1}) and 13.7×10^4 Bq ^{14}C -PEG 4000 (5.92×10^8 Bq g^{-1}).

Conditions were the same as described above. Intracellular volume, deduced from the differential partitioning of $^3\text{H}_2\text{O}$ and ^{14}C -PEG, allowed the calculation of the intracellular benzoate concentration. Intracellular pH was deduced from the internal and external benzoate concentrations as described by Padan *et al.* (1981).

Calculations. For the glucose and glycerol-fed cultures, the ATP yield is defined as $Y_{\text{ATP}} = D \times 1000 / [2 \times (q_{\text{Acet}} + q_{\text{Lact}} + q_{\text{Succ}} + q_{\text{Etol}})]$ where q_{Acet} , q_{Etol} , q_{Lact} , and q_{Succ} are the specific rates (expressed in $\text{mmol g}^{-1} \text{h}^{-1}$) of production of acetate, ethanol, lactate and succinate, respectively. D is the dilution rate (h^{-1}). Y_{ATP} is expressed as g cell per mol ATP produced.

The energetic efficiency (Eff) corresponds to $\text{Eff} = D \times 1000 / (q_{\text{Glyc}} \times Y_{\text{ATP}})$ where q_{Glyc} is the specific rate of glycerol consumed. Eff is expressed as mol ATP formed per mol glycerol consumed.

For the glycerol-fed culture, the specific rate of formation of NAD is $q_{\text{NAD}} = q_{\text{PPD}} + q_{\text{Lact}} + 2q_{\text{Etol}} + 2q_{\text{Succ}}$ where q_{PPD} is the specific rate of production of PPD.

For PDH activity, the specific rate of formation of NADH is $q_{\text{NADH}} = 3q_{\text{Acet}} + 3q_{\text{Etol}} - q_{\text{Form}} + 2q_{\text{Lact}} + 2q_{\text{Succ}} + 8.035\mu$. q_{Form} is the specific rate of formation of formate, 8.035 is the amount, expressed in $\text{mmol (g cells)}^{-1}$, of the NADH requirement for the biomass formation with glycerol as substrate (Ingraham *et al.*, 1983).

For the glucose-fed culture, the specific rate of formation of NAD is the same as described above. The specific rate of formation of NADH is $q_{\text{NADH}} = 2q_{\text{Acet}} + 2q_{\text{Etol}} - q_{\text{Form}} + q_{\text{Lact}} + q_{\text{Succ}} + 14.678\mu$ where 14.678 is the amount, expressed in $\text{mmol (g cells)}^{-1}$, of the NADH requirement for the biomass formation with glucose as substrate (Ingraham *et al.*, 1983).

The redox recovery is defined as $100(q_{\text{NAD}}/q_{\text{NADH}})$.

Chemicals. All chemicals, enzymes and co-enzymes were purchased from Sigma. The radiochemical compounds, $^3\text{H}_2\text{O}$ and ^{14}C -PEG were purchased from Amersham, and ^{14}C -benzoate from ICN Pharmaceuticals. 3-HPA was synthesized from acrolein by the method of Hall & Stern (1950).

RESULTS

Comparison of the glycerol- and glucose-fed continuous cultures

(i) Fermentation profile. *E. agglomerans* was grown anaerobically at pH 7 in chemostat culture at a dilution rate of 0.05 h^{-1} under glycerol or glucose limitation. Fermentation profiles, shown in Table 1, showed that acetic acid, ethanol and formic acid were the main products of the glucose fermentation. Lactic and succinic acids were only produced as by-products of the fermentation. Very low amounts of PPD and 2,3-butanediol were measured. In contrast, with glycerol as carbon source, PPD was the major fermentation product. Acetic acid, ethanol and formic acid were the main by-products, while lactic and succinic acids were produced in small quantities. No significant microbial energetic changes were observed when using these two different substrates, the ATP yields being almost similar. Biomass concentration was higher with glucose than

Table 1. Fermentation product profiles of *E. agglomerans* grown anaerobically under glucose- and glycerol-limited continuous cultures

Data are given with a 3% precision. Carbon recovery is defined as the amount of carbon that is retrieved in biomass and analysed products. Redox recovery is defined in Methods.

	<i>D</i> (h ⁻¹):		0.05	0.15	0.25	0.31
	Substrate:	Glucose	Glycerol	Glycerol		
Residual conc (g l ⁻¹)		< 0.1	< 0.1	0.2	1.0	1.2
Biomass (g l ⁻¹)		1.6	1.05	1.06	0.97	0.82
<i>Y</i> _{ATP} (g mol ⁻¹)		6.44	6.89	7.83	7.92	7.02
<i>Eff</i> (mol mol ⁻¹)		2.3	0.71	0.63	0.59	0.57
Product yields (mol mol ⁻¹)						
1,3-Propanediol		< 0.013	0.44	0.52	0.55	0.57
Acetate		0.70	0.21	0.20	0.20	0.20
Ethanol		0.73	0.23	0.16	0.10	0.07
Formate		1.365	0.40	0.31	0.24	0.21
Lactate		0.066	0.020	0.055	0.080	0.090
Succinate		0.050	0.035	0.014	0.013	0.012
2,3-Butanediol		< 0.013	0	0	0	0
Carbon recovery (%)		89	100	100	99	98
Redox recovery (%)		95	94	98	97	98

with glycerol because of a greater efficiency of ATP generation per mol substrate. A good redox balance was obtained, with a reducing equivalent recovery of 94% on glycerol and 95% on glucose.

(ii) Enzymic study. Table 2 shows the enzyme profile of *E. agglomerans* grown in chemostat cultures. In contrast to the glucose-limited culture, glycerol leads to a 30–120-fold increase in the levels of Glyc DH, DHA kinase, Glyc DA and PPD DH. The expression of the structural genes of these enzymes, encoded by the *dha* regulon, is subject to catabolite repression or is induced by glycerol in *Klebsiella pneumoniae* (Lin, 1976). A similar regulation seemingly occurs in *E. agglomerans*. Moreover, activities of glycerol kinase and G3P DH were not detected indicating that this metabolic route is not present under anaerobic conditions. Glycolytic enzymes exhibited higher levels of activity on glucose in comparison with the glycerol-limited culture. This is particularly pronounced for GAP DH. PFL activity was lower in the glycerol culture while PDH activity was detected in both cultures, which suggested that PFL is not the only enzyme generating acetyl-CoA from pyruvate. Formate dehydrogenase activity, belonging to the formate hydrogen-lyase complex, was not detected, which is in accordance with the findings of De Vos *et al.* (1983) who reported the inability of *E. agglomerans* to decompose formate into CO₂ and H₂, and those of Ewing & Fife (1972) who described an extremely low gas production by this strain. Acetic-acid-producing enzymes were threefold higher on glucose than on glycerol. The ethanol production pathway exhibited a high level of aldehyde dehydrogenase activity on glucose while a

lower ADH activity was observed on glucose, which shows that the most reduced substrate gave highest ADH activity as described by McPhedran *et al.* (1961).

(iii) Intracellular nucleotide and metabolite concentrations and pH. The NAD:NADH ratio was slightly lower on glycerol (2.8) than on glucose (3.1) (Table 2). The ATP level was similar for the two cultures, while the ADP level was higher on glucose. DHAP and FBP concentrations were higher on the glycerol- than on the glucose-fed culture although the carbon flow at that level was lower. GAP concentration, in equilibrium with DHAP, remained low in both cultures. Transmembrane pH of *Clostridium acetobutylicum* was shown to be inverted when glycerol was co-added to a glucose-limited chemostat culture (Girbal *et al.*, 1994). To investigate whether the same occurred in *E. agglomerans*, the ΔpH was measured on glucose- and glycerol-fed cultures. An almost similar positive transmembrane pH was found in both conditions despite the different redox states of the substrates.

Effect of the dilution rate on the glycerol-fed culture

(i) Carbon flow distribution. As depicted in Table 1, increasing dilution rate resulted first in an incomplete substrate exhaustion particularly at *D* = 0.25 h⁻¹ and 0.31 h⁻¹. For such growth rates, biomass concentration decreased concomitantly with the ATP yield and the energetic efficiency. In terms of carbon flow distribution, we paid attention to three branching points. When growth rate was increased, the glycerol entering the cell was preferentially directed towards the PPD formation

Table 2. *In vitro* enzyme levels of *E. agglomerans* grown in continuous cultures fed with 20 g l⁻¹ glucose or glycerol

Activities are expressed in $\mu\text{mol mg}^{-1} \text{min}^{-1}$. Nucleotide and intermediate metabolite concentrations are expressed in $\mu\text{mol (g dry cell wt)}^{-1}$. Data in parentheses are SD deduced from eight determinations. ND, Not determined.

Substrate:	<i>D</i> (h ⁻¹):		0.31
	0.05		
	Glucose	Glycerol	Glycerol
Glycerol catabolism enzymes			
Glycerol dehydrogenase	0.16 (0.028)	13.03 (2.88)	5.36 (0.23)
Dihydroxyacetone kinase	0.305 (0.021)	9.34 (1.23)	3.80 (0.40)
Glycerol dehydratase	0.0045 (0.0018)	0.18 (0.04)	6.15 (0.99)
1,3-Propanediol dehydrogenase	0.078 (0.021)	9.16 (2.2)	8.33 (1.36)
Central axis enzymes			
Glyceraldehyde-3-phosphate dehydrogenase	5.51 (0.37)	2.31 (0.1)	3.37 (0.29)
Pyruvate kinase	1.35 (0.09)	0.80 (0.08)	1.04 (0.15)
Pyruvate formate lyase	9.35 (1.50)	3.42 (0.31)	4.95 (0.86)
Pyruvate dehydrogenase	0.074 (0.005)	0.16 (0.03)	0.13 (0.027)
Acid producing enzymes			
Phosphotransacetylase	19.11 (2.67)	5.31 (0.8)	11.57 (3.03)
Acetate kinase	51.3 (4.7)	17.06 (3.01)	30.29 (1.54)
Lactate dehydrogenase	1.26 (0.09)	1.33 (0.20)	0.68 (0.04)
Ethanol producing enzymes			
Aldehyde dehydrogenase	0.030 (0.008)	0.0035 (0.0012)	0.0022 (0.0006)
Alcohol dehydrogenase	0.205 (0.014)	0.94 (0.17)	0.52 (0.08)
Nucleotide levels			
NADH	1.39 (0.28)	2.38 (0.40)	2.26 (0.39)
NAD ⁺	4.28 (0.39)	6.78 (0.53)	7.79 (0.83)
ATP	4.43 (0.59)	4.49 (0.39)	6.33 (0.57)
ADP	2.03 (0.42)	0.70 (0.28)	2.19 (0.41)
Intermediate metabolites			
Dihydroxyacetone phosphate	6.93 (0.83)	9.64 (0.81)	17.35 (2.13)
Glyceraldehyde 3-phosphate	0.55 (0.34)	0.95 (0.39)	1.70 (0.50)
Fructose 1,6-bisphosphate	1.13 (0.49)	6.91 (0.62)	16.57 (1.92)
Transmembrane pH			
ΔpH	0.46 (0.08)	0.37 (0.07)	ND

route (Fig. 1): 44% at $D = 0.05 \text{ h}^{-1}$ and 57% at $D = 0.31 \text{ h}^{-1}$. At the pyruvate level, lactate synthesis was stimulated by increasing D despite the shift of the carbon flow to acetyl-CoA. Finally, acetyl-CoA distribution towards acetate formation was favoured to the detriment of ethanol production, which resulted in a drop in the ethanol molar conversion yield from 0.23 to 0.07 when D was raised from 0.05 h^{-1} to 0.31 h^{-1} (Table 1). As a consequence, the relative ratio between the specific rates of production of PPD and ethanol was enhanced from 1.9 at $D = 0.05 \text{ h}^{-1}$ to 8.1 at $D = 0.31 \text{ h}^{-1}$. Similar results were reported for *K. pneumoniae* (Streekstra *et al.*, 1987; Ogbe Solomon *et al.*, 1994) and *Citrobacter freundii* (Boenigk *et al.*, 1993). The contribution of the two parallel enzymic complexes, PFL and PDH, in the global carbon flow generating acetyl-CoA is illustrated in Fig. 1. It shows that *in vivo* PDH activity catalysed 9% and 22% of the pyruvate flow towards acetyl-CoA at $D = 0.05 \text{ h}^{-1}$ and 0.31 h^{-1} , respectively.

(ii) **Enzyme activities.** Evolution of glycerol catabolizing enzyme activities were different in the reductive or the oxidative branch (Table 2). Activities of Glyc DH and DHA kinase decreased 2.5-fold when D increased from 0.05 h^{-1} to 0.31 h^{-1} . By contrast, activity of Glyc DA showed a 34-fold increase while the level of PPD DH remained stable. Such an increase of Glyc DA activity as a result of increasing the growth rate has been reported previously in *C. freundii* grown in glycerol-limited continuous culture: it increased from 0.05 U mg^{-1} at $D = 0.08 \text{ h}^{-1}$ to 1.3 U mg^{-1} at $D = 0.36 \text{ h}^{-1}$ (Boenigk *et al.*, 1993). The preferential distribution of glycerol towards the PPD pathway at high dilution rates, reported above, was thus correlated with the increase of the relative ratio of Glyc DA to Glyc DH. Central axis enzymes presented a uniform 1.4-fold increase except for PDH activity which decreased slightly. Enzymes associated with acetic acid formation showed a two-fold increase at high dilution rate. In contrast, LDH presented a twofold decrease of activity between

Fig. 1. Quantitative flow schemes at $D = 0.05 \text{ h}^{-1}$ (a), 0.15 h^{-1} (b) and 0.31 h^{-1} (c). Values are expressed in $\text{mmol g}^{-1} \text{h}^{-1}$. Abbreviation: PEP, phosphoenolpyruvate.

$D = 0.05 \text{ h}^{-1}$ and 0.31 h^{-1} , although *in vivo* activity was significantly enhanced. Levels of aldehyde dehydrogenase and ADH showed a 1.6- and 1.8-fold drop in activity, respectively, at high growth rate.

(iii) Intracellular nucleotide and metabolite concentrations. A slight increase in the NAD pool was observed, while the NADH pool was not affected (Table 2). Consequently, the relative ratio NAD:NADH increased from 2.85 at $D = 0.05 \text{ h}^{-1}$ to 3.45 at $D = 0.31 \text{ h}^{-1}$. ATP and ADP pools presented respectively a 1.4- and 3.1-fold increase resulting in a 2.2-fold decrease of the ATP:ADP ratio. Triose-phosphates and FBP concentrations increased significantly at the higher dilution rate, while the GAP concentration remained low.

DISCUSSION

In *K. pneumoniae* anaerobic pathways of glycerol dissimilation involve the utilization of PFL to catalyse the oxidation of pyruvate to acetyl-CoA (Streekstra *et al.*, 1987). When grown in batch culture under low initial glycerol content, and hence low glycerol uptake rate, *E. agglomerans* was thought to exclusively use PFL to funnel pyruvate to acetyl-CoA: a perfect relationship between the molar amount of formate and acetate plus ethanol was established (Barbirato *et al.*, 1995). Nevertheless, this correlation was not verified when batch cultures were performed with higher glycerol concentrations. In the chemostat culture, an unbalanced relationship was noticed between the molar amount of formate and acetate plus ethanol produced in addition

to the lack of formate dehydrogenase activity, suggesting the utilization of a parallel pathway for the conversion of pyruvate to acetyl-CoA. PDH activity is commonly observed under aerobic conditions whereas PFL, which shows extremely high sensitivity to molecular oxygen (Abbe *et al.*, 1982), is expressed and is only active under strict anaerobiosis (Sawers & Böck, 1988). However, PDH was shown to be active under anaerobiosis in *Streptococcus mutants* (Carlsson *et al.*, 1985) and it was revealed that an important part of the pyruvate flow was shifted to PDH at high NAD:NADH ratios in *Enterococcus faecalis* (Snoep *et al.*, 1990). *In vitro* PDH activity was detected in *E. agglomerans* in both glucose- and glycerol-fed cultures. Moreover, the redox balance was better equilibrated by supposing PDH activity, and the intracellular NAD:NADH ratio was also favourable to the *in vivo* activity of the enzyme. These results argue for the presence of PDH activity in *E. agglomerans*: between 9% and 22% of the pyruvate flow to acetyl-CoA is shifted to PDH. CO_2 production from the decarboxylation of pyruvate by PDH was less than expected and its utilization by carboxylating steps in the metabolism is suspected, as well as its absorption by the feed medium with the formation of carbonate (Zeng, 1995). PDH activity thus occurred in *E. agglomerans*, as depicted in Fig. 2.

At the pyruvate branching point, distribution of the carbon flow through LDH, PFL and PDH activities occurred. The proportion of the pyruvate flow shifted to PFL represented 87% at $D = 0.05 \text{ h}^{-1}$ and only 58% at $D = 0.31 \text{ h}^{-1}$. Such behaviour probably results from a

Fig. 2. Metabolic pathways involved in glycerol assimilation by *E. agglomerans*. Abbreviations: G1,3P, glyceraldehyde-1,3-biphosphate; PEP, phosphoenolpyruvate; acetyl-P, acetyl phosphate. Enzymes: 1, Glyc DA; 2, PPD DH; 3, Glyc DH; 4, DHA DH; 5, GAP DH; 6, pyruvate kinase; 7, LDH; 8, PFL; 9, PDH; 10, phosphotransacetylase; 11, acetate kinase; 12, aldehyde DH; 13, ADH.

carbon flow conversion limitation at the level of PFL. This phenomenon is particularly pronounced at high dilution rate when glycerol is provided in excess. PFL activity is known to be activated by pyruvate (Knappe & Sawers, 1990) and inhibited by triose-phosphates, GAP and DHAP (Takahashi *et al.*, 1982). In comparison to levels reached in the glucose-fed culture, intracellular concentrations of triose-phosphates and FBP were higher in the culture supplied with glycerol. This is surely linked to the lower GAP DH activity measured in the latter culture, suggesting that this metabolic step is limiting. One can suspect that the relative drop in *in vivo* PFL activity, when dilution rate increases, results from a partial inhibition of PFL by intracellular triose phosphates, particularly DHAP. The enhanced *in vivo* PDH activity thus may allow the decarboxylation of the excess pyruvate (Hansen & Henning, 1966) and hence the prevention of any deleterious effect by that compound (Collins, 1972), and also the increase in the global rate of NADH generation, limited at the level of the GAP DH, thus contributing to the redox balance. The increase of *in vivo* LDH activity may result from an intracellular accumulation of pyruvate (Tarmy & Kaplan, 1968).

High rates of glycerol dissimilation thus involve both a preferential distribution towards the PPD metabolic pathway and a limitation by GAP DH. As glycerol is the branching point of the reductive and oxidative pathways, the carbon flux through the reductive branch is not exclusively controlled by the glycolytic flux. The PPD pathway acts as a pacemaker of the oxidative metabolism by increasing the regeneration, and hence the availability, of the NAD necessary at the glycolytic level to minimize the limiting step. This explains why a high NAD:NADH ratio is observed despite the fact that the organism is grown on a reduced substrate. Despite this 'overflow metabolism' (Streekstra *et al.*, 1987), an accumulation of triose phosphates occurred, indirectly causing an enhanced pyruvate flow distribution toward PDH and LDH. Parameters such as pyruvate and triose phosphate intracellular concentrations and the NAD:NADH ratio could play a key role in this proposed regulation. Obviously, this hypothetical regulation scheme at the level of the pyruvate branching point needs to be confirmed.

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