The fructose diphosphate/phosphate regulation of carbohydrate metabolism in low G+C Gram-positive anaerobes

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Introduction

In the 1920's, Orla Jensen used lactic acid production as a means of classifying Gram-positive bacteria. Based on his classification scheme, "homofermenters" produced only lactic acid, while the "heterofermenters" produced ethanol and carbon dioxide as well as lactic acid. This scheme was later supported by different pathways of hexose catabolism (DeMoss et al., 1951). Homofermenters utilized the Embden-Meyerhoff enzyme aldolase, while the heterofermentative scheme employed glucose-6-phosphate dehydrogenase and phosphoketolase (Sokatch, 1969).

By the 1950's, it became apparent that lactic acid production was not always a reliable taxonomic tool (Gunsalus and Niven, 1942; White et al., 1955), and by the 1970's the validity of phenotypic traits as phylogenetic indicators was being questioned (Woese, 1987). Based on the observation that rRNA is a complicated molecule relatively free from lateral transfer and environmental selection pressure, 16S rRNA sequencing has become a standard basis of bacterial classification and has allowed bacteriologists to construct phylogenetic schemes of evolution.

The Gram-positive bacteria seem to share a common evolution and have been divided into two major groups commonly referred to as either low or high mol % G+C. The low G+C group of fermentative Gram-positive bacteria includes the streptococci, lactococci, lactobacilli, clostridia, and butyrvibrios, and the phylogenetic relatedness of these bacteria is supported by the observation that many share a pattern of regulatory enzymes and 'energy signals'. This scheme of fructose diphosphate (FDP)/phosphate regulation affects fermentation endproducts. ATP dissipation by non-growth energy spilling reactions, and catabolite regulation.

Phylogeny

In the past, the classification of Gram-positive, fermentative organisms was primarily based upon cell morphology and spore formation (Hardie, 1986), but considerable phenotypic diversity still existed. Gram-positives that produced significant quantities of lactate were often simply defined as "lactic acid bacteria", even though this group included organisms of different morphology and phenotype (Ingram, 1975). While the boundaries of what constituted a "lactic acid bacterium" remained somewhat vague, this definition has survived for more than a century.

With the introduction of 16S rRNA cataloging, the relatedness of lactic acid bacteria became more apparent (Woese, 1987). Streptococci, lactobacilli, and 'clostridial types' have a relatively deep line of descent, often referred to as the low G+C Gram-positive anaerobes. Recent work has subdivided the genus Lactobacillus into three distinct clusters (Collins et al., 1991) and the genus Streptococcus into three new genera (streptococci, lactococci and enterococci) (Scheifer and Kilpper-Bülz, 1987). The clostridia have proved to be a more heterogeneous group (Collins et al., 1994). A large number of strains are closely related to the 'traditional' spore-forming, solvent-producing clostridia (e.g. C. butyricum, C. acetobutylicum), but over 20 other clusters of 'clostridial' organisms have been identified. These clusters include such diverse bacteria as the peptostreptococci, ruminococci, butyrovibrios and eubacteria.

FDP/phosphate-dependent lactic acid production

Many species of lactic acid bacteria produce L-lactate, but Wolin (1964) noted that the NADH-dependent L-lactate dehydrogenase (L-LDH) of the

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ruminal bacterium *Streptococcus bovis* could not convert pyruvate to L-lactate. The L-LDH of *S. bovis* required fructose 1,6-diphosphate (FDP) for activation, and similar enzymes have been found in enterococci (Hardman and Pritchard, 1987), lactobacilli (Hensel *et al.*, 1977), clostridia (Vancanneyt *et al.*, 1990), and butyrvibrios (Van Gylswyk, 1977). FDP-activated NAD-dependent L-LDHs have only been found in Gram-positive bacteria (Garvie, 1980).

FDP-activated L-LDHs normally exist as inactive monomers or dimers (Mayr *et al.*, 1980). FDP induces internal conformational changes in the enzyme after binding to a site distinct from both the NADH and pyruvate sites. This allosteric modification decreases the $K_m$ for pyruvate (Mayr *et al.*, 1982), and in some bacteria, the $K_m$ for NADH (Crow and Pritchard, 1977). L-LDH affinity for FDP is not constant, and *Lactobacillus casei* requires tenfold more FDP than *Lactobacillus curvatus* (Hensel *et al.*, 1977). The pH optima of L-LDHs usually are less than 5.5 (Mayr *et al.*, 1980; Russell and Hino, 1985), and at low pH, FDP is not required as an activator. Protons appear to facilitate tetramer formation, and this pH-induced subunit association promotes internal conformational changes similar to those caused by FDP (Mayr *et al.*, 1982). Divalent cations, such as Co$^{2+}$ and Mn$^{2+}$, also can induce subunit association and activation (Mayr *et al.*, 1980; Hardman and Pritchard, 1987), but the physiological role of these activators is not clear. Some L-LDHs are not activated by FDP, but many of these enzymes have similar molecular weights, amino acid compositions, and antigenic properties (Crow and Pritchard, 1977; Garvie, 1980). These non-FDP activated L-LDHs appear to be permanently bound together as active tetramers (Mayr *et al.*, 1980), and thus do not require FDP. Wolin (1964) noted that phosphate stabilized the *S. bovis* L-LDH during extraction and purification, but phosphate can also be an inhibitor of L-LDH activity (Yamada and Carlsson, 1975). Free phosphate increases the requirement for FDP, and appears to be a competitive inhibitor of FDP binding (Jonas *et al.*, 1972).

The pyruvate kinases (PK) of lactic acid bacteria are often allosterically regulated in a manner similar to the L-LDH. These bacteria can have more than one PK (Crow and Pritchard, 1976), and while FDP is not always the sole activator (Collins and Thomas, 1974; Crow and Pritchard, 1976), all PKs appear to be activated by "early" glycolytic intermediates e.g. glucose-6-phosphate (Abbe and Yamada, 1982), fructose-6-phosphate, or galactose-6-phosphate (Thomas, 1976). The PKs of enteric bacteria are often regulated by ATP, ADP, or AMP, but these signals have little effect on the PKs of lactic acid bacteria (Collins and Thomas, 1974). Streptococcal PKs are strongly inhibited by phosphate (Collins and Thomas, 1974; Crow and Pritchard, 1976; Abbe and Yamada, 1982).

Pyruvate-formate lyase (PFL) provides an alternative route of pyruvate catabolism in lactic acid bacteria. PFL is very sensitive to reductions in pH and has a pH optimum as high as 7.5 (Lindmark *et al.*, 1969). PFL is unaffected by FDP or phosphate, but is strongly inhibited by the triose phosphates glyceraldehyde-3-phosphate and dihydroxyacetone phosphate (Takahashi *et al.*, 1982). These latter intermediates are in equilibrium with FDP and tend to increase with FDP.

The FDP/phosphate regulation of lactic acid bacteria allows some to shift their fermentation endproducts in an 'energy-dependent' fashion (fig. 1). When energy is limiting (continuous culture), FDP and triose phosphate pools are low, and intracellular phosphate concentrations are high (Thomas *et al.*, 1979; Mason *et al.*, 1981; Fordyce *et al.*, 1984). Under these conditions, the L-LDH is not active, and pyruvate is converted to acetyl-CoA and formate via PFL. NADH arising from glycolysis is oxidized by alcohol dehydrogenase, and flux through acetate kinase of this 'heterofermentative' scheme provides additional ATP. When carbohydrate is plentiful, glycolytic flux can exceed the capacity of

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**Fig. 1.** The catabolism of glucose by lactic acid bacteria showing key regulatory enzymes and energy signals.
Under these conditions, FDP and triose phosphate concentrations increase (Thompson and Torchia, 1984; Lohmeier-Vogel et al., 1986). FDP activation of L-LDH allows cells to convert excess pyruvate to lactic acid, resulting in classic "homofermentation" (Devries et al., 1970; Thomas et al., 1979).

This regulation allows lactic acid bacteria to maximize their 'rate' of ATP production under both energy-limiting and energy-excess conditions. When energy is limiting, the pathway through PFL is sufficient, and acetate kinase increases the amount of ATP produced per hexose. The PFL pathway, however, is not designed for high rates of hexose catabolism. The lactate pathway decreases ATP per hexose, but this high capacity scheme can provide very high rates of ATP production (fig. 2) (Hungate, 1979).

If lactic acid bacteria are grown at low pH, however, regulation by FDP and phosphate can be circumvented. When extracellular pH is low, intracellular pH declines (Cook and Russell, 1994b), PFL is inhibited, and the LDH is activated in an FDP-independent fashion (Russell and Hino, 1985). This pH-dependent regulation of LDH and PFL promotes pyruvate conversion to lactic acid, and reduces acetate, formate and ethanol production (Rhee and Pack, 1980; Finlayson, 1986). The ability of lactic acid bacteria to decrease their intracellular pH decreases ATP produced per hexose, but the low pH gradient (ApH) prevents undissociated fermentation acid anions from accumulating inside the cell (fig. 3). If intracellular pH did not decline, undissociated, membrane-permeable fermentation acids would cross the cell membrane and dissociate in the more alkaline interior in accordance with the Henderson-Hasselbalch equation (Russell, 1992).

Many clostridia have FDP-dependent LDHs, but lactic acid is not normally a major endproduct of clostridial fermentations. Clostridia typically produce weak acids (e.g. butyrate and acetate) and solvents (e.g. butanol and acetone). Clostridia and lactic acid bacteria have different patterns of intracellular pH regulation. Clostridia buffer their intracellular pH decline to a greater extent, and the increase in ApH restricts them to a less acidic niche (Huang et al., 1985). Most clostridia lack PFL, and instead utilize pyruvate-ferrioxin oxidoreductase (PFO) (Uyeda and Rabinowitz, 1971; Meinecke et

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**Fig. 2.** The ATP production of *Streptococcus bovis* in continuous culture showing the amount produced by glucose conversion to either lactate or acetate formate and ethanol (data taken from Russell and Baldwin, 1979).

**Fig. 3.** The effect of extracellular pH on the intracellular pH (a) or acetate anion accumulation (b) of *S. bovis* and *Escherichia coli* (data taken from Russell, 1992 and Kaback, 1990).
al., 1989). High PFO activity and low LDH expression circumvent the influence of FDP and phosphate, and keep clostridia from producing lactate, a strong acid that would decrease pH even more.

**FDP-stimulated ATPase activity**

Because low G+C Gram-positive anaerobes are not normally respiratory, the primary purpose of the F$_1$F$_0$ ATPase is to create a proton motive force. The F$_1$F$_0$ ATPase has a relatively high affinity for ATP (Rimpiläinen et al., 1988) and is inhibited by the Δp that it creates (Maloney, 1977, 1990). ATPase regulation is usually thought to be transcriptional, and reductions in pH can induce ATPase synthesis (Kobayashi, 1985; Belli and Marquis, 1991). Recent work with *S. bovis* indicated that the F$_1$F$_0$ ATPase could also hydrolyse 'excess' ATP. If *S. bovis* was starved for nitrogen or treated with the antibiotic chloramphenicol, the rate of ATP production (glycolysis) exceeded the rate of ATP utilization (protein synthesis). The accumulation of intracellular ATP promoted lactic acid production, and a non-growth mechanism of ATP spilling (Russell and Strobel, 1990; Russell, 1993a).

Energy-limited *S. bovis* cells spilled little, if any, ATP, but a sudden increase in energy caused a rapid increase in non-growth energy dissipation (Cook and Russell, 1994a). Because the non-growth ATP hydrolysis was enhanced by protonophores and completely inhibited by DCCD, it appeared that the F$_1$F$_0$ ATPase was catalysing a futile cycle of protons through the cell membrane (fig. 4). Excess energy caused an increase in intracellular ATP, but these accumulations were often transient (Cook and Russell, 1994a). Based on the observation that energy spilling cells were always homolactic, it appeared that FDP might be a regulatory signal for the F$_1$F$_0$ ATPase. This conclusion was supported by recent observations: (1) energy spilling cells had high intracellular FDP, and (2) the F$_1$F$_0$ ATPase activity could be increased 2-fold by FDP addition (Emond and Russell, 1996). FDP-stimulated energy spilling has only been characterized in *S. bovis*, but *L. cre;vovis* and *L. lactis* also dissipate ATP in non-growth reactions (Otto, 1984; Fordyce et al., 1984).

The ability of energy spilling reactions has not been precisely defined, but there are several potential advantages (Russell and Cook, 1995). Bacteria are often provided with a transient supply of energy, and bacteria that can re-initiate growth quickly have a distinct growth advantage. A high rate of energy source turnover might allow cells to maintain higher intracellular concentrations of critical precursor metabolites (Marr, 1991). Because the ΔG’p of ATP hydrolysis can be as much as 3-fold higher than Δp (Cook and Russell, 1994a), the ATPase has the potential to distort the cell membrane via dielectric effects (Russell and Cook, 1995). In this light, energy spilling reactions, by decreasing membrane resistance, may serve as a “safety valve”. Lastly, energy spilling, by turning over ATP, may provide the ADP which is needed for normal glycolytic catabolism. It has long been recognized that *E. coli* and other bacteria can produce the toxic endproduct methylglyoxal (Cooper, 1984) and that this alternative pathway of hexose catabolism, which does not require ADP, is fostered by an imbalance of catabolism and anabolism (Russell, 1993b).

**Phosphate-dependent PEP conservation**

The viability of bacteria has often been correlated with an energized membrane (Dawes, 1985), but the membrane potential of lactic acid bacteria declines rapidly when sugar is depleted (Otto et al., 1984). The ability of lactic acid bacteria to remain viable for many hours, or even days, after starvation, is regulated by intracellular phosphate (fig. 1). Lactic acid bacteria commonly use the phosphoenolpyruvate (PEP) phosphotransferase system (PTS) as a method of transport, and the PTS does not require either a membrane potential or ATP. The PTS is driven by PEP, and lactic acid bacteria conserve a PEP pool by rapidly inhibiting PK. When exogenous energy sources are depleted, FDP declines, inorganic phosphate accumulates, and PK inhibition prevents further conversion of PEP to pyruvate (Thompson and Thomas, 1977). While both FDP and phosphate can affect PK activity, $^{31}$P NMR and $^{14}$C fluorography experiments indicated that phosphate was the more important regulator (Lohmeier-Vogel et al., 1986). Cells treated with iodoacetate...
only depleted their PEP pools when phosphate declined (Thompson and Torchia, 1984), and PEP concentrations were more closely correlated with changes in phosphate than FDP.

**FDP-dependent inducer expulsion**

Bacteria often utilize some energy sources preferentially, and sequential patterns of energy source utilization minimize protein synthesis and maximize growth rates (Magasanik, 1961). In enteric bacteria, catabolite regulation is often mediated by cAMP-dependent control of transcription, as well as PTS-mediated inhibition of active transport ( inducer exclusion) (Postma et al., 1993). In low G+C Gram-positive anaerobes, the PTS is the dominant method of sugar transport, and some PTS sugars can inhibit the activity and synthesis of other PTS systems (Thompson, 1987). In these bacteria, cAMP is not a key regulatory molecule, and PTS-mediated inducer exclusion appears to be due to a straightforward competition for HPr, a non-sugar-specific soluble phosphocarrier of the PTS.

In 1980, Reizer and Panos demonstrated that *Streptococcus pyogenes* has a unique mechanism of catabolite regulation (Reizer and Panos, 1980). When *S. pyogenes* and other lactic acid bacteria transport the non-metabolizable lactose analog, methyl-β-D-thiogalactopyranoside (TMG), the intracellular pool of TMG-6-P is stable for long periods of time. If glucose is added, however, the TMG-6-P is dephosphorylated and expelled. Based on these results, it appeared that "inducer expulsion" involved a glucose-dependent activation of a sugar phosphate phosphatase (Reizer et al., 1983; Thompson and Chassy, 1983a,b; London et al., 1985; Sutrina et al., 1988; Ye et al., 1994). Because inducer expulsion could not be triggered by 2-deoxyglucose (2-DG), a non-metabolizable glucose analog, it appeared that inducer expulsion was indeed an energy-dependent phenomenon (Cook et al., 1995).

When glucose is rapidly taken up by lactic acid bacteria, FDP accumulates (Thompson and Torchia, 1984) and intracellular phosphate declines (Mason et al., 1981). FDP activates an ATP-dependent protein kinase (Reizer et al., 1984), and the work of Deutscher and Saier (1983) and Reizer et al. (1983) showed that this kinase was able to phosphorylate HPr in a unique position, serine 46. A mutant form of HPr (HPrS46D) having a negatively charged aspartyl group at position 46 elicited TMG expulsion in the absence of FDP, suggesting that the phosphorylation of HPr at this unique site was regulating inducer expulsion. A mutant HPr with a neutral alanine at position 46 could not trigger expulsion of TMG, even if FDP was present (Reizer et al., 1989). Recent work in *L. lactis* (Ye and Saier, 1995) and *S. bovis* (Cook et al., 1995) has demonstrated that a membrane-associated, sugar phosphate-phosphatase is stimulated 10-fold by HPr(ser-P). Since HPr(ser-P) can be dephosphorylated by a phosphate-activated phosphatase (Reizer et al., 1989, 1993), phosphate and FDP are both determinants of inducer expulsion.

All bacteria possessing a PTS have HPr, but HPr is not always able to facilitate inducer expulsion. Only the metabolite-activated protein kinase appears to phosphorylate HPr at serine 46, and this protein has only been demonstrated in Gram-positive bacteria. *Lactobacillus brevis* does not use a PTS system per se (Reizer et al., 1988), but this bacterium still has HPr, a metabolite-activated protein kinase, and a glucose-dependent mechanism of TMG expulsion (Romano et al., 1987). Until recently, inducer expulsion had only been demonstrated in lactic acid bacteria, but *Clostridium acetobutylicum*, a bacterium with an FDP-dependent L-LDH, also expelled TMG when glucose was added (Diez-Gonzalez and Russell, 1996).

**Conclusions**

Because rRNA genes encode relatively complicated molecules which appear to evolve slowly and are not laterally transferred from one species to another, changes in rRNA are thought to provide an evolutionary clock that runs at a more or less constant speed. True phylogenists have been dismayed by the observation that many simple biochemical traits (e.g. antibiotic resistance) can be easily changed or transferred from one species to another, and microbial physiology has been treated as only the 'flesh' that adorns the true 'phylogenetic skeleton' (Kandler, 1994). Given the observation that the FDP/phosphate regulatory web (1) confers many metabolic advantages, (2) is a complicated scheme, and (3) only appears to be found in bacteria.
sharining a common 16S rRNA ancestry, it may be possible to relate these aspects of macrophysiology to the phylogeny of other low G+C Gram-positive anaerobes.

References


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Carbon metabolism and its regulation in *Streptomyces* and other high GC Gram-positive bacteria

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**Introduction**

The high GC Gram-positive bacteria are a diverse group of bacteria, which typically have GC molar ratios in the 60-70\% range. They include a large number of organisms of commercial or medical importance, ranging from human pathogens such as *Mycobacterium tuberculosis* to antibiotic-producing *Streptomyces* species.

Carbon catabolite repression (CR) is the phenomenon whereby the synthesis of proteins is repressed by the presence of a catabolite, often generated from a rapidly metabolizable exogenous carbon source such as glucose. CR has been observed for organisms from many genera within the high GC Gram-positive bacteria, e.g., *Mycobacterium* (Bowles and Segal, 1965), *Eubacterium* (Sharak-Genther and Bryant, 1987), *Bifidobacterium* (Degnan and Mac-