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Regulation of metabolic shifts in *Clostridium acetobutylicum* ATCC 824

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Abstract

Alcohol formation was initiated in continuous cultures of *Clostridium acetobutylicum* under distinct steady-state conditions: (i) in glucose-limited cultures established at low operating pH with formation of butanol, ethanol and acetone (induction of the solventogenesis) in which cells contained normal levels of NADH and a high level of ATP and butyric acid; and (ii) by increasing the NADH pressure at neutral pH in glucose-limited cultures after addition of Neutral red, or in glucose–glycerol or glucose–glycerol–pyruvate grown cultures, with a strictly alcohologenic metabolism (no acetone produced) associated with high levels of intracellular NADH and various levels of ATP. These two different metabolic shift systems are correlated with the expression of different genes involved in the solvent-forming pathways and the electron flow distribution. A high NADH level leading to butanol and ethanol formation was accompanied by increased activities of the NADH-dependent alcohol and butyraldehyde dehydrogenases, and ferredoxin:NAD(P)$^+$ reductases, and by decreased activities of the NADH:ferredoxin reductase. This last group of enzymes constitutes the key enzymes regulating electron flow, since no change in hydrogenase activity was observed. On the other hand, classical solventogenesis appears to be characterized by high levels of expression of the NADPH-dependent alcohol and butyraldehyde dehydrogenases, and of the two enzymes involved in the acetone-forming pathway, while the ferredoxin:NAD(P)$^+$ reductases were not synthesized. A decrease of the in vitro hydrogenase activity explains the lower hydrogen generation. In addition, the regulation of the intracellular pH was different between the alcohologenic culture grown at neutral pH and the solventogenic cultures grown at low pH. An inversion of the transmembrane pH gradient was observed during the production of alcohol at neutral pH and was related to a lower in vivo specific rate of hydrogen production while in the cultures grown at low pH the transmembrane pH generation was not linked to the F_1F_0 ATPase activity.

Keywords: Clostridium acetobutylicum; Metabolic shift; Alcohol formation; Hydrogenase; NADH

1. Introduction

The complex anaerobic metabolism of *Clostridium acetobutylicum* has been studied in considerable detail in recent years, though the physiological state associated with the transition from the acidogenic to solventogenic phase and the factors triggering this

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metabolic shift are still not totally understood. In batch cultures, the initiation and sustained production of solvents are associated with a low extracellular and intracellular pH and a high undissociated butyric acid concentration [1–3]. In continuous cultures, ATP and NAD(P)H availabilities appear to play a key role in product selectivity. High ATP concentrations related to low ATP demand or high efficiency of ATP generation leads to enhanced solvent production (i) for glucose-sufficient cultures at a low pH with biomass recycling [4,5]; (ii) for iron-, nitrogen-, or phosphate-limited cultures [6–8]; and (iii) during shifts induced on phosphate-limited cultures by lowering the pH or adding organic acids [9]. Production of both ethanol and butanol was associated with increased availability of reducing power (i) when the in vivo activity of the hydrogenase was decreased by CO gassing [4,10–12], or by adding Methyl viologen [9]; (ii) during a shift in solvent production induced by lowering the pH when acetyl-Coenzyme A (CoA) was first converted to acetone (a pathway consuming no reducing energy), creating a redox imbalance [9].

Table 1
Fermentation parameters for continuous phosphate-limited steady-state cultures of *C. acetobutylicum* [a]

Parameters	Value for following data set [b]					
	Reference [c]	Ia [d]	Ib [d]	Ic [d]	II	III
Biomass concentration ($g\ l^{-1}$)	0.93	1.06	1.06	1.07	1.00	1.18
pH	6.5	6.5	6.5	6.5	6.5	4.4
Dilution rate (h^{-1})	0.049	0.050	0.052	0.051	0.048	0.049
Neutral red (mM)	–	–	–	–	1	–
Fed substrate concentration (mM)						
Glucose	167	83	56	28	161	168
Glycerol	–	163	163	163	–	–
Pyruvate	–	–	57	114	–	–
Residual concentration (mM)						
Glucose	0	0	0	0	0	0.23
Glycerol	–	65	33.5	0	–	–
Pyruvate	–	0	0	0	–	–
Specific consumption rate (q) (mmol h^{-1} g^{-1}) [e]						
Glucose	8.72	3.94	2.71	1.33	7.69	6.97
Glycerol	–	4.65	6.43	7.92	–	–
Pyruvate	–	0	2.78	5.42	–	–
In vivo flux through the GAPDH (mmol h^{-1} g^{-1})	16.2	10.9	10.5	8.2	13.5	13.6
Speficic production rate (q) (mmol h^{-1} g^{-1}) [e]						
Ethanol	0.26	1.30	1.66	0.71	0.76	0.3
Butanol	0.01	3.86	4.59	3.92	2.51	3.35
Acetone	0.02	0	0	0	0	2
Acetate	3.67	0.42	0.44	0.51	0.33	0.45
Butyrate	6.09	0.66	0.91	2.04	3.67	0.9
CO_2	16.6	11	13.14	13.5	14.74	15.6
H_2	19.1	7.24	6.29	7.92	6.38	11.4
NAD(P)H production via Fd oxidoreductase	−3.41	3.72	6.66	5.25	5.3	2.16

[a] Cultures were with a feed concentration of carbon of 995 mM.
[b] Values in columns 'Reference' and 'Ia' are taken from Vasconcelos et al. [13]; values in 'Ib' and 'Ic' from Girbal and Soucaille [14]; values in column 'II' from Girbal et al. [15]; values in 'III' from Girbal et al. [16].
[c] Acidogenic culture on glucose alone at pH 6.5 taken as a reference.
[d] Data sets correspond to different P/(P + G) values: a, 0 g/g; b, 0.33 g/g; c, 0.67 g/g.
[e] Determined with an average accuracy of ±5%.

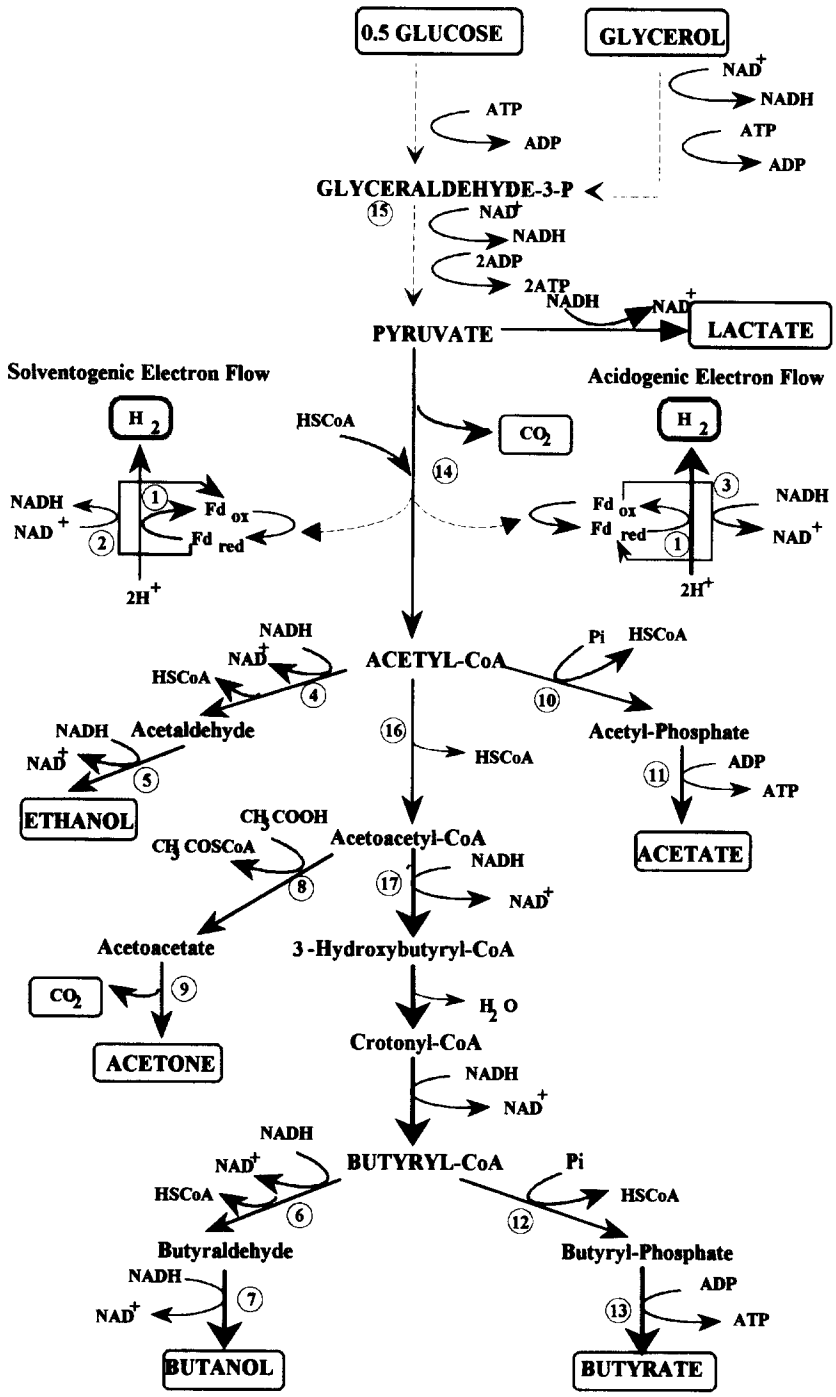

Fig. 1. Metabolic pathways of *Clostridium acetobutylicum* (from [13]) 1, hydrogenase; 2, ferredoxin–NAD reductase; 3, NADH–ferredoxin reductase; 4, acetaldehyde dehydrogenase; 5, ethanol dehydrogenase; 6, butyraldehyde dehydrogenase; 7, butanol dehydrogenase; 8, CoA-transferase; 9, acetoacetate decarboxylase; 10, phosphotransacetylase; 11, acetate kinase; 12, phosphotransbutyrylase; 13, butyrate kinase; 14, pyruvate ferredoxin oxidoreductase; 15, glyceraldehyde-3-phosphate dehydrogenase; 16, thiolase; 17, 3-hydroxybutyrylCoA dehydrogenase.

Since a chemostat culture of *C. acetobutylicum* on glucose alone at neutral pH produced only acids, the metabolic flexibility was studied by changing the overall degree of reduction of the substrate (using mixtures of glucose, glycerol and pyruvate), or by adding Neutral red, an artificial electron carrier, or by lowering the operating pH from 6.5 to 4.4. The existence of two different mechanisms for the shift toward butanol production has thus been demonstrated with two distinct patterns of expression of the enzymes involved in the distribution of the electron flow and in the solvent-forming pathways.

2. Flexibility of carbon and energy fluxes

2.1. Growth of C. acetobutylicum at neutral pH on substrate mixtures

The metabolism of *C. acetobutylicum* was first manipulated by using a mixture of glucose and glycerol (Table 1: experiment Ia). Cultures grown on glucose alone produce acids (acetic and butyric) and molecular hydrogen. In contrast, cultures grown on glucose and glycerol (with a glycerol/glucose molar ratio of 1.96) produce mainly alcohols with only low production of acids and hydrogen, and no acetone. Glycerol is a more reduced substrate than glucose: for the same amount of carbon, glycerol metabolism liberates twice as much NADH than glucose (Fig. 1). The reducing equivalent excess provided by the conversion of glycerol to pyruvate must be oxidized through the NADH-consuming pathways. Surprisingly, this reducing power was not used to form molecular hydrogen, a compound normally generated to eliminate the excess of reducing energy provided by glycolysis. On the contrary, part of the reduced ferredoxin produced via the pyruvate:ferredoxin oxidoreductase was used to generate NADH, leading to a low hydrogen production. A seven-fold increase of the intracellular NADH concentration was observed for the mixed substrate culture and was associated with a three-fold decrease of the in vivo specific hydrogen production rate. In parallel, at the phosphorylation level, although the ATP + ADP pool remained constant (about 3.2 μmol per g dry weight of cells), a 2.5-fold increase in ATP concentration occurred when butanol production was induced. The metabolic shift, when glycerol was metabolized, was characterized by high intracellular content of both NADH and ATP.

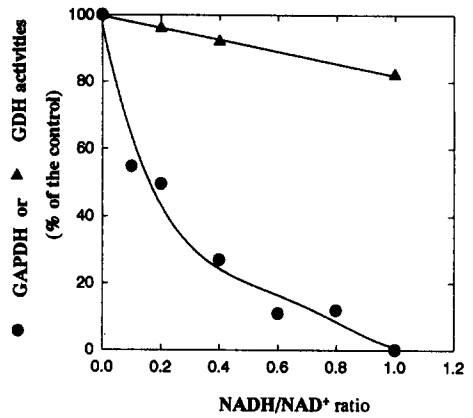

Fig. 2. Influence of the NADH/NAD$^+$ ratio on the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and glycerol dehydrogenase (GDH) activities in *C. acetobutylicum* strains. The symbols indicate the amount of GAPDH and GDH remaining in the presence of a given NADH/NAD$^+$ ratio (from [14]). The concentration of NAD$^+$ was 0.5 mM.

Although the steady-state continuous culture on glucose alone was carbon-limited, it was carbon-sufficient on a glucose–glycerol mixture (residual glycerol). A probable influence of the high NADH/NAD$^+$ ratio on the glycerol metabolism was investigated. The effect of the NADH/NAD$^+$ ratio on the in vitro activities of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an enzyme known to be affected by the NADH/NAD$^+$ ratio in other clostridia [17], and of the glycerol dehydrogenase (GDH), the first enzyme of glycerol metabolism that uses NAD$^+$ as cofactor, is shown on Fig. 2. Significant inhibition of GAPDH by a high NADH/NAD$^+$ ratio was observed with no measurable activity at a NADH/NAD$^+$ ratio greater than 1. During glucose–glycerol cultures, the in vivo NADH/NAD$^+$ ratio has been shown to be as high as 0.75, a level at which 90% inhibition of activity would be expected. On the other hand, GDH was not significantly affected by the NADH/NAD$^+$ ratio.

In order to confirm in vivo this regulation, continuous cultures were established under the same conditions as for glucose–glycerol cultures, except that part of the glucose was replaced by pyruvate (while maintaining constant glycerol concentration and total

amount of carbon in the feed medium). Pyruvate is a more oxidized substrate than glucose and enters the central metabolism downstream of GAPDH. Two different pyruvate/(glucose + pyruvate) ratios (this ratio will be called P/(G + P) and is expressed in grams per grams) of 0.33 (experiment Ib) and 0.67 (experiment Ic) were used. Carbon distribution to the end-products was only slightly changed for the P/(G + P) value of 0.33. However, the highest P/(G + P) value resulted in a decrease of carbon flow towards the alcohols, but the yield (ethanol + butanol) remained higher than 35%. The specific glycerol consumption rate increased with glucose substitution by pyruvate: for a P/(G + P) value of 0.67, the enhanced $q_{glycerol}$ led to glycerol limitation. The in vivo flux through the GAPDH (see Table 1) was constant for the two non-limited cultures (P/(G + P) values of 0 and 0.33) and was related to the same $NADH/NAD^+$ ratio (Table 2). So, with a P/(G + P) value of 0.33, the microorganism consumed more glycerol to finally reach the same flux through the GAPDH and the same $NADH/NAD^+$ ratio. A P/(G + P) of 0.67 resulted in a lower flux through the GAPDH and a lower $NADH/NAD^+$ ratio. This suggests that for this degree of pyruvate substitution the microorganism would be able to consume more glycerol if provided in the feed before reaching the maximum flux capacity of GAPDH fixed by the $NADH/NAD^+$ ratio. It is noteworthy that the $NADH/NAD^+$ ratio obtained with a P/(G + P) value of 0.67 still remained 3.6 times higher than the ratio observed in the acidogenic culture on glucose alone. On the other hand, pyruvate addition and the related increase in glycerol consumption were associated to a lower ATP generation. Furthermore, when the substitution of glucose by pyruvate was increased, the intracellular concentration of ATP diminished while ADP increased (Table 2). These results obtained at neutral pH with continuous cultures on glucose–glycerol–pyruvate mixtures show that alcohol production can occur under conditions in which a high $NADH/NAD^+$ ratio but a low ATP pool are observed.

2.2. Growth of C. acetobutylicum at neutral pH on glucose in presence of Neutral red

The metabolic flexibility of *C. acetobutylicum* was studied in continuous culture at neutral pH in presence of Neutral red (1 mM) (Table 1: experiment II). Neutral red is an artificial electron carrier which can replace ferredoxin in the oxidoreduction reactions catalysed by the enzymes involved in the distribution of the electron flow. The carbon distribution into the products and the associated electron flow were modified in the steady-state continuous culture performed in the presence of dye. Butanol production was induced and the ethanol yield was increased 3-fold but no acetone was produced. Concomitantly, the total acid yield was 2-fold lower, but the effect on acetic acid yield was 7-fold more pronounced than was seen for the butyric acid yield. The presence of the artificial electron carrier favored the NADH-dependent pathways (alcohols and butyric

Table 2
Nucleotide levels of continuous phosphate-limited steady-state cultures of *C. acetobutylicum*

Nucleotide	Concentration [a] (μmol (g dry cell mass)$^{-1}$) (S.D.) for following data set ($n = 4$)				
	Reference [b]	Ia	Ib	Ic	III
ATP	0.62 (0.1)	1.6 (0.1)	0.9 (0.1)	0.3 (0.1)	1.30 (0.1)
ADP	2.50 (0.3)	1.8 (0.2)	2.3 (0.3)	2.7 (0.4)	2.05 (0.2)
NAD^+	6.8 (2.0)	6.7 (1.9)	8.0 (2.2)	6.4 (2.0)	13.0 (3.6)
$NADP^+$	0.41 (0.18)	0.63 (0.20)	0.24 (0.12)	0.21 (0.10)	0.44 (0.20)
NADH	0.97 (0.15)	5.05 (1.30)	6.3 (1.8)	3.2 (0.6)	1.30 (0.24)
NADPH	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2
$NADH/NAD^+$	0.14	0.75	0.79	0.5	0.1

[a] Values in columns 'Reference' and 'Ia' are from Vasconcelos et al. [13]; values in 'Ib' and 'Ic' from Girbal and Soucaille [14]; values in column 'III' from Girbal et al. [16].
[b] Acidogenic culture on glucose alone at pH 6.5 taken as a reference.

acid). The specific rate of NAD(P)H production from ferredoxin ($q_{NAD(P)H\ from\ ferredoxin}$) was positive in the culture with Neutral red. This positive value indicates a deficit in reduced cofactor, and quantifies the part of reducing energy that the ferredoxin $NAD(P)^+$ reductases catalysed to form NAD(P)H at the expense of molecular hydrogen generation (decrease of the specific hydrogen production rate by a factor of 3 in experiment II). Just after the addition of Neutral red to an acidogenic culture, analysis of the instantaneous in vivo activity changes for the hydrogenase and ferredoxin oxidoreductases shows that the simultaneous utilization of ferredoxin and dye alters the electron flow from hydrogen to NAD(P)H production [15]. Thus, in this case, a higher rate of NAD(P)H production should be generated within the cell.

2.3. Growth of *C. acetobutylicum* at low pH on glucose

The third manipulation of *C. acetobutylicum* metabolism was performed by changing the operating pH from 6.5 to 4.4 (Table 1: experiment III). At pH 6.5, the culture was acidogenic with 60% of the carbon from substrate recovered as organic acids. At pH 4.4, this percentage dropped to 10.8%, while 47.7% of the carbon from glucose consumption were recovered as solvents. This solventogenic metabolism can be characterized by a molar distribution of 6:3.5:0.5 for butanol, acetone and ethanol respectively. The two experiments exhibited a small variation of the $NADH/NAD^+$ ratio (Table 2). The total adenylated nucleotide concentrations were similar at

Table 3
Enzymatic activities from cell extracts of continuous phosphate-limited steady-state cultures of *C. acetobutylicum*

Enzyme	Specific activity [a] (μmol min^{-1} mg^{-1}) for following data set				
	Reference [b]	Ia	Ic	II	III
Solventogenic pathways					
Acetaldehyde dehydrogenase					
NADH	0.061	0.103	0.107	0.176	0.077
NADPH	0.028	0.044	0.013	0.021	0.029
Ethanol dehydrogenase					
NADH	0.0078	0.039	0.032	0.052	0.002
NADPH	0.0013	0.010	0.012	0.025	0.023
Butyraldehyde dehydrogenase					
NADH	< 0.0004	0.016	0.038	0.0867	0.003
NADPH	< 0.0004	< 0.0004	0.0125	< 0.0004	0.016
Butanol dehydrogenase					
NADH	0.0041	0.031	0.022	0.059	0.014
NADPH	0.0015	0.0099	0.017	0.039	0.046
Coenzyme A transferase					
Acetate	0.0019	0.015	0.008	0.0030	0.983
Butyrate	< 0.0003	< 0.0003	< 0.0003	0.0008	0.034
Acetoacetate decarboxylase	0.21	0.28	0.029	0.426	2.31
Hydrogenase and coupling enzymes					
Hydrogenase					
Hydrogen uptake	5.95	8.16	6.87	2.58	2.91
Hydrogen evolution	0.44	0.68	0.43	0.34	0.066
Ferredoxin NAD^+ reductase	0.0326	0.293	0.208	0.094	< 0.0004
Ferredoxin $NADP^+$ reductase	0.0512	0.199	0.203	0.091	< 0.0004
NADH ferredoxin reductase	0.168	0.024	0.0335	0.0252	0.0178
NADPH ferredoxin reductase	< 0.0003	< 0.0003	0.0006	< 0.0003	< 0.0003

[a] Values in columns 'Reference' and 'Ia' are from Vasconcelos et al. [13]; values in 'Ic' from Girbal and Soucaille [14]; values in 'II' from Girbal et al. [15]; values in 'III' from Girbal et al. [16].
[b] Acidogenic culture on glucose alone at pH 6.5 taken as a reference.

both pH values although the ATP/ADP ratio was 2.6-fold higher at pH 4.4 than at pH 6.5 (corresponding to a 2-fold increase of the intracellular ATP concentration).

These experiments showed two distinct types of solventogenic metabolism. The metabolism induced by changing the overall degree of reduction of the substrate (experiments Ia, Ib and Ic) and after the addition of Neutral red (experiment II) could be termed 'alcohologenic' (production of butanol and ethanol but no production of acetone) in regard to the classical termed solventogenic metabolism obtained at low operating pH (experiment III), producing alcohols and acetone. In addition, the onset of the alcohologenic metabolism can be related to the high NAD(P)H-variable ATP concentrations, while the classical solventogenic metabolism is associated to low NAD(P)H-high ATP and butyric acid concentrations.

3. Regulation of the expression of enzymes involved in the solventogenic pathways and orientation of the electron flow

The in vitro enzymatic activity values (reflecting the in vivo level of an enzyme) measured for alcohologenic and solventogenic cultures of *C. acetobutylicum* are reported in Table 3. The in vitro activities detected during an acidogenic culture performed at neutral pH on glucose alone are presented for comparison.

3.1. Regulations in alcohologenic continuous cultures

For the different steady-state continuous cultures producing alcohols (but not acetone) obtained during growth on substrate mixtures (experiments Ia and Ic) and in the presence of Neutral red (experiment II), quite similar in vitro activities were detected for the enzymes responsible for the orientation of both carbon and electron flows towards alcohol formation. In cultures grown at neutral pH on mixtures of glucose, glycerol (with or without pyruvate), the ethanol production appears to be controlled at the genetic level, via higher activities of the NADH-dependent ethanol dehydrogenase, since the in vitro acetaldehyde dehydrogenase activities measured in both physiological and non-physiological directions were comparable to those obtained during the acidogenesis. After addition of Neutral red to an acidogenic culture, ethanol formation was correlated to higher activity levels of the NADH-specific ethanol and acetaldehyde dehydrogenases. Whatever the system chosen to induce alcohologenic metabolism, butanol production was related to the induction of the NADH-dependent butyraldehyde dehydrogenase and to higher activities of the NADH-specific butanol dehydrogenase.

Coenzyme A transferase and acetoacetate decarboxylase, two enzymes involved in the acetone-producing pathway, were detected at very low levels in alcohologenic cultures (in comparison with the in vitro activities measured for these two enzymes in the acetone-producing culture obtained at low pH). These low values explained the absence of acetone production in these cultures.

The deviation of the electron flow towards the production of reduced coenzymes (cofactors of the alcohol forming pathways) at the expense of hydrogen synthesis was not related to a change at the hydrogenase level, but was apparently due to high activities of the ferredoxin NAD^+ reductase which are associated with low in vitro activities of the NADH ferredoxin reductase. To understand the mechanism of transition from an acidogenic to an alcohologenic metabolism induced by the addition of Neutral red, the apparent kinetic parameters of the enzymes involved in the distribution of electron flow were established and used to simulate the in vivo enzymatic activity changes just before the addition of the dye (when ferredoxin is the only substrate) and immediately after the addition of Neutral red (when both ferredoxin and Neutral red are potential substrates) [15]. Neutral red addition had no effect on the pyruvate:ferredoxin oxidoreductase, neither on the NADH ferredoxin reductase nor on the hydrogen uptake activity. On the contrary, the simultaneous use of ferredoxin and dye led to a 36% increase of the simulated in vivo activity of the ferredoxin NAD reductase and a 83% decrease of the simulated in vivo hydrogen evolution activity. Since the regeneration of oxidized Neutral red and ferredoxin via hydrogen production was diminished, the microorganism must have directed the electron flow towards the

second regenerating system, the 'ferredoxin' NAD^+ reductase. The higher $NADH/NAD^+$ ratio which must be generated might, thereafter, be the signal for the increased expression of the ferredoxin NAD^+ reductase and NADH-dependent butyraldehyde and alcohol dehydrogenase genes, and the decreased expression of the NADH ferredoxin reductase gene.

3.2. Regulations in a solventogenic culture

A different enzymatic pattern was obtained in the continuous solventogenic culture grown at pH 4.4 (Table 3: experiment III).

The increase of the ethanol yield at pH 4.4 compared to pH 6.5 was associated with a higher expression of the NADPH-dependent ethanol dehydrogenase. The acetaldehyde dehydrogenase was still more active with NADH as a cofactor than with NADPH and the level of expression remained unchanged between acidogenesis and solventogenesis. Initiation of butanol production was related to the induction of the NADPH-dependent butyraldehyde dehydrogenase and to a higher expression of the NADPH-dependent butanol dehydrogenase. The induction of acetone production was accompanied by a high expression of the Coenzyme A transferase, mainly active with acetate as co-substrate. The acetoacetate decarboxylase, already present at pH 6.5, exhibits a 10-fold increase of in vitro activity at pH 4.4.

Under solventogenic conditions, lower in vitro hydrogenase activities were detected in both directions but the hydrogen evolution activity was decreased more than the hydrogen uptake activity. This could partially explain the lower hydrogen production detected at pH 4.4. The results obtained for the ferredoxin nicotinamide oxidoreductase activities at pH 4.4 are quite surprising: no ferredoxin NAD(P) reductase activities were detected, although the positive value of the in vivo specific NAD(P)H production rate indicates that reduced coenzymes are produced from reduced ferredoxin. A hypothetical mechanism of NAD(P)H synthesis from reduced ferredoxin, via a membrane-bound electron and proton transport system has been proposed [16]. This mechanism takes also into account the observation that at pH 4.4 the F_1F_0 ATPase was not involved in the transmembrane pH generation (see below).

4. Relation between the type of metabolism and the transmembrane pH gradient value

The values of the transmembrane pH gradient (ΔpH), transmembrane electrical gradient ($\Delta\psi$), and proton motive force (PMF) during the different types

Table 4
Proton motive force and its components in continuous phosphate-limited steady-state cultures of *C. acetobutylicum*

	Value for following data set [a]				
	Reference [b]	Ia	Ic	II	III
ΔpH [c]	0.097 ± 0.029	−0.344 ± 0.052	−0.325 ± 0.064	−0.259 ± 0.180	1.103 ± 0.110
Chemical component $-61.\Delta$pH	−6.0 ± 1.8	+21.0 ± 3.2	+19.8 ± 3.9	+15.8 ± 11.0	−67.3 ± 6.7
Electrical component $\Delta\Psi$ (mV)	−140.5 ± 11.3	−176.8 ± 1.9	ND	ND	−90 ± 35
Proton motive force [d] PMF (mV)	−146.5 ± 13.1	−155.8 ± 5.1	ND	ND	−157.3 ± 41.7

[a] Values in columns 'Reference', 'Ia', 'Ic' and 'II' are from Girbal et al. [18]; values in 'III' from Girbal et al. [16].
[b] Acidogenic culture on glucose alone at pH 6.5 taken as a reference.
[c] The transmembrane ΔpH represents the difference between intracellular pH and extracellular pH. The positive values were obtained from the [^{14}C]benzoate distribution and the negative values from the [^{14}C]benzylamine distribution.
[d] The proton motive force was the sum of the chemical and electrical components.
ND, not determined.

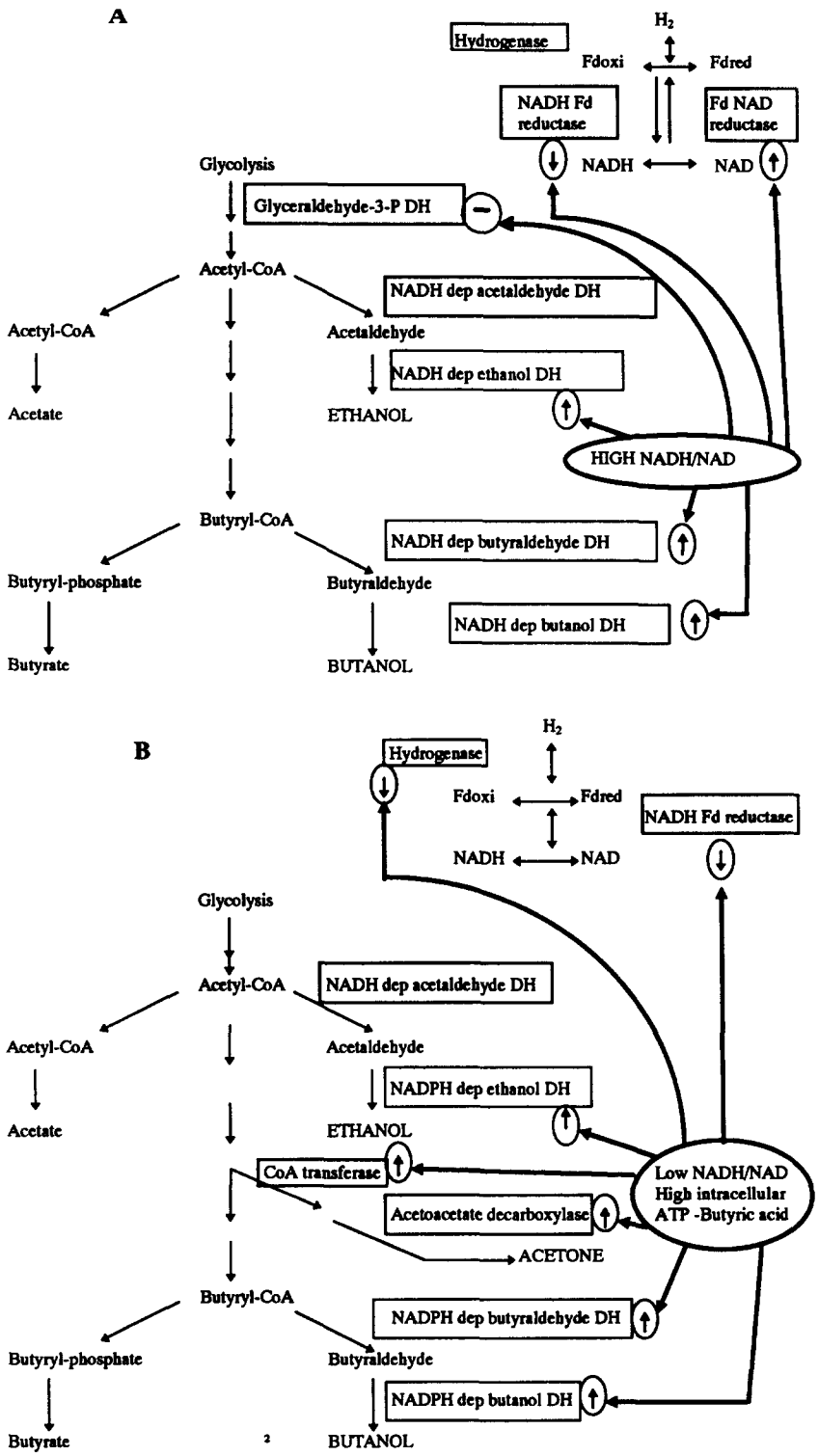

Fig. 3. Schemes of the regulatory mechanisms involved in carbon and electron flow orientation in continuous phosphate-limited steady-state cultures of *C. acetobutylicum*. **(A)** During alcohologenic metabolism (from [14]); **(B)** during solventogenic metabolism at pH 4.4. (↑) high-level enzyme expression; (↓) low-level enzyme expression; (−) inhibition at the enzymatic level.

of metabolism of *C. acetobutylicum* are reported in Table 4.

4.1. ΔpH regulation in alcohologenic cultures

During alcohologenic metabolism induced at neutral pH by using mixtures of substrate (experiments Ia, Ic) or by adding Neutral red (experiment II), the cells maintained their intracellular pH at a lower value than the extracellular pH, resulting in a negative ΔpH. These cultures exhibited lower in vivo specific hydrogen production rate than the acidogenic culture (Table 1), this latter culture showing a positive ΔpH. Since two protons are needed for H_2 production with reduced ferredoxin as an electron donor, it was postulated [18] that the hydrogenase is involved in the alkalinization of the cytoplasm. The ΔpH measurement in cells taken from an acidogenic culture (exhibiting an initially positive ΔpH) and gassed during the ΔpH measurement with carbon monoxide (a hydrogenase inhibitor) led to a negative ΔpH, thus confirming the role of hydrogenase in proton consumption and ΔpH generation in *C. acetobutylicum* cells grown at neutral pH.

However, this inversion of ΔpH (more acidic inside) did not seem to create energetic problems for the cells. Similar PMF values were measured in alcohologenic cells and acidogenic cells. In fact, an increase of the electrical component ($\Delta\psi$) compensated for the decrease of the chemical potential associated to the negative ΔpH. The interconversion of the two components of the PMF appears to be at least partially related to changes in K^+ distribution between inside and outside the membrane [16].

4.2. ΔpH regulation in a solventogenic culture

The decrease of the operating pH from 6.5 to 4.4 inducing a solventogenic metabolism was related to an increase of the ΔpH from 0.1 at pH 6.5 to 1.1 at pH 4.4. Such a high ΔpH was still measured in cells treated with *N,N'*-dicyclohexylcarbodiimide, an ATPase inhibitor, and in cells gassed with carbon monoxide, a hydrogenase inhibitor [15]. It can therefore be concluded that at pH 4.4 neither the ATPase nor the hydrogenase are involved in the maintenance of the ΔpH.

5. Conclusions

Distinct conditions exist under which alcohol formation may be initiated in continuous cultures of *C. acetobutylicum*: (i) glucose-sufficient cultures, and glucose-limited cultures performed at low operating pH, or with addition of butyric acid [4,16], result in the formation of butanol, ethanol and acetone (induction of the solventogenesis) and are associated, under steady-state conditions, with a normal or low level of NADH and a high intracellular level of ATP and butyric acid; and (ii) decreasing the in vivo activity of the hydrogenase by carbon monoxide, Methyl viologen, iron limitation or utilization of glucose–glycerol mixtures leads to an alcohologenic metabolism (no acetone produced) and is correlated, under steady-state conditions, with high intracellular levels of both ATP and NADH [4,9,17]. In addition, the transitory state of a glucose-limited culture after addition of Neutral red (which generates a strong decrease of the in vivo hydrogen evolution activity) suggests a possible effect of NADH on the induction of butanol and ethanol production [15]; (iii) use of mixtures of glucose–glycerol and a more oxidized substrate like pyruvate induces the formation of alcohol and is related, under steady-state conditions, to a high NADH concentration but a low ATP concentration [14]. From these data it can be concluded that the shift toward an alcohologenic metabolism is related to a high NADH concentration.

These two different metabolic shift mechanisms are correlated with the expression of different genes involved in the solvent forming pathways and the electron flow distribution (Fig. 3A, B). A high NADH level leads to butanol and ethanol formation and is accompanied by increased activities of the NADH-dependent alcohol and butyraldehyde dehydrogenases, and ferredoxin $NAD(P)^+$ reductases. These latter enzymes constitute the key enzymes regulating electron flow since no change in hydrogenase activity was observed. On the other hand, classical solventogenesis appears to be characterized by low NADH and high intracellular ATP and butyric acid concentrations and by high levels of expression of the NADPH-dependent alcohol and butyraldehyde dehydrogenases, and of the two enzymes involved in the acetone-formation pathway. In contrast, the ferredoxin $NAD(P)^+$ reductases are not synthesized, and

a decrease of the in vitro hydrogenase activity explains the lower hydrogen generation.

Finally, the regulation of the intracellular pH is different between the alcohologenic culture grown at neutral pH and the solventogenic cultures induced at low pH values. An inversion of the transmembrane pH gradient was observed during the production of alcohol at neutral pH and was related to a lower in vivo specific rate of hydrogen production [18] while in the cultures grown at low pH the transmembrane pH generation was not linked to the F_1F_0 ATPase activity. [16].

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