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The diversion of lactose carbon through the tagatose pathway reduces the intracellular fructose 1,6-bisphosphate and growth rate of *Streptococcus bovis*

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Abstract Twenty strains of *Streptococcus bovis* grew more slowly on lactose (1.21 ± 0.12 h^{-1}) than on glucose (1.67 ± 0.12 h^{-1}), and repeated transfers or prolonged growth in continuous culture (more than 200 generations each) did not enhance the growth rate on lactose. Lactose transport activity was poorly correlated with growth rate, and slow growth could not be explained by the ATP production rate (catabolic rate). Batch cultures growing on lactose always had less intracellular fructose 1,6-bisphosphate (Fru1,6P_2) than cells growing on glucose (6.6 mM compared to 16.7 mM), and this difference could be explained by the pathway of carbon metabolism. Glucose and the glucose moiety of lactose were metabolized by the Embden-Meyerhoff-Parnas (EMP) pathway, but the galactose moiety of lactose was catabolized by the tagatose pathway, a scheme that by-passed Fru1,6P_2. A mutant capable of co-metabolizing lactose and glucose grew more rapidly when glucose was added, even though the total rate of hexose fermentation did not change. Wild-type *S. bovis* grew rapidly with galactose and melibiose, but these galactose-containing sugars were activated by galactokinase and catabolized via EMP. On the basis of these results, rapid glycolytic flux through the EMP pathway is needed for the rapid growth (more than 1.2 h^{-1}) of *S. bovis*.

Proprietary or brand names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product, and exclusion of others that may be suitable

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Introduction

Streptococcus bovis is a rapidly growing bacterium that inhabits the rumen of cattle and the colon of humans (Hardie 1986). *S. bovis* numbers in the rumen are generally low, but can increase dramatically if large amounts of readily fermented carbohydrate are added to the diet (Hungate et al. 1952). Hungate (1979) concluded that the success of *S. bovis* was based on its "ability to generate more ATP per unit of time when sugar was plentiful". *S. bovis* cells growing anaerobically consume sugars such as glucose, maltose, and sucrose at rates exceeding 80 μmol hexose mg $protein^{-1}$ h^{-1}, a value 15% greater than that for anaerobic *Escherichia coli*, similar to that for *Zymomonas moblis*, and much higher than those of other lactic acid bacteria (Stouthamer 1979; Forrest, 1967).

Most strains of *S. bovis* cannot utilize pentoses but can grow rapidly (more than 1.5 h^{-1}) on a variety of hexose and hexose disaccharides (Russell and Baldwin 1978). *S. bovis* also grows on lactose, but the growth rate is nearly 40% slower than on other sugars (Cook et al. 1995). Some lactic acid bacteria that utilize permeases for transport excrete galactose when growing on lactose (Poolman 1993), and Gilbert and Hall (1987) indicated that *S. bovis* had a lactose operon and permease similar to the one found in *E. coli*. Later studies with whole cells and membrane vesicles, however, demonstrated that *S. bovis* had a phosphotransferase system (PTS) for lactose and utilized the whole molecule for energy production and growth (Cook et al. 1995).

Streptococci typically catabolize hexoses via the Embden-Meyerhoff-Parnas (EMP) pathway, and most disaccharides are eventually converted to glucose 6-phosphate or fructose 6-phosphate (Thompson 1987). When lactose is transported by the PTS, phospho-β-galactosidase produces free glucose and galactose 6-phosphate. The glucose moiety is converted into glucose 6-phosphate via a glucokinase, but galactose 6-phosphate must be metabolized via the tagatose path-

way (Poolman 1993). In the tagatose pathway, tagatose 6-phosphate and tagatose bisphosphate are intermediates rather than fructose 6-phosphate and fructose bisphosphate (Fru1,6P_2). Tagatose bisphosphate is converted to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate by a unique aldoase that only has activity for tagatose bisphosphate (Crow and Thomas 1982).

Because the slow growth rate of *S. bovis* on lactose could be correlated with a diversion of carbon through the tagatose pathway, we hypothesized that the growth rate was being regulated by the availability of early glycolytic intermediates rather than by ATP generation *per se*. The following experiments measured the ability of *S. bovis* to (1) transport glucose and lactose, (2) generate ATP and (3) couple ATP production with cell growth.

Materials and methods

Cell growth

Streptococcus bovis isolates of ruminal origin JB1 (ATCC 700410), 26, 58AXY2, RS1, K27FFA, R45S1, R21096C, isolates of septicemic origin 1314, 2703, 43143, 43144, 49133, 49147, 6, 6448, 9410, V1388, 1499, V1477, and a JB1 strain lacking a glucose PTS were grown in batch culture unless otherwise specified. Cultures were grown under anaerobic conditions in glass tubes with butyl rubber stoppers and aluminum crimp seals at 39 °C in basal medium containing (l^{-1}) 292 mg K_2HPO_4, 292 mg KH_2PO_4, 480 mg $(NH_4)_2SO_4$, 480 mg NaCl, 100 mg $MgSO_4 \cdot 7H_2O$, 64 mg $CaCl_2 \cdot 2H_2O$, 600 mg cysteine hydrochloride, 1 g Trypticase (BBL Microbiology Systems, Cockeysville, Md., USA), and 0.5 g yeast extract (Difco Labs, Detroit, Mich., USA). The medium was adjusted to pH 6.7 and the final pH was never less than 6.5. Sugars [glucose, lactose (4-*O*-β-galactosyl-glucose), melibiose (6-*O*-α-galactosyl-glucose), cellobiose (4-*O*-β-glucosyl-glucose), maltose (4-*O*-α-glucosyl-glucose), or galactose] were provided as energy sources at a concentration of 2 g/l. *S. bovis* JB1 was also grown in glucose- or lactose-limited continuous culture (22 mM hexose in the medium reservoir) under O_2-free CO_2 (360-ml culture vessel, 39 °C, 0.1–1.65 h^{-1} dilution rates). At least 98% of the medium turned over between samplings (approximately four culture vessel volumes). *S. bovis* JB1^{PTS} was selected in a medium containing 2 g/l lactose and 2 g/l 2-deoxyglucose. After seven transfers, the glucose PTS activity of the culture was reduced by 95%, the culture was plated on agar containing lactose and 2-deoxyglucose, and an isolated colony was picked as the source of future studies (Kearns and Russell 1996). Growth in batch culture was monitored by the increase in absorbance (600 nm, 1-cm light path), and by measurement of protein concentration in continuous culture (see below).

Toluene-treated cells

Cells were harvested (10 ml) during exponential growth (absorbance approximately 1.0 at 600 nm) by centrifugation (10 000 *g*, 10 min, 4 °C) and washed twice in 100 mM sodium potassium phosphate buffer (pH 7.2) containing 5 mM $MgCl_2$ and 2 mM dithiothreitol. Cells were then resuspended in 2 ml of the same buffer in a microcentrifuge tube, which was placed on ice until use. The cell suspension (500 µl) was treated with 15 µl toluene/ethanol mix (1:9, v/v) as previously described (Martin and Russell 1986).

PTS assays

Phospho*enol*pyruvate-dependent sugar phosphorylation was assayed enzymatically by a method that measured pyruvate production via lactic dehydrogenase (Martin and Russell 1986). The PTS assays were performed at 39 °C and corrections were made for non-specific NADH oxidase activity by omitting sugar. Specific activities were determined under substrate-excess conditions. Each assay typically was repeated three times and contained 10 µl toluenized cells (approximately 10 µg protein), 0.5 unit L-lactic dehydrogenase, 0.15 µmol dithiotheitol, 1 µmol NADH, 20 µmol phospho*enol*pyruvate, 5 µmol sugar, 1 µmol $MgCl_2$, and 66 µmol phosphate/ml.

Fermentation rate and growth yield

Bacterial heat production was measured as described previously (Russell and Strobel 1990) with an LKB 2277 bioactivity monitor equipped with semi-conducting Peltier elements as thermopiles and gold flow cells. The instrument was calibrated with an internal heat source, and the flow cell was sterilized with 95% ethanol and 1 M HCl. The flow cell temperature was set at 39 °C. The lactate production rate was estimated from the enthalpy (ΔH) of glucose conversion to lactate (87.6 J $mmol^{-1}$ glucose or 195.4 J $mmol^{-1}$ lactose) and the conversion factor 0.278 mW J^{-1} h^{-1}). Growth yield (Y_{ATP}) in batch and continuous culture was calculated from fermentation end-products and cell protein. For each 1 mol lactate it was assumed that 1 mol ATP was produced via substrate-level phosphorylation. For each 2 mol formate + 1 mol acetate + 1 mol ethanol it was assumed that 3 mol ATP were produced.

Protonmotive force

The pH gradient across the cell membrane (ΔpH) and the electrical potential ($\Delta\Psi$) were determined by methods employing silicone oil centrifugation and the distribution of [^{3}H]tetraphenylphosphonium bromide and [^{14}C]benzoate as previously described (Russell and Strobel 1990). The intracellular volume (4.3 µl/mg protein) was estimated from the difference between [^{14}C] polyethyleneglycol and ^3H_2O, and corrections were made for extracellular contamination. ΔpH and $\Delta\Psi$ were eliminated by treating the cells with a combination of nigericin (5 µM) and valinomycin (5 µM).

ATP

Samples for ATP determination were prepared as previously described (Russell and Strobel 1990) and assayed with a luminometer (model 1250, LKB Instruments Inc., Gaithersburg, Md.) to measure light output of a luciferin/luciferase mix (Sigma Chemical Co., St. Louis, Mo.).

Intracellular potassium

Exponentially growing cultures (4 ml) were centrifuged through 0.3 ml silicone oil. The cell pellets and supernatant samples (10 µl) were digested at room temperature for 24 h in 3 M HNO_3, and insoluble cell debris was removed by centrifugation (13 000 *g*, 15 min). The potassium concentration was determined by flame photometry (Cole-Parmer 2655-00 digital flame analyzer; Cole-Parmer Instrument Co., Chicago, Ill.). Corrections were made for extracellular contamination in cell pellets. The intracellular volume was estimated as described above.

Intracellular fructose 1,6-bisphosphate

A 1-ml sample of *S. bovis* culture was placed into a prepared microcentrifuge tube containing silicone oil (equal-parts mixture of Dexter Hysol 550 and 560) layered on top of 100 µl 10% perchloric acid plus 0.01 mg/ml methyl orange. After centrifugation (13 000 *g*, 1 min), the supernatant and silicone layers were removed, the cell pellet was vortexed, and the cells were incubated at

0 °C for 15 min. After centrifugation to remove cell debris (13 000 *g*, 5 min), 75 µl of this extract was removed and 50% K_2CO_3 was added, 5 µl at a time, until the indicator dye turned yellow (above pH 4.5). After centrifugation to remove precipitate, samples were analyzed for Fru1,6P_2. Known quantities of Fru1,6P_2 were also subjected to the extraction procedure and analyzed to determine recovery. The Fru1,6P_2 assay contained (ml^{-1}) 0.5 U α-glyceraldehyde-phosphate dehydrogenase, 2.5 U triosephosphate isomerase, 0.2 µmol NADH, 250 µmol triethanolamine and 2 µmol $MgCl_2$ (pH 8.5). Samples were equilibrated in the spectrophotometer, and the reaction was initiated by the addition of 1 U Fru1,6P_2 aldolase to each cuvette. Fru1,6P_2 content was determined from the change in absorbance at 340 nm, assuming the relationship of 2 mol NADH/mol Fru1,6P_2. When samples were analyzed without freezing, recovery of Fru1,6P_2 standards averaged 95%, and final values were adjusted on the basis of standards. Freezing reduced recovery by as much as 30%.

Other assays

Lactate, ethanol, fermentation acids, and sugars in cell-free supernatant samples were analyzed by high-pressure liquid chromatography (87H BioRad column, 0.5 ml/min 85 mM H_2SO_4, refractive index detector, 50 °C). Cells were treated with 0.2 M NaOH (100 °C, 10 min), and protein was determined by the Lowry method (Lowry et al. 1951). Galactokinase was assayed by monitoring ADP production from ATP in the presence and absence of galactose, using toluene-treated cells. ADP was measured by monitoring NADH disappearance in a coupled assay using pyruvate kinase and lactate dehydrogenase. All enzymes were obtained from Sigma Chemical Co., St. Louis, Mo., USA.

Results

Growth rate

Twenty strains of *S. bovis* grew more rapidly on glucose than on lactose. Glucose provided a mean growth rate of 1.67 ± 0.12 h^{-1}, and the growth rate on lactose was only 1.21 ± 0.12 h^{-1} (Fig. 1). No strain grew as fast on lactose as on glucose. *S. bovis* JB1, the strain selected as a model, grew 40% more slowly on lactose than on glucose, and repeated transfers (approximately 200 generations) did not eliminate this difference. Even cultures that were selected in continuous culture (approximately 200 generations) always grew more slowly on lactose than on glucose.

PTS activity

All *S. bovis* strains tested had lactose PTS activity. When these were expressed as hexose equivalents, the average lactose and glucose PTS activities were 703 ± 312 nmol compared to 810 ± 313 nmol hexose transported mg $protein^{-1}$ min^{-1} respectively (Fig. 1). *S. bovis* JB1 had lactose and glucose PTS activities of 880 nmol and 920 nmol hexose equivalent mg $protein^{-1}$ min^{-1} respectively. The variation in PTS activities was large, and the correlation coefficients for lactose or glucose PTS activities and growth rates were −0.37 and 0.14, respectively.

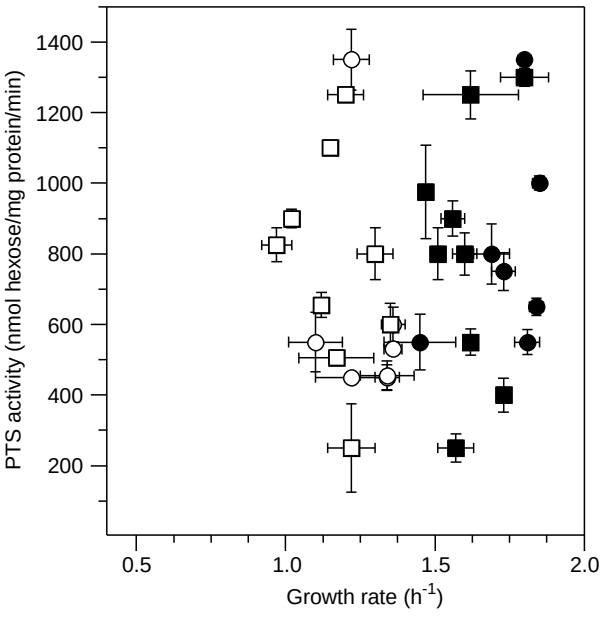

Fig. 1 Relationship between glucose (●, ■) or lactose (○, □) phosphotransferase (*PTS*) activity and growth rate for twenty *Streptococcus bovis* strains isolated from ruminal (●, ○) and human (■, □) environments

Catabolic rate

Exponentially growing cultures of *S. bovis* were always homolactic, and galactose was never detected as an end-product. The catabolic rates of *S. bovis* JB1 for glucose and lactose were similar (Fig. 2). When growing (on glucose or lactose) *S. bovis* JB1 cultures were treated

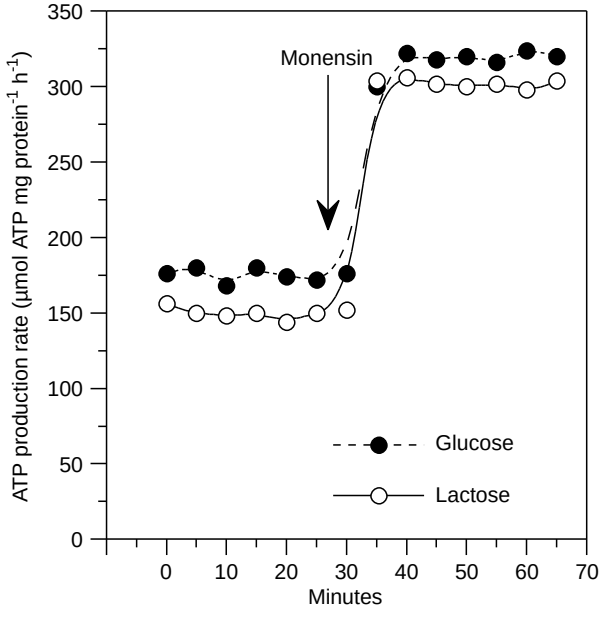

Fig. 2 ATP production rates of *S. bovis* JB1 cultures growing on glucose or lactose. Monensin (0.5 µM) was added at 30 min to determine whether cells were capable of increased sugar transport and catabolism of each sugar

with low concentrations of monensin (a sodium/proton antiporter), the rates of glucose and lactose catabolism increased by as much as 50%, and this change occurred within 5 min after uncoupler addition. Intracellular ATP and protonmotive force of glucose and lactose cultures decreased, and the growth rates were reduced by 42% and 35% respectively.

Growth efficiency

S. bovis JB1 cultures that were growing exponentially on lactose grew less efficiently than cultures growing on glucose, and the Y_{ATP} was 32% lower (8.3 g compared to 12.1 g protein mol ATP^{-1}). Continuous culture experiments indicated that the difference in yield was being caused by a decrease in the theoretical maximum growth yield (Y_{ATPmax}, or 1/slope in Fig. 3) rather than an increase in maintenance energy *per se* (Fig. 3). The lactose-dependent decrease in Y_{ATP} was only observed at growth rates greater than 0.5 h^{-1}.

Glycolytic flux

A *S. bovis* JB1 mutant deficient in glucose PTS activity ($JB1^{PTS}$), but retaining a facilitated diffusion mechanism of glucose transport, grew at the same rate on lactose as did wild-type cultures. *S. bovis* $JB1^{PTS}$ was able to use lactose and glucose simultaneously even if the glucose concentration was greater than 50 mM. When the glucose-PTS-deficient mutant was provided with lactose, glucose caused an increase in growth rate (Fig. 4) even though the total rate of hexose utilization did not increase (data not shown).

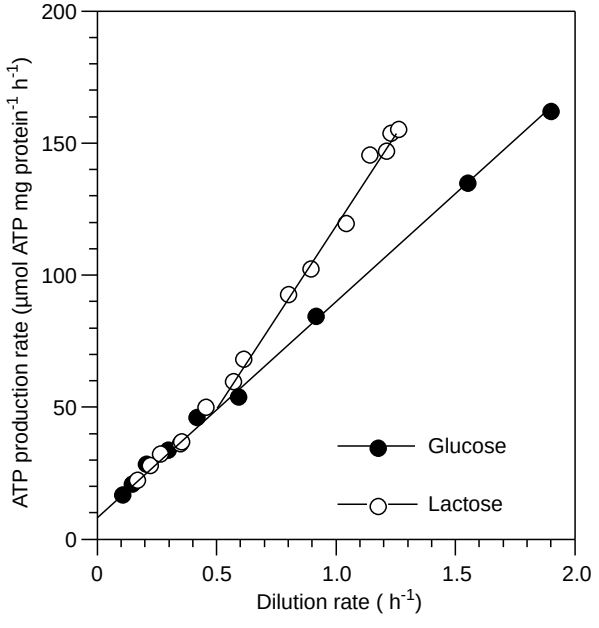

Fig. 3 ATP production rates of glucose- or lactose-limited *S. bovis* JB1 continuous cultures. The intercept on the vertical axis represents maintenance energy, and the slope equals $1/Y_{ATP}$

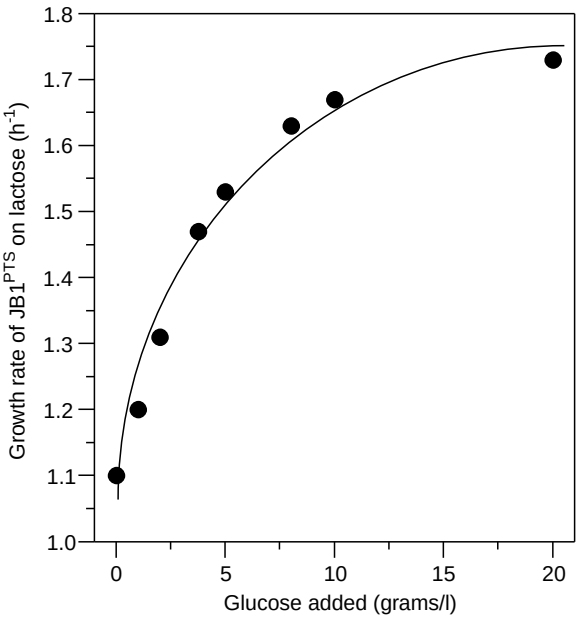

Fig. 4 Effect of glucose addition on growth rate of *S. bovis* $JB1^{PTS}$

Intracellular pools and protonmotive force

Cells growing on glucose and lactose had similar levels of intracellular ATP (4.7 ± 0.6 mM compared to 4.0 ± 0.4 mM), potassium (610 ± 10 mM compared to 640 ± 15 mM) and protonmotive force (−137 ± 8 mV compared to −134 ± 10 mV), but the Fru1,6P_2 concentrations of lactose-grown cells were at least three-fold lower (Fig 5). *S. bovis* JB1 grew rapidly on glucose, galactose, melibiose, maltose, and cellobiose and, in each case the amount of intracellular Fru1,6P_2 was at least 2.5-fold greater than the amount found with lactose. Cells growing with glucose and lactose had low galactokinase activities (less than 20 nmol mg protein^{-1} min^{-1}), but

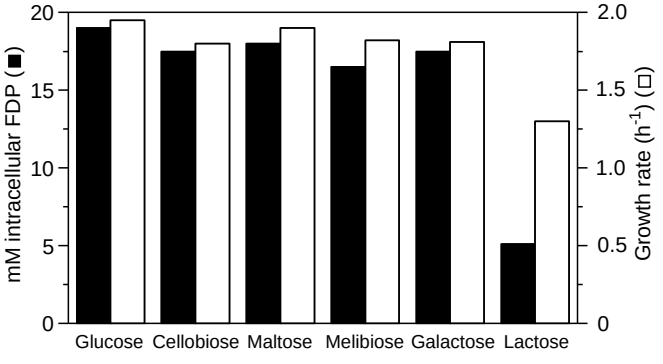

Fig. 5 Intracellular fructose 1,6-bisphosphate (*FDP*) and growth rate of *S. bovis* JB1 growing on a variety of sugars (2 g/l initial concentration)

galactose- and melibiose-grown cells had high activities (980 and 750 nmol mg protein^{-1} min^{-1} respectively).

Discussion

It has often been assumed that ATP production is the rate-limiting step in bacterial growth (Marr 1991), but Marr (1991) concluded that "the rate of growth of *E. coli* is set by the flux of precursor metabolite ... rather than the flux of ATP". *S. bovis* cultures always grew slowly on lactose, but there was little indication that transport or catabolic rate (ATP production rate) was the cause. Transport activity could be increased by monensin, an antiporter that decreases protonmotive force and increases ATPase activity (Russell and Strobel 1990), and this result supported the idea that the catabolism was not rate-limiting. Since the EMP and tagatose pathways provide the same amount of ATP from homolactic fermentation (Poolman 1993), the ATP production rates were similar. Lactose-limited continuous cultures had lower Y_{ATP} values when the growth rates were greater than 0.5 h^{-1}, and this result indicated that ATP was in excess, and some other aspect of anabolism was limiting.

S. bovis JB1 has a PTS-mediated inducer exclusion mechanism that prevents simultaneous glucose and lactose utilization, but this bacterium has two mechanisms of glucose transport, a PTS and a facilitated diffusion mechanism (Russell 1990). Because mutants lacking the membrane component of the glucose PTS can utilize glucose and lactose simultaneously (Cook et al. 1995), it was possible to ascertain the effect of supplemental glucose on cells utilizing lactose. On the basis of the observation that glucose increased growth rate via a mechanism that did not involve increased ATP generation, it appeared that cells growing on lactose were limited for carbon, rather than ATP.

S. bovis cultures grew as rapidly with either galactose or melibiose as with glucose. Galactose and melibiose cultures had had high galactokinase activity, and galactose 1-phosphate can enter the EMP following conversion to glucose 6-phosphate (Thompson 1987). Galactose 6-phosphate, arising from lactose 6-phosphate (produced during PTS transport), cannot be converted to early glycolytic intermediates (Thompson 1987), and must be fermented via the tagatose pathway. The galactose 6-phosphate/tagatose 6-phosphate isomerase is an inducible enzyme in *S. bovis* JB1, and this organism does not have β-galactosidase activity (Cook et al. 1995). These observations support the idea that the galactose portion of lactose can only be fermented via the tagatose pathway.

The flux of lactose carbon through the tagatose pathway was correlated with a low intracellular FDP concentration. When *S. bovis* was grown on other sugars, the tagatose pathway was not used, intracellular FDP was higher, and the growth rate was faster. When glucose was added to a mutant culture growing on lactose, the growth rate increased even though there was no increase in the overall hexose consumption rate or ATP production rate. On the basis of these observations, the diversion of carbon through the tagatose pathway decreased the growth rate via a mechanism involving early glycolytic intermediates rather than ATP *per se*.

Low-(G + C) gram-positive anaerobes have evolved catabolic regulatory schemes and patterns of gene expression that are Fru1,6P_2-dependent (Chauvaux 1996, Russell et al. 1996), but the evolution of these mechanisms has not been elucidated. Did bacteria like *S. bovis* develop Fru1,6P_2-dependent regulatory schemes and then increase intracellular Fru1,6P_2? A more more likely avenue would involve regulation as a later development. Fru1,6P_2 is is not a branch point in metabolism or an anabolic precursor, but glucose 6-phosphate and fructose 6-phosphate are needed to synthesize peptidoglycan and teichoic acids (Archibald et al. 1993). Virtually all eubacteria have peptidoglycan, but low-(G + C) gram-positive bacteria have much more cell-wall material than do gram-negative bacteria (as much as 25% of the dry weight). Further work is needed to define the role of early glycolytic intermediates in other low-(G + C) gram-positive organisms, but results with *S. bovis* indicate that high glycolytic flux is needed for rapid growth on glucose and lactose.

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