Pyruvate catabolism during transient state conditions in chemostat cultures of *Enterococcus faecalis* NCTC 775: importance of internal pyruvate concentrations and NADH/NAD⁺ ratios

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NADH/NAD⁺ ratios and internal pyruvate concentrations were determined during switches between aerobic and anaerobic steady-state conditions of glucose-limited chemostat cultures of *Enterococcus faecalis*. During the switch experiments, changes in catabolic fluxes were observed: transition from anaerobic to aerobic conditions resulted in a complete and instantaneous conversion of glucose into acetate and CO₂ via the pyruvate dehydrogenase complex, while during a switch from aerobic to anaerobic conditions the culture became homolactic. A similar switch to a homolactic fermentation was observed upon release of the limitation by addition of a glucose pulse to the culture. In sharp contrast to this, a pyruvate pulse resulted in an increase of both pyruvate formate-lyase and pyruvate dehydrogenase complex activity. Furthermore, acetoin was formed during a pyruvate pulse, probably due to a dramatic increase in internal pyruvate concentration. Regulation of the catabolic fluxes over the various pyruvate-catabolizing enzymes is discussed in view of the observed changes in internal pyruvate concentrations and NADH/NAD⁺ ratios.

Introduction

Research on regulation of glycolysis in streptococci has been focused mainly on the role of the glycolytic intermediates fructose 1, 6-bisphosphate (FBP), glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP) (for a review see Yamada, 1987). Thus, it was found that high intracellular concentrations of FBP were a prerequisite for high lactate dehydrogenase (LDH) activities and resulted in a homolactic fermentation. This could be achieved by growing streptococci either at high dilution rate, or in glucose-excess cultures, or at low culture-pH values. A role for GAP and DHAP in switches from homolactic to mixed acid fermentations was suggested by their inhibition of pyruvate formate-lyase (PFL) under glucose-excess conditions. In addition, PFL is extremely sensitive to oxygen and is completely inhibited under aerobic conditions.

Little attention has been paid to the role of the

pyruvate dehydrogenase complex (PDC) in the catabolism of anaerobically grown lactic acid bacteria, since it has been assumed that this enzyme complex is inactive during anaerobiosis. Recently, it has been shown that this assumption cannot be generalized as, under a number of growth conditions, high anaerobic activities of PDC were observed in *Enterococcus faecalis*, which resulted in a significant effect on overall product formation (Snoep *et al.*, 1990). These activities could be attributed to the low sensitivity of this particular PDC for NADH and its high level of expression under anaerobic conditions (Snoep *et al.*, 1992). It was found that distribution of the catabolic fluxes over PDC and PFL in *E. faecalis* was correlated with the redox potential of the NAD⁺/NADH couple (Snoep *et al.*, 1991).

In addition to LDH, PFL and PDC, a fourth enzyme that can use pyruvate as a substrate, α -acetolactate synthase (α -ALS), is present in streptococci (Starrenburg & Hugenholtz, 1991). As compared to the other enzymes, α -ALS of *Lactococcus lactis* has a very low affinity ($K_m = 50$ mM) for pyruvate, as was shown by *in vitro* experiments with the purified enzyme (J. L. Snoep, M. J. Teixeira de Mattos, M. J. C. Starrenburg & J. Hugenholtz, unpublished results).

From the above, it is to be expected that besides the

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Abbreviations: α -ALS, α -acetolactate synthase; DHAP, dihydroxyacetone phosphate; FBP, fructose 1,6-bisphosphate; GAP, glyceraldehyde 3-phosphate; LDH, lactate dehydrogenase; PDC, pyruvate dehydrogenase complex; PFL, pyruvate formate-lyase.

concentration of the glycolytic intermediates GAP and DHAP, the concentration of pyruvate and the NADH/NAD⁺ ratio will all play a regulatory role in the activity and synthesis of the various enzymes that have pyruvate as a substrate. In this paper we report on changes in these parameters brought about either by transitions between anaerobic and aerobic steady-state conditions or by relief of energy-source-limited growth in chemostat cultures of *E. faecalis*. The effects observed were related to changes in catabolic fluxes.

Methods

Micro-organism and growth conditions. *Enterococcus faecalis* NCTC 775 was maintained on beads in a reinforced clostridial medium (Oxoid) with 50% (w/v) glycerol at -20 °C. Organisms were cultured in a 700 ml Modular Fermenter series III (LH Engineering Co.) as described previously (Snoep *et al.*, 1990). Growth media were simple salts media as specified by Evans *et al.* (1970) with the addition of yeast extract (0.5% w/v), biotin (1 mg l⁻¹), *p*-aminobenzoic acid (25 µg l⁻¹), nicotinamide (2 mg l⁻¹), thiamin (2 mg l⁻¹), pantothenate (2 mg l⁻¹) and cysteine.HCl (50 mg l⁻¹).

Analyses. Steady-state bacterial dry weight was measured by the procedure of Herbert *et al.* (1971). Pyruvate, lactate, formate, acetate and acetoin were determined by HPLC as described previously (Snoep *et al.*, 1990). CO₂ production and O₂ consumption by the cultures were determined by passing the effluent gas from the fermenter through an Infralyt carbon dioxide analyser (Junkalor, Dessau, Germany) and an oxygen analyser (Taylor Servomex type OA 272). Protein was assayed according to the biuret method (Gornall *et al.*, 1949).

NADH oxidase activity measurement, *in vitro*. Cell-free extracts were prepared as described previously (Snoep *et al.*, 1990). NADH oxidase was measured by following the oxidation of NADH in 50 mM-sodium phosphate buffer, pH 7.0.

Pulse experiments. The anaerobic glucose-limited cultures were pulsed by injecting a sterile solution of glucose or pyruvate into the culture vessel by means of a syringe, to give an end concentration of 20 mM or 50 mM, respectively. Simultaneously the medium pump was switched off. Samples were then taken at regular time intervals. Cells and supernatant were separated by centrifugation in an Eppendorf centrifuge, and growth rates calculated from the change in bacterial dry weight during the pulse.

NADH/NAD⁺ ratios. Levels of nucleotides were measured by first extracting the nucleotides from a culture sample and then assaying for the nucleotides in the neutralized, filtered extract, as described previously (Snoep *et al.*, 1990). For glucose-excess conditions, NADH was extracted at room temperature at a pH value of 12.5.

Internal pyruvate determination. Cells sampled directly from chemostat cultures were spun through silicone oil into 25% (w/v) trichloroacetic acid, after which the supernatant and oil were suctioned off. Intracellular pyruvate concentrations were determined essentially as described by Friedemann (1957); an internal standard was included in the assay for calibration. When pyruvate was present in the chemostat effluent a correction was made for adhesion of culture fluid to the bacterial cells [1–2 µl (mg dry wt)⁻¹; this value was determined with cells which had been harvested from a glucose-limited chemostat culture and subsequently starved for 2 d. These cells were mixed with a known pyruvate concentration and spun down through silicone oil within 10 s].

Results

Steady-state conditions

E. faecalis was grown in glucose-limited chemostat cultures at a dilution rate of 0.1 h⁻¹, pH 6.0 under aerobic and under anaerobic conditions. The specific rates of product formation and substrate consumption were highly dependent on the culture conditions (Table 1). Since changes in product formation rates reflect changes in *in vivo* enzyme activities, it could be deduced that under anaerobic conditions PFL was responsible for the major part of oxidation of pyruvate into acetyl-CoA, whereas under aerobic conditions this oxidation was catalysed by the PDC only.

Under anaerobic conditions, re-oxidation of NADH was achieved by formation of lactate and ethanol, whereas under aerobic conditions this was achieved mainly by reduction of oxygen. Due to these different mechanisms for oxidation of NADH a higher Y_{Glc} was observed in the aerobic cultures as more acetate was produced. A somewhat higher Y_{ATP} value was found in the anaerobic culture compared to the aerobic steady-state value. The different mechanisms for oxidation of reducing equivalents in the presence or absence of oxygen were also manifest in the NADH/NAD⁺ ratios measured under these conditions. Under aerobic conditions the NADH/NAD⁺ ratio was much lower than that found under anaerobic conditions (Table 2).

The steady-state internal pyruvate concentration was not influenced much by the presence or absence of oxygen. Under anaerobic conditions it was 2.3 µmol (g dry wt)⁻¹, whereas under aerobic conditions it was 2.0 µmol (g dry wt)⁻¹.

Switch from anaerobic to aerobic conditions

Clearly, large differences in both steady-state catabolic fluxes and NADH/NAD⁺ ratios of aerobic cultures compared to anaerobic cultures were found. These parameters were followed during a transition from anaerobic to aerobic conditions (a typical experiment is shown in Fig. 1).

An immediate increase of *in vivo* PDC activity from 0.8 mmol (g dry wt)⁻¹ h⁻¹ to 6.9 mmol (g dry wt)⁻¹ h⁻¹ was observed upon switching from anaerobic to aerobic conditions. Lactate and formate were no longer being formed (washed out) and all glucose was converted into acetate (Table 3), indicating that PDC was the major pyruvate-catabolizing enzyme under these conditions. Surprisingly, an initial increase in the NADH/NAD⁺ ratio was found upon switching to aerobic conditions, followed by a slow decrease to the steady-state aerobic value [with an essentially constant NAD(H) pool]. On

Fig. 1

Fig. 2

Fig. 1. Effect of a switch from anaerobic to aerobic growth conditions of a glucose-limited chemostat culture ($D = 0.1 \text{ h}^{-1}$, pH 6.0) of *E. faecalis* on the NADH/NAD⁺ ratio (●), and on production of lactate (△), formate (▲), ethanol (○) and acetate (□).

Fig. 2. Effect of a switch from aerobic to anaerobic growth conditions of a glucose-limited chemostat culture ($D = 0.1 \text{ h}^{-1}$, pH 6.0) of *E. faecalis* on the NADH/NAD⁺ ratio (●), and on production of lactate (△), formate (▲) and acetate (□).

Table 1. Specific rates of substrate utilization and product formation in chemostat cultures of *E. faecalis* NCTC 775

Cells were grown at $D = 0.1 \text{ h}^{-1}$, pH 6.0. The specific rates, q , are expressed in $\text{mmol (g dry wt)}^{-1} \text{ h}^{-1}$, Y_{Glc} in g dry wt per mol of glucose consumed, and Y_{ATP} in g dry wt per mol of ATP produced. %C, percentage carbon recovery (carbon output/carbon input $\times 100\%$); %H, redox balance expressed in percentage terms ($q_{\text{NAD}^+}/q_{\text{NADH}} \times 100\%$). Glc, glucose; Lac, lactate; For, formate; Ac, acetate; EtOH, ethanol; Acet, acetoin.

	q_{Glc}	q_{Lac}	q_{For}	q_{Ac}	q_{EtOH}	q_{CO_2}	q_{Acet}	q_{O_2}	Y_{Glc}	Y_{ATP}	%C	%H
Anaerobic	-3.4	4.2	1.4	0.7	1.5	0.8	0.0	0.0	29.4	14.1	94	100
Aerobic	-2.9	0.9	0.0	4.2	0.0	5.0	0.2	4.9	34.5	10.3	93	91

Table 2. Effect of aeration on the NADH/NAD⁺ ratio and on the total NAD(H) pool

Cells were grown, glucose-limited, at $D = 0.1 \text{ h}^{-1}$, pH 6.0, under anaerobic or aerobic conditions. NAD(H) concentrations are expressed as $\mu\text{mol (g dry wt)}^{-1}$. SD values are given in parentheses, $n = 3$.

	NAD ⁺	NADH	NADH/NAD ⁺	NAD(H)
Anaerobic	8.80 (1.12)	8.10 (0.76)	0.92	16.9
Aerobic	10.7 (2.60)	0.72 (0.54)	0.07	11.4

the other hand, when an aerobic culture was grown anaerobically for 1 h and subsequently switched back to aerobic conditions, an immediate and fast decrease in the NADH/NAD⁺ ratio (from 1.47 to 0.44 in 15 min) occurred. The initial changes in product formation were probably due to regulation of enzyme activity rather than

regulation of enzyme synthesis, as essentially the same effect on catabolic fluxes was observed during a transition in the presence of chloramphenicol ($50 \mu\text{g ml}^{-1}$) (data not shown).

Switch from aerobic to anaerobic conditions

During aerobic growth, acetate was the main product, but a complete switch to lactate formation (Table 3) occurred upon switching the culture to anaerobic conditions (a typical experiment is shown in Fig. 2). Concomitantly, an immediate and rapid increase was observed in the NADH/NAD⁺ ratio [with essentially constant NAD(H) pool] (Fig. 2). The internal pyruvate concentration remained more or less constant.

Glucose pulse to anaerobic glucose-limited cultures

Upon addition of glucose, catabolism was shifted from a mixed acid to a homolactic fermentation (Table 3),

Table 3. Specific production rates of transient state cultures of *E. faecalis* NCTC 775

Cells were grown, prior to the switch or pulse, glucose-limited, at $D = 0.1 \text{ h}^{-1}$, pH 6.0, under anaerobic and under aerobic conditions. Pulse procedures are described in Methods. The specific rates, q , are expressed in $\text{mmol (g dry wt)}^{-1} \text{ h}^{-1}$. Glc, glucose; Pyr, pyruvate; Ac, acetate; For, formate; Acet, acetoin.

	Switch from anaerobic to aerobic	Switch from aerobic to anaerobic	Pulse with Glc	Pulse with Pyr
q_{Ac}	6.9	0	0	8.2
q_{For}	0	0	0	3.2
q_{Lac}	0	5.5	12.1	4.4
q_{Acet}	0	0	0	0.8

which was accompanied by an immediate increase in the specific glucose uptake rate [from 3.4 to 7.0 $\text{mmol (g dry wt)}^{-1} \text{ h}^{-1}$]. The growth rate did not increase much and varied between 0.12–0.15 h^{-1} with Y_{ATP} values of 9.9 and 12.4 g dry wt $(\text{mol ATP})^{-1}$. Concomitant with the increase in catabolic rate, the internal pyruvate concentration was found to increase after 5 min from 2.3 to 6.6 $\mu\text{mol (g dry wt)}^{-1}$. This high concentration was still found after 30 min. A small increase (from 0.91 to 1.1) in the NADH/NAD⁺ ratio relative to the steady-state value was measured on addition of glucose, whereas the total NAD(H) pool remained constant at 20 (± 2) $\mu\text{mol (g dry wt)}^{-1}$.

Pyruvate pulse to anaerobic glucose-limited cultures

In contrast to the glucose pulse, a much more complex effect was observed upon addition of pyruvate to glucose-limited cultures. Rather surprisingly, pyruvate was immediately taken up by the cells at an initial specific rate of 13.6 $\text{mmol (g dry wt)}^{-1} \text{ h}^{-1}$ and converted into lactate, acetate, formate, CO₂ and acetoin (Fig. 3, Table 3). The growth rate did not change on pulsing with pyruvate and a Y_{ATP} during the pulse of 12.2 g dry wt $(\text{mol ATP})^{-1}$ could be calculated. No change in NADH/NAD⁺ ratio was observed after 30 min but the internal pyruvate concentration increased dramatically to 64 $\mu\text{mol (g dry wt)}^{-1}$ after 5 min.

Discussion

Pyruvate is the final product of glycolysis and is thus situated at the central branchpoint in catabolism of streptococci. In *E. faecalis*, four enzymes that use pyruvate as a substrate are known: LDH, PFL, PDC and α -ALS. Regulatory mechanisms of catabolic fluxes through these enzymes are most satisfactorily elucidated by comparing the characteristics of the enzymes *in vitro* with their specific activities *in vivo*. In this study, particular attention has been paid to two important

Fig. 3. Effect of a pyruvate pulse to a glucose-limited chemostat culture of *E. faecalis* ($D = 0.1 \text{ h}^{-1}$, pH 6.0) on consumption of pyruvate (∇), and on production of lactate (Δ), formate ($\blacktriangle$), ethanol ($\circ$), acetoin ($\blacksquare$) and acetate ($\square$).

parameters: the NADH/NAD⁺ ratio and the internal pyruvate concentration.

When isolated, PDC is inhibited by high NADH/NAD⁺ ratios and *in vivo* studies in chemostat cultures do show a correlation between the redox potential of the NAD⁺/NADH couple and the distribution of the catabolic flux over PDC and PFL (Snoep *et al.*, 1991). This result is in agreement with the observation that under steady-state aerobic conditions, in which very low NADH/NAD⁺ ratios are measured, PDC has a high *in vivo* activity. On switching from aerobic to anaerobic conditions, an immediate and rapid increase in NADH/NAD⁺ ratio is observed with a concomitant complete inhibition of PDC. However, upon switching from the anaerobic to the aerobic state, an immediate increase in PDC activity is observed while the NADH/NAD⁺ ratio also shows an initial increase. This initial increase is surprising. Although the q_{NADH} increases, due to PDC activity, one would expect the NADH/NAD⁺ ratio to

decrease as an electron acceptor with a much higher midpoint potential is introduced. Of course, there has to be a mechanism in the cell to interact with oxygen in order to detect its presence. It is known that dihydro-lipoamide dehydrogenase (E3), the enzyme component of PDC that is inhibited by NADH, can react directly with oxygen (Massey *et al.*, 1969). So, the apparent conflict observed between the high PDC activity together with a high NADH/NAD⁺ ratio can be understood by assuming a direct reaction of oxygen with E3. In this way, E3 will not be over-reduced to the, inactive, four-electron reduced form.

Although E3 can react with oxygen, NADH oxidase is thought to be responsible for the major part of NADH oxidation under aerobic conditions. This is also manifest from the slow change in NADH/NAD⁺ ratio upon switching from anaerobic to aerobic conditions. As shown by the *in vitro* activity of the NADH oxidase in cell-free extracts, the amount of this enzyme in aerobically grown cells [0.7 U (mg protein)⁻¹] is much greater than in anaerobically grown cells [0.1 U (mg protein)⁻¹]. The slow change in NADH/NAD⁺ ratio upon switching from anaerobic to aerobic conditions can be explained by the presence of low concentrations only of NADH oxidase.

It is clear that the NADH/NAD⁺ ratio plays an important role in regulation of the catabolic fluxes. Correlations between this parameter and catabolic fluxes are found not only in steady-state anaerobic conditions but also during switch experiments between aerobic and anaerobic states. However, it was found that oxygen itself can also directly influence enzyme activity. It should be realized that oxygen may not only react with E3, but is also known to inactivate PFL (Yamada, 1987).

The effect of relief of energy-source limitation on the NADH/NAD⁺ ratio was small, yet large effects on the catabolic fluxes were observed. A glucose pulse resulted in a switch to a homolactic fermentation. This is probably due to an increase in FBP, GAP, and DHAP concentration resulting in activation of LDH and inhibition of PFL, respectively, as observed in glucose excess cultures (Yamada, 1987). However, no effect of intermediates on PDC activity is known. Our data suggest that the inhibition of PDC is caused by the increase in NADH/NAD⁺ ratio.

A pyruvate pulse resulted in an increase of the activity of all four enzymes. Since no change in NADH/NAD⁺ ratio was measured some other regulatory parameter has to be responsible for this effect. Since all steady states were glucose-limited, it is not surprising to find low internal pyruvate concentrations under these conditions. During switches between the aerobic and anaerobic states, no great fluctuations were found in internal pyruvate concentrations. However, relief of the limita-

tion by addition of either glucose or pyruvate resulted in dramatic changes in the internal concentration of this intermediate (a roughly 28-fold increase upon addition of pyruvate). The unit – $\mu\text{mol (g dry wt)}^{-1}$ – that is used to express the internal pyruvate concentration can be transformed into mM by dividing through the internal volume. Of course this internal volume is not a constant, but this calculation only serves to compare the internal pyruvate concentration with the K_m values of the enzymes. By assuming an internal volume of 1.7 $\mu\text{l (mg dry wt)}^{-1}$ (Kashket, 1985), an internal concentration of about 38 mM-pyruvate is calculated after a pyruvate pulse. Although this concentration might be saturating for PDC, PFL and LDH, the apparent K_m for pyruvate of α -ALS in cell-free extracts of *E. faecalis* was found to be 40 mM (data not shown). It has been suggested that the function of α -ALS is to remove excess pyruvate (Collins, 1972). The low affinity of α -ALS for pyruvate is probably the reason why no *in vivo* activity of the enzyme is observed under energy-source-limiting conditions. Only during a pyruvate pulse is some activity observed, as reflected by acetoin production.

From the above, it is clear that regulation of pyruvate catabolism is complex. In addition to what is known about regulation of LDH and PFL by the glycolytic intermediates FBP, GAP and DHAP, we have shown now that NADH/NAD⁺ ratios and internal pyruvate concentration play an important role in regulating of PDC and α -ALS, respectively.

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