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Physiology of pyruvate metabolism in *Lactococcus lactis*

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Abstract

Lactococcus lactis, a homofermentative lactic acid bacterium, has been studied extensively over several decades to obtain sometimes conflicting concepts relating to the growth behaviour. In this review some of the data will be examined with respect to pyruvate metabolism. It will be demonstrated that the metabolic transformation of pyruvate can be predicted if the growth-limiting constraints are adequately established. In general lactate remains the major product under conditions in which sugar metabolism via a homolactic fermentation can satisfy the energy requirements necessary to assimilate anabolic substrates from the medium. In contrast, alternative pathways are involved when this energy supply becomes limiting or when the normal pathways can no longer maintain balanced carbon flux. Pyruvate occupies an important position within the metabolic network of *L. lactis* and the control of pyruvate distribution within the various pathways is subject to co-ordinated regulation by both gene expression mechanisms and allosteric modulation of enzyme activity.

Introduction

The description of pyruvate metabolism in homofermentative lactic acid bacteria appears to be simple. More than 90% of pyruvate is converted to lactate during industrial sugar fermentations. However, in an increasing number of cases diversion of this simple conversion has been observed, leading to production of a number of other metabolites. In fact, pyruvate is the key metabolic intermediate in lactic acid bacteria and metabolism is strongly regulated in the process of pyruvate production and conversion. In this overview, a variety of nutitional factors are mentioned that effect the activity of enzymes involved in pyruvate metabolism. These changes in activity can lead to important variations in end product formation.

Lactic acid bacteria (LAB), unlike many of the other widely studied bacteria of industrial importance, metabolise sugars predominantly to generate biochemical energy. Anabolic precursor metabolites are obtained from other components of the medium. It is, therefore, the energy generating aspect of the catabolic pathways which needs to be considered when examining metabolic regulation. The 'growth supporting' substrates (a variety of sugars) can best be defined as those compounds able to be fermented at rates and by pathways which can provide the necessary flux of biochemical energy to facilitate the inter-conversion and synthesis of cell material from preformed nitrogen-containing organic matter. Other carbon substrates may be metabolised and partially catabolised in so much as their consumption has an influence on the energy status of the cell.

Metabolic pathways of sugar fermentation

Before examining the factors specifically regulating pyruvate metabolism it is important to briefly review the catabolic pathways involved in sugar metabolism. Most of the sugars are taken up by the cells via either PEP-dependent phosphotransferase systems (PTS) involving coupled transport and phosphorylation of the sugar (Thompson, 1978, 1979; Yamada, 1987; Benthin et al., 1993), or via permease systems (Thompson et al., 1985; Crow & Thomas, 1984; Romano et al., 1987) in which sugar transport is followed by kinase-

mediated phosphorylation of the free sugar within the cytosol (Bisset & Anderson,1974). The contribution of each transport mechanism to overall sugar uptake has never been adequately assessed and speculation tends to predominate as to which mechanism is functional. Most probably a variety of uptake mechanisms contribute to the global transport and relative proportions of each system will depend upon the prevailing nutritional environment.

Once phosphorylated, sugars are catabolised by relatively simple linear pathways (Figure 1) whose composition depends upon the nature of the sugar. Many monosaccharides enter central metabolism via glucose-6-phosphate (G6P) and their catabolism follows a classical glycolysis to pyruvate with a net gain of 2 ATP per sugar once transport requirements have been taken into consideration. The vast majority of sugars enter the central pathways as G6P though fructose enters as FDP (due to the synthesis of F1P by the PTS^{fru}) and the Gal6P moiety of lactose transported via the PTS^{lac} enters glycolysis at the level of triose-phosphates after transformation via the tagatose pathway. Galactose entering the cytoplasm via permease transport or via the hydrolysis of lactose by β-galactosidase (Farrow, 1980) enters metabolism as G6P but requires the operation of the Leloir pathway involving galactose kinase, UDP-glucose epimerase/transferase and the P-glucose isomerase.

Regulation of homolactic metabolism

Glycolysis generates the ATP necessary for the biosynthesis of cell material but also NADH, which in the absence of respiratory activity needs to be recycled via reactions involving the reduction of metabolic intermediates to liberate the fermentation end-products. This reducing equivalent wastage occurs primarily at the level of pyruvate and under non-limiting growth conditions involves the production of lactic acid. The allosteric control of many enzymes of glycolysis by FDP has been reported (see below) and hence phosphofructokinase and fructose diphosphate aldolase might be expected to play an important role in glycolytic regulation. Indeed, in *L. lactis*, FDP aldolase has been studied in some detail (Crow & Thomas, 1982) and found to have an affinity for FDP of 1.1 mM. Measured intracellular pools of FDP are an order of magnitude higher than this value under conditions of homolactic fermentation and hence the enzyme operating under substrate saturated conditions might contribute significantly to

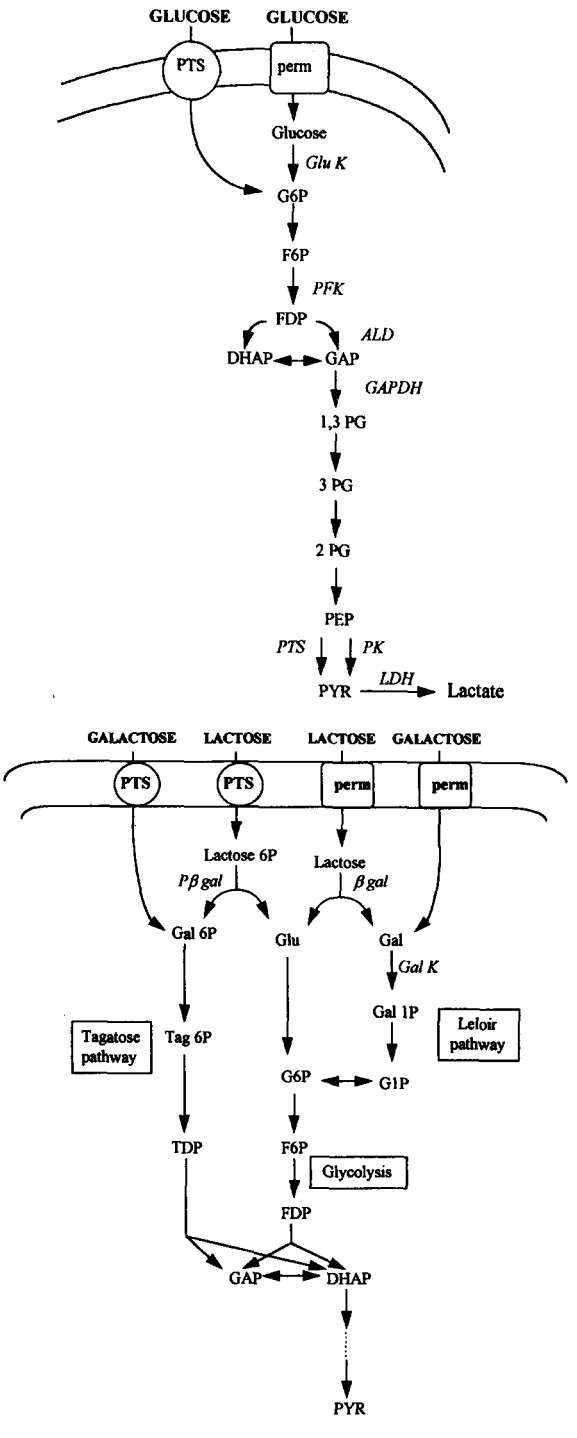

Figure 1. Metabolic pathways involved in sugar fermentation by *Lactococcus lactis* during growth on glucose (A) and on galactose or lactose (B). PTS: phosphotransferase system; perm: permease; (P)β gal: (Phospho) β galactosidase; Gal K: galactokinase; Glu K: glucokinase; PFK: phosphofructokinase; PK: pyruvate kinase; LDH: lactate dehydrogenase.

the control of pathway flux. The controlling influence of this key enzyme, however, has never been studied and it is generally believed that the glyceraldehyde-phosphate dehydrogenase (GAPDH) exerts most influence over the flux through glycolysis. Poolman et al. (1987a) studied this enzyme and estimated a flux control coefficient of 0.9 when applying metabolic control analysis. It should be remembered that the sum of the flux control coefficients of each enzyme in a linear pathway as defined by metabolic control theory (Kell & Westerhoff, 1986) should be equal to 1. This indicates an extremely important role for this enzyme but the results were obtained with non-proliferating cells and under conditions (use of enzyme inhibitors) which might have led to over-estimation of the control coefficient of GAPDH. Metabolic control theory gives a precise quantification of the contribution of each enzyme to control of carbon flux but the values obtained cannot be extrapolated to growth conditions other than those used experimentally. Furthermore, it is often seen that diminishing the activity of any enzyme has a significant effect on carbon flux while increasing the activity of that same enzyme has a more constrained effect. This can be interpreted as evidence that many enzymes share the control, i.e., protein conservation has evolved a metabolic structure in which no single enzyme is produced in significant excess under conditions in which high carbon throughput is favoured. Despite such arguements, it is clear that GAPDH has a major role in regulating carbon flux through glycolysis in *L. lactis*. Many related homolactic bacteria implicated in oral pathology posses an NADPH-dependent GAPDH activity (Yamada, 1987; Crow & Wittenberg, 1979) though this enzyme has not been found in *L. lactis*. The genetic organisation of the glycolytic enzymes is different to that frequently encountered among aerobic Gram-positive bacteria (Cancilla et al., 1995) though the implications for this from a regulation viewpoint are not yet clear.

The enzymes more closely associated with pyruvate metabolism have been examined and shown to be subject to considerable biochemical regulation. The pyruvate kinase which, together with the PTS mechanism, generates the pyruvate, has been shown to be subject to activation effects by various glycolytic metabolites: FDP, G6P, F6P, Tag6P, Gal6P, TagDP but also by GAP and DHAP. This mechanism modifies the affinity of the enzyme (Thomas, 1976a) for PEP from a low affinity (Km = 5 mM) to a high affinity status (Km = 0.14 mM) leading to a low intracellular PEP concentration (Thompson, 1978; Thompson & Torchia, 1984; Lohmeier-Vogel et al., 1986). In other words, when the initial stages of glycolysis, or parallel pathways, are operating rapidly, high concentrations of C_6 compounds and both GAP and DHAP are observed and the pyruvate kinase is activated. To understand why such a mechanism has developed, it is necessary also to take into account that the PTS provides the second reaction generating pyruvate and that these same activators are implicated in the control of PTS sugar transport. Thus, in order to assure an efficient transformation of PEP to pyruvate under substrate excess conditions in which permease transporters are probably dominant, an antagonistic control is exerted leading to a shift in reactions favouring the pyruvate kinase and hence regenerating the ATP necessary for direct intracellular phosphorylation of the permeated sugar. This antagonistic control is also seen in the manner in which inorganic phosphate and ATP inhibit the pyruvate kinase activity (Collins & Thomas, 1974; Thompson & Torchia, 1984) but activate the PTS.

The catabolism of sugars leads to pyruvate, a major branch point metabolite whose further metabolism determines the nature of the fermentation. Since *L. lactis* strains are generally considered to be homolactic, the normal pathway accounting for the vast majority of the generated pyruvate involves the reductive transformation of pyruvate to lactate via lactate dehydrogenase (LDH). This reaction enables the recycling of the reduced coenzymes produced in glycolysis thus maintaining energetic equilibrium. As for other key enzymes, phosphorylated metabolites play an important role in regulating this activity: both FDP and TagDP are known to activate the enzyme (Thomas, 1976b) while PEP and inorganic phosphate have a negative effect on LDH activity (Yamada, 1987; Konings et al., 1989). Indeed, LDH is a tetrameric protein stabilised by a phosphate with an affinity for pyruvate of approximately 2 mM (Thomas et al., 1980). The various subspecies of *L. lactis* used in the dairy industry seem to be extremely similar as regards the LDH activity and recent molecular characterisation of the *ldh* gene shows no significant difference in the putative amino acid structure (Swindell et al., 1994). Interestingly, the *ldh* gene is part of an operon containing the genes for phosphofructokinase and pyruvate kinase enzymes expressed to high levels but also subject to strict control by the phosphate potential of the cell (Llanos et al., 1992, 1993).

From these data a model has been developed to explain the homolactic behaviour of the strain which is coherent with the majority of the experimental observa-

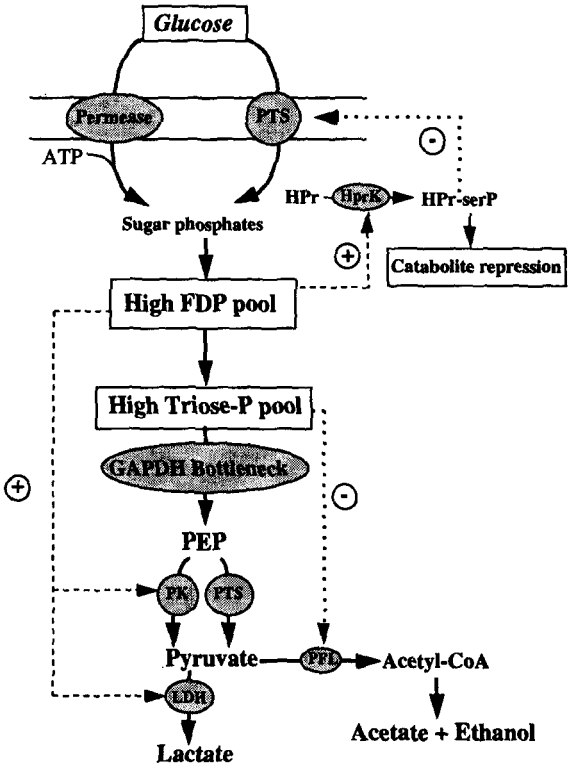

Figure 2. Central role of FDP in co-ordinating glycolytic flux both upstream and downstream of the rate-limiting GAPDH during homolactic fermentation of glucose by *L.lactis*. HPrK: HPr kinase; GAPDH, glyceraldehyde dehydrogenase; PK, pyruvate kinase; PFL, pyruvate-formate lyase; LDH, lactate dehydrogenase. ⊕, activation of an enzyme activity; ⊖, inhibition of an enzyme activity.

tions. When substrate is in excess, the flux through the catabolic pathways is important and adequate to supply the ATP required for cell growth. Under such conditions the synthesis of cell biomass from other organic compounds present in the medium limits growth. The regulation of glycolysis has been correlated with the high level of FDP which activates both pyruvate kinase and lactate dehydrogenase, and a low level of PEP, presumably resulting directly from the activation of these enzymes. The result is a linear metabolism of sugar conversion to lactate of constant thermodynamic efficiency. The glycolytic pathways articulate around GAP dehydrogenase whose controlling influence (Poolman et al., 1987) would lead to a situation in which all metabolites downstream of the triose phosphates would tend to be present at relatively low concentrations, phenomena accentuated by the activated PK and LDH activities. Thus the upper portion of the catabolic pathways can be envisaged as being substrate saturated with correspondingly high pools of phosphorylated sugars, while the lower pathway, common to all pathways of sugar catabolism, is substrate limited due to the GAP dehydrogenase bottleneck. Within such a model, FDP clearly plays a key role modulating the metabolic potential of the cell by a cascade of allosteric, post-translational and catabolite repression phenomena (Figure 2), but other phosphorylated metabolites are also implicated in the modulation of enzyme activity. Indeed FDP has less effect on PK than other metabolites (Thomas, 1976a): G6P is the principal activator (more significant effect on Vm at lower concentrations) in a hierarchic control cascade which can be expressed as G6P > F6P > DHAP > GAP > FDP. The confusion which has developed in this respect is linked to the fact that FDP is the metabolite whose intracellular concentration is highest and therefore easiest to measure leading to an over-simplification of the phenomena involved. Recent progress as regards the role of FDP as an alarmone controlling the carbon catabolite repression cascade via the phosphorylation of HPr in Gram-positive bacteria (Ye et al., 1994; Veyrat et al., 1994; Saier et al., 1996) needs also to be taken into account, though further research is necessary to identify the extent to which modulation of gene expression reinforces the biochemical control of enzyme activity within *L. lactis*.

Mixed acid fermentation

Under certain conditions, *L. lactis* deviates significantly from a homolactic fermentation and minor products (<10% of total carbon consumption) derived from pyruvate become far more important. Such a metabolism is quite distinct from a heterolactic metabolism in which glycolysis is replaced by a variant of the pentose pathway involving a phosphoclastic enzyme generating directly a C_3 and a C_2 residue from pentose-P (Figure 3). In the case of mixed acid fermentation (Figure 4) glycolysis continues to generate pyruvate, but further metabolism involves a considerably diminished flux through LDH. This response was first observed in carbon-limited chemostat cultures in which presumably other organic nutrients are in excess. Under such conditions, pyruvate is metabolised via either pyruvate formate lyase (PFL) or pyruvate dehydrogenase (PDH) to give either formate or CO_2respectively, and acetate + ethanol mixtures. Thus, the mixed acid fermentation and the extent of the deviation of pyruvate away from lactate will result

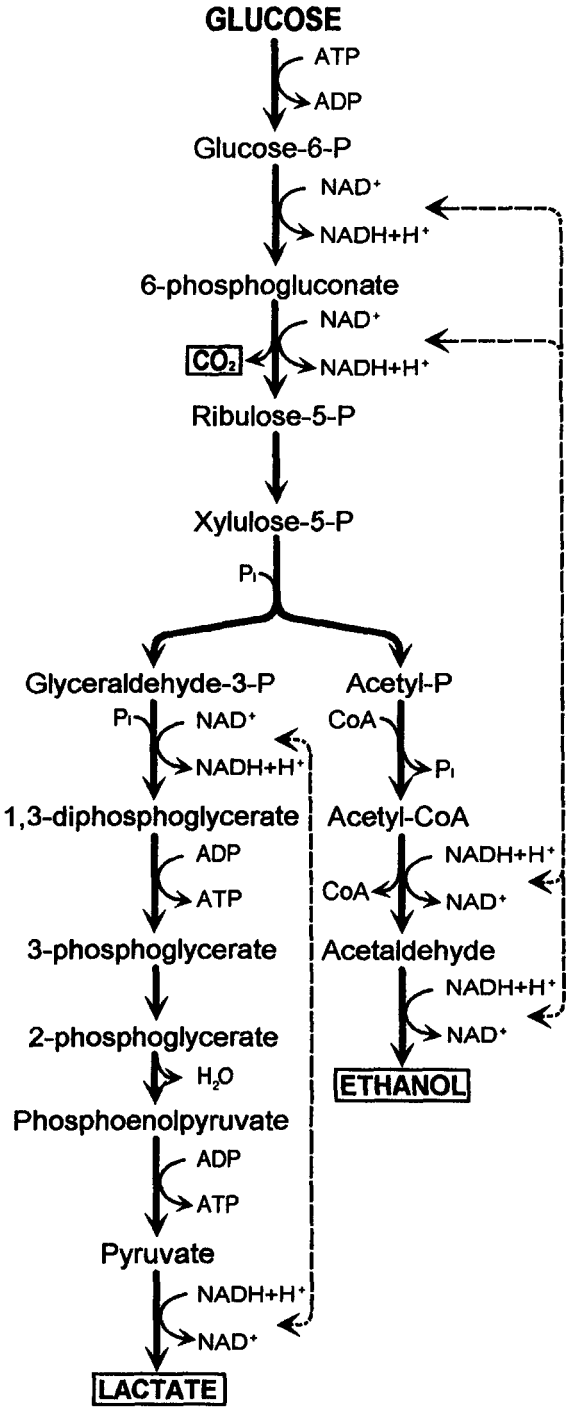

Figure 3. Heterofermentative metabolism of sugars by lactic acid bacteria.

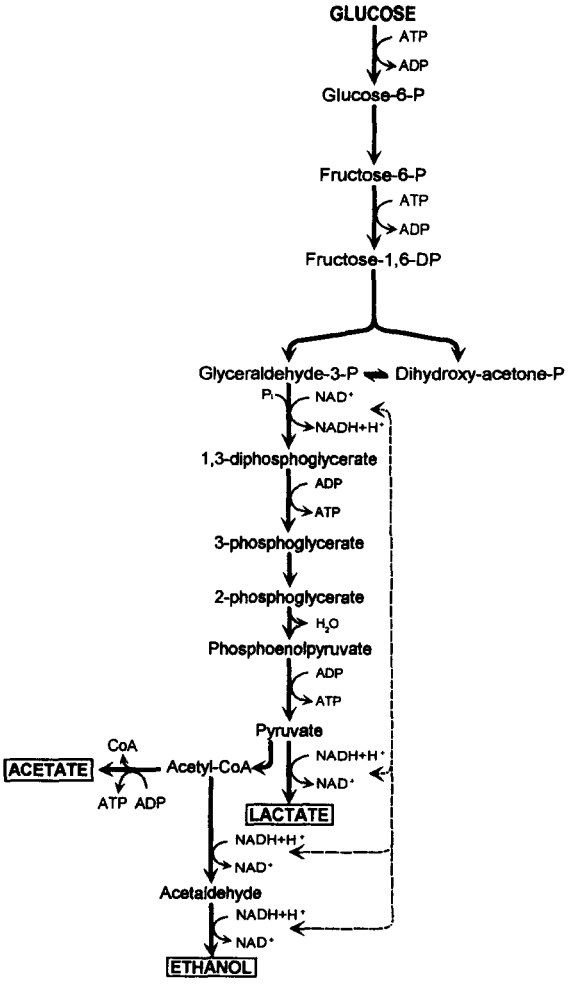

Figure 4. Mixed acid fermentation of sugars by *L. lactis*.

from the competition which will occur between LDH and PFL or PDH.

In strictly anaerobic conditions, pyruvate formate lyase activity is usually detected though the extreme sensitivity of this enzyme to oxygen (Takahashi et al., 1982; Abbe et al., 1982) requires extensive attention to analytical techniques. The enzyme transforms pyruvate to formate and acetyl-CoA and is inhibited by both GAP and DHAP (Thomas et al., 1980; Takahashi et al., 1982). This inhibition of PFL activity by triose phosphates, present at high intracellular concentrations during homolactic fermentations, is probably sufficient to explain why the majority of pyruvate is transformed to lactate under conditions in which the LDH is activated. This orientation was thought to be reinforced by a difference in affinity for pyruvate: the PFL has been shown to have a lower affinity (Km = 7 mM, Thomas

et al., 1980) than LDH (Km = 2 mM), though recent work in our laboratory indicates that this value may be falsely high. Under strict anaerobic conditions, we have found that the PFL activity of a *L. lactis* strain isolated from vegetal matter has a Km for pyruvate of 1 mM. The biochemical control of PFL has been most extensively studied in *E. coli* (Knappe & Sawers, 1990) in which activity is controlled by enzymatic activase/deactivase activities which modify the redox state of the enzyme. As opposed to the radical form of the enzyme that is irreversibly deactivated by oxygen, the non radical form of PFL is insensitive to oxygen, this constituting a protection mechanism against oxygen. However, this modulation of the active form of the protein appears not to exist in the lactic bacteria (Yamada, 1987) making the enzyme irreversibly deactivated by oxygen. For the moment little is known of the factors governing the expression of this activity in *L. lactis* though subject to complex transcriptional control in *E. coli* (Knappe & Sawers, 1990).

The alternative reaction involves a reductive decarboxylation of pyruvate via the pyruvate dehydrogenase complex yielding acetyl-CoA and CO_2. The significant difference between this reaction and that of the pyruvate formate lyase is that a CO_2 rather than formate is produced and hence an additional reduced coenzyme is generated. In gram-negative bacteria, the PDH activity is subject to a variety of allosteric regulations but no evidence for this exists in gram- positive species for which the only known inhibition is related to the biochemical redox potential. The sensitivity to this factor, as represented by the NADH:NAD ratio, is linked to the inactivation of Enzyme 3 (Figure 5) of the complex (Snoep et al., 1992a) and the variation between species depends upon the relative amount of this protein within the enzyme complex. In the case of *Enterococcus faecalis*, this component is strongly expressed and hence the PDH remains active under anaerobic conditions while Enzyme 3 is only weakly expressed in *L. lactis* and is hence rapidly inactivated under anaerobic conditions (Snoep et al., 1993a).

Irrespective of the enzyme involved in acetyl-CoA formation, further metabolism of this intermediate to give either acetate or ethanol involves either the phosphotransacetylase / acetate kinase, or the aldehyde and alcohol dehydrogenases respectively. Acetate formation generates a supplementary ATP while ethanol production enables two reduced co-enzymes to be recycled. When pyruvate formate lyase is employed, reducing equivalent equilibrium can be maintained by an equimolar partition of acetyl-CoA between acetate and

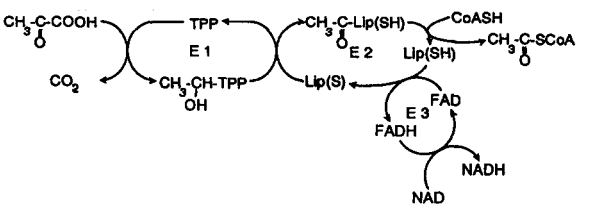

Figure 5. Biochemical structure of the multienzyme pyruvate dehydrogenase complex. E 1: pyruvate decarboxylase; E 2: dihydrolipoamide succinyltransferase; E 3: dihydrolipoamide dehydrogenase.

ethanol with a net increase in ATP gain of 50% relative to a homolactic fermentation for that part of the pyruvate deviated towards the mixed acid fermentation. No obvious gain can be expected from using the PDH since the additional NADH synthesised requires that all acetyl-CoA is converted to ethanol to maintain coenzyme equilibrium. However, since PDH activity is only observed under aerobic conditions in which oxygen is present, additional pathways of reduced coenzyme wastage may be involved (see below). The mixed acid fermentation is likely to predominate when anaerobic conditions are encountered if sugar consumption is rate limiting, i.e., when the rate of formation of new cell material is limited by the availability of ATP rather than the availability of anabolic carbon metabolites.

Influence of carbon source limitation on pyruvate metabolism

In conditions of carbon excess, i.e. batch fermentations, sugars are generally metabolised to yield a homolactic fermentation. However, under conditions in which glucose availability is limited, e.g., carbon-limited chemostat conditions, a diminished production of lactate is observed (particularly at low dilution rates in which true carbon- limitation occurs) with a corresponding increase in the quantity of fermentation products derived from the action of pyruvate formate lyase: formate plus acetate/ethanol mixtures (Thomas et al., 1979). This shift from a homolactic fermentation towards a mixed acid fermentation can be correlated with a decrease in lactate dehydrogenase specific activity, but also with a significantly lower intracellular concentration of this enzyme's principle activator, FDP (Thomas et al., 1979). Work with non-proliferating cells (Thompson, 1978; Thompson & Torchia, 1984; Lohmeier-Vogel et al., 1986) also demonstrated that the intracellular pools of both GAP and DHAP were

also significantly diminished. Since these metabolites are responsible for an inhibitory deactivation of PFL in many organisms, it would appear that a concerted control of pyruvate metabolism operates under carbon limitation, so as to redirect carbon away from the energetically less favourable pathway of lactate formation. This would effectively increase ATP production under conditions in which sugar metabolism is inadequate to supply the anabolic pathways with the required biochemical energy.

Despite the importance of this shift, little work has been published as regards the expression of PFL activity in *L. lactis*. In *Streptococcus mutans*, specific activity of PFL was seen to increase by a factor of five under carbon limitation, further consolidating the cells potential to modify its homolactic fermentation (Thompson & Gentry- Weeks, 1994). Thus it would seem unlikely that GAPDH has the same controlling constraint on carbon flux through glycolysis when growth is carbon-limited. Indeed, the modified metabolite pools (low FDP, DHAP, GAP, high PEP...) are more coherent with a pathway bottleneck at the level of pyruvate and it would be logical to assume that sugar transport may become the major controlling influence, though this reflects the lack of available substrate rather than the specific activity of the transporter itself. The low FDP pool and high PEP potential would in fact lead to maximal activity of the PTS mechanism, most probably the major transporter at low substrate concentrations.

Mixed acid fermentation under carbon excess conditions

Galactose metabolism results in a fermentation end product profile in which significant amounts of C_2 compounds are produced, though lactate remains the major product (Thomas et al., 1980). As was the case for glucose-limited chemostat cultures, this shift was correlated to a diminished flux through lactate dehydrogenase and an increase in pyruvate formate lyase activity. Again, the diminished metabolite pools upstream of GAPDH (FDP, GAP, DHAP) would consolidate the observed changes in enzyme activity.

The metabolism of galactose is believed to be directly related to the pathway employed and the corresponding transport system. A homolactic fermentation has been attributed to PTS transport and the tagatose pathway while a mixed acid fermentation involves permease activity coupled to the Leloir pathway. However, this hypothesis is incomplete since *L. lactis* subsp *cremoris* retains a mixed acid fermentation in the absence of Leloir pathway, i.e. all galactose is metabolised via the tagatose pathway (Thompson, 1980; Thomas et al., 1980). Furthermore, lactose in strains harbouring the lactose plasmid is metabolised via both pathways but retains a homolactic fermentation. The phenomena really provoking this shift are almost certainly the flux through the pathways and the effects of the corresponding metabolite pool concentrations on enzyme activities rather than the use of specific pathways.

Maltose metabolism deviates significantly from the homolactic fermentation and constitutes an interesting metabolic model since the general requirements outlined above are not entirely satisfied. Maltose is transported via a permease and most probably phosphorylated by an inorganic phosphate dependent phosphorylase to yield glucose–1-P and glucose, both of which will be further metabolised via glycolysis after transformation to glucose–6-P (Qian et al., 1994; Sjoberg & Hahn-Hagerdal, 1989). While FDP pools are low (and inorganic phosphate high) explaining the diminished flux through lactate dehydrogenase, the triose-phosphate pools remain high provoking a strong inhibition of the PFL. The result of such a metabolism is that the shift towards a mixed acid fermentation is less pronounced than during carbon-limited cultures or galactose-grown cells (Lohmeier-Vogel et al., 1986; Sjoberg & Hahn-Hagerdal, 1989). Interestingly, such a situation provokes some accumulation of exopolysaccharides, due principally to the low activity of the phosphoglucomutase activity (Qian et al., 1994).

The metabolism of pentose sugars presents a somewhat different situation and is often considered to involve a truely heterolactic fermentation, i.e. phosphoketolase activity leading to C3- and C2-unit formation directly from the pentose-phosphate (Kandler, 1983). The capacity to metabolise pentose sugars appears to be strain dependent and the extent to which mixed acid fermentation occurs perhaps reflects the different capacities of the strains used (Kandler, 1983; Ishizaki et al., 1992; Westby et al., 1993). In our laboratory, growth was found to be possible for *L. lactis* on ribose, xylose and gluconic acid, all of which lead to pentose-phosphate formation though ribose and xylose are taken up by permeases while gluconate is transported via a PTS mechanism (Thompson & Gentry-Weeks, 1994). The metabolism via phosphoclastic pathway has not been examined at the enzyme level but end-product profiles were not those to be expected from a classical heterolactic fermentation. Moreover, the close agreement between formate concentration and the amount of acetate and ethanol produced indicate

Table 1. Batch growth of Lactococcus lactis NCDO 2118 on defined media containing all essential amino acids. Influence of sugar uptake rate on growth and end-product profiles as expressed as the % of pyruvate recovered as lactate or as products of pyruvate-formate lyase activity (formate, acetate and ethanol)

Substrate	Specific rates		% Pyruvate distribution	
	Sugar uptake (mmol C_6/g.h)	Growth (h–1)	LDH	PFL
Glucose	15.0	0.55	93	7
Galactose	9.3	0.20	67	33
Lactose	5.1	0.17	3	97

that a mixed acid fermentation occured and hence pentose phosphates must presumably be directed back into glycolysis by transketolase/transaldolase reactions of the pentose pathway.

It is often remarked that the contribution of the mixed acid fermentation only exceeds 10% of carbon flux under conditions in which the growth rate is considerably affected. This, at first sight, suggests that this shift might be growth-rate related. In our hands, the correlation is not so straight forward since a number of substrates showing roughly the same growth rate show a significant variation in the extent to which the mixed acid fermentation operates (Figure 6). Preliminary results suggest that the causative phenomenon is the rate at which sugars can be taken up by the cells. Thus, under growth conditions in which sugar metabolism via the homolactic pathway is inadequate to yield the ATP necessary, the mixed acid fermentation is progressively activated. The mechanisms governing gene expression remain to be determined, but it should be remembered that the gene for lactate dehydrogenase is carried on the same operon as the genes coding for key glycolytic enzymes, phosphofructokinase and pyruvate kinase (Llanos et al., 1992, 1993).

The hypothesis that the flux through the central pathways determines the cells capacity to metabolise pyruvate provides a unified theory that explains equally well the behaviour of *L. lactis* on certain sugars and chemostat behaviour. In both cases, sugar uptake plays a key role though one can be viewed as a biological limitation, while in the chemostat the fermentation protocol determines the behaviour, i.e., in batch culture the sugar transport activity may impose a limitation on the resulting carbon flux, while in the chemostat the substrate availability (residual concentration) is limiting. To examine further this concept, we have investigated the manner in which the metabolism of lactose is perturbed in *L. lactis* strains lacking the lactose-PTS and associated tagatose pathways enzymes. Certain strains of *L. lactis* isolated from vegetable matter do not possess the plasmid encoding such proteins (Crow et al., 1983; McKay et al, 1972). For a long time these strains were believed to be unable to grow at the expense of lactose. However, the use of minimal medium (Cocaign-Bousquet et al., 1995) thus overcoming certain problems associated with the use of complex media, has enabled us to demonstrate clearly that such strains are able to grow on lactose, albeit at considerably diminished growth rates. If the biochemical model proposed above is correct, it would be expected that such strains, in which lactose permease is the only manner to take up lactose, would have slow rates of sugar consumption and show a mixed acid fermentation. Indeed, such was the case with virtually no lactate at all being produced. Interestingly, the growth rate of the strain on lactose is similar to that on galactose (Table 1) but much lower than that obtained on glucose, for which a homolactic fermentation was seen. Galactose metabolism yields mixtures of lactate and formate/acetate/ethanol. Examination of the sugar uptake rate shows that lactose is taken up at considerably slower rates than galactose and one might postulate that the extent to which the metabolism is limited by uptake capacity is greater in the case of lactose. The additional energy derived from the increased production of acetate is sufficient however to sustain similar growth rates (Table 1) and the estimated Y_{ATP} remains constant. How then does the cell control this shift? Intracellular metabolite measurements have shown a good correlation between the concentrations of FDP and triose-P and pyruvate flux through LDH and PFL, phenomenon accentuated by the modified expression of each enzyme. Similar shifts have been seen in strains having undergone mutation selection to inactivate P-β-galactosidase activity and/or the lactose PTS transport (Demko et al, 1972; Crow & Thomas, 1984). Mixed acid fermentation was seen in these strains, as was the case for *S. mutans* lacking a PTS^{gluc} activity (Thompson & Gentry-Weeks, 1994), however no details concerning the effect on sugar consumption or the degree of the diversification of the fermentation are available. Leblanc et al. (1979) observed that the loss of the plasmid encoding the PTS^{lac}, P-β-Gal and the tagatose pathway in dairy lactococcal strains grown on galactose diminished lactate production from 90% of products recovered to 75% with a corresponding increase in acetate/ethanol production. No data was given for lactose. Of course, from a practical point of

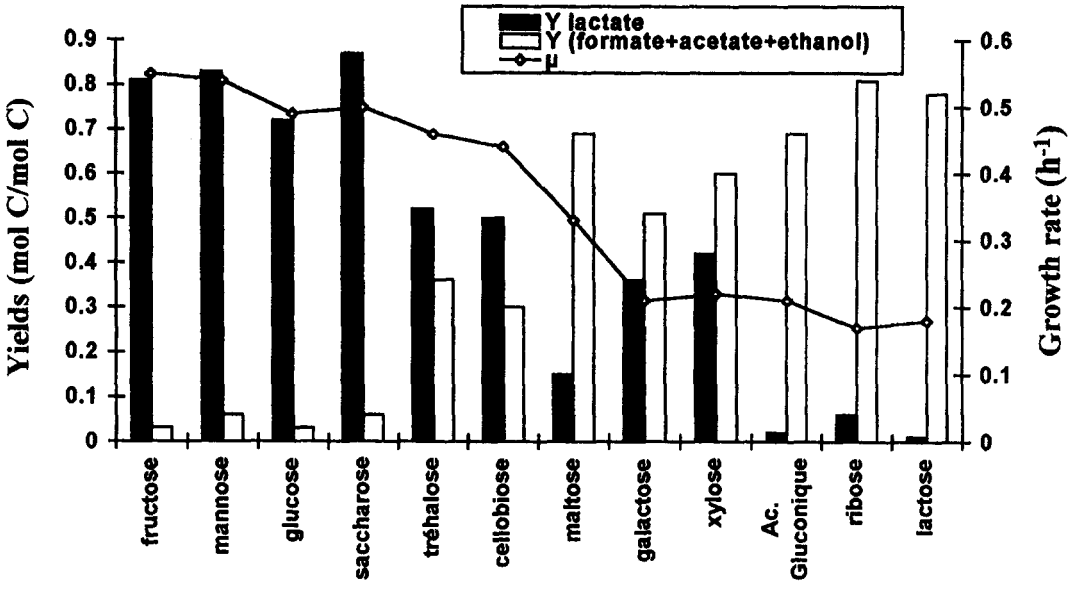

Figure 6. Effect of substrate on growth rate and fermentation profile during batch cultivation of *Lactococcus lactis* NCDO 2118 in a defined medium.

view, the strains thus produced would remain minority within a population due to the greatly diminished growth potential and be unlikely to result in significant spoilage of product. One might however consider whether such strains have any potential role to play in metabolic engineering strategies since they possess phenotypes virtually identical to those of *ldh*$^-$ strains which now form the basis of many genetic engineering strategies for volatile compound overproduction (Hugenholtz et al., 1994). Such strategies aim at directing pyruvate through the acetolactate synthase (ALS) reaction towards diacetyl (Platteeuw et al., 1995; Benson et al., 1996) by deletion or attenuation of the usual pyruvate consuming activities but grow slowly. Growth of the vegetable strains on lactose may facilitate just such a modified metabolism, with the additional advantage that such strains express all the enzymes necessary for the synthesis of branched chain amino acids and hence possess the high affinity ALS (Godon et al., 1992) as well as the low affinity enzyme habitually associated with acetolactate synthesis in *L. lactis* (Marrug et al., 1994). It is too early to assess the feasibility of such a nutritional approach to flavour compound production but the use of 'natural' strains may avoid the public's unfavourable reception to genetic engineering for food compounds.

If the concept is accepted that sugar (and hence energy) limitation leads to loss of homolactic fermentation, then the unspoken but implicit correlative is that under such conditions, the organic matter necessary for cell synthesis is in excess. This was examined using progressively simplified media in our laboratory with the vegetal strain. It was seen that removing amino acids from the media progressively diminished growth rate and hence, might be expected to shift the extent to which energy availability limited cell proliferation. Indeed, the observed end- product profile was seen to favour lactate production as the media was simplified though the homolactic behaviour was never completely restored. This adds further evidence to the model but suggests that a delicate equilibrium between carbon and energy flux towards biomass is involved rather than a simple relationship between sugar catabolism and pyruvate fermentation. Of course, the logical extrapolation of such a hypothesis is that whenever a homolactic fermentation is observed, growth is being limited either by the availability of other organic components of the growth medium, or the cells capacity to assimilate such compounds.

The physicochemical environment

Effect of aeration

In the mixed acid fermentation, only the competition between LDH and PFL have been discussed. Since PFL is inactivated by oxygen one might ask how *L.*

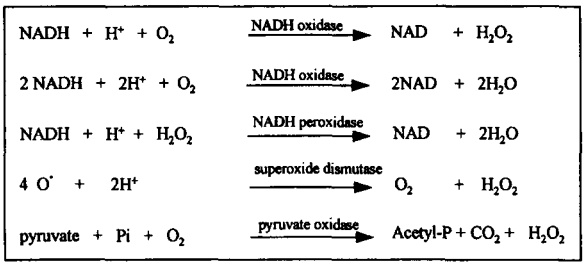

Figure 7. Enzymes involved in oxygen metabolism in lactic acid bacteria.

lactis responds to the presence of air. In conditions of excess substrate, metabolism of sugars leading to either a homolactic fermentation (glucose, lactose) or mixed acid fermentation (galactose), is sometimes deviated towards an increased production of acetate at the expense of lactate and/or ethanol (Smart & Thomas, 1987). Certain strains are reported to accumulate small amounts of pyruvate. This shift towards acetate production is related to the induction of various enzymes able to oxidise NADH (NADH oxidase, NADH peroxidase) in aerated cultures (Smart & Thomas, 1987; Hanson and Haggstrom, 1984; Cogan et al., 1989). These enzymes are able to recycle the reduced coenzymes directly without recourse to carbon compound oxido-reduction reactions (Figure 7). The requirement to produce reduced end-products is diminished and acetate production can be favoured, thus yielding more ATP (Condon, 1987). This metabolic response should not be confused with respiration since no electron transport phosphorylation is involved though an effective but indirect energetic gain is achieved due to the increased production of acetate. Competition between lactate and/or aldehyde and alcohol dehydrogenases and the O_2 consuming NADH oxidising enzymes is responsible for any shift towards acetate production though this may also involve a modified level of *ldh* gene expression since lactate dehydrogenase has been shown to diminish to approximately half the anaerobic activity in some strains (Smart & Thomas, 1987). The consequence of this is an increased availability of pyruvate. The PFL activity is inactivated by O_2 and hence an alternative pathway of pyruvate disposal is necessary. One might be tempted to ask whether the oxygen consumption is to provoke an increased ATP yield or to remove the oxygen from the medium thus enabling an active PFL to be synthesised. However, in the absence of an active PFL, other enzymes are able to metabolise pyruvate, notably the pyruvate dehydrogenase complex, but also in certain LAB a pyruvate oxidase (Sedewitz et al., 1984a, b; Zitzelberger *et al.*, 1984). However, no evidence has yet been presented to suggest that this enzyme exists in *L. lactis*. The action of NADH oxidase has been demonstrated in many micro-organisms and this enzyme is widespread but not ubiquitous within LAB. Two forms of this enzyme exist, one of which leads to H_2O production while the other yields H_2O_2. The first type is difficult to distinguish from the H_2O_2-producer since frequently an NADH peroxidase exists, though separate enzyme activities have been demonstrated in *S. mutans* (Higuchi et al., 1993). Superoxide dismutase activity is widespread in LAB and also induced in aerated cultures. This enzyme, like Mn^{2+} cations, effectively removes the toxic radical oxygen (Archibald & Fridovich, 1981a, b).

As regards the oxygen effect, the enormous variety of media used to cultivate *L. lactis* most probably account for the somewhat variable results obtained. As yet, it cannot really be decided whether such variation is due to the strain variability as regards either the oxidising enzymes themselves or their capacity to take up and assimilate amino acids, peptides, bases, etc., or to the media composition used. While some authors claim an increase in acetate production, other have observed no significant influence of aeration (Smart & Thomas, 1987, Starrenburg & Hugenholtz, 1991). Once again it is necessary to take into account the extent to which each culture is energy-limited or carbon-limited and to recall that unlike many bacteria, these two fluxes essential for cell growth are quite separate and independently controlled in *L. lactis*.

Aeration might be expected to improve cell growth in certain media but it must be remembered that both NADH oxidase and superoxide dismutase lead to H_2O_2 production which may lead to autoinhibition in certain strains (Grufferty & Condon, 1983). The homofermentative LAB in general, and *L. lactis* in particular, do not possess a catalase activity (Zitzelberger et al., 1984) and only NADH peroxidase can avoid H_2O_2 accumulation. It is therefore essential to maintain a correct balance and though details are not available, one might expect that the enzyme possessing the highest affinity for NADH would be the peroxidase, thereby avoiding an excessive accumulation of H_2O_2 in those strains possessing both NADH oxidase and NADH peroxidase activity.

Effect of pH

Little is known of the effect of pH on the metabolism of *L. lactis* but in *Enterococcus faecalis* the partition between pyruvate flux passing via the pyruvate dehydrogenase and the PFL is strongly influenced by the broth pH. It must be remembered that many acid- producing bacteria do not maintain pH homeostasis but rather a constant pH gradient across the cell membrane. This is certainly the case for *L. lactis* (Cook & Russell, 1994) in which intracellular ATP was also seen to decrease as pH diminished. However, this effect appears to be dependent upon the manner in which the experiments are undertaken since intracellular pH can be maintained in washed cells when the external medium is acidified with mineral acids (Poolman et al, 1987b). The PDH is dominant at pH values of 5.5–6.5 while the PFL becomes dominant at pH > 7. This shift was attributed to an effect of redox potential rather than pH itself and involved changes in the specific activity of each enzyme, i.e., the concentration of enzyme (Snoep et al., 1990, 1991). Such effects were no doubt further enhanced by the effect a modified redox potential would have on the various dehydrogenase enzymes, all of whose activity will depend upon the ratio of oxidised to reduced coenzymes. Furthermore, the nutritional requirements were also shown to influence this distribution since addition of lipoic acid under acidic conditions further accentuated the flux through the PDH at the expense of PFL (Snoep et al., 1993b). As mentioned above, the PDH has a somewhat different structure in *L. lactis* and hence coexistence of significant activity of these two enzymes will be restricted to a limited range of nutritional environments as regards both oxygenation and acidity.

Minor fermentation products

If the major pathways described above account for the vast majority of the carbon flux from sugars, much interest is being stimulated by the minor by-products which take on considerable importance as flavour compounds in natural food products (Figure 8). When lactose is consumed in complex media (Kaneko et al., 1990, 1991) or in lipoate-limited synthetic media (Cogan et al., 1989), some strains produce, in the presence of oxygen, alternative products such as diacetyl or acetoin. These compounds are synthesised from pyruvate and are only produced in significant quantities under conditions in which the normal metabolism of pyruvate is perturbed. Such by-products are synthesised via a specific pathway involving acetolactate synthase (ALS), enzyme containing a similar first subunit to the PDH complex, which is responsible for pyruvate decarboxylation into hydroxy-ethyl-TPP. The product of the ALS reaction involving two moles of pyruvate is acetolactate; this compound can accumulate in the medium in certain conditions (Jordan & Cogan, 1988) but is unstable and subject to spontaneous transformation to diacetyl via a purely chemical reaction in the presence of O_2. The acetolactate synthase normally implicated in diacetyl production is an isoenzyme possessing a low affinity for pyruvate (Km = 40–50 mM) and hence unlikely to be operative under normal growth conditions in which the pyruvate pool is low (Snoep et al., 1992b). It is believed that this enzyme may play a role in detoxifying pyruvate and the gene coding for this enzyme has been cloned and sequenced (Marugg et al., 1994).

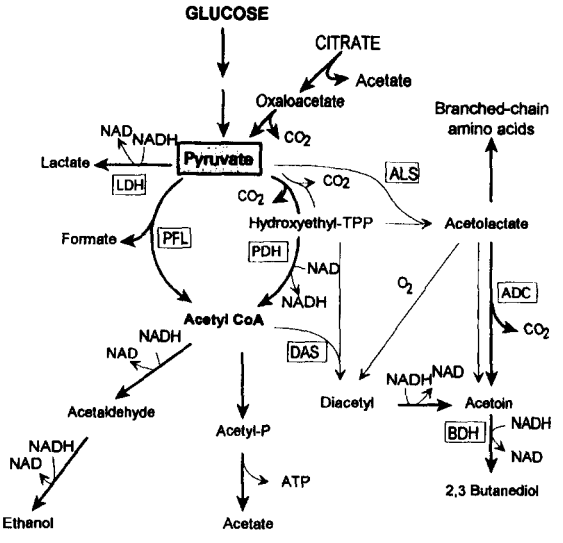

Figure 8. Pyruvate metabolism in *Lactococcus lactis*. LDH: lactate dehydrogenase; PDH: pyruvate dehydrogenase; PFL: pyruvate formate lyase; ALS: acetolactate synthase; ADC: acetolactate decarboxylase; BDH: butanediol dehydrogenase; DAS: diacetyl synthase.

If this enzyme is normally associated with diacetyl production, *L. lactis* also possess a second acetolactate synthase activity coded by the *ilv BN* genes (Godon, 1992) which is involved in the initial reaction of the pathway of branched-chain amino acid synthesis and repressed by the presence of isoleucine. The possible role of this enzyme in diacetyl production has never been investigated in detail since most strains of *L. lactis* require branched-chain amino acids for growth. The strain *L. lactis* NCDO 2118 has no such auxotro-

phy (Loubiere et al., 1996) and expresses this enzyme under certain growth conditions, but lacks many of the other characteristics considered essential for obtaining detectable diacetyl accumulation i.e. citrate consumption capacity. Though *L. lactis* NCDO 2118 is apparently prototroph for branched-chain amino acids by the single omission technique, minimal medium (containing only five amino acids) requires either a suitable supplement of these amino acids (Cocaign-Bousquet et al., 1995) or threonine (Loubiere et al., 1996), whose deamination by the *ilv A* gene product yields ketobutyrate. This compound is a substrate of acetolactate synthase, but has also been implicated in the regulation of *ilv B* expression, inducing higher levels of expression in *Corynebacterium glutamicum* (Eggeling et al., 1987) and *Salmonella tiphymurium* (Shaw & Berg, 1980) when added to the growth medium. This type of regulation may provide a useful strategy for diacetyl production in strategies employing the acetolactate synthase of the amino acids pathway (Benson et al., 1996), thus avoiding the necessity to provoke a tenfold increase in the pyruvate concentration.

Acetolactate may also be biologically transformed to less financially interesting compounds than diacetyl. As mentioned above, acetolactate is an intermediary compound in branched-chain amino acid synthetic pathways, but may also be decarboxylated to acetoin by the acetolactate decarboxylase coded by *ald B*. This enzyme is activated by valine, leucine and isoleucine (Monnet et al., 1994; Phalip et al., 1994) in *L. lactis diacetylactis*. Furthermore, the expression of the gene is activated by the presence of leucine, thus providing the basis of an interesting metabolic phenomena in which the pyruvate flux into amino acid synthetic pathway can be oriented towards carbon metabolites when the amino acids are present (Godon, 1992). The result is acetoin production, which in turn may be reduced to butanediol by a dehydrogenase activity (Crow, 1990). In *L. lactis* two butanediol dehydrogenase activities exist leading to two isomers of butanediol. This same enzyme is able to reduce acetoin to butanediol but also diacetyl into acetoin and is most probably the enzyme which previously known as acetoin reductase or diacetyl reductase. A further enzyme activity, the diacetyl synthase, has been proposed to explain the production of diacetyl from pyruvate and acetyl CoA (Kaneko et al., 1989, 1990, 1991) but this pathway is no longer considered to play any significant role in diacetyl production in *L. lactis*. This conclusion is supported by C^{13}-NMR analysis (Verhue & Tjan, 1991; Ramos et al., 1994).

In addition to this group of products derived from pyruvate, research in our laboratory has recently shown that *L. lactis* produces trace amounts of succinate, presumably via a reductive TCA cycle and though few details of TCA cycle activities are available as yet the anaplerotic reaction PEP carboxylase has been demonstrated. Indeed when precise carbon balances are established (feasible on the defined media used) it is clear that a variety of non-identified minor products accumulate in the media whose physiological role and possible commercial value remain to be determined.

Effects of auxiliary substrates on pyruvate metabolism

Citrate metabolism

Citrate is present in milk at concentration of 8–9 mM and is cometabolised together with the sugars by many strains of LAB including certain *L. lactis*. The strain specific nature of citrate metabolism is due to the presence in certain strains of a plasmid encoding for citrate permease (Sesma et al., 1990; Smith et al., 1992). This metabolism of citrate is important since it generates an additional source of pyruvate without the production of reduced co- enzymes. This occurs since citrate is metabolised via citrate lyase yielding acetate and oxaloacetate, whose further metabolism via oxaloacetate decarboxylase leads to the production of pyruvate (Figure 8). The result of such a metabolism is that under conditions of aerobiosis and in acidic environments, the fermentation of a reconstituted milk medium leads to a significant increase in flavour compound production in the presence of citrate (Drinan et al., 1976; Hugenholtz & Starrenburg, 1992). Indeed, it is now known that citrate can be metabolised in the absence of a sugar, producing acetate, formate and flavour compounds, though such a metabolism occurs only in a narrow range of pH values (Starrenburg & Hugenholtz, 1991).

The increase in diacetyl production during citrate metabolism can be correlated to an increase in acetolactate synthase activity and a partial repression of butanediol dehydrogenase (Cogan, 1981). While this latter activity is derepressed immediately after complete depletion of citrate and is hence directly related to the presence of citrate, the effect on acetolactate synthase activity is less clear. While it is possible that citrate has a direct effect on enzyme expression the increased pyruvate pool resulting from citrate metabolism may also be involved. Certainly such an

increase will favour the flux through acetolactate synthase.

Amino acid metabolism

For many years, amino acids were considered uniquely as a source of assimilable carbon for biomass formation but recent work in our laboratory has shown that certain essential amino acids may also contribute to the fermentation pattern. This was most obvious for serine in minimal medium for which ^{14}C-serine was predominantly recovered as lactate (Loubiere et al., 1996) and as such may play a role as an auxiliary substrate somewhat akin to citrate. Furthermore, the depletion of serine in such media led to a shift in the acetate/ethanol ratio with ethanol appearing as a product only after total removal of serine. Other amino acids are known to be catabolised but this generally is restricted to decarboxylation reactions, e.g. arginine conversion to ornithine with a corresponding gain in ATP (Poolman, 1993).

Conclusion

Pyruvate occupies a key position within the metabolic network of *L. lactis* and as such is subject to considerable regulation. Current understanding of this metabolic node is adequate to envisage some degree of exploitation within a biotechnological context, but at a scientific level we are only able to see the consequences of the regulation mechanisms involved. This absence of mechanistic understanding is most obvious at the level of gene expression for which little is known. Biochemical knowledge is more advanced, though certain areas remain rather speculative, and a more structured approach to metabolic control within a modelisation framework may promote a more rigorous interpretation of the current data. Since pyruvate metabolism represents the convergence of catabolic and fermentation pathways, while also being generated directly via the various PTS sugar transport mechanisms, it would seem likely that we will need to incorporate extensive energy-signalling mechanisms into such models before the details become apparent. This information is essential for the next generation of lactic acid bacteria, constructed pragmatically via genetic engineering strategies, to satisfy market demand. The fact that such strains do not yet meet with public approval should not block the basis research necessary to construct customised cell factories.

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